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Evaluation of Radionuclide Induced Damage in Marine Invertebrates

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By

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ABSTRACT

Limited studies have been carried out to assess the potential effects of ionising radiation on marine organisms. Therefore the general aims of this thesis were, (a) to assess the cytotoxic, genotoxic and developmental effects of ionising radiation on the embryo-larvae of two ecologically relevant marine invertebrates *Mytilus edulis* and *Platynereis dumerilii*, (2) to assess the effects of an environmentally relevant cocktail of radionuclides (3) to monitor the potential impact of radiation in the natural environment and finally (4) attempt to predict the potential effects of radiation at a population level.

Following validation of developmental stages and mammalian based cytotoxic and genotoxic assays, chromosomal aberrations (Cabs), sister chromatid exchanges (SCEs) and proliferation rate index (PRI), on the embryo-larvae of *M.edulis* and *P.dumerilii,* the embryo-larvae stages were exposed to a reference radionuclide, tritium, (0.37, 3.7, 37 & 370 kBq/ml). Low doses of radiation delivered by tritium were shown to be detrimental to the development of embryo-larvae with an increase in abnormality for *P.dumerilii* and an increase in mortality for *M.edulis*. Tritium increased the induction of chromosomal aberrations and sister chromatid exchanges, in exposed embryo-larvae, indicating that tritium is potentially genotoxic. Cytotoxic effects (reduction in the cell proliferation rate) were also observed following exposure of embryo-larvae to tritium.

In collaboration with the Royal Devonport Dockyard (DML) investigations on the cytotoxic, genotoxic and developmental effects of a cocktail of radionuclides (radioactive liquid waste diluted to 1.8, 3.2, 5.6, 18%) were carried out. All embryo-larvae exposed to 18% radioactive waste were dead within 24h. Both species exhibited increased abnormality, SCEs and Cabs and a reduction in PRI in dilutions 1.8-5.6%. In general *M.edulis* appeared to be more sensitive to ionising radiation than *P.dumerilii* embryo-larvae.

Following experiments on the embryo-larvae stages of the two marine invertebrates studies were carried out to assess the effects of ionising radiation on adult life stages. Following validation of mammalian based genotoxic assays (comet and micronucleus assays) on adult *M.edulis,* the mussels were exposed to a reference radionuclide, tritium, (0.37, 3.7, 37 & 370 kBq/ml) in an attempt to assess the genotoxic effects of ionising radiation on the adult life stage. An increase in the levels of single strand breaks (comet assay) and in the induction of micronuclei (micronucleus assay) in haemocyte cells was observed in adults exposed to tritium.

In collaboration with the Royal Devonport Dockyard (DML) a field study was carried out to assess the use of the genotoxic assays (comet and micronucleus assays) as biomarkers of exposure to radiation in adult mussels transplanted to an area of radionuclide discharge. Statistical analysis detected no correlation between the health of the deployed mussels and the levels of environmentally realistic radioactivity.

After development of *P.dumerilii* embryo-larvae to sexual maturity there appeared to be no affect on the number or sex of the worms that reached adulthood in comparison to control worms. Although at the highest concentration of tritium (370 kBq/ml) there was a reduction in the number of eggs produced from sexually matured females.

In conclusion, from the current studies it can be stated that ionising radiation is cytotoxic and genotoxic to sensitive embryo-larvae stages of marine invertebrates. However further studies need to be carried out to correlate the effects seen at molecular levels with the potential long-term effects observed at population and community levels of these species.

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of philosophy has the author been registered for any other University award.

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Jha, A.N., Hagger, J.A., Hill, S.J. (2000). Tributyltin induces cytogenetic damage in the early life stages of the marine mussel, *Mytilus edulis*. Environmental and Molecular Mutagenesis 35:4, 343-350.

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Chapter 1

Introduction

1.1 Electromagnetic & Ionising Radiation

Radioactivity is a part of nature with natural processes continuously producing new radioisotopes. However, ever since its discovery, the influence of radioactivity on man and his well being has been in question. With advances in technology, nuclear processes have been developed that has led to ionising radiation playing an important role in modern civilisation. Nuclear processes such as fission and fusion have meant that nuclear energy can be used for both energy production and weapon manufacturing, although, this has occasionally led to contamination of the environment by radioactivity and has posed an increasing threat to human health. Large acute doses of radiation can cause effects, such as opacities in the lens of the eye that can lead to cataract, and temporary or permanent sterility. In severe cases of whole body irradiation, acute syndromes, such as damage to bone marrow, gastrointestinal tract, lungs and nervous system, can lead to death within a short period of time after exposure. At low doses, radiation exposure can also plausibly induce severe health effects, such as malignancies, which are statistically detectable in a population (AMAP, 1998). Before assessing the effects of radiation to organisms it is important to understand the nature and activity of radiation as well as possible sources of potential exposure (Valković, 2000).

The sources of radiation can be divided into natural and anthropagenic. Natural background radiation is ascribable to cosmogenic and primordial radionuclides, as mentioned in section 1.2. Primordial radionuclides are derived from internal radioactive sources in the Earth that were generated at the time the planet was formed (Kennish, 1997), and the majority of radiation received by humans is due to the spontaneous rearrangements of unstable radionuclides occurring from natural sources (Clarke, 1997).

Atoms commonly lose or gain one or more negatively charged electrons (which orbit the nucleus) in the course of a chemical reaction, or through physical processes to become a positively or negatively charged ion and the process by which this occurs is known as ionisation. Isotopes are atoms of the same chemical that have the same number

of protons but different numbers of neutrons present in the nucleus. They still have the same chemical properties but differ in their nuclear mass. Some isotopes are unstable and therefore the instability of the nucleus is remedied by a change in the ratio of protons to neutrons. This change in ratios is accompanied by the emission of particles and energy, which is known as radioactivity and the unstable forms of isotopes are called radionuclides (Clarke, 1997). Thus, radioactivity denotes the process by which a radionuclide undergoes spontaneous disintegration (decay) of its unstable nucleus with the emission of one or more radiations and the formation of a daughter nuclide.

1.1.1 Types of radiation

Radiation emitted from a radioactive substance can be in the form of particles (alpha, beta and neutron particles) and electromagnetic waves (gamma rays and X-rays). If these particles and rays are sufficiently energised they will produce positively and negatively charged ion pairs when penetrating matter, this is know as ionising radiation.

1.1.1.1 Alpha particles

Alpha particles, which are released by the unstable nucleus, consist of two protons and two neutrons. They are relatively slow moving, positively charged particles that have a large mass and are intensely ionising when passing through biological tissue, however, they possess low penetrating power. They lose their energy (within the range of 4 MeV – 8 MeV) in a short distance and are stopped by a few centimetres of air or only 40μm of soft tissue. They are, however, intensely ionising in the matter, through which they pass and can cause more damage to living tissue than particles with a longer path such as beta particles (Kennish, 1997). Nuclei emitting alpha particles e.g. polonium-210 are therefore of biological consequence if they are taken into the body for example by ingestion or inhalation (Bishayee *et al.*, 2000).

1.1.1.2 Beta particles

Beta particles e.g. tritium have greater penetrating capacity than alpha particles with a energy range of 0.5 – 3.5 MeV. They are formed by the spontaneous conversion of a neutron to a proton, or vice versa, and are ejected from the nuclei of radioactive atoms. Negatively charged beta particles are termed negatrons or electrons and are formed when a neutron has been changed into a proton. Positively charged beta particles are called positrons and these occur when a proton has been converted into a neutron. Negatrons are more frequently emitted from radionuclides than positrons. β-particles vary widely in their energy but lose most of it within a relatively short distance and can be screened by a few millimetres of Perspex or 40 mm of tissue. Like the α-particles, their biological significance is greatest if the β-emitter is taken into the body (Kennish, 1997).

1.1.1.3 Neutron particles

Neutron particles can be generated naturally in the atmosphere via cosmic ray interaction, as well as anthropogenically during fission reactions in nuclear reactors and at sites of nuclear detonations. These particles comprise of an electron and a proton and have a much greater range than either alpha and beta radiation. Their great kinetic energy (ranging from hundreds of MeV down to a fraction of an eV) and penetrating capacity are linked to their large mass and chargeless state (Kennish, 1997).

1.1.1.4 Gamma rays and X-rays

The emission of gamma rays commonly accompanies the release of alpha and beta particles during the decay of many radionuclides. Beta-emitters can also release energy in the form of gamma photons. Gamma rays are released from the nuclei of radioactive atoms whereas X-rays arise from the electron shells. Both gamma rays and X-rays have very short wavelengths (high frequencies) and great penetrating power and are strongly ionising to organismal tissue through which they pass. Gamma rays occupy the highest range in the

electromagnetic spectrum, well beyond visible light, ultraviolet and X-rays. Gamma rays are very energetic photons, with energies between 1MeV – 10GeV and wavelengths of the longest gamma radiation are less than 10^{-10}m. X-rays on the other hand, have energies ranging from 124 eV upwards and wavelengths of 10^{-8}m to about 10^{-11}m. Living tissues need to be shielded from γ radiation by a considerable thickness of heavy material such as lead or concrete to absorb the radiation (Kennish, 1997).

1.1.2 Mode of action and penetration of radionuclides

The mode of action of particles (α and β) varies from that of photons (γ- and X-rays). When α- or β-particles travel through matter, their electric charges (positive or negative) cause ionisation of atoms in the matter, this is called a direct effect. The track of α-particles is generally short and straight whereas, β-particles usually scatter, frequently producing a wavy track.

In comparison γ- and X-rays act indirectly. Photons can produce ionisation in three ways, the photoelectric effect, the Compton effect and pair production. The *photoelectric effect* occurs when the photon striking an electron in the innermost shell (K shell) has energy equal to or slightly higher than that of the electron. The electron is then released from the atom and its energy is equal to that of the photon diminished by the K-shell binding energy. The *Compton effect* occurs when a photon strikes an electron in the L-shell (the next to innermost shell) with energy much in excess of that of the electron. The electron is then knocked out, but only part of the photon energy is transferred to the electron. The remainder is reradiated as a photon of lower energy. *Pair production* occurs when a photon having energy greater that 1.02 MeV strikes the nucleus and disintegrates into an electron and a positron. The positron loses energy by ionising atoms of the matter. Eventually it collides with an electron and annihilates itself, producing two photons, each having an energy of 0.511 MeV and travelling in opposite directions (Zakrzewski, 1997).

The interaction of particles with matter results in the excitation of the electron followed by ionisation. Ionisation is the most important transfer of energy from ionising particles to mater. The penetration of ionising radiation through tissue depends on the type of radiation (i.e., its mass and charge) and also its energy. The amount of damage to the tissue is related to the linear energy transfer (LET). When a particle or a ray travels through matter, it gradually loses energy transferring it to the matter. The initial energy of the incoming radiation (Emax) divided by the thickness of the matter required to dissipate all the energy is referred to as the linear energy transfer which is expressed in energy units (keV) per unit of path length (μm) (Zakrzewski, 1997). The LET is affected by the velocity and the charge of the ionising particles. Alpha particles, neutrons and protons are high LET radiations and X- and γ rays and fast electrons are low LET radiations. Particles with a high LET are more damaging per unit of dose than low LET radiations. In practical terms it means that the different types of particles and rays, each has different penetrating abilities, (Figure 1.1.). α-particles, have a high LET, are very damaging to tissue but do not penetrate more than a few micrometers and are stopped by paper. Whereas β-particles can be stopped by aluminium, γ-radiation is weakened by lead, but it is never totally blocked and neutrons will pass through lead but will be stopped by concrete.

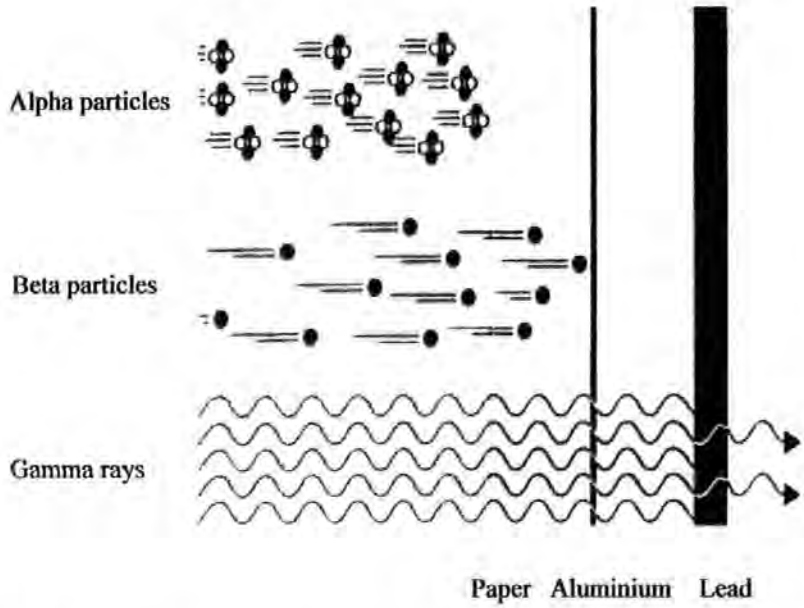


Figure 1.1. Penetrating powers of ionising radiations (adapted from Valković, 2000).

1.2 Environmental radioactivity

In 1894 Wilhelm Conrad Röntgen discovered X-rays, when observing that a fluorescent screen would glow some metres away from an electrical discharge tube, this led to the discovery of radioactivity by the French physicist Henri Becquerel in 1896. Much work has since been carried out into the science of radioactivity and the sources of radionuclides.

Natural background radiation is ascribable to cosmogenic and primordial radionuclides. Cosmic radionuclides form by the interaction of primary cosmic rays with matter in the atmosphere and on the surface of the Earth. Most of these radionuclides exist in minute quantities, the exception being tritium (^{3}H) and carbon-14 (^{14}C), although four of them (^{3}H, ^{7}Be, ^{14}C and ^{22}Na) contribute significantly to the dose received by humans (AMAP, 1998). Terrestrial gamma rays may also be formed naturally contributing to background radiation. Primordial radionuclides are derived from internal radioactive sources in the Earth that were generated at the time of formation of the planet. Detectable primordial radionuclide of significance include potassium-40 (^{40}K), uranium-238 (^{238}U) and thorium-232 (^{232}Th) (Kennish, 1997). ^{40}K is a β- and γ-emitter with a half-life of 1.3 x

10^9 years. It occurs in rocks and soil, as well as muscles of animals. The abundance of ^{40}K in the environment makes it a major source of both internal and external doses from naturally occurring radiation. ^{40}K in rocks, soils and building materials is also a major contributor to external background radiation (AMAP, 1998). According to UNSCEAR (1988), about 40% of the average annual dose to humans from external radiation is due to ^{40}K in the surroundings. Other natural sources of radiation occurs from the disintegration of ^{238}U, ^{232}Th and, to a lesser extent, ^{235}U. Uranium is encountered in certain rocks, soils and phosphate deposits. Radon is produced by the gaseous decay of ^{238}U and ^{232}Th. 54% of the Earth's background radioactivity is due to the two isotopes of radon (^{222}Rn and ^{220}Rn). The occurrence of radon is not uniformly distributed around the globe but occurs in areas where the soil is rich in thorium. Radon, a noble gas, is an α-emitter and as such is very un-reactive, therefore if inhaled it will not persist in the lungs long enough to cause any damage. However, it can decompose into polonium isotopes 218 and 216 (^{218}Po, ^{216}Po), that may be trapped in the lungs and can cause damage to the tissue. Another naturally occurring radionuclide is rubidium-87 (^{87}Rb), which is an β-emitter with a half-life of 4.89 x 10^{10} years. It occurs in certain minerals, seawater and water of many mineral springs and salt lakes (MacKenzie, 2000).

Seawater is naturally radioactive, largely due to the presence of potassium-40, but it also contains decay products of uranium and thorium and receives a continuous input of tritium through the activity of cosmic rays. Table 1.1, show the levels of natural radioactivity in surface seawater (Clark, 1997). Heavy radionuclides have a low solubility in water and tend to be adsorbed on to particulate matter, therefore accumulating in sediments. Naturally, fine sediments with a large surface area will adsorb more than coarse sediments, thus while oceanic seawater has a radioactivity of about 12.6 Bq/L, marine sands have a radioactivity of 200-400 Bq/Kg and muds 700-1000 Bq/Kg. In some parts of the world marine sands produce high levels of natural radioactivity. At one popular bathing beach at Guarapari, near Rio de Janeiro, the visitor is exposed to a dose rate of 20 μGy/h

(Clark, 1997), and in coastal areas of Kerala and Tamil Nadu in India, which is populated by over 1,000,000 people, doses of up to 6 μSv/h can be received (Valković, 2000).

Table 1.1. Natural levels of radioactivity in surface seawater (Clark, 1997).

Radionuclide	***Concentration (Bq / L)***
Potassium-40	11.84
Tritium (^{3}H)	0.022-0.11
Rubidium-87	1.07
Uranium-234	0.05
Uranium-238	0.04
Carbon-14	0.007
Radium-228	$(0.0037\text{-}0.37) \times 10^{-2}$
Lead-210	$(0.037\text{-}0.25) \times 10^{-2}$
Uranium-235	0.18×10^{-2}
Radium-226	$(0.15\text{-}0.17) \times 10^{-2}$
Polonium-210	$(0.022\text{-}0.15) \times 10^{-2}$
Radon-222	0.07×10^{-2}
Thorium-228	$(0.007\text{-}0.11) \times 10^{-3}$
Thorium-230	$(0.022\text{-}0.05) \times 10^{-4}$
Thorium-232	$(0.004\text{-}0.29) \times 10^{-4}$

1.3 Anthropogenic radioactivity

At the beginning of the 20th century, mankind's ability to create artificial radioactive sources was limited to chemical isolation and concentration of natural radionuclides. The development of linear accelerators was the next progression of the use of nuclear power, these producing beams of particles that could also be used to artificially

transmute nuclei (AMAP, 1998). However, the greatest change in the nuclear industry was the application of nuclear fission. In the 1940s, nuclear fission allowed humans the ability to produce large quantities of artificial radionuclides that were then used for both peaceful and military purposes. The fission process itself, and the high neutron flux densities achieved in nuclear weapons explosions and fission reactor cores, led to the production of large quantities of fission and activation products. In most situations the most radiologically important fission products, in the short term, are ^{89}Sr, ^{90}Sr, ^{131}I and ^{137}Cs, although only ^{90}Sr and ^{137}Cs are important in the long term due to their yields, half-lives and chemical properties. Typical activation products include ^{51}Cr, ^{54}Mn, ^{55}Fe, ^{60}Co, ^{63}Ni, ^{64}Cu, ^{65}Zn, ^{69}Zn, ^{110}Ag, ^{109}Cd, ^{134}Cs, ^{236}U and ^{239}U (AMAP, 1998). However, Figure 1.2 shows that it is in fact natural sources of radioactivity that account for the largest annual doses received by the human population and not as we might believe anthropogenic sources (Valković, 2000).

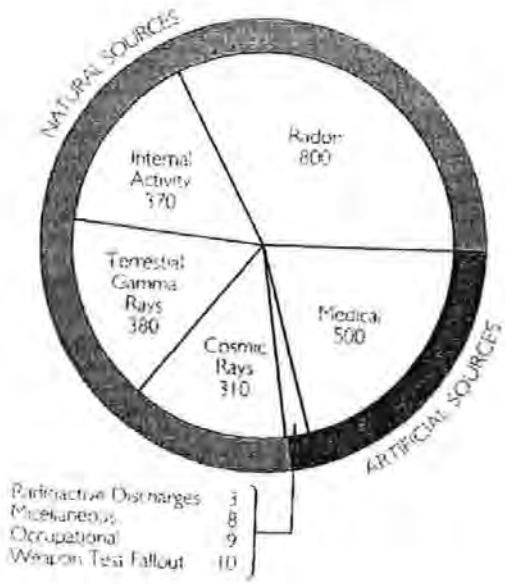


Figure 1.2. Natural and artificial sources of radiation to which the human population is exposed in the UK. Average annual dose in μSv (Clark, 1997).

1.3.1 Nuclear warfare and testing

Nuclear weapons explosions have provided the largest inventory of both fission and activation products in the global environment, and many of these have been, and remain, detectable world-wide (AMAP, 1998). It has been estimated that there have been 905 nuclear explosions with a cumulative energy yield of some 366 megatons of Trinitrotoluene (TNT) equivalent in the period between the testing of the first nuclear weapon on the 16th of July 1945 and the 30th of June 1978 (Woodhead, 1984).

Nuclear devices are basically of two types, fission (the "atomic" bomb) and fusion (the thermonuclear or "hydrogen" bomb). Uranium-235 and plutonium-239 are the essential ores in the production of fission reactions and will produce over 100 radioisotopes with varying half-lives ranging from fractions of a second to millions of years. Fusion of light elements (isotopes of hydrogen) produces smaller quantities of radioisotopes but it does require a fission device to trigger the fusion reaction. Small nuclear explosions are usually produced by fission while larger explosions generally involve both fission and fusion (Valković, 2000).

The fallout pattern from an explosion depends on the yield of the device, the time of year of the explosion, the latitude of the site and whether or not the fireball intersects the surface of the earth. The limited test ban was proceeded by a period of intensive testing during 1961 and 1962, this led to a maximum annual deposit in the northern hemisphere during 1963. A substantial fraction of the atmosphere tests have and are still being carried out on small islands or coral atolls, therefore local and intermediate fallout enters the marine environment directly (Simon, 1997).

1.3.2 Nuclear power industry

Any human activity utilising radionuclides or their associated radiations is likely to generate some form of active waste. Many factors, including the site of production, the physical and chemical nature of the waste, the spectrum of radionuclides involved and the

waste management strategies adopted, will determine whether there will be any consequential contamination of the marine environment. Of the many possible sources of waste, the nuclear fuel cycle (ore mining and uranium extraction; uranium enrichment and fuel fabrication; reactor operation; spent fuel reprocessing) is by far the most significant in terms of both the total activity involved and its concentration at the various stages (Woodhead, 1984). The major sources of contamination of the marine environment are reactor operation and fuel reprocessing at coastal sites, which typically use a mixture of plutonium and uranium oxides. Another source of radiation pollution is the disposal of packaged radioactive wastes from a variety of sources into the deep ocean. Figure 1.3., indicates the UK nuclear establishments giving rise to the principal discharges of liquid radioactive waste (MAFF, 1995).

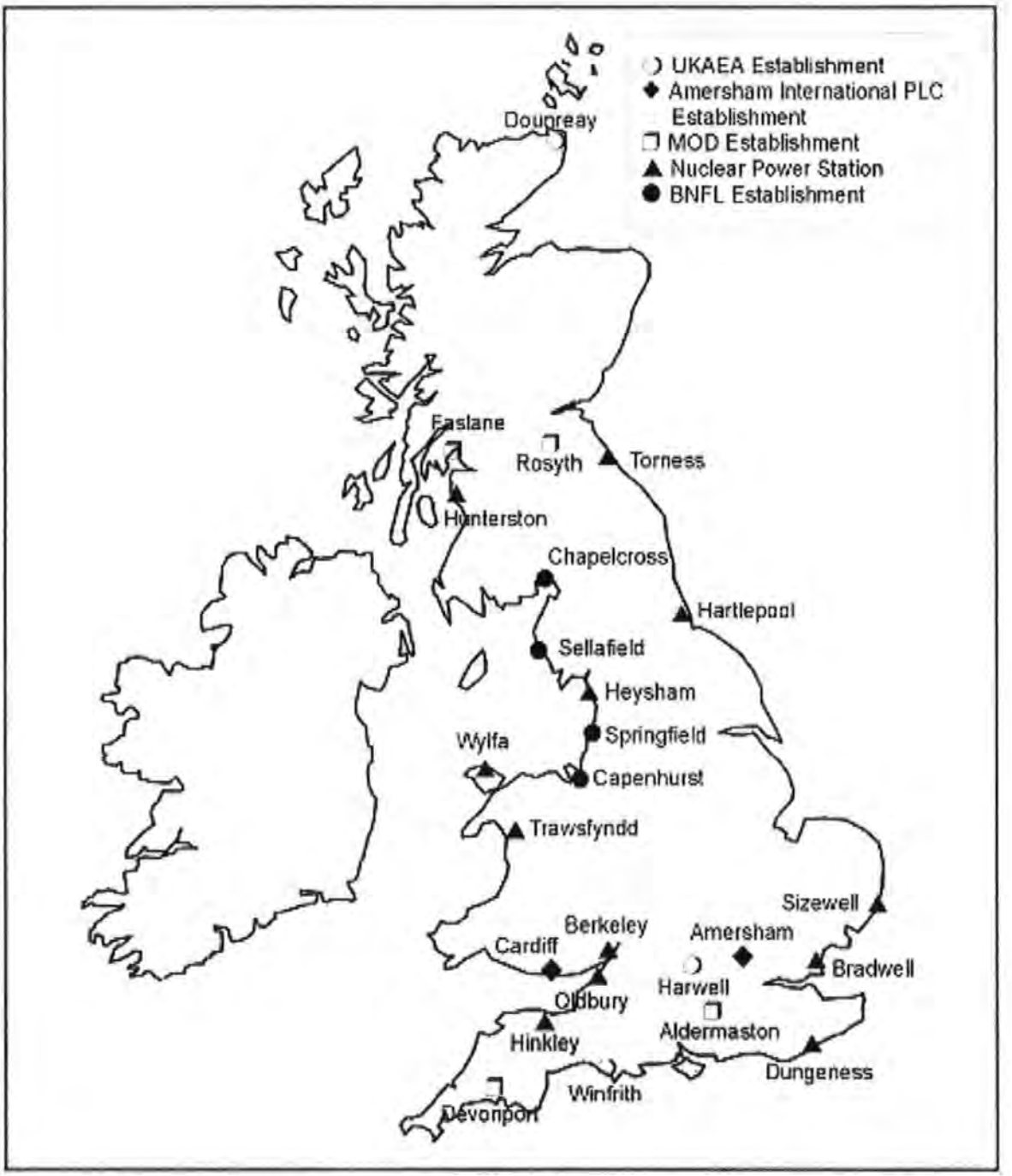


Figure 1.3. UK nuclear establishments giving rise to principal discharges of liquid radioactive waste. (Adapted from MAFF, 1995). (BNFL = British Nuclear Fuels Plc; UKAEA = United Kingdom Atomic Energy Authority; MOD = Ministry of Defence).

1.3.3 Nuclear accidents

In addition to discharges of radionuclides in the environment as a result of various human activities, several undesirable accidents have taken place in different parts of the world causing great concern for human and environmental health.

At Three Mile Island near Harrisburg, Pennsylvania, a partial meltdown occurred in 1979, but due to the fact that no fire or explosion took place most of the radioactive contamination was confined to the reactor containment building. However, a considerable proportion of the radiation (^{131}iodine) did escape into the environment and unofficial surveys have indicated an elevation in the incidence of leukemia and other cancers, (thyroid) within a 20mile radius of the plant. The cleanup took nearly 10 years and the cost exceeded $1 billion (Zakrzewski, 1997).

On April 26, 1986, a complete meltdown of the unit 4 reactor (a RBMK-1000 graphite moderate light-water reactor) in the Chernobyl nuclear power complex took place. The accident destroyed the reactor core and part of the building in which the core was housed. The Chernobyl accident involved the largest short-term release from a single source of radioactive materials to the atmosphere ever recorded. Of the materials released from the reactor core, four elements have dominated the short-term and long-term radiological situation in the affected areas of the USSR. These are iodine (primarily ^{131}I), caesium (^{134}Cs, ^{137}Cs), strontium (primarily ^{90}Sr) and plutonium (^{239}Pu, ^{240}Pu). In addition, highly radioactive fuel fragments (hot particles) were released. The destroyed reactor released a very large amount (10^{19} becquerels) of radioactive material into the environment (Valković, 2000). Fire and explosions accompanied the meltdown, and the result was deemed a major catastrophe. 135,000 people were evacuated from the surrounding area, 31 people died, 1000 suffered immediate injuries and the projected increase in deaths from cancer was as high as 100,000. According to Soviet estimates, the amount of debris released into the atmosphere amounted to 7000 kg containing 50-100 million curies. The

degree of fallout depended on the wind direction and was as widespread as the Arctic Circle to the North, Greece to the South and Great Britain to the West (Zakrzewski, 1997).

There have been several cases of radioactive contamination of the environment during the period of operation of nuclear powered ships. In 1961, a submarine with a damaged reactor returned to its base on the Kola Peninsula and local contamination occurred. In 1965, a local release of radioactive material was reported during an accident with a submarine reactor in the Severodvinsk shipyard (AMAP, 1998). A large release of liquid radioactive waste (74TBq) occurred in 1989 from a Northern Fleet submarine. A fire broke out in the stern section of the Komsomolets nuclear submarine causing the submarine to sink of the Norwegian coast. The wreck contained one nuclear reactor and two nuclear warheads, one of which was fractured. The radionuclide inventory included 1.5PBq ^{90}Sr, 2PBq ^{137}Cs, about 16TBq ^{239}Pu in the two warheads and 5TBq of actinides in the reactor core (Valković, 2000). An area of about 1.0km^2 was believed to be contaminated by radioactive waste. During accidents involving submarine nuclear reactors, personnel have been affected by gamma radiation that can result in high doses causing development of acute radiation sickness. In addition beta radiation from radioactive gases entering the hull of the submarine during major accidents can cause acute radiation damage to skin (AMAP, 1998).

1.3.4 Medical and other uses of radiation

One of the best uses of radiation occurs in the medical industry, such as the use of radiography and radiotherapy. Radiotherapy is the treatment of disorders by means of ionising radiation. Ionising radiation provides an alternative to surgery by preventing the cancer cells from proliferating indefinitely so that the malignant uncontrolled growth is stopped. The use of X-rays for diagnosis of disorders is now standard procedure in most hospitals due to use of radiation. Any medical exposure of radiation should be justified by weighing the diagnostic or therapeutic benefits they produce against the radiation

detriment they might cause, taking into account the benefits and risks of available alternative techniques that do not involve medical exposure (Valković, 2000).

Gamma radiation from cobalt-60 or caesium-137 has been used in sterilisation plants to sterilise plastics, syringes, needles etc. Radiation has also been used as a pesticide as well as for pasteurising food, disinfecting against food poisoning agents such as *Salmonella* and disinfecting meat against tapeworms (Thornburn, 1972). Radiation, in particular radium and tritium, have also been used to produce luminous clocks and watches.

1.3.5 Radiation protection limits for humans

The International Commission on Radiological Protection (ICRP) was established by the second International Congress of Radiology (ICR) in 1928, and since its inception the ICRP has been the one internationally recognised body responsible for recommending values of dose limits for exposure to ionising radiation (Martin and Harbison, 1986). In its Publication 26, ICRP distinguishes between two types of effects that may be induced by ionising radiation, stochastic effects and non-stochastic effects. Stochastic effects are those for which the probability of an effect occurring is regarded as a function of dose, without threshold e.g. the induction of cancers and genetic effects. Non-stochastic effects are effects for which the severity if the effect varies with the dose and for which a threshold may therefore exist e.g. cataracts, impairments of fertility. The aim of radiation protection as stated by the ICRP is to prevent detrimental non-stochastic effects and to limit the probability of stochastic effects to levels deemed to be acceptable (Martin and Harbison, 1986). To achieve this the ICRP set a dose equivalent limit of 0.5 Sv (0.15 Sv lower limit) in a year for all tissues to prevent non-stochastic effects and to limit stochastic effects the annual dose equivalent limit for uniform irradiation of the whole body is set at 50 mSv. The limit for members of the general public as set by the ICRP in March 1985, is an annual effective dose equivalent of 1 mSv (Martin and Harbison, 1986).

1.4 Effects of ionising radiation on biological systems

Figure 1.4. demonstrates the penetrating power of ionising radiation in tissue. γ and *n* radiation penetrates easily, β-particles penetrate a few centimetres at maximum and α-particles do not penetrate the dead layer of the skin.

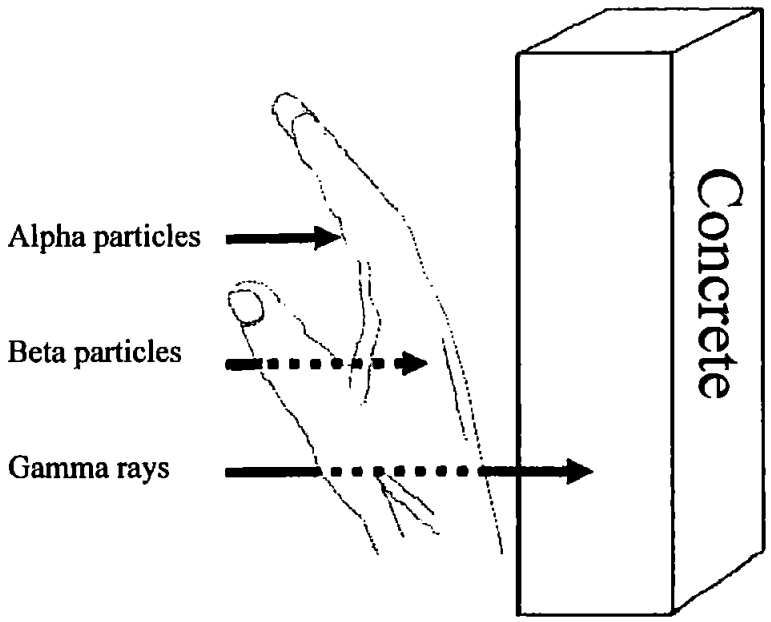


Figure 1.4. Penetrating powers of ionising radiation in tissue (Valkovic, 2000).

Radiation may damage biologically important molecules in two ways, directly or indirectly. Direct action, as the name implies, is the alteration of a biological molecule through deposition of energy in it, as the result of a primary interaction with the radiation. Indirect action takes place when the primary action of the radiation is with water. The biological molecule being attacked by the highly reactive products then radiolysis, giving rise to free radicals such as hydroxyl compounds (Coggle, 1983). In general the processes leading to radiation damage within cells can be considered to take place in four stages (Martin and Harbison, 1986). The initial physical stage last only a fraction of a second and is when energy is deposited in the cell causing ionisation. In water the process may be written as:

$$H_20 \xrightarrow{\text{radiation}} H_2O^+ + e^-$$

The physico-chemical stage is when the ions interact with other water molecules resulting in a number of new products. For example, the positive ion dissociates:

$$H_2O^+ \rightarrow H^+ + OH$$

The negative ion, the electron, attaches to a neutral water molecule that then dissociates:

$$H_2O + e^- \rightarrow H_2O^-$$

$$H_2O^- \rightarrow H + OH^-$$

Thus the products of the reactions are H^+, OH^-, H and OH. The two former ions that are present to quite a large extent in ordinary water, take no part in subsequent reactions. The other two products, H and OH, are called free radicals, that is they have an unpaired electron and are chemically highly reactive. Another product is hydrogen peroxide, H_2O_2, which is a strong oxidising agent and is formed by the reaction:

$$OH + OH \rightarrow H_2O_2$$

The third stage is the chemical stage in which the reaction products interact with the important organic molecules of the cell. The fourth stage is the biological stage where the chemical changes can affect an individual cell in a number of ways. For example they may result in, the early death of a cell, the prevention or delay of cell division or a permanent modification which can be passed on to daughter cells (Martin and Harbison, 1986).

The direct and indirect effect of radiation on biological molecules results in a wide range of biological effects at various levels of biological organisation. Table 1.2., shows some of the types of damage that may arise in mammalian systems due to the interaction with ionising radiation (Coggle, 1983).

Table 1.2 Possible types of radiobiological damage in mammalian systems (Coggle, 1983).

Level of biological organisation	Important radiation effects
Molecular	Damage to macromolecules such as proteins (including enzymes), carbohydrates, lipids and nucleic acids (RNA and DNA), and interference with the metabolic pathways
Subcellular	Damage to cell membranes, nucleus, chromosomes, cell organelles e.g. mitochondria and lysosomes
Cellular	Inhibition of cell division; cell death; transformation to a malignant state
Tissue; Organ	Disruption of such systems as the central nervous system, the bone marrow and intestinal tract may lead to the death of animals; induction of cancer
Whole animal	Death; "radiation lifeshortening"
Populations of animals	Changes in genetic characteristics due to gene and chromosomal mutations in individual members of the species; changes in population structure (gene and enzyme frequencies)

1.4.1 The effects of radiation at molecular levels

The first evidence to show that ionising radiation could have an effect on biological systems was provided by H.J.Muller in 1927. Through his breeding experiments using the fruit fly, *Drosophila melanogaster*, he demonstrated that x-rays could induce changes in genetic material. In the following decades it was established that radiation could affect proteins, including enzymes, and other molecules, such as carbohydrates, lipids and nucleic acids (DNA and RNA), causing physio-chemical and biochemical effects. Physio-chemical changes are manifested as a decrease in molecular weight due to fragmentation of polypeptide chains, changes in solubility, disorders of the secondary and tertiary structure, cross linkage and the formation of aggregates, as well as the destruction of amino acids in the chain. Biochemical damage caused by radiation will result in the enzyme losing its ability to carry out its function (Coggle, 1983).

DNA is now considered to be the primary target for the action of ionising radiations (UNSCEAR, 1986). The major types of damage that radiation can cause to nucleic acids are single strand breaks (ssb), double strand breaks (dsb) and base damage (bd). The number of single strand breaks produced is linearly related to the dose of radiation, however, the repair of single strand breaks is usually rapid and efficient, probably due to excision repair mechanisms (Coggle, 1983). A significant proportion of single strand breaks that occur as a result of exposure to radiation are induced indirectly via radical formation at deoxyribose following the loss of a hydrogen atom which makes the DNA backbone unstable. Damage to the DNA bases such as ring saturation can also result in destabilisation of the glycosidic bond (linking bases to the sugar molecule). In comparison, double strand breaks can be formed by a single ionising event or by the coincidence of random single strand breaks on complementary strands. As with the single strand breaks, double strand breaks of DNA can be repaired but there is more chance of a misrepair or error prone repair to occur, giving rise to a mutation. Furthermore, the induction of dsb has been directly related to the production of chromosomal aberrations and cell death

(Fairbairn *et al.*, 1995). Radiation is also believed to produce DNA base damage at similar amounts as single strand breaks (Banáth *et al.*, 1999).

While a large number of studies have been carried out in mammalian systems, both under *in vivo* and *in vitro* conditions to evaluate or determine the effects of different qualities of radiation, there is limited information available in the literature pertaining to effects of ionising radiations in the natural biota (non mammalian species such as fish, invertebrates and plants) (Abbott and Mix, 1979; Evans *et al.*, 1959; Harrison and Anderson, 1994; Ilyinskikh *et al.*, 1998; Knowles and Greenwood, 1994; Strand *et al.*, 1977). Sugg *et al.* (1996), detected an increase in the frequency of DNA strand breaks in catfish obtained from the cooling ponds at Chernobyl. The increase in genetic damage was directly related to the concentration of radiocesium in the fish. Meyers-Schöne *et al.* (1993), also detected an increase in the number of strand breaks in freshwater turtle species that inhabited a lake that received both radionuclide and chemical inputs. No correlation could be made to link the DNA damage and one specific genotoxin, although there was significantly higher concentrations of radionuclides and mercury (Hg) in the turtles from the contaminated site. Theodorakis, *et al.* (1997), have investigated the DNA integrity (number and type of strand breaks in liver and blood cells) in mosquito fish exposed to radionuclides from two radionuclide-contaminated sites in the US Department of Energy's Oak Ridge Reservation. Fish from contaminated sites were reported to have more structural DNA damage than fish from clean sites. They also concluded that the analysis of double strand breaks were a better discriminator for contamination than single strand breaks using gel electrophoresis. It is generally expected that double stranded DNA breaks, rather than other types of DNA damage (i.e. single-strand breaks, base deletions, etc.) are the most important immediate lesion caused by ionising radiation (Hahnfeldt *et al.*, 1992).

1.4.2 The effects of radiation at subcellular levels

Ionising radiation is delivered to biological material in discrete energy deposition events. If such an energy deposition occurs within the genetic material of the cell this can result in the physical breakage and /or the alteration of the DNA molecule. Since the DNA molecules are packaged in chromosomes, disruption of DNA can lead to chromosome breaks and alterations unless they are repaired (Geard, 1982). The effects of ionising radiation on chromosomes can be analysed using a light microscope and the type of damage reflects at what stage of the cell cycle the damage occurred (See Figure 1.5.). The cell cycle is a set of biochemical events that is responsible for the duplication of a cell. The cell cycle is traditionally divided into two phases, interphase and mitosis (also called M-phase) and each of these phases are made up of several parts. Interphase consists of three parts, the G_1 phase, the S phase, and the G_2 phase. G_1 is an active period, when the cell synthesises its vast array of proteins, including enzymes and structural proteins needed for growth. In G_1 each chromosome consists of a single molecule of DNA and its proteins. After G_1 follows the S phase where all the DNA within the nucleus is replicated, resulting in two molecules of DNA. G_2 phase is a preparationary period preceding mitosis, during this phase the proteins of the spindle are organised. Mitosis is the process of cell division; it is a continuous process divided into prophase, metaphase, anaphase, telophase and cytokinesis.

As well as damage to chromosomes ionising radiation has also been shown to cause changes in other subcellular organelles such as the stimulation mitochondria activities and an increase in the production of Bax which is involved in the induction of apoptosis of damaged cells (Gong *et al.*, 1998).

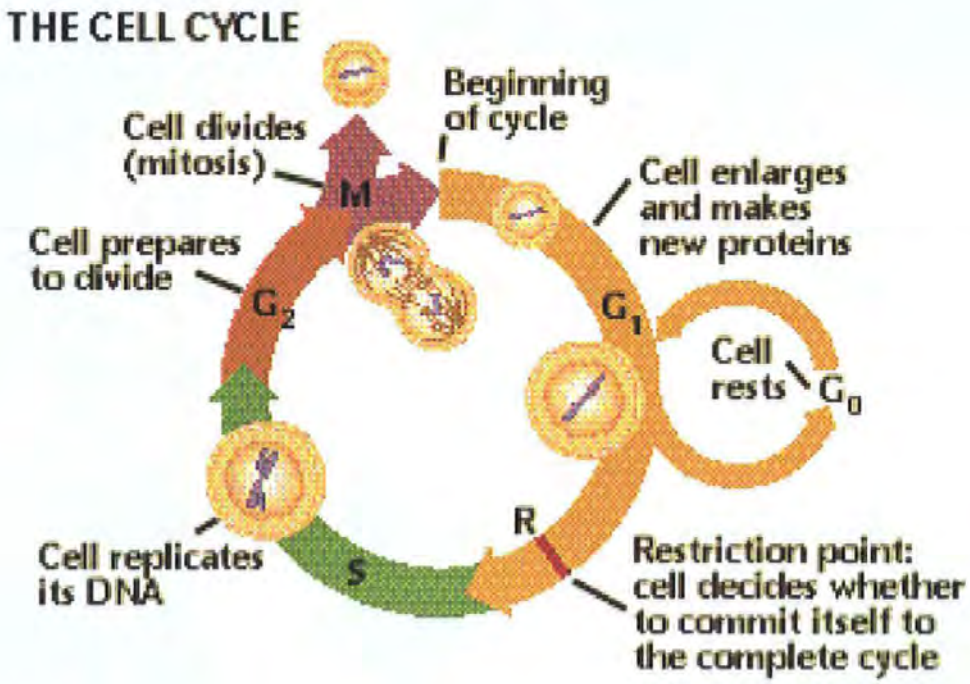


Figure 1.5. Cell cycle. Picture credited to Dimitry Schidlovsky.

http://www.sciam.com/0996issue/0996weinbergdiagram3.html

If the DNA is disrupted prior to its replication (i.e. in the G_1 phase of the cell cycle) both strands of DNA can be replicated and then appear at mitosis as chromosomal breaks, dicentrics or as translocations. After chromosomes have replicated (irradiation occurred in S and G_2 cells) the possibilities for chromosomal alterations are multiplied and changes can now be seen at the chromatid and chromosome level. The dose delivered by ionising radiation is received in cells as individual energy deposition events and the rate of energy deposition per unit distance traversed, the LET (linear energy transfer) is a common parameter in radiation studies. Different sources of ionising radiation can result in the production in cells of low LET (less than 10 keV/μm energy deposition events) or high LET (greater than 40 keV/μm). The dose response for chromosomal aberration induction is highly dependent on LET, however for low LET radiations a curvilinear response is typically found. This is when there is a greater than linear increase in aberrations as the dose increment increases.

Ultimately, cells containing aberrations will not be allowed to proceed through the cell cycle, will not genetically divide and will therefore be eliminated. Therefore, chromosomal aberrations are of great significance, to organisms, resulting in reduced potential of survival and reproduction (Chandley, 1981). In addition it is now well established that induction of chromosomal aberrations play an important role in the initiation and promotion of cancer, one of the major causes of human deaths in western countries. The types and frequencies of induced chromosomal aberrations depends on the mutagen used and the stage of the cell cycle treated. Ionising radiation and chemicals which directly produce DNA strand breaks induce chromatid aberrations in the G_2 stage of the cell cycle and chromosome aberrations in the G_1 stage (Natarajan *et al.*, 1994). The formation of chromosomal aberrations due to radiation has long been known, and the dose response/aberration induction scenario has been extensively reviewed. This has led to chromosomal aberrations being used as reliable parameter for biological dosimetry, to estimate absorbed radiation doses in humans that have been suspected or accidentally exposed to radiation (Bender and Gooch, 1966). In cases of radiation accidents, where no physical dosimetry is feasible, chromosomal aberrations (especially the frequencies of dicentrics and rings) have been used as a biological dosimeter (Natarajan *et al.*, 1994). Biological dosimetry is a diagnostic methodology for the measurement of the individual dose absorbed in the case of accidental overexposition to ionising radiation. Such information gives a valid contribution to understanding of the action of ionising radiation or pharmaceuticals on cells and, in return, can be of value to human radioprotection and chemoprotection. Radiation dose-response curves are of fundamental importance both in practical radiotherapy and as the basis of more theoretical considerations concerning the potential benefit to be gained from modified dose-fractionation schedules or of the effects of dosimetric and biological variability. The steepness of the dose-response curve is a key parameter and quantitative measures of steepness derived from clinical data are strongly needed (Bentzen and Tucker, 1997).

A variety of radionuclides and cells have been used to construct dose response curves for biological dosimetry. For example, a reference dose-response curves for chromosomal aberrations was constructed by Jha and Sharma (1992) for peripheral blood lymphocytes after exposed to ^{60}Co γ-rays and 100keV X-rays and Moquet, *et al.* (2001) produced a dose response curve in human lymphocytes *in vitro* in order to compare complex rearrangements produced by high LET radiation (plutonium-239 alpha particles). Construction of these *in vitro* dose response curves are considered to be important for the estimation of absorbed radiation doses in accidental and occupational exposures (IAEA, 1986).

1.4.3 Cellular effects of radiation

Viruses, bacteria, plant and animal cells can be killed by ionising radiation, and in general, as the dose of radiation increases so does the proportion of cells being killed (Coggle, 1983). Wang *et al.* (1999a) reported that the occurrence of radiation-induced cell death, in the limb buds of embryonic mice, was due to apoptosis (programmed cell death) and in a further study also concluded that radiation induced apoptosis in the brain cells of embryonic mice (Wang *et al.*, 1999b). Furthermore, more apoptotic cells were found in the brains of mice exposed prenatally to tritium than to X-rays. However a radiation killed cell may not exhibit any signs of damage until it attempts to divide and as such may not die for weeks or months after exposure to radiation. Therefore cell death due to radiation can be defined as the loss of proliferative ability. It has been shown in eukaryotes that ionising radiation can cause a temporary decrease in the rate of DNA synthesis per cell and that this decrease is due to the cycle being halted at S-phase known as mitotic delay (Rowley *et al.*, 1999). Yeast, especially *Saccharomyces cerevisiae,* have been used to study the cellular effects of ionising radiation especially in relation to cell cycle control (Bennett *et al.*, 2001; Dolling *et al.*, 2000; Jaruga *et al.*, 1995).

1.4.4 The effects of radiation at the tissue level

Early studies on rat testes lead to the discovery that the dividing cells were markedly affected by radiation whilst the non-dividing cells appeared undamaged. This led to the theory of tissue radiosensitivity (Bergonie and Tribondeau, 1906). It was suggested that the radiosensitivity of a tissue is directly proportional to its mitotic activity and inversely proportional to the degree of differentiation of its cells. This generally meant that actively dividing tissues were "radiosensitive" and non-dividing tissues were "radioresistant". Therefore, in mammals, the liver, kidneys, muscles, brain, bones, cartilage and connective tissue are often classified as radioresistant tissues since all these tissues in adults exhibit little or no active cell division and are composed of mature, specialised cells. In contrast, the cells of the bone marrow, the germinal cells of the ovary and testis, the epithelium of the intestine and the skin are all considered to be radiosensitive tissues (Coggle, 1983). It is also important to point out that early embryonic stages of development are going to be extremely radiosensitive due to the high turnover of proliferating cells and high mitotic activity. Irradiation (X-ray, neutron, cobalt gamma) induces a series of events in the gastrointestinal tract such as GI haemorrhage, endotoxemia, bacterial infection, anorexia, nausea, vomiting, diarrhoea, and loss of electrolytes and fluid. These symptoms are probably due to a rapid modification of the intestinal motility and to the structural alteration of the intestinal mucosa (cell loss and altered crypt integrity) (Somosy *et al.*, 2002).

Strand *et al.* (1973) demonstrated that the immune system of rainbow trout was significantly effected by exposure to tritiated water resulting in increased susceptibility to infection by *Chrondrococcus columnaris* disease. The developing central nervous system (CNS) is know to be particularly vulnerable to teratogenic insults throughout the embryonic and fetal developmental periods, the human central CNS exhibited the greatest sensitivity in early stages of prenatal development. Gao *et al.* (1999) showed that rats exposed to chronic β radiation from $^{3}H_2O$ had reduced postnatal learning ability and

memory behaviour and a decrease in the development of the brain. Furthermore, the effects of the chronic exposure to tritiated water were greater than that from the same dose of acute X and γ rays. After the atomic explosions in Hiroshima and Nagasaki, many cases of microcephalus and mental retardation were observed in newborn children, it was believed that the radiation had impeded the growth of the CNS.

1.4.5 The effects of radiation at individual level

Organismal responses that influence population structure and dynamics (such as survival, growth, and reproduction) are useful ecological parameters as they are often affected by changes in molecular physiology (Theodorakis, *et al.*, 1997). Puffer fish (*Fugu niphobles*) reared in tritiated water experienced a decrease in egg hatchability (Ichikawa and Suyama, 1974). Those larvae that did hatch were rather inactive and tended to lie on the bottom of the tank in comparison to controls that actively swam. Radiation exposed larvae were morphologically stunted in size and had swollen abdomens. Female gammarids (*Gammarus duebeni*) also exhibited a reduction in the egg production rate and the total number of eggs laid after exposure to sub-lethal doses of x-rays (Hoppenheit, 1973). The observed number of malformed embryos of the mosquitofish (*Gambusia affinis*) was elevated in fish from sites contaminated with radionuclides in comparison to control sites (Theodorakis *et al.*, 1997). Anderson *et al.* (1990) found that effects on reproduction and genetic material was observed at low doses cesium-137 in the polychaete worm *Neanthes arenaceodentata*, whereas only high doses resulted in acute mortality and decreased life span. They concluded that cellular effects of ionising radiation were markedly more sensitive endpoints than whole animal bioassays. Knowles and Greenwood, (1994) also found that reproductive performance was influenced by gamma radiation although no effect on growth rate of the polychaete, *Ophryotrocha diadema*, was observed at similar dose rates.

1.5 Effects of ionising radiation on ecosystems

The procedures for the protection of humans from ionising radiation are well developed, with a system in place to limit the effects of individuals based on recommendations from the International Commission on Radiological Protection (ICRP). At present, an internationally accepted method for assessing the environmental impact of ionising radiation does not exist and up to now the approach taken has relied on recommendations from the ICRP first made in 1977, and modified in 1990 (Copplestone *et al.*, 2001). The ICRP states that the standard of environmental control needed to protect humans will ensure that other species will not be put at risk (ICRP, 1991). However, this statement can be challenged due to the lack of cited evidence from the ICRP to support it, because it fails to protect environments where no humans inhabit (e.g. oceans) and biota in certain habitats might be exposed to more harmful doses below the recommended human exposure limits (Copplestone *et al.*, 2001). Furthermore, the environment is a complex interaction of fauna and flora and the interaction of radiation with this environment may present changes in the rates and ratios of uptake and exposure of radionuclides to various organisms. Figure 1.6. represents a summary of the transfer pathways of radionuclides in ecosystem components and emphasises the complexity of potential uptake routes of radionuclides such as the chemical form of the radionuclide , sorption and leaching in soils, and root uptake by plants. Essentially radionuclides behave chemically in the same way as their non-radioactive naturally occurring isotopes, but the possibility of bioaccumulation and biomagnification in food chains has greater significance if the substance accumulated is radioactive.

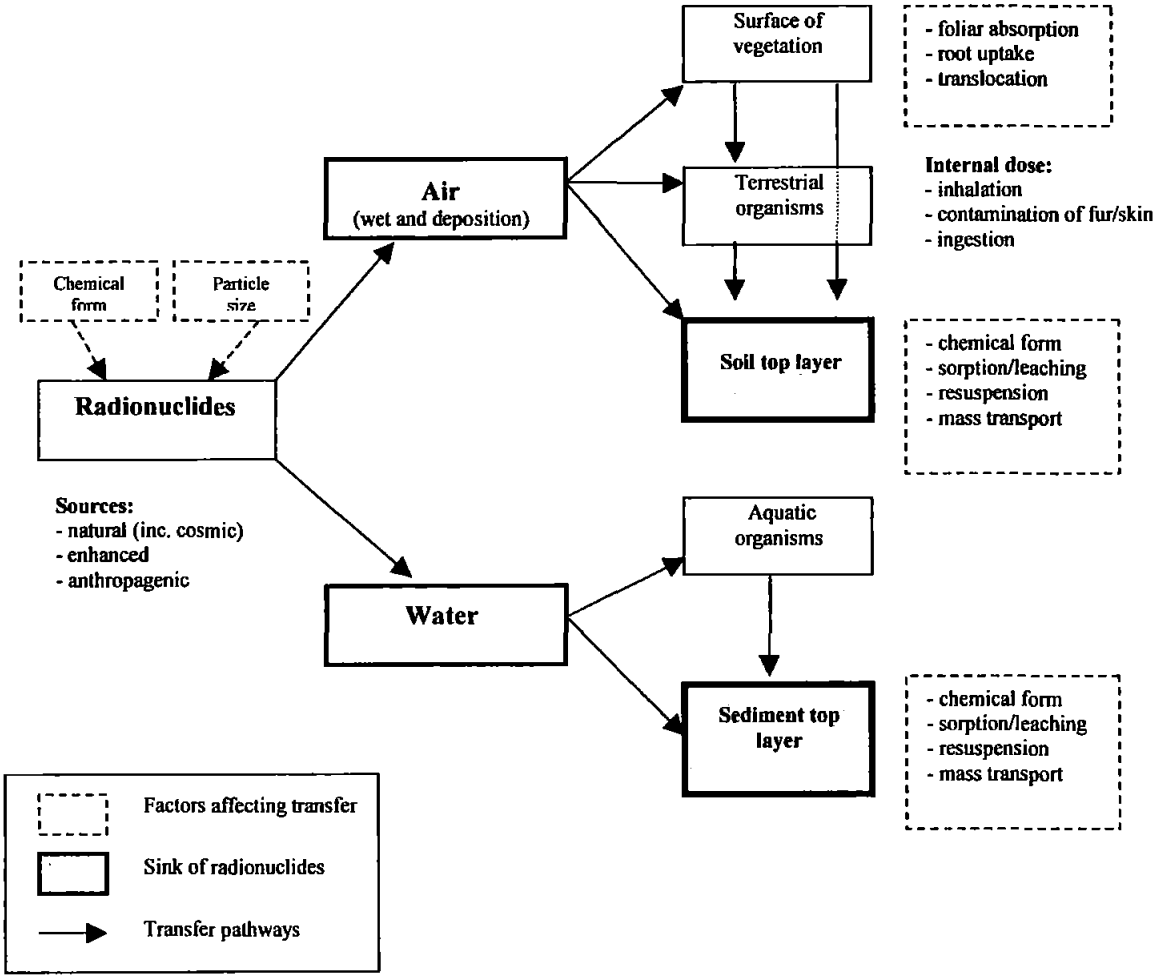


Figure 1.6. Summary of the transfer pathways of radionuclides in different ecosystem components and potential factors that may influence their distribution (Copplestone *et al.*, 2001).

1.5.1 Terrestrial ecosystem

In terrestrial ecosystems, soil characteristics such as organic matter content, clay mineral content, drainage, nutrient status and pH can have a significant effect on the mobility of radionuclides in natural ecosystems (Kennedy *et al.*, 1990). Plants obtain nutrients and radionuclide contamination from the soil and therefore the rate of uptake of radionuclides from the soil is determined by the rate at which the plants' roots absorb different elements or compounds and the activity concentrations of radionuclides in the soil solution. Many radionuclides are either taken up by plant roots, at very low rates, or form

strong bonds with various soil constituents. Therefore, the rate of plant uptake of many radionuclides is low compared with nutrient ions. The main exceptions are radiostrontium and radiocaesium. Radiocaesium also has a further exposure route from the soil due to uptake by fungal hyphae (AMAP, 1998).

Radionuclides released from the Chernobyl accident have been shown to express late effects in plants although the mechanisms leading to these effects vary greatly according to the tissue or systems of organs under investigation. Furthermore the mechanisms themselves seem to differ in complexity ranging from damage to the chromatic structure, to distortion of functioning of the complicated regulating systems of organism (Grodzinsky, 1995). The sensitivity of plants to radiation damage can vary within a 1000-fold range. The pattern of radiation damage to a forest exposed to γ-rays for 6 months showed that at less than 2 Rads per day there was no effect but as the dose increased the pines were destroyed followed by the oaks, then the evergreen shrubs (heath) and finally the sedges. In addition, a negative correlation was found between the size of the chromosomes and the radio-sensitivity of the plants. The plants that had the larger chromosomes showed the greatest damage in comparison to plants with smaller chromosomes that were more radio-resistant. (Woodwell, 1967). Radionuclides present in coniferous woodlands reside for longer (3-5 years) than deciduous woodlands (6 months) due to the decomposition of a thicker mat of leaf litter (Schell *et al.*, 1996). Combined alpha, beta and gamma irradiation was found to produce higher levels of genotoxicity in barley pollen than similar doses from an external source of gamma radiation, furthermore, a dose of 50-200 mSv was needed to double the mutation rate of the barley pollen (Bubryak *et al.*, 1992).

Invertebrates have often been used to study the effects of radionuclides under experimental conditions although accumulation is generally low, except for invertebrates that are involved in decomposition processes (Copplestone *et al.*, 2001). A study carried out in the former Soviet Union found that the earthworm *Lumbricus terrestris* was the most

sensitive group of soil mesofauna after soil irradiation. A parallel decline in the centipede population was also detected and this factor was attributed to the decline in the earthworm prey population rather than direct effects of radiation exposure (Krivolutski *et al.*, 1980). In a long-term field experiment with plutonium-239 in chernozem soils an obvious decrease in soil macrofaunal populations was apparent after three years, the population density of earthworms and insect larvae had halved and that of microarthropods had decreased by a factor of 5.5 (Krivolutsky, *et al.*, 1992). Wood lice have also been shown to particularly accumulate actinides (typical concentration factors of 0.3) (Copplestone *et al.*, 1999). The southern green stink bug, *Nezara viridula* (L.) was rendered partially sterile after being exposed to ionising radiation of <10 Gy, females laid nonviable eggs in high proportions and had significantly lower fecundity than controls (Dyby and Sailer, 1999). Male pupae of the tobacco hornworm, *Manduca sexta* (L.) experienced an induction in mortality and malformations in the emerging adult insects after exposure to gamma radiation from an external source (Seth and Reynolds, 1993). Ionising radiation has also been used as quarantine treatments against tephritids (fruit flies) (Hallman and Worley, 1999; Hallman and Thomas, 1999). Spiders inhabiting salt marshes were found to accumulate high levels of ^{137}Cs from the soft tissues of their prey, although the actinides, $^{239}+^{240}Pu$ and ^{241}Am were not transferred to the spiders in this way (Copplestone 2001).

Reindeer, *Rangifer tarandus*, in the Arctic obtain substantially high concentrations of radiocaesium during the winter due to consumption of radioactive lichens. Other game such as roe deer, *Capreolus capreolus*, and moose, *Alces alces*, also experience an increase in radiocaesium contamination due to consumption of highly contaminated fungi. Approximately 80% of the radiocaesium taken in by the wildlife is absorbed through the gut wall and then is distributed within the animal tissues. Radiocaesium is readily transferred to milk, meat and offal, all of which is then eaten by humans (AMAP, 1998).

1.5.2 Aquatic ecosystem

In comparison to land, radioactivity in water is quickly diluted and translocated. Translocation takes place through bulk water movement (currents), by sedimentation of particulate matter, and in association with living organisms. The spread of activity is more complex in the sea than in fresh water, simply because there is a larger volume of seawater. However, distribution of radioactivity in the sea is not uniform due to some areas having little or no mixing (Thornburn, 1972).

Complex interactions of physical, chemical and biological factors act to disperse, dilute or concentrate radioactive substances in estuarine and marine environments. Strong temperatures and salinity gradients were shown to be the main forcing factor that influenced the bottom circulation of radioactive waste deposited in the Japan sea (Cetina *et al.*, 2000). The effect of sediment size, pH, temperature and conductivity on the transfer of uranium from sediment to water was studied from the Jucar River (Spain). Total uranium activity, uranium ratios, and distribution factors were found to vary with pH and changes in uranium activity was probably due to leaching and dilution which depends on pH and salinity (Rodriguez-Alvarez and Sanchez, 1999). Salinity was also shown to exhibit a strong correlation with the concentrations of tritium, uranium and fluoride in lake water of Lake Obuchinuma in Japan (Kimura, *et al.*, 1997). As radionuclides behave the same as other chemicals in the same column of the periodic table of elements, radionuclides such as ^{45}Ca, ^{90}Sr, ^{140}Ba, ^{226}Ra and ^{45}Ca behave like calcium and ^{40}K, ^{86}Rb and ^{137}Cs behave like potassium. Consequently, ^{90}Sr accumulates largely in shells, exoskeletons or bones, and ^{137}Cs collects in the soft tissue of an organism's body (Kennish, 1997).

Radionuclides accumulate in sediments of shallow seawater systems where detritus feeders facilitate the recycling of radioactive substances through biotic compartments. Aside from the uptake of radionuclides by organism from bottom sediments, radionuclides enter biotic compartments by organismal uptake directly from seawater or from other organisms via ingestion.

Algae are able to acquire large concentrations of substances from the surrounding water. *Porphyra umbilicalis*, has attracted particular attention as it is eaten in quantity by a proportion of the human population in Britain. Samples of *Porphyra* collected near the outfall of the nuclear processing plant Sellafield were found to have accumulated 10 times the concentration of caesium-137 than that found in the surrounding water, 400 times the concentration of zirconium-95 and niobium-95, 1000 times the concentration of cerium-144, and 1500 times the concentration of ruthenium-106. Other seaweeds in the area also accumulated radionuclides but in different ratios. *Entermorpha*, *Ulva* and the fucoids also accumulated large amounts of plutonium-239 (Hunt and Smith, 1999).

In grazing food webs, herbivores accumulate radioactive material largely from consumption of primary producers. Omnivores and carnivores, in turn, obtain radionuclides by consuming herbivores and other prey that contain the contaminants, as well as obtaining substances directly from seawater and sediments (Kennish, 1997). The flow of ^{137}Cs through marine systems provides an example of this. The radionuclide moves from the water compartments to sediments where it is available to detritivores and bottom feeders. Bottom-dwelling plants and phytoplankton accumulate the cesium which moves up through the food chain via herbivore consumers, primary consumers (e.g., carnivorous invertebrates and small fish) and finally secondary consumers (i.e., large fish). Cesium concentration factors for marine algae, molluscs, crustaceans, and fish typically average 10, 10, 50, and 30, respectively (Preston, 1983).

Other factors need to be considered when assessing the effect of radiation on estuarine organisms and that is the interaction of radiation with salinity and temperature. Experiments were carried out on the blue crab and it was found that radiation did interact with salinity and temperature resulting in an alteration of ionic regulation (Engel, 1973). Salinity and temperature significantly affected the ionic composition and free amino acid level of haemolymph in the crabs and radiation also interacted significantly with these factors to further alter the regulatory pattern of ionic exchange and thus osmoregulatory

control. The accumulation rate of cesium-137 in macroalgae species was influenced by temperatures between 6-16°C furthermore the bioaccumulation rate in isopods and macroalgae increased in lower salinities whereas the bioaccumulation rate in fish species was higher in brackish water (Topcuoglu, 2001). Uptake rates of cesium in the green mussel (*Perna viridis*) were shown to be inversely related to the ambient salinity, uptake increased by about two-fold when the salinity was reduced from 33 to 15‰ (Ke *et al.*, 2000). However lower temperatures significantly reduced the uptake of 11 radionuclides by the brittle star *Ophiothrix fragilis*, although the lower temperatures also had little effect on the loss rates of the same radionuclides (Hutchins *et al.*, 1996).

1.6 Environmental monitoring and the use of "sentinel" & surrogate organism

The concept of biomonitoring is generally considered to have originated during the mid-1970s (Goldberg, 1975) and by the late 1970s, was widely used, primarily because of the development of "mussel watch" programs which were funded by both federal and state agencies (Mix, 1986). Biomonitoring involves the identification of pollutant and measurement of contaminants in the tissues of indigenous species to derive information about, or establish correlations with, pollutant levels in the surrounding environment (Mix, 1986). Since 1986, the Mussel Watch Project of the National Oceanic and Atmospheric Administration (NOAA) has chemically analysed mussels and oysters collected annually from coastal sites throughout the USA. The Mussel Watch Project monitors concentrations of over 70 chemical contaminants in the tissues of bivalve molluscs (primarily mussels and oysters) and sediments. More than 350 coastal sites of the continental United States, off Hawaii, several Caribbean islands, and in the Great Lakes are regularly monitored and the Mussel Watch Project has been developed by numerous other countries such as the United Kingdom, France, Malaysia and Australia to name but a few.

Biomonitors were originally proposed in order to identify and quantify xenobiotics in the environment so that possible public health problems and effects on indigenous

organisms could be identified. Furthermore, reliable measurements of pollutants in water were technically difficult, expensive and provided little information on long term levels or effects, whereas levels of pollutant in tissues of organisms provided some information about possible exposure levels received (Mix, 1986). The use of natural biota or wild species as sentinel or surrogate species for the evaluation of genotoxic effects offers the possibility of expanding our understanding and response to environmental health concerns. Invertebrate representatives from many different phyla have been used for biomonitoring studies in both terrestrial and aquatic ecosystems. More than 90% of all the species on the plant are invertebrates and thus they play a vital role in the Earth's ecosystem (Jha, 1998). The effects of contaminants on invertebrates may affect human health due to accumulation of toxicants in the food chain although potentially more harm might be caused in an ecological context via significant alterations in the long term survival of the exposed population and therefore the natural balance of the ecosystem. However, basic criteria has been adopted to optimise the choice for the most appropriate species.

Ideal sentinel or surrogate species, (1) are sessile, as always being in the same place they show what is happening in that place, (2) inhabit environments with varying degrees of water quality, (3) provide an integrated index of pollution levels, (4) provide information relative to the bioavailability of particular pollutants, (5) are thought to metabolise organic chemicals very slowly, if at all, (6), are relatively easy to locate and sample, (7) have populations of a size sufficient to withstand continuous sampling generally available or, if not, animals from a clean site or even a hatchery, can be established at the site of interest and (8) have a cosmopolitan distribution, in particular, as it permits comparisons to be made between populations inhabiting different environments, in different geographic areas (Mix, 1986).

1.6.1 Test organisms

1.6.1.1 *Platynereis dumerilii* (Audouin & Milne-Edwards, 1833)

Polychaetes (phylum Annelida, class Polychaeta) have also been shown to have potential for compliance and marine environmental quality monitoring purposes, by regulatory and research groups (Pocklington and Wells, 1992). Polychaetes are useful biomonitors as they are readily available, they are easy to sample and they are abundant. There are over 86 families of polychaetes consisting of over 8000 different species, resulting in a wide diversity of lifestyles and body shapes. Polychaetes play a fundamental role in the food chain as prey for a wide range of commercially important fish species and for wading birds. They are numerically abundant, digestible and contain a high-energy content (Hutchinson *et al.*, 1995). Furthermore polychaetes play an important role in the flow of energy in the marine and coastal ecosystem. They have representatives in different trophic levels, including sedentary, mobile and tube-building species, and they have also been used widely as ecotoxicological test organisms. The usefulness of polychaetes in ecotoxicological studies has been reviewed by Pocklington and Wells, (1992). One of the major advantages of using polychaetes as biomonitors of pollution is their short life span, which allows the effects of contamination to be examined at population and community level as well as the typical individual level (Pocklington and Wells, 1992).

Platynereis dumerilii (Genus : Nereide) is a marine polychaete worm that belongs to the phylum Annelida. *P.dumerilii* reproduce by sexual reproduction between epitokes (figure 1.7.). Epitokes are reproductively capable, pelagic individuals who have adapted to living in bottom burrows or tubes. Epitokous individuals arise from a non-reproductive atoke either by direct transformation of the entire individual or by transformation and separation of the posterior end of the atoke (Ruppert and Barnes, 1994). As the organisms develop and mature they shed gametes into the coelom usually in the form of gametogonia or primary gametocytes and final maturation of the gametes occurs in the coelomic fluid. When the worm is mature the coelom is packed with eggs or sperm. Polychaete

reproduction is regulated by a number of factors including hormones and environmental stimulus, and of particular significance is photoperiod that has been shown to play an important role in the process of gametogenesis (Olive, 1999a). Oocyte growth may be stimulated by photoperiodic changes and this is thought to involve enhanced rates of binding of the protein vitellogenin to the oocyte oolemma membrane and subsequently enhanced rates of protein incorporation into the oocyte cytoplasm (Olive, 1999a). When conditions are optimal the epitokous polychaetes swim to the surface to shed the eggs and sperm. This synchronous behaviour is known as spawming or "nuptial dance" and optimises the congregation of large numbers of sexually mature individuals in a relatively short time and thus increases the likelihood of fertilisation. Evidence suggests that the females release a pheromone that attracts the males, which in turn release sperm that stimulates the female to release the eggs, thus increasing the possibility of fertilisation. Once the adults have released their gametes they die, and although the biological significance of semelparity in polychaetes are not fully understood it is believed to have evolved in conjunction with the adoption of epitoky (Olive, 1999a). After the gametes have been fertilised, the embryo rapidly develops into a top-shaped trochophore larva, which are pelagic and often inhabit the surface microlayers of the sea (Dorresteijn and Eich, 1991). Within 24h polychaete metamorphosis transforms the trochophore into the juvenile body form. The larvae remain planktonic for a few days until they settle out onto substrate such as rocks and seaweeds, at which stage they are actively seeking food. Following a few weeks (3-4) the worms produce tubes by producing secretions and cementing foreign material, such as food and faecal matter. Tube secretions are commonly produced by glands on the ventral surfaces of the worms segments. The tube provides protection to the worm and also allows the worm to inhabit hard, bare surfaces such as rock, shells or corals (Ruppert and Barnes, 1994).

As well as being used in field monitoring several polychaetes have been shown to be useful laboratory bioassay organisms (Harrison and Anderson, 1994; Nusetti *et al.*,

1998) with particular suitability for cytogenetic techniques (De Boeck and Kirsch-Volders, 1997; Dixon *et al.*, 1999). *P.dumerilii* has proven to be very adaptive to laboratory culture (Hauenschild and Fischer, 1969) and thus has been used extensively to study the role of sex pheromones (Hardege *et al.*, 1998; Zeeck *et al.*, 1998), egg development (Dorresteijn and Eich, 1991; Kluge *et al.*, 1995), eye development (Rhode, 1992), chromosomes (Jha *et al.*, 1995a,b) as well as field studies (Grant, 1989). Toxicity testing involving developmental alterations of *P.dumerilii* has also been carried out (Beckmann *et al.*, 1995; Jha *et al.*, 1996; 1997; Hutchinson *et al.*, 1998; Palau-Casellas and Hutchinson, 1998).

Figure 1.7. Sexually mature male (red and white) and female (yellow) *P.dumerilii*.

1.6.1.2 *Mytilus edulis*

1.6.1.2 *Mytilus edulis*

Clams (*Mya arenaria* and *Mercenaria mercenaria*), oysters (*Crassostrea gigas* and *Ostrea edulis*) and especially mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) are the organisms of choice in most biomonitoring studies. Shellfish from the phylum Mollusca and the class Pelecypoda (Bivalvia) are thought to be particularly valuable for this purpose. The phylum Mollusca comprises one of the largest and most successful phyla within the animal kingdom. Over 100,000 species of living molluscs have so far been described (more than twice the number of vertebrates) and a rich fossil record extends back to the Cambrian (Seed, 1983). They have successfully adapted to both terrestrial and aquatic environments and have penetrated a wider range of habitats than virtually any other animal group (Seed, 1983). Bivalve molluscs are exclusively aquatic taxon of extremely wide distribution in fresh, brackish and marine waters around the globe. They are particularly abundant in the highly productive marine coastal areas. They have been exploited for food since prehistoric times and they have been cultivated systematically in various parts of the world for many centuries. The genus *Mytilus* is widely distributed throughout the world. *Mytilus edulis* (see figure 1.8.) is boreal, but eurythermic and is distributed from northern Norway (where summer temperatures hardly surpass 4°C to 5°C) to the Bay of Biscay (where summer temperatures may reach 23°C at Arachon). It is found on both coasts of the North Atlantic, including the North and Baltic seas (His *et al.*, 1999). Principal spawning of *M.edulis*, around the coasts of Europe, takes place from March to late June. This is followed by a non-reproductive period during the summer and gametogenesis resumes in October or November and continues through the winter (Bayne, 1976).

M.edulis is gonochoristic and the reproductive anatomy is fairly simple. The gonad consists of a mass of follicles, which develop fully at the period of sexual maturity, at which time the sexual products make up a significant part of the body. The sexual products grow within genital ducts, the diameters of which increase progressively during gametogenesis. The various small ducts converge on larger gonoducts, through which the

Sexual maturation is governed by exogeneous factors, such as temperature, lunar cycle and, in particular, nutritional factor, and endogeneous factors, such as genetics and hormones (His *et al.*, 1999).

Male bivalves usually spawn first, as they are more sensitive to stimulation, this induces other males to also spawn followed by females until the entire population spawns simultaneously. Spawning in the field is never initiated by one single event but generally by a series of stimuli, such as thermal changes caused by falling and rising tides, mechanical agitation by waves and currents, changes in salinity and differences in pressure between high and low water. The development of bivalves consists of an embryonic phase followed by a larval phase after which the settlement and metamorphosis into the adult occurs (His *et al.*, 1999).

Due to its prolific distribution and its importance in mariculture, *M.edulis* has been used extensively as a model species for pollution studies. Numerous field and laboratory studies have been carried out on *M.edulis* to investigate the effects of a wide variety of compounds on most aspects of the mussel's biology.

Figure 1.8. Adult bivalve mollusc, *M.edulis*.

1.7 Genotoxic and cytotoxic biomarkers

1.7 Genotoxic and cytotoxic biomarkers

Biomarkers are defined as the "biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples, or at the level of whole organisms, to provide evidence of exposure and/or effects from one or more contaminants (Depledge, 1994). Cytotoxic agents are contaminants that affect the ability of the cell to divide whereas genotoxicity tests contribute to the assessment of whether a chemical has the potential to cause somatic or germ-cell effects in animals (i.e. the potential to induce cancer or heritable mutation) (Elliot, 1994). In general there is a continuum of events between the first interaction of a xenobiotic and DNA and mutation, but they maybe divided into four broad categories (Shugart 1990). The first is the formation of adducts, the next is that the toxic contaminant may cause secondary modifications of DNA such as strand breaks, changes in the minor base composition, or an increase in the rate of DNA repair. The third stage is when the structural perturbations to the DNA become fixed. At this stage affected cells often show altered function and several cytogenetic assays are available, including chromosomal aberrations and sister chromatid exchanges (SCEs). Finally damage caused by toxicants may lead to the creation of mutant DNA which leads to alterations in gene function. Cytotoxic assays analyse effects on cell cycle regulation and may include the proliferation rate index (PRI) and mitotic delay and index. Table 1.3. indicates some examples of the most frequently used biomarkers of genotoxic and cytotoxic effects of pollutants at different levels of biological organisation.

Table 1.3. Examples of different genotoxic and cytotoxic biomarkers at various levels of biological organisation.

Level of organisation	Observed genotoxic effects	References
Molecular/Biochemical	- DNA adduct formation	Jones and Parry, 1992
	- Increase in the frequency of DNA strand breaks	Singh *et al.*, 1988; Fairbairn *et al.*, 1995
Cytogenetic	- Occurrence of sister chromatid exchanges	Dixon and Clarke, 1982; Jha *et al.*, 1996
	- Induction of chromosomal aberrations	Natarajan *et al.*, 1994; Solomon *et al.*, 1991
	- Increase in the expression of micronuclei	Heddle *et al.*, 1983; Scarpato *et al.*, 1990; Venier *et al.*, 1997

Level of organisation	Observed cytotoxic effects	References
Cellular	- Inhibition of cell cycle observed using either the proliferative rate index or the mitotic index	Jha *et al.*, 2000a; Lamberti *et al.*, 1983

1.7.1 DNA adducts

Adduct formation is the result of covalent binding of chemical environmental pollutants to DNA. DNA adduct formation integrates xenobiotic uptake, metabolism and macromolecular repair, and is the initial event in chemical carcinogensis (Livingstone, 1993). Thus, it is currently being used in humans as a biomarker for exposure to environmental and occupational carcinogens and has been successfully adapted for use in aquatic organisms (Jones and Parry, 1992; Liu *et al.*, 1991; Malmström *et al.*, 2000). In its simplest form DNA adducts are a measure of genetic damage as a result of exposure to carcinogens although the development of ^{32}P-postlabelling has allowed the extremely sensitive detection of certain classes of carcinogen-DNA adducts in organisms exposed to nonradioactive environmental or laboratory carcinogens (Dunn *et al.*, 1987). However genetic damage (DNA adducts) created by hydroxyl radicals, which may be produced via the action of free radical agents and by radiation, can also be analysed in DNA from aquatic organism (Chipman and Marsh, 1991).

1.7.2 Comet assay or Single Cell Gel electrophoresis

Rydberg and Johanson (1978), were the first to directly quantitate DNA damage in individual cells by lysing cells embedded in agarose, on slides, under mild alkali conditions to allow partial unwinding of DNA. After neutralisation, the cells were stained with acridine orange and the extent of DNA damage was quantitated by measuring the ratio of green (indicating double-stranded DNA) to red (indicating single stranded DNA) fluorescence using a photometer (Singh *et al.*, 1988). This technique involved numerous critical steps and was therefore not widely used. Also in 1978, Cook and Brazall observed that cells treated with 2 M NaCl and anionic detergents produced "nucleoids" composed of 50-100 kb loops of DNA attached to a proteinaceous network, this led to the development of the halo assay (Roti and Wright, 1987). The halo assay involved the relaxation of supercoiled DNA due to DNA strand breaks which allowed for the expansion of the halo DNA loops attached to nuclear matrix proteins of individual cells (Olive, 1999b).

In 1984, Östling and Johanson improved the sensitivity of technique for detecting DNA damage in isolated cells by developing a microgel electrophoresis technique. As in previous assays the cells were embedded in agarose gel on microscope slides, lysed by detergents and high salt, and then electrophoresed for a short period under neutral conditions. Electrophoresis acted to pull the negatively charged damaged DNA away from the nucleoid towards the anode. Cells with increased DNA damage displayed increased migration of DNA resulting in a comet like appearance, hence the name "Comet assay" (Singh *et al.*, 1988). The comet assay may also be known as the single cell gel assay (SCG) or microgel electrophoresis (MGE) (Fairbairn *et al.*, 1995). Östling and Johanson microgel electrophoresis assay used neutral conditions for lysis and electrophoresis that permitted the detection of double-stranded DNA breaks but could not detect single-stranded ones. Singh *et al.* (1988) modified the microgel electrophoresis technique to permit the evaluation of single-stranded DNA breaks and alkali-labile sites by using alkaline

condition. Alkali allow the DNA duplex to denature and unwind, and single strands of DNA can then migrate independently (Olive *et al.*, 2001).

The comet assay has advantages over other cytogenetic assays that need to have cells with high proliferation rates as interphase cells may be analysed. Additionally there is no need to have an organism with a suitable karotype as damage is detected at the DNA level. Due to these reasons and many more, the comet assay is being extensively used as an early indicator of genotoxic contamination in a vast number of organisms.

1.7.3 Sister Chromatid Exchanges (SCEs)

Sister chromatid exchanges (SCEs) are the reciprocal interchange of DNA at homologous loci between sister chromatids at the four-strand stage during the replication of chromosomal DNA. The occurrence of SCEs was first detected from the transformation of small ring chromosomes in maize following cell divisions (McClintock, 1938). Taylor (1958) was the first to demonstrate the exchange of genetic material between sister chromatids of the same chromosome. He used tritiated thymidine to demonstrate the semi-conservative mode of chromosomal replication in root tips of *Vicia fabia*. Although initially demonstrated in plant chromosomes, after ^{3}H-thymidine labelling, the SCEs technique was quickly adapted for mammalian systems, with human blood lymphocytes and Chinese hamster ovary (CHO) cell lines being extensively used (Perry and Evans, 1975). To date, the analysis of SCEs has been carried out in a variety of numerous marine organisms including fish (Kligerman, 1979), polychaetes (Jha *et al.*, 1996; Pesch *et al.*, 1981), sea urchins (Anderson *et al.*, 1994a), and molluscs (Brunetti *et al.*, 1986; Dixon and Clarke, 1982; Harrison and Jones, 1982).

The modern method of SCE analysis involves exposing cells or organisms to two DNA replication cycles to 5-bromodeoxyuridine (BrdU), a chemical analogue of the DNA-nucleotide base thymine. After two cell cycles the chromosome possess one chromatid that is unifilarly substituted with BrdU and its sister chromatid is bifilarly substituted. After

staining with the fluorochrome plus giemsa (FPG) technique, that incorporates staining with the fluorochrome Hoechst 33258 and Giemsa, the chromatids appear differentially stained due to the presence or absence of BrdU (Latt *et al.*, 1981; Perry and Evans, 1975).

Chromosomes that have undergone SCEs are not regarded as damaged in the conventional sense since they are morphologically intact. Nevertheless, SCEs occur at sites of mutational events including chromatid breakage. They have been shown, both *in vitro* and *in vivo*, to be extremely sensitive indicators of chromosomal damage, often at mutagen concentrations several orders below those at which significant increases in structural and numerical aberrations (e.g. chromosome breaks, deletions and structural rearrangements) are apparent (Dixon and Clarke, 1982). Eckl (1995), made a direct comparison between SCEs, micronuclei and chromosomal aberrations in rat hepatocytes and found that the SCEs were the most sensitive genotoxic endpoint after exposure to contaminated water samples. SCEs has proven to be a sensitive indicator of genotoxicity but the use of SCEs as a cytogenetic end point has been the subject of some criticism because to date, the molecular mechanism of SCEs formation and its biological meaning are not completely understood and some non-genotoxic agents have also been shown to induce SCEs (Tucker and Preston, 1996). However, in order to produce a SCEs, a lesion must pass through the S-phase and then the SCE can be detected during the following cell mitosis. Thus increased strand breakage during the S-phase lead to increased SCEs.

1.7.4 Chromosomal aberrations (Cabs)

Chromosomal alterations have been studied extensively for nearly a century. Cytogenetic anomalies are now know to be of many types and to be induced by a variety of agents by several different mechanisms, some of which are partially understood but remain to be elucidated (Tucker and Preston, 1996). The induction of chromosomal alterations was first analysed in the 1930s. Chromosomal alterations were induced in pollen microspores of *Tradescantia* after exposure to X-rays (Sax, 1938). By the 1960's, the analysis of

structural aberrations was being used to quantify the level of exposure to ionising radiation in humans (Bender and Gooch, 1966). Chromosomal aberrations ultimately will lead to cell lethality due to losses of chromosomal material at mitosis or inhibition of accurate chromosome segregation at anaphase. Generally, if chromosomal aberrations arise in germ cells they can lead to dominant lethality, perinatal mortality or congenital malformation whereas, if the aberrations are induced in somatic cells they may lead to malignancy (Kirkland, 1990). The analysis of chromosomal aberrations is also a very important parameter for risk assessment, as there is a clear association between chromosome rearrangement and with initiation and promotion of cancer (Solomon *et al.*, 1991). The types and frequencies of induced chromosomal aberrations depend on the mutagen used and the stage of the cell cycle treated as described earlier. In general ionising radiation and chemicals which directly produce DNA strand breaks induce chromatid aberrations in the G_2 stage and chromosome aberrations in the G_1 stage (Natarajan *et al.*, 1994).

1.7.5 Fluorescent *in situ* hybridization (FISH)

The relatively recent development of fluorescent based staining methods (FISH, fluorescent *in situ* hybridization) particularly whole chromosome painting, has heralded a significant improvement in the ability of metaphase-based cytogenetic analyses to be used in the process of risk assessment (Tucker and Preston, 1996). This technique allows sites of specific nuclei acid sequences to be detected in metaphase spreads or interphase nuclei (Dixon and Wilson, 2000). This technique is used for the detection of target molecules with a system of coupled antibodies and fluorochromes. The detection of nucleotidic sequences on a combed DNA molecule is performed indirectly, by first hybridizing the seeked nucleotidic sequences (the probes) with the combed DNA (also called the matrix DNA or target). If the probes are synthesised with incorporated fluorescent molecules or antigenic sites that can be recognised with fluorescent antibodies, the direct visualisation of the relative position of the probes is possible. The method facilities the identification of

specific chromosomes or chromosome arm regions in species which have proved largely intractable to DNA banding, such as marine invertebrates (Dixon and Wilson, 2000). An example of this approach has been carried out in a study into the phenomenon of Robertsonian polymorphism in the common European dog-whelk *Nucella lapillus* (Pascoe *et al.*, 1996). A FISH painting probe has also been prepared and used to establish a dose-response curve for ionising radiation induced chromosome interchange aberrations in yellow bellied slider turtle (*Trachemys scripta*) fibroblasts (Ulsh, *et al.*, 2000).

1.7.6 Micronucleus assay (Mn)

Evans *et al.*, (1959) were the first authors to suggest counting cells with micronuclei (Mn) as a method for the evaluation of cytogenetic damage and Schmid (1975) and Heddle (1973) both independently proposed the micronucleus test as an alternative to the laborious and complex counting of aberrations in metaphases. Micronuclei are expressed in dividing cells that contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term "micronucleus". Figure 1.9. represents the mechanisms involved in the formation of micronuclei.

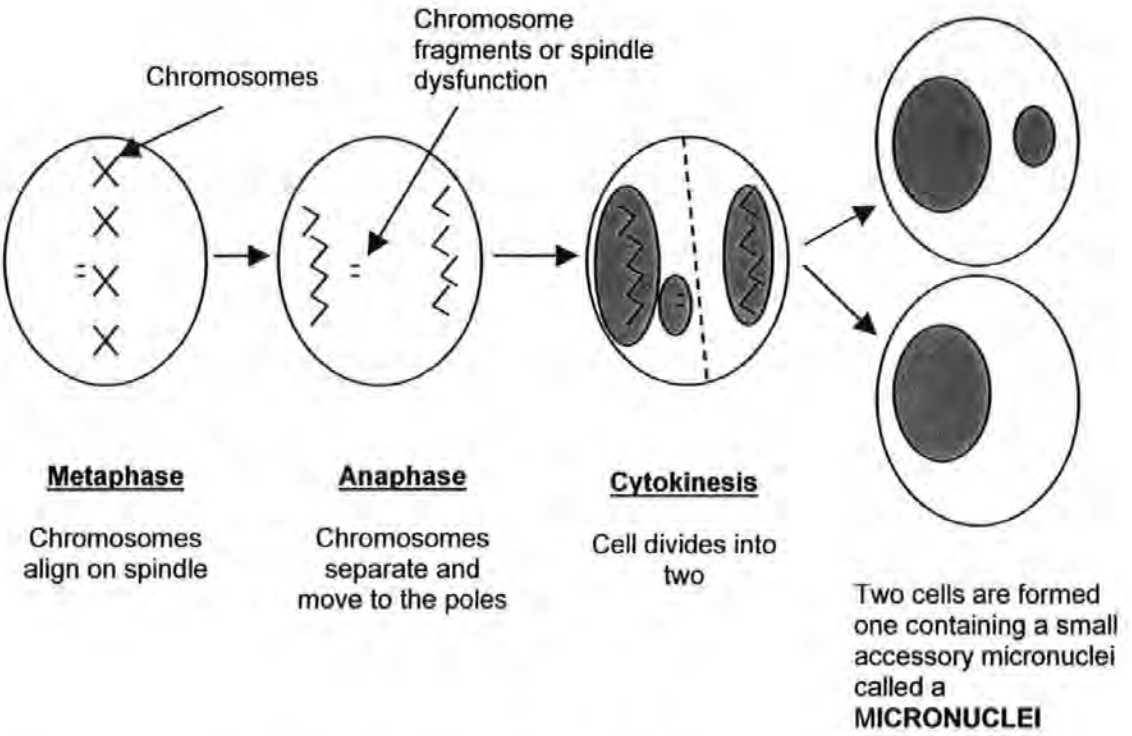


Figure 1.9 Formation of a micronuclei during cell division.

Micronuclei provide a convenient and reliable index of both chromosome breakage and chromosome loss (Fenech 1996). Micronuclei are also known as Howell-Jolly bodies by haematologists and because they are caused by chromosomal aberrations (Countryman and Heddle, 1976) are considered to be a sensitive indicator of genotoxic risk of exposure to mutagenic agents (Rodgers and Baker, 2000). The micronucleus assay was initially developed in dividing mammalian cells and its use in bone marrow and peripheral blood erythrocytes is now one of the best established *in vivo* cytogenetic assay in the field of genetic toxicology. Micronuclei are only expressed in dividing cells and the assay can not be used in nondividing cell populations in which the cell division kinetics is not well controlled or understood. In mammalian systems, the cytokinesis-blocked micronucleus assay has been developed to block cells that have completed one nuclear division from performing cytokinesis, using cytochalasin-B. Consequently cells will have a binucleated appearance and the accumulation of virtually all dividing cells will occur regardless of their degree of synchrony and proportions of dividing cells. Micronuclei are then scored in

binucleated cells only, which enables reliable comparisons of chromosome damage between cell populations that may differ in their cell division kinetics.

The micronucleus assay has several advantages over other cytogenetic assays. Heddle (1973) stated that the scoring micronuclei were more than ten times faster than scoring chromosomal aberrations at metaphase. Other advantages of the micronucleus assay are that the number of scorable cells is virtually unlimited, little formal training is needed as the end-point is easily recognisable and a suitable karyotype is not required. Problems associated with the interpretation of other cytogenetic tests such as the significance of chromosomal gaps or sister chromatid exchanges are avoided with the micronucleus test. One limitation of the micronucleus assay is when agents cause neither chromosomal breakage or lagging chromosomes, for example, aberrations that involve chromosomal rearrangement without the occurrence of an acentric fragment such as translocation or inversion, these will not be detected (Heddle *et al.*, 1983).

As with many other mammalian based assays, the micronucleus test has been successfully adapted to aquatic organisms. Induction of micronuclei in fish has been widely used due to advantages over other cytogenetic assays such as chromosomal aberrations and sister chromatid exchanges. As well as being time consuming these tests are not very effective due to the relatively large number of very small chromosomes (Ayllon and Garcia-Vazquez, 2000) and, as in cold water fish, the cells have a low mitotic activity which results in fewer scorable metaphase spreads (Hooftman and De Raat, 1982). The micronucleus assay has been extensively used in haemocytes and gill cells of many mollusca species, including the freshwater zebra mussel *Dreissena polymorpha* (Mersch and Beauvais, 1997; Pavlica *et al.*, 2000), the oyster *Crassostrea gigas* (Burgeot *et al.*, 1995), the Mediterranean mussel *Mytilus galloprovinciallis* (Majone *et al.*, 1987,1988; Scarpato *et al.*, 1990; Venier *et al.*, 1997) and the common blue mussel *Mytilus edulis* (Bolognesi *et al.*, 1999; Dopp *et al.*, 1996; Wrisberg and Rhemrev, 1992). Many micronuclei studies have been carried out on molluscs in laboratory conditions but due to

sedentary nature of molluscs the micronucleus test has also been proposed as a potential biomarker of pollutant exposure in field studies (Mersch and Beauvais, 1997). Although the micronuclei frequency in haemocytes from mussels were shown to be seasonally dependent (Wrisberg and Rhemrev, 1992) and many authors have reported high variability between individuals (Mersch and Beauvais, 1997). The incidence of micronuclei has also been linked to the induction of leukemia cells in the clam *Mya arenaria,* suggesting that the micronucleus test is a very good indicator of the potentially life threatening consequences of genotoxic exposure (Dopp *et al.*, 1996).

1.7.7 Proliferative Rate Index (PRI)

This ability to identify unequivocally cells which have replicated one, twice or more than three times in the presence of BrdU has led to the use of this technique being used for cell cycle and cell proliferation analyses. Originally proposed by Schneider and Lewis (1981), for application to actively proliferating and highly differentiated mammalian cells *in vitro*, the PRI have also been adapted for use in aquatic environments (Jha *et al.*, 2000a; Lamberti *et al.*, 1983).

Differential staining of metaphase chromosomes is possible due to the substitution of 5-bromodeoxyuridine (BrdU) for thymidine in DNA via semi conservative replication. After one replication cycle in the presence of BrdU, both chromatids contain one parental strand of DNA and one BrdU substituted DNA strand. After staining with a fluorochrome plus giemsa (FPG) both these chromatids appear the darkly stained. Following two cell replications in the presence of BrdU, one chromatids' DNA is unifilarly substituted with BrdU and the sister chromatid is bifilarly substituted with BrdU. The sister chromatid that contains only BrdU substituted DNA displays diminished Giemsa staining, therefore for cells that have replicated twice in the presence of BrdU will have one chromatid that is darkly stained and the other sister chromatid will be lightly stained, giving rise to a harlequin appearance. After three or more replication cycles, in the presence of BrdU,

some chromosomes will contain DNA that is completely substituted with BrdU and therefore appear entirely light in appearance and other chromosomes will resemble cells that have only divided twice.

The PRI has been used extensively as a cytotoxic assay in embryo-larvae stages of marine invertebrates, due to the rapid turnover of developing cells (Hutchinson *et al.*, 1998; Jha *et al.*, 1996; 1997; 2000). Martínez-Expósito *et al.* (1994), have used sister chromatid differentiation (SCD) in order to determine the cell proliferation kinetics of gill tissue in the mussel *Mytilus galloprovincialis*.

1.7.8 Mitotic delay

Ultimately, cells containing aberrations will not be allowed to proceed through the cell cycle, will not genetically divide and will therefore be eliminated. However DNA damage is repairable although this will lead to a delay in the cell cycle whilst the error is recognised and repaired. The delay in the mitotic cell cycle can be used as a useful indicator of genotoxicity. It is important that the potential of a chemical or radiation to cause cell cycle delay be addressed when carrying out cytotoxic assays that depend on cell cycle kinetics (Kirkland, 1998). Upon exposure of cells to radiation delivered at a continuous low dose rate, the cell proliferation was sustained although the cells exhibited a constant doubling time (or mitotic delay) that was independent of the total dose but dependent on the dose rate (Yi *et al.*, 1994). Exposure to ionising radiation was also shown to temporarily block eukaryotic cell cycle progression at the G_2/M boundary (G_2 delay). The delay probably provided time for repair of DNA damage before chromosome segregation and is thus an active response, indicative of a checkpoint control function (Rowley and Zhang, 1996). Experiments have also been carried out using mitotic delay on human lymphocytes to provide some insight into the problem of the variability of the adaptive response (Salone, *et al.*, 1996).

1.8 Outline of thesis

While there is growing concern over the discharge of radionuclides in the environment, limited studies have been carried out to assess the effects of ionising radiation on marine organisms, specially the marine invertebrates that play an important role in the ecosystem. Therefore the general aim of this thesis were, (a) to assess the effects of ionising radiation on the embryo-larvae of two ecologically relevant marine invertebrates *Mytilus edulis* and *Platynereis dumerilii*, (2) to assess the effects of an environmentally relevant cocktail of radionuclides (3) to monitor the potential impact of radiation on the natural environment and finally (4) attempt to predict the potential effects of radiation at population level.

The validation of developmental stages and mammalian based cytotoxic and genotoxic assays (chromosomal aberrations, sister chromatid exchanges and proliferation rate index) was carried out on the embryo-larvae of *M.edulis* and *P.dumerilii* after exposure to potential (tributyltin) and reference (Methylmethane sulphonate) genotoxins (chapter 3). Following validation of the techniques, the embryo-larvae stages were exposed to a reference radionuclide (tritium) in an attempt to assess the effects of ionising radiation on early life stages (chapters 4 and 5). In collaboration with the Royal Devonport Dockyard (DML) investigations into the effects of a cocktail of radionuclides (environmentally relevant ionising radiation) were carried out (chapter 6).

Following experiments on the embryo-larval stages of the two marine invertebrates studies were carried out to assess the effects of ionising radiation on adults. Validation of mammalian based genotoxic assays (comet and micronucleus assays) was carried out on adult *M.edulis* after exposure to a reference genotoxin (Ethylmethane sulphonate) (chapter 7). Following validation of the techniques the adult *M.edulis* were exposed to a reference radionuclide (tritium) in an attempt to assess the effects of ionising radiation on the adult life stage (chapters 7). In collaboration with the Royal Devonport Dockyard (DML) a field study was carried out to assess the applications of the genotoxic assays (comet and

micronucleus assays) as biomarkers to radiation exposure in adult mussels transplanted to an area of radionuclide discharge (chapter 8). The potential effect of ionising radiation on adult *P.dumerilii* was carried out in chapter 9. Due to relatively short life span of the worm (3-6 months) attempts were made to assess the potential population effect, in terms of growth, development and reproductive success, of tritium. Figure 1.10. represents a general overview of the chapters and experiments carried out during the thesis. Overall the general hypothesis that is tested during the studies is that ionising radiations induce genotoxic and cytotoxic responses in embryo/larvae and adults life stages of two marine invertebrates and that these responses can be detected using molecular, cytogenetic and cytological methods.

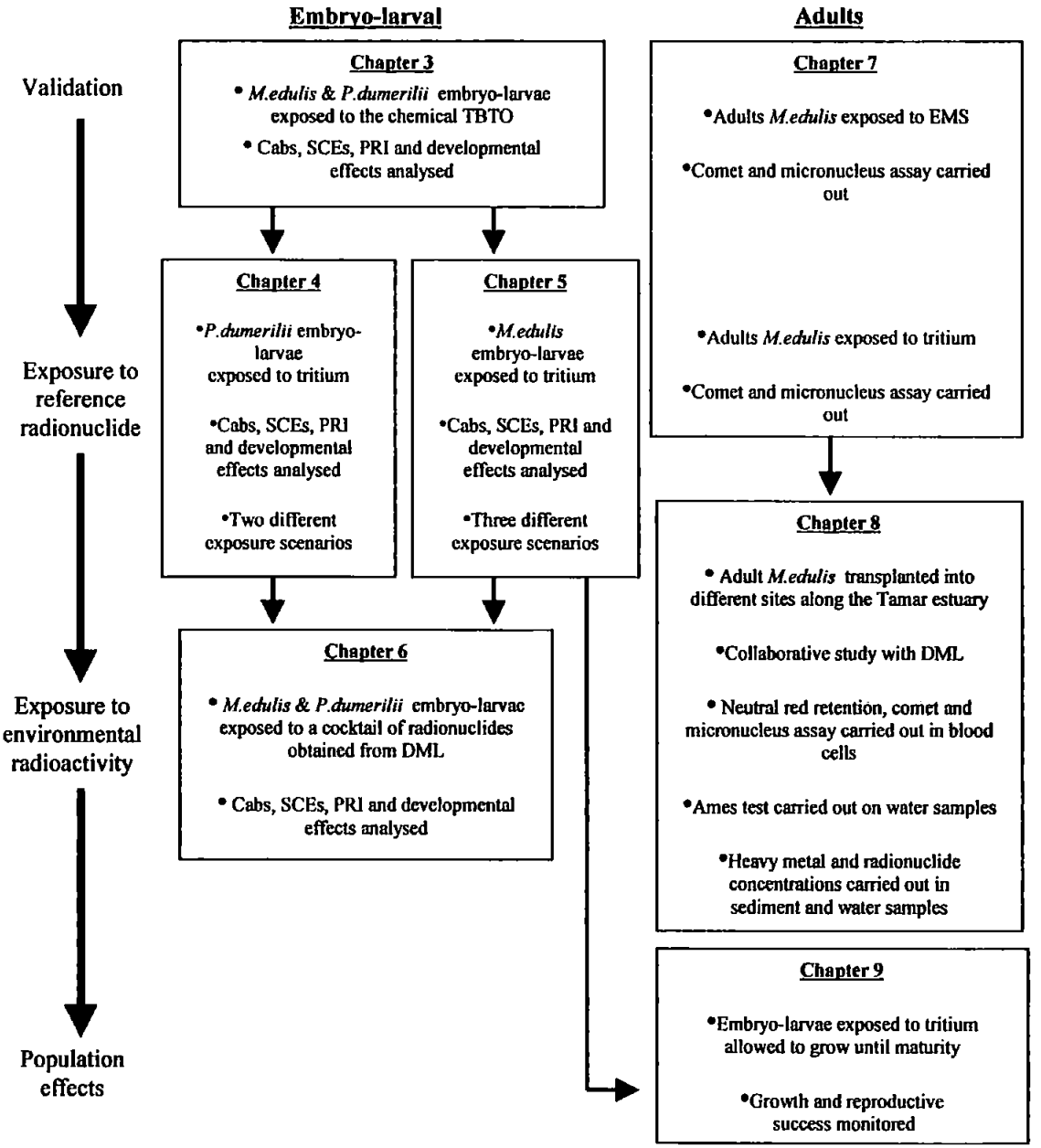


Figure 1.10. Overview of PhD chapters and experiments.

Chapter 2

Materials and Methods

2.1 Organisms and maintenance

2.1.1 *Platynereis dumerilii*

P.dumerilii were initially cultured at Brixham Environmental Laboratory (AstraZeneca), Devon, UK. and then transferred to the University of Plymouth, Devon in April 1996. An additional batch of animals was also obtained from Dr. Adriann Dorresteijn, University of Mainz, Germany in 2000. Culturing of *P.dumerilii* has been described elsewhere in detail (Hauenschild and Fischer, 1969; Hutchinson *et al.*, 1995, Jha *et al.*, 1995b). Stock cultures were maintained in 10 μm filtered natural seawater with a constant temperature of 20 ± 1°C, a salinity range of 35-37‰, oxygen concentration of 7.8-8.2 mg/l and pH range of 7.8-8.2. Photoperiod manipulation was set at 16h light followed by 8h of darkness, with continuous low-level illumination, once every 4 weeks to simulate a full moon phase (Hutchinson *et al.*, 1995; Jha *et al.*, 1995). As mentioned in section 2.2.1. fertilisation of gametes was carried out in 250 ml beakers containing 100 ml of filtered (10 μm) seawater. Following fertilisation the embryo-larvae were placed in plastic tanks (20 x 10 x 10 cm; length, width, depth respectively) containing 1 litre of filtered seawater and were initially fed on a diet of finely ground spinach (1 ml every 2 days). When the worms were visible without the use of a microscope they were transferred to larger plastic tanks (60 x 30 x 15 cm; length, width, depth respectively) with a continuous air supply and a sponge filter to help maintain clean aerated conditions essential for optimal health and growth. The adult worms were fed a mixed diet of fish flake (Waltham®, Elland, UK.) and chopped spinach. A spinach concentration of 10 g per 100 ml distilled water was prepared and 20 ml of spinach solution per added to each tank containing approx. 100 worms. Faecal material was removed and the water changed approx. once a week.

2.1.2 *Mytilus edulis*

Adult *M.edulis* were collected from the tidal zone, Whitsand Bay, Cornwall. Mussels approx. 50 mm in length were removed from the rocks and immediately transported to the aquarium where they were placed into filtered (10 μM) natural seawater at a constant temperature of 15 ± 1°C, a salinity range of 35-37‰, oxygen concentration of 7.8-8.2 mg/l and pH range of 7.8-8.2. *M.edulis* was fed Liquifry® (Interpret, Surrey, UK) twice a week. Liquifry® consists of 70% waterlife inert food (a suspension of plankton and other marine derived proteins, vitamins and mineral salts), 28% liquifry marine (dextrin, pea flower, whole egg, yeast and spinach), 1% selco and 1% *Haematococcus* species (algae). Water was renewed once a week.

2.2 Spawning organisms

2.2.1 *Platynereis dumerilii*

Spawning of *P.dumerilii* is controlled by simulated lunar photoperiod manipulation (Grant, 1989). Adult worms live in tubes, produced from mucous secreted from the body of the growing worms combined with faecal and food matter, until they undergo epitoky into heteronereid forms, with the mature females appearing yellow and the males have a white anterior and a red posterior (Hauenschild and Fischer, 1969). Following a lunar photoperiod (artificially manipulated and naturally occurring) sexually mature worms emerge from their tubes and swim to the surface of the water where they perform a "nuptial dance" which leads to spawning (Hardege *et al.*, 1998). The adults are left in the gamete suspension to aid mixing of the sperm and eggs. A variety of factors confirms that fertilisation has occurred. Typically, fertilised eggs secrete a jelly coat to prevent polyspermy occurring, following this the embryos change colour to a grey/blue in contrast to the yellow coloration of unfertilised eggs, the embryos also appear less granular until finally the first cleavage occurs (Hutchinson *et al.*, 1995). Figure 2.1. shows the difference between unfertilised eggs with no cleavage and fertilised eggs after a period of cleavage.

Spawning is completed and fertilisation is achieved within an hour, after which the sexually matured worms die naturally. The fertilised eggs were passed through a 150µm sieve to remove the adult worms and any other material. The fertilised eggs were then poured into an 80µm sieve and washed thoroughly with seawater in order to remove any excess sperm. The percentage of fertilisation was evaluated prior to each experiment and only organisms that produced over 95% fertilisation rates were used.

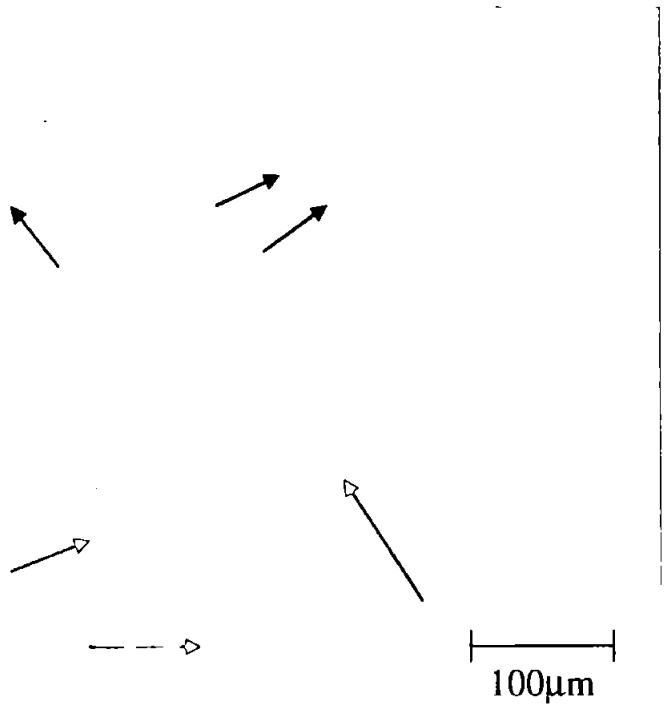


Figure 2.1. *P.dumerilii*: Newly fertilised (→) and unfertilised (→) eggs.

2.2.2 *Mytilus edulis*

Adult mussels collected during the spawning season (see section 1.6.2 for dates) were washed and any epifauna and flora removed from their outer shells. The mussels were placed into individual 250 ml glass beakers containing aerated filtered (10 µm) seawater (salinity ≃ 35‰). The beakers were than transferred to a water bath at a set temperature of 18-19°C. It was ensured that the temperature never exceeded 20°C as per ASTM guidelines (ASTM, 1997). Water temperature and oxygen levels were adjusted, by renewal of seawater and aeration using an air stone, at regular intervals to maintain

optimum spawning conditions. Gametes released by the adults are easily recognisable. The males produce a white discharge that clouds the water whereas female mussels release a mass of pink/orange eggs which sink in the bottom of the spawning chamber (beakers). *M.edulis* eggs are approx. 40-50 μm in diameter (ASTM, 1997) and the number per batch can vary greatly between 200,000 to 1,500,000 per female. The eggs and sperms were passed through an 80 μm sieve in order to obtain a homogenous suspension and to remove any faecal or other extraneous material. The number and viability (actively swimming) of the sperm was checked using a haemocytometer. Following analysis of eggs using a light microscope the number of fertilised eggs produced was calculated per ml. The number of embryos were adjusted to the range of 20-50 eggs / ml before the sperm was added at approx. 10^5 to 10^7 sperm/ml (ASTM, 1997). Fertilisation was carried out at 15 ± 1°C in order to limit and prevent any lethal damage occurring to the embryos. The percentage of fertilisation was calculated, Figure 2.2. illustrates the difference between unfertilised and fertilised eggs. After the eggs had been fertilised the embryo suspension was poured into a 30 μm sieve and rinsed with filtered seawater to remove any excess sperm. The suspension was then backwashed into a 2 L beaker. The embryos were mixed using a plastic perforated plunger in order to obtain a homogeneous suspension, they were then divided equally into acid washed, 1litre test vessels at a concentration not exceeding 30 embryos / ml (ASTM, 1997). The percentage of fertilisation was evaluated prior to each experiment and only organisms that produced over 90% fertilisation rates were used.

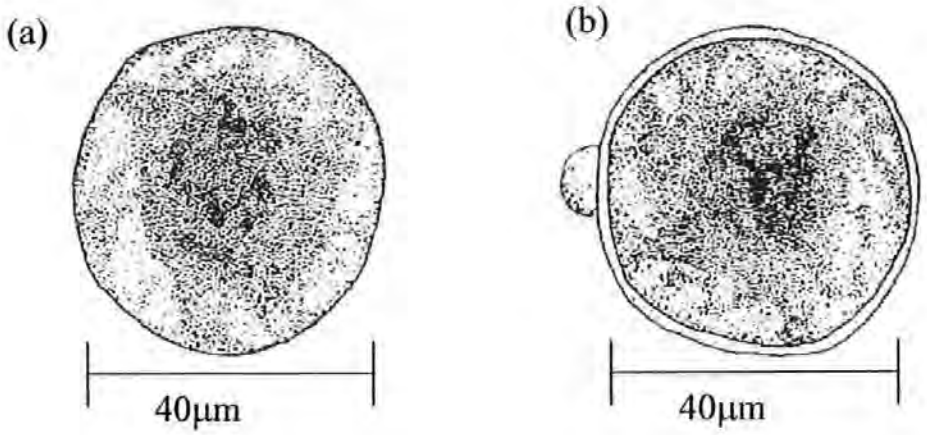


Figure 2.2. (a) unfertilised and (b) fertilised *M.edulis* egg with distinct polar body indicating point of sperm entry (adapted from ASTM, 1997).

2.3 Embryo-larvae exposures

An "integrated approach" investigating developmental, cytotoxicity and genotoxicity effects of ionising radiation and chemicals on embryo-larvae of *P.dumerilii* and *M.edulis* was adopted. An integrated approach simultaneously analyses/evaluates the effects of an agent at different levels of biological organisation. Embryo-larvae stages of development are relatively more sensitive to toxicants when compared to juvenile and adult stages (Hutchinson *et al.*, 1998; Jha *et al.*, 2000b). Hence, it is important to understand how and to what extent early life stages might be affected by ionising radiation and other potentially (geno) toxic chemicals. It is important to recognise the most sensitive life stages and species in an attempt to assess the hazard to the environment. In order to carry out the various developmental, cytotoxic and genotoxic assays, using early life stages, embryo-larvae need to be obtained. For experimental purposes, the spawning and fertilisation of both *P.dumerilii* and *M.edulis* embryos can be achieved under laboratory condition and the embryo-larvae stages of development used for diverse studies. Spawning of gametes from *P.dumerilii* and *M.edulis* has been described in detail in section 2.2. and morphological descriptions of embryo-larvae at developmental stages aged 24-72h are

described in section 2.3.1. Cytotoxicity was assessed using the proliferative rate index (PRI) as described in section 2.3.4. Genotoxicity was assessed using chromosomal aberrations (Cabs) and sister chromatid exchanges (SCEs) as described in sections 2.3.2. and 2.3.5. respectively. A summary of the general protocol followed is described in figure 2.3.

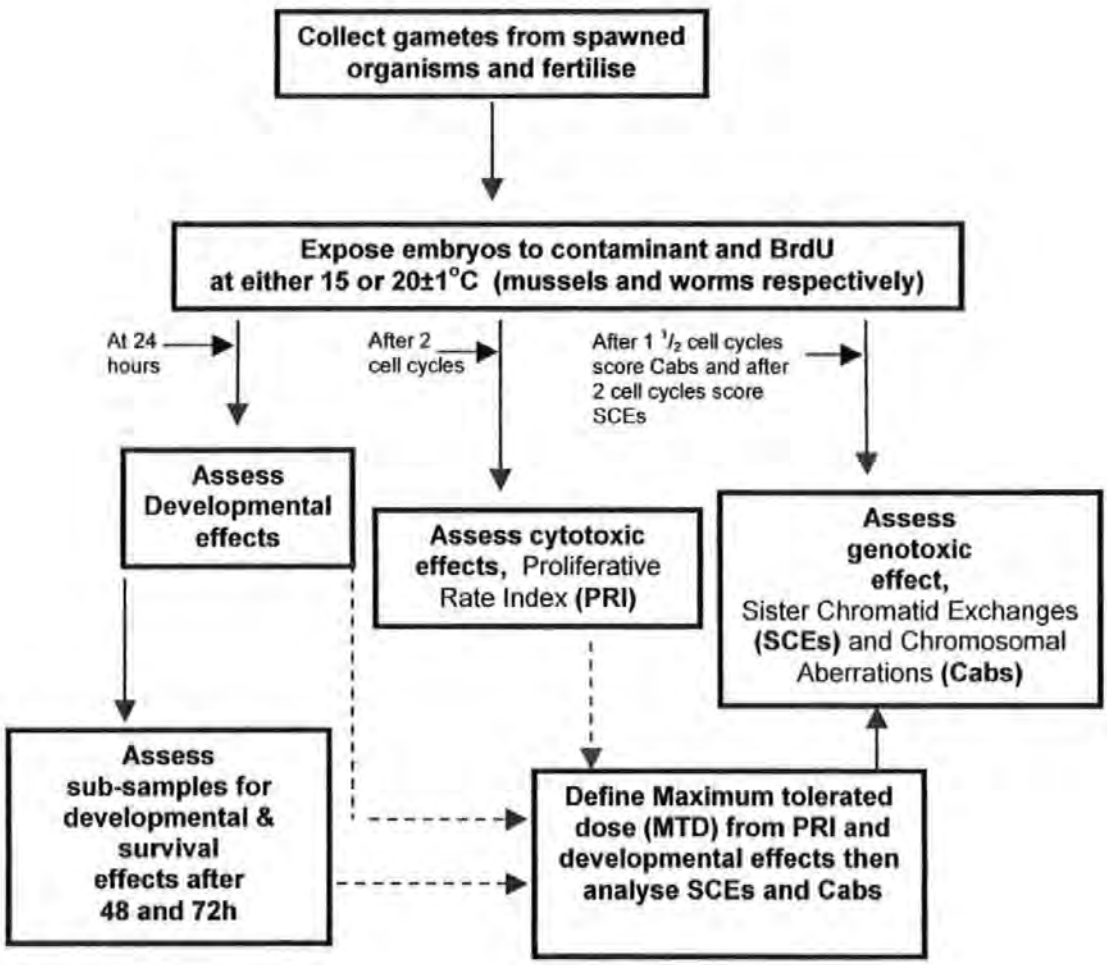


Figure 2.3. Summary of generalised protocol for exposure of embryo-larvae to radionuclides and chemicals. (Adapted from Hutchinson *et al.*, 1998; Jha *et al.*, 2000b).

2.3.1 Developmental life stages

After fertilisation and during exposures the embryo-larvae were maintained in cooled incubators (Sanyo Gallenkamp Plc., UK.) under controlled temperatures (20°C and 15°C for *P.dumerilii* and *M.edulis* respectively) and photoperiod (16h light : 8h dark).

Developmental effects of ionising radiation (radionuclides) and chemicals were assessed every 24h until 72h for growth of *P.dumerilii* and *M.edulis* embryo-larvae. Approximately 1/3 of the embryo-larvae were examined for developmental abnormalities, survival and swimming behaviour every 24h after which the embryo-larvae were fixed in a 10% formalin/seawater solution for further examination of morphological developmental abnormalities as described in sections 2.3.1.1. and 2.3.1.2.

2.3.1.1 *Platynereis dumerilii*

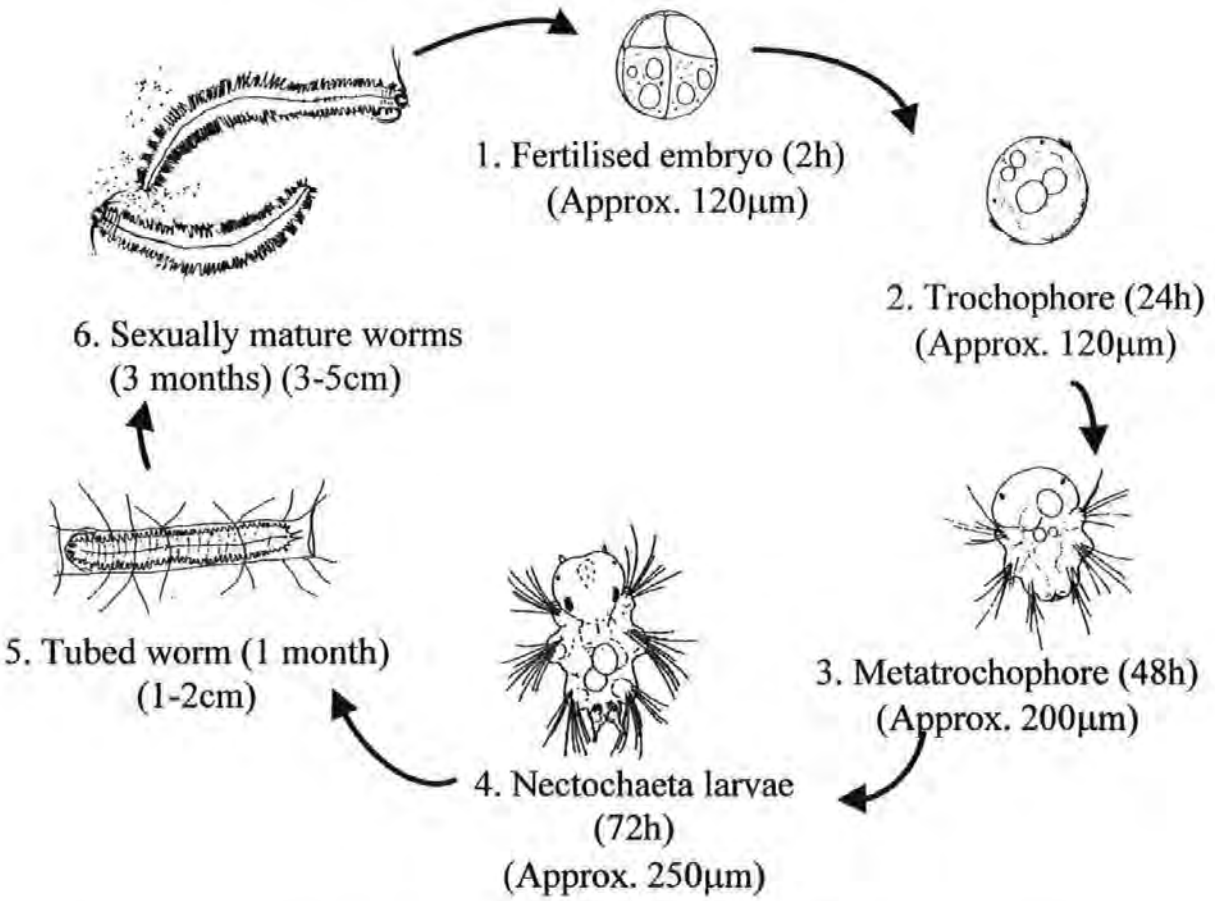


Figure 2.4 Life cycle/history of *P.dumerilii* (Adapted from Hutchinson *et al.*, 1995).

Developmental stages of *P.dumerilii* have been well documented (Hauenschild and Fischer, 1969; Dorresteijn, 1990). At 24h, normal embryo-larvae of *P.dumerilii* should have reached the free swimming trochophore stage (Figure 2.4.). Morphologically

trochophore larvae are spherical in shape and generally 120 μm in diameter. They have distinct ciliary bands, prominent fat droplets and are actively swimming. Abnormal trochophore larvae generally lack any ciliary bands causing locomotion or swimming to be hindered. Another characteristic of abnormal trochophores is the replacement of several fat droplets with just one of two larger vacuoles (Figure 2.5.b). At 48h the larvae should have developed to the metatrochophore (multi-segmented) stage (Figure 2.4.). The metatrochophore stage of polychaete development is characterised by the appearance of a segmented body, a wide head region, eyespots and chaetae (Figure 2.5.c). Abnormal metatrochophores generally lack one or more of these features (Figure 2.5.d). 72h normal polychaete larvae will have progressed to the nectochaeta stage of development (Figure 2.4.), they can be identified by the development of three segments, distinct parapodia and eyespots. As with the previous stages of development abnormal embryo-larvae are distinguished by a lack of one or more of these features (Figure 2.5.f). Dead embryo larvae at all stages of development appear as a mass of degenerating cells held together loosely by a membrane (Hutchinson *et al.*, 1995).

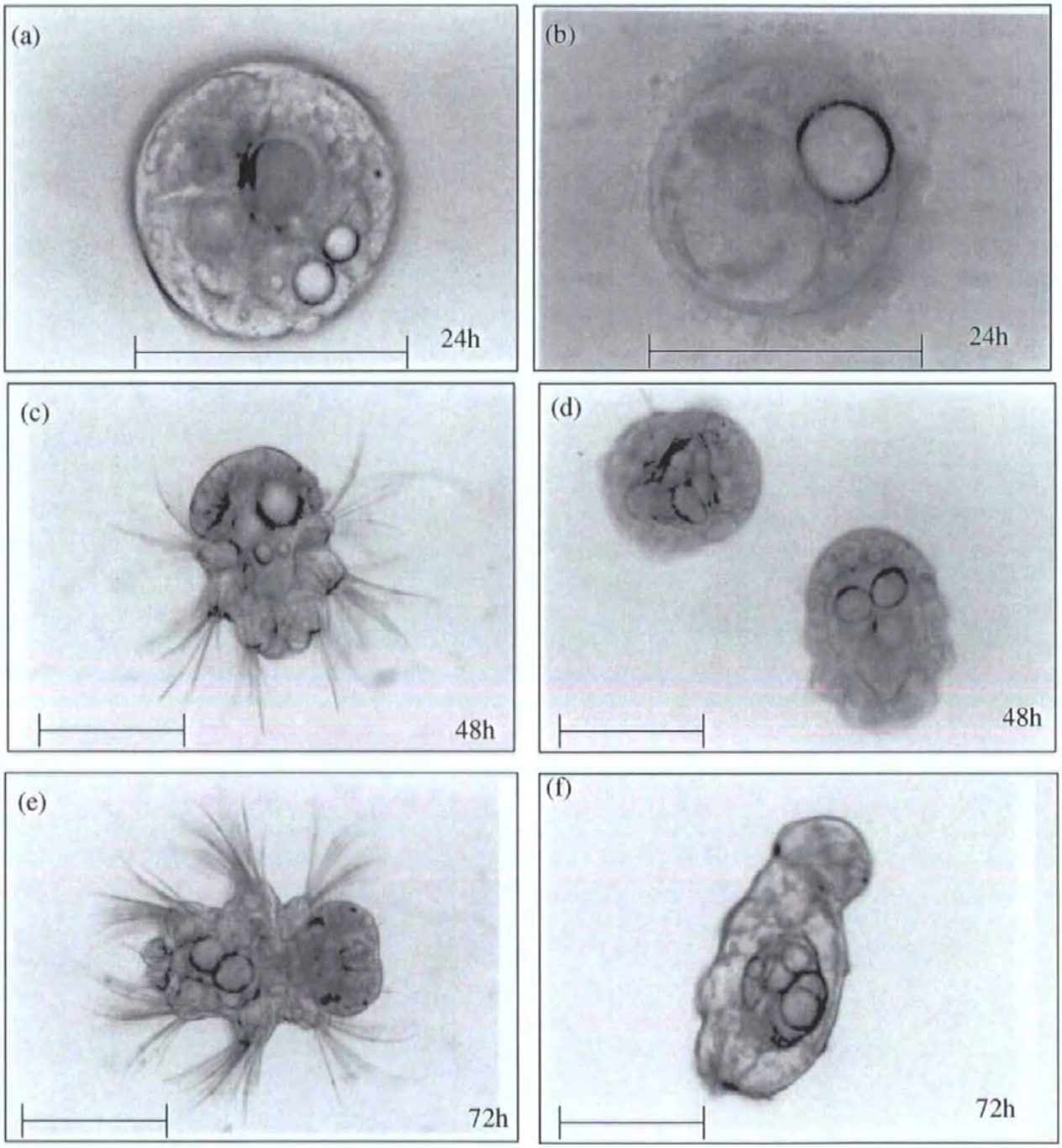


Figure 2.5. The developmental stages of *P.dumerilii* embryo-larvae. Photos a, c & e represent normal embryo-larvae at 24, 48 and 72h respectively and b, d & f represent morphologically abnormal embryo-larvae at 24, 48 & 72h respectively. Scale bar = 100µm.

2.3.1.2 *Mytilus edulis*

Like *P.dumerilii*, the developmental stages of *M.edulis* have been well characterised and are temperature dependent (15 ± 1°C) (ASTM, 1997), they develop faster at higher temperatures although not exceeding upper limits of temperature (20°C). The development of *M.edulis* consists of an embryonic phase followed by a larval phase (Bayne, 1976). The first larvae stage occurs at approx. 24h and the larvae are known as trochophores (Figure 2.6).

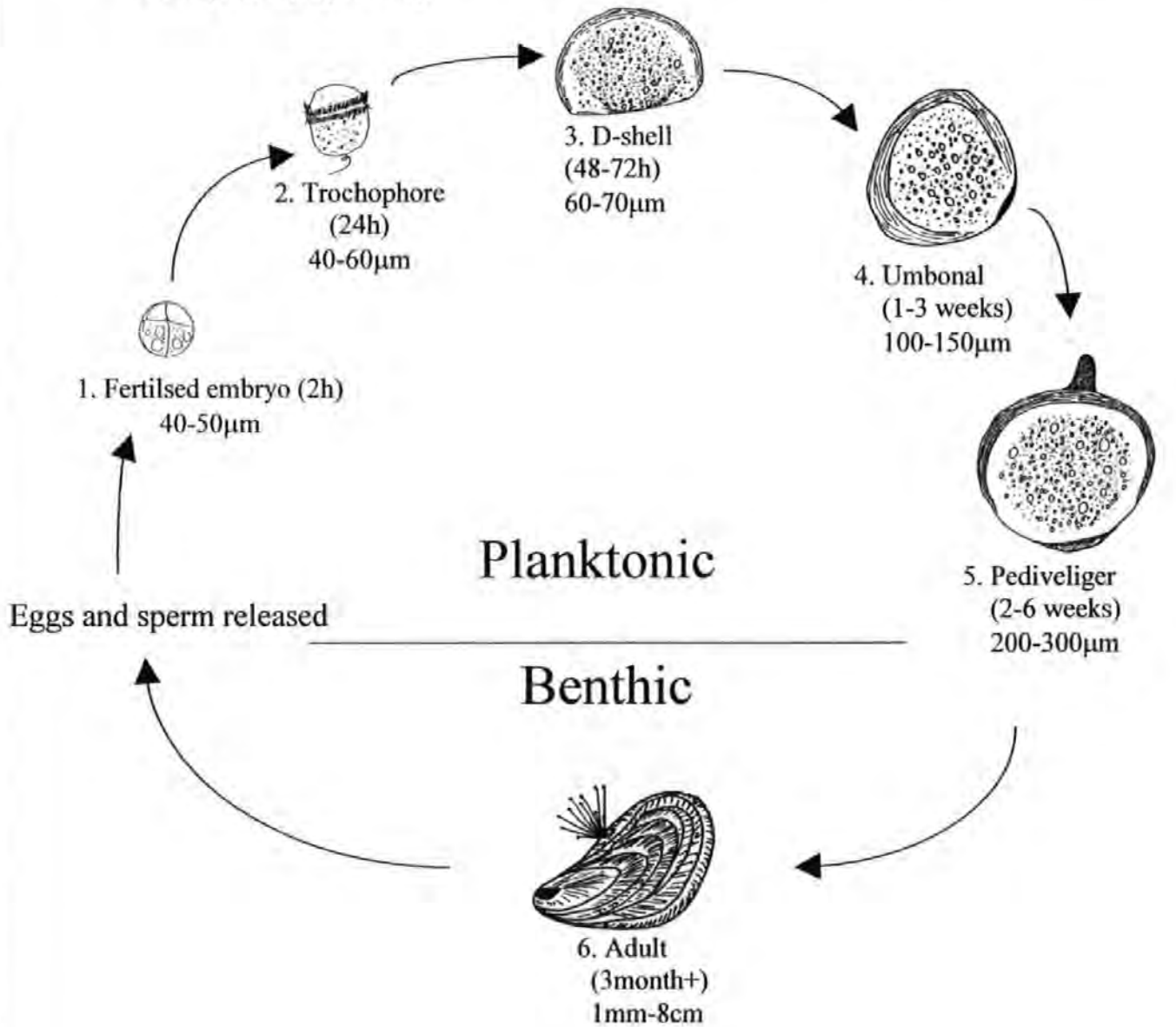


Figure 2.6. Life cycle/history of *M.edulis* (adapted from Bryan, 1976).

This stage is defined as free swimming larvae, with a circular band of short cilia (prototroch) and a flagellum. Abnormal larvae are usually misshapen (not spherical in shape or symmetrical) and may lack cilia and/or the flagellum, resulting in abnormal swimming behaviour (Figure 2.7.b). The second larvae stage is the veliger, which is usually formed after 48h in *M.edulis* (15°C). A dorsal thickening of the ectodermis, secreted by the shell gland, forms an initial organic cuticle that spreads over the entire body. A straight dorsal hinge gives the larvae the characteristic capital D shape, hence the name "D-larva" (Figure 2.7.c). The shell may at this stage begin to calcify, forming the prodissoconch I and the prototroch may have developed to form the velum. The larvae are approx. 60-70 μm in size and begin to feed at this stage. At 72-96h (depending on survival due to lack of food), as the larvae grow beyond 100μm a secondary shell is secreted (the prodissoconch II) and the umbo begins to form. The larvae at this stage are known as "umboned larva" (figure 2.6.). As the larvae grows to a size of 200-300 μm, an "eye spot" appears within the shell and the foot is formed, the larvae at this stage are termed "eyed larva" and "pediveliger". As the ciliated foot grows and becomes functional the larva becomes capable of both pelagic and benthic modes of life, either swimming by use of the velum (e.g. to feed), or creeping along the hard substrate with its foot (e.g. to explore for a suitable site for settlement). Once it has attained this stage it is ready to metamorphose. During metamorphosis the velum disappears and the foot develops into a specialised form, labial palps and gills are developed and the final shell (the dissoconch) is secreted (His *et al.*, 1999).

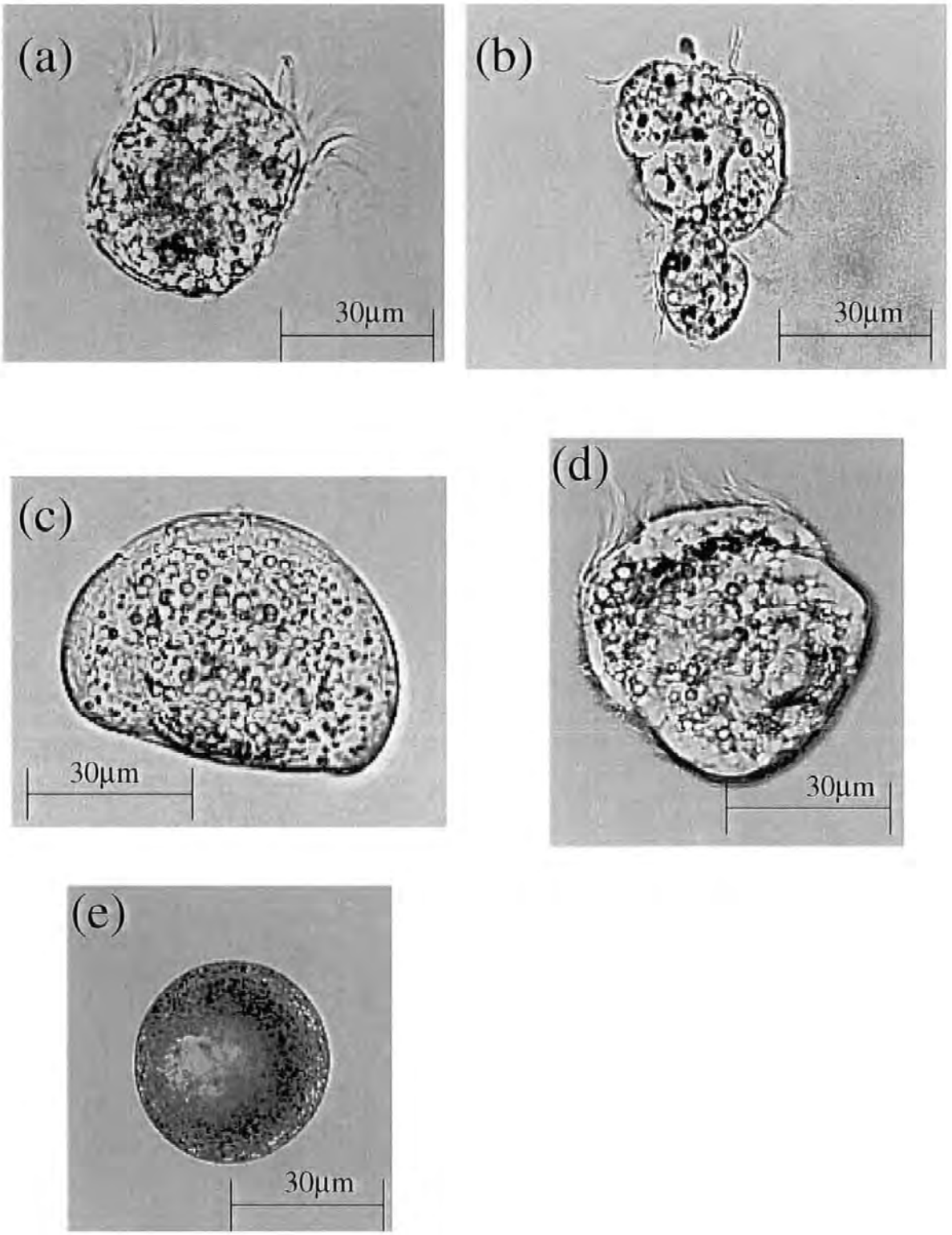


Figure 2.7. The developmental stages of *M.edulis* embryo-larvae. Photos a, and c represent normal embryo-larvae at 24h and 48h+ respectively and b and d represent morphologically abnormal embryo-larvae at 24h and 48h+. (e) represents an unfertilised egg.

The different abnormalities of D-larvae are shown in figure 2.8., they include convex hinges, incomplete or damaged shells and a protruding mantle.

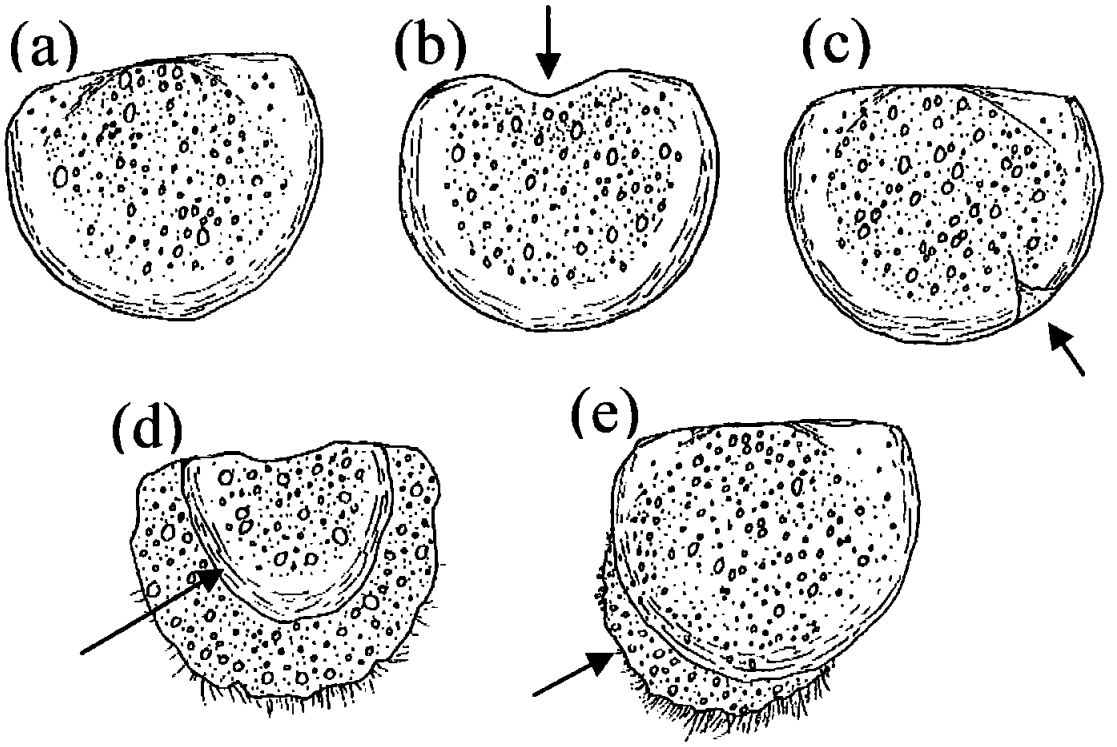


Figure 2.8. Different abnormalities observed in D-larvae of *M.edulis* (a) Normal D-larva; (b) convex hinge; c) indented shell margin; (d) incomplete shell; (e) protruding mantle. (Based on diagrams from His *et al.*, 1997).

Dead trochophores appear as a mass of degenerating cells with no cilia or flagellum movement. Dead D-larvae may lack cilia movement, lose clarity of internal organs and become transparent (Bayne, 1976; His *et al.*, 1999).

2.3.2 Preparation of metaphase chromosome spreads from embryo-larvae

Details of slide preparation from embryo-larvae of mussels and worms have been described in literature (Harrison and Jones, 1982; Jha *et al.*, 1995; 1996; 2000a; 2000b). Embryos (age depended on exposure scenarios) were exposed to radionuclides or test chemicals for 1½ or 2 cell cycles (for the analysis of Cabs and SCEs respectively) as summarised in figure 2.3. SCEs analysis requires cells that have replicated at least twice in

the presence of BrdU as described in figure 2.9. Cabs are analysed after 1½ as it is important that they have replicated at least once in the presence of the contaminant in order for any potential break to develop into an aberration. Although it is vital that the cell does not proceed through a subsequent division (2 cell divisions) and therefore the aberration might be eliminated from the cell or that the cell may have been broken down. Using SCD staining, earlier studies (Jha *et al.*, 2000a) have indicated that the cell cycle of *M.edulis* at approximately 15°C the average generation time (Ivett and Tive, 1982) of the embryo-larvae cells between 12 and 24h post fertilisation is approximately 3.8h. Using SCD staining the average generation time (Ivett and Tive, 1982) of the embryo-larvae cells of *P.dumerilii* between 12 and 24h post fertilisation is approximately 3.0h at approximately 20°C (appendix I). Harvesting of cells during this period provides optimal chromosome morphology to analyse cytogenetic damage as the cells are larger and the chromosomes are less condensed than at later stages (Jha *et al.*, 1996). After the appropriate incubation time the embryos were placed into 0.025% (w/v) solution of colchicine (Sigma, CAS Nº 64-86-8) for 30 min at room temperature to arrest the cells at metaphase. The embryos were then placed for 10 min into a series of hypotonic solutions. The hypotonic solutions were made up with seawater / KCl (0.56%) in the following ratios 2:1, 1:1, 1:2 and 1:3. After the hypotonic solutions were completed, the embryos were transferred into conical centrifuge tubes using glass pasteur pipettes. The embryos were centrifuged for 5-6 min at 2000 rpm. After centrifugation the supernatant was discarded and a few drops of cold (4°C) Carnoys fixative (1:3 (v/v) glacial acetic acid/methanol) was added. The embryos were left for a minimum of 24h in the fixative to ensure that all the material had been fixed. Approx. 4 drops of the fixed embryos were placed into labelled microscope slides using glass pasteur pipettes. A few drops of 60% (v/v) glacial acetic acid (in distilled water) were placed on top of the fixed embryo solution. The slide was then placed onto a hot plate (40°C) were it was occasionally rotated to ensure an even distribution of embryos on the slide. When the slide was dry it was checked for metaphase spreads using a phase-contrast microscope. The

diploid chromosome number (metaphase stage of mitosis) for both *P.dumerilii* and *M.edulis* is (2n) = 28.

2.3.3 Sister chromatid differential (SCD) staining of chromosomes

The methods followed to obtain sister chromatid differential staining was adopted from a method proposed by Goto *et al.*, (1975) with some modifications and has been described in literature (Czarnetzki, 1978; Dixon and Clarke, 1982; Jha *et al.*, 1997; 2000a; 2000b).

In order to identify cells which have divided once, twice, or thrice or more times (PRI) and to analyse sister chromatid exchanges (SCEs), differential staining of chromosomes is required. This is achieved by allowing the embryo-larvae to grow in 1.0 x 10^{-5} M of the chemical 5–bromo-2′-deoxyuridine (BrdU) (Sigma, CAS Nº: 59-14-3) for two cell cycles, simultaneously with exposure to the radionuclides and reference chemicals. Following exposure, the slides were processed as described in section 2.3.2.

Following examination of metaphase spreads using a phase contrast microscope the slides were processed for sister chromatid differential (SCD) staining (Goto *et al.*, 1975). The slides were placed into coplin jars and rinsed twice with distilled water. A 0.025% (w/v) solution of Hoechst 33258 also known as BisBenzimide H 33258 (Sigma, CAS Nº: 23491-45-4) prepared in phosphate-buffered saline (PBS) (Sigma) was added to the jars and the slides were incubated for 20 min in the dark at room temperature. The slides were rinsed twice in distilled water and allowed to air-dry. A few drops of PBS were placed onto the slides and a coverslip added. The slides were placed onto a hotplate at approx. 55°C and simultaneously exposed to a blacklight (F18W-BLB lights, Sylvania, UK for 25 min, after which the slides were rinsed in distilled water and the coverslip was removed. The slides were stained for 15 min in 10% (v/v) Giemsa stain (Giemsa stain (CAS Nº: 51811-82-6)/Giemsa buffer pH 6.4 both obtained from BDH), after which the slides were rinsed twice in distilled water and left to air-dry. When the slides were dry a few drops of DPX

mountant for microscopy (BDH) were added followed by a coverslip. The DPX covered slides were left to harden prior to microscopic analysis.

2.3.4 Analysis of Proliferative Rate Index (PRI)

Prior to scoring, slides were coded, randomised and then scored blind. Metaphase spreads were examined using a bright field microscope at a final magnification of x 1000. Metaphases were classified, as described in figure 2.9., as first (M1), second (M2) and third or subsequent (M3+) division cells.

Examples of each cell division are shown in figure 2.10. At least 200 complete metaphases were scored per treatment, 100 each from two replicates. PRI was calculated using the formula (based on a method proposed by Lamberti *et al.*, 1983):

$$PRI = ((1 \times M1) + (2 \times M2) + (3 \times M3)) / \text{Number of cells analysed}$$

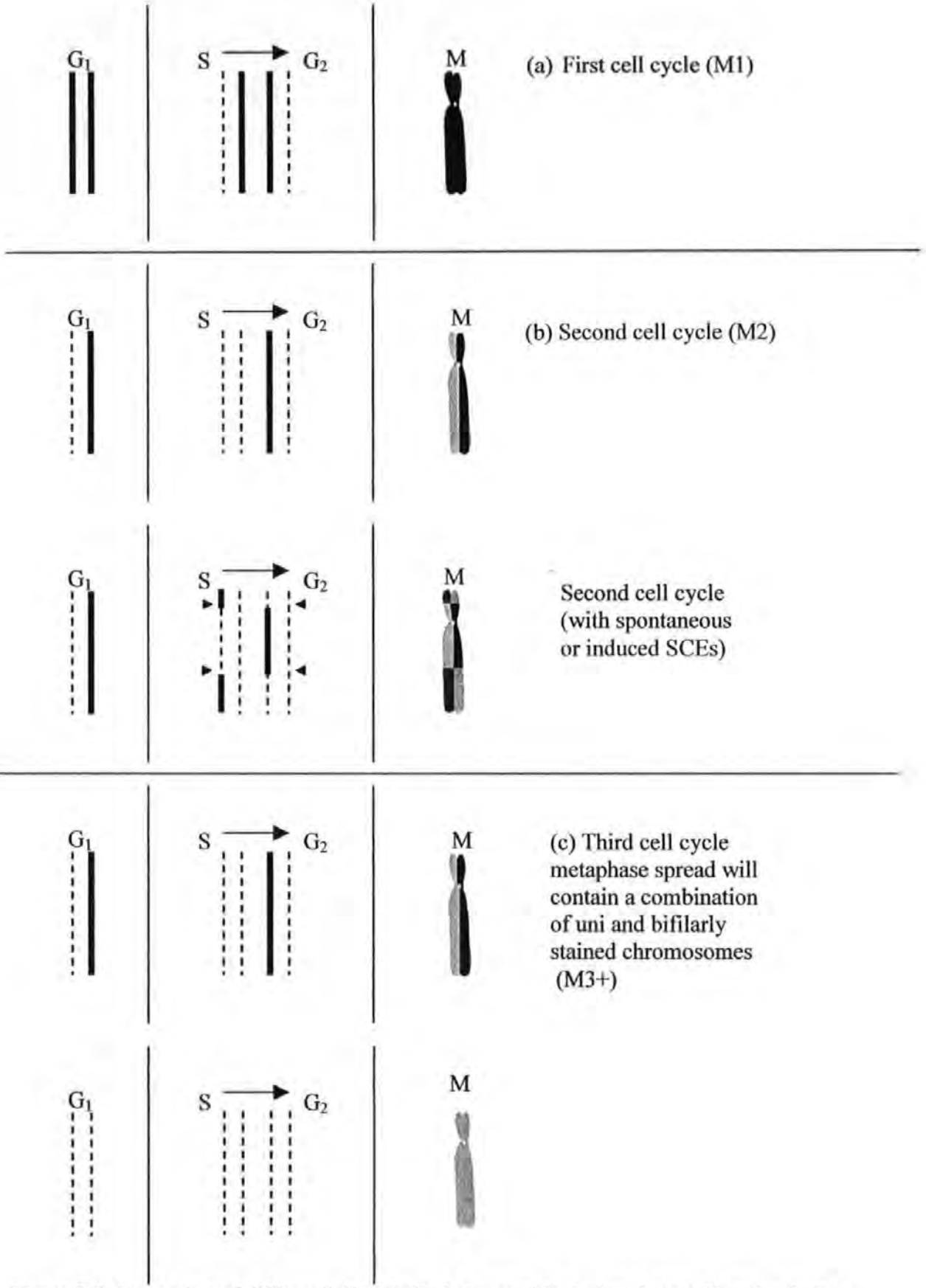


Figure 2.9. Formation of differentially labelled chromatids during three cell cycles in the presence of BrdU. G_1, Pre-synthesis stage; S, DNA synthesis period; G_2, post-synthesis period; M, metaphase (adapted from Dixon, 1983).

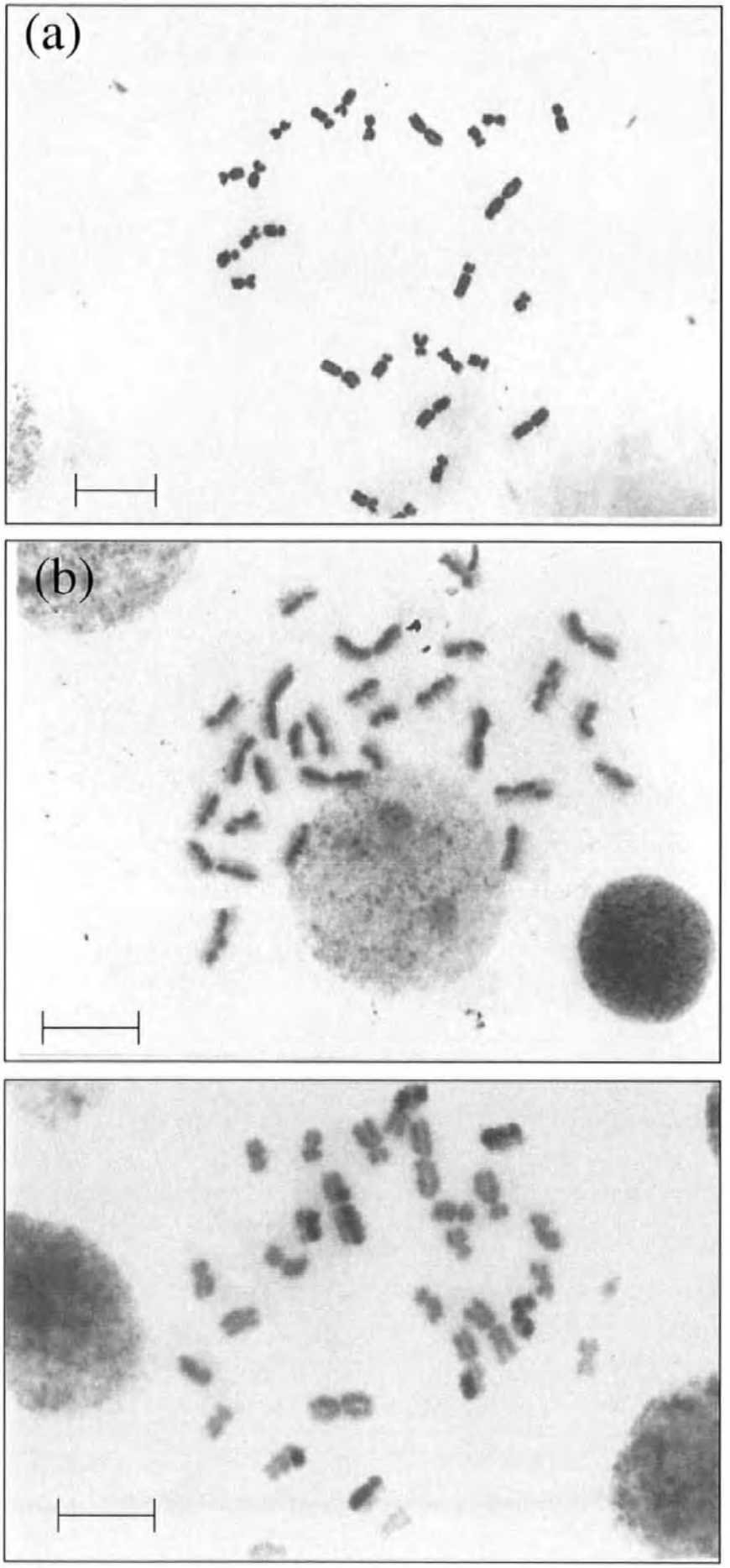


Figure 2.10. First (a), second (b) and third (+) (c) division cells from embryo-larvae of *P.dumerilii* following sister chromatid differential (SCD) staining. Scale bar = 10μm.

2.3.5 Sister Chromatid Exchanges (SCEs)

Sister chromatid exchanges are visually scored only in the 2nd cell cycle, (as previously described in figure 2.9) when sister chromatids are differentially stained. The method for obtaining differentially stained sister chromatids has been previously described in section 2.3.3. SCEs were scored at the same time as PRI. Only complete (28 chromosomes) 2nd cell division metaphases were analysed for the occurrence of SCEs. 50 metaphases per treatment were analysed for SCEs, 25 from each of two replicates, and the results were expressed as the SCEs frequency per cell. Examples of SCEs are shown in figure 2.11.

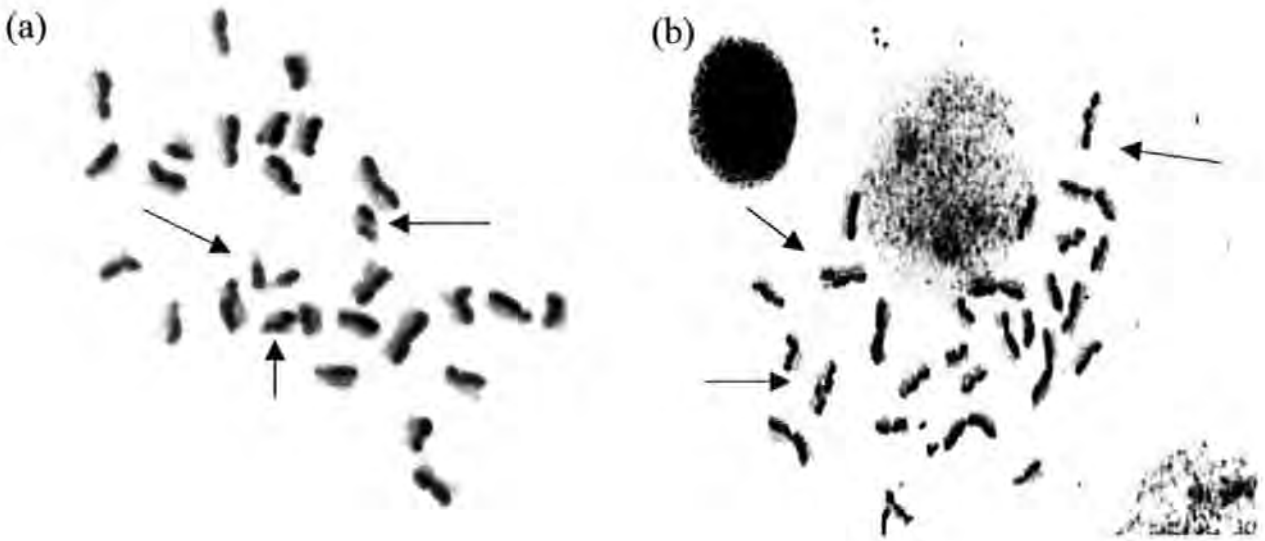


Figure 2.11. Sister chromatid exchanges (→) in metaphase spreads from embryo-larvae of (a) *M.edulis* (b) *P.dumerilii.*

2.3.6 Chromosomal aberrations

2.3.6.1 Preparation of metaphase spreads for the analysis of chromosomal aberrations (Cabs)

Embryos (age depended on exposure scenarios) were exposed to radionuclides or test chemicals for 1½ cell cycles (for the analysis of Cabs). *P.dumerilii* has a cell cycle of 3.0h and *M.edulis* has a cell cycle of approx. 3.8h as mentioned in section 2.3.2. and

appendix 1. After the appropriate incubation time the embryos were processed for preparation of metaphase spreads as describe in section 2.3.2.

After fixation of metaphase spreads onto the slides, the slides were stained for 25 min in 10% (v/v) Giemsa stain (Giemsa stain/Giemsa buffer), after which the slides were rinsed twice in distilled water and left to air-dry. When the slides were dry a few drops of DPX were added followed by a coverslip. The DPX covered slides were left to harden for 24h prior to examination using phase contrast microscopy.

2.3.6.2 Scoring criteria for chromosomal aberrations

Prior to scoring, slides were coded and randomised and then they were scored blind. Only metaphase cells that were intact and well spread were used for analysis. 200 cells were scored from each treatment, 100 from each of two replicates as recommended by the United Kingdom Environmental Mutagen Society (UKEMS) (Kirkland *et al.*, 1990). Table 2.1. and table 2.2. shows the different types of structural chromosomal and chromatid aberrations respectively: classification and definitions adapted by the UKEMS (Kirkland *et al.*, 1990). Chromosomal observations were expressed as the percent aberrant cells (including heavily damaged cells) and total aberrations (chromosome plus chromatid type excluding gaps).

Table 2.1 Structural chromosome type aberrations: classification and definitions

(Kirkland, 1990)

Aberration Type	Diagrams	
(1) Chromosome gap	 (*csg*, G)	A non staining region or achromatic lesion at the same locus in both chromatids with minimal misalignment of the chromatids
(2) Chromosome break	 (*ace*)	A discontinuity at the same locus in both chromatids giving an acentric fragment (ace)
(3) Chromosome exchange		Involving two or more loci in the same or different chromosomes
(a) Interchange, between chromosomes e.g. Dicentric with associated fragment	 (*dic + ace*)	Resulting from an asymmetrical exchange that also produces a fragment which is not scored as a separate event

Aberration Type	Diagrams	
(b) Intrachange, within a chromosome		
(i) between arms (inter-arm intrachange) e.g. Centric ring with fragment	 (*csr + ace*)	One fragment should be allocated to each ring and not scored as a separate event.
(ii) within an arm (intra-arm intrachange)	 (*cs min*, M)	Acentric rings are formed and are generally small and called minutes (cs min or M) or interstitial deletions

Table 2.2 Structural chromatid type aberrations: classification and definitions

(Kirkland, 1990)

Aberration Type	Diagrams	
(1) Chromatid gap	 (*ctg, g*)	A non staining region or achromatic lesion in which there is minimal misalignment of the chromatids
(2) Chromatid break	 (*ctb, c*)	A discontinuity in which there is a clear misalignment of the chromatid
(3) Isochromatid break	 (SU)	Showing complete rejoining or sister union (SU) of broken ends
	 (Nud) (Nup)	Incomplete rejoining (non-union, Nu) either proximally (p) or distally (d). Fragments may be aligned or displaced

Aberration Type	Diagrams	
(4) Chromatid exchange (*cte*)		
(a) Interchange between chromosomes		
(i) Asymmetrical	 (*qr*)	An acentric fragment and a dicentric chromatid are produced if rejoining is complete, sometimes called a quadriradial (*qr*)
(ii) Symmetrical	 (*qr*)	Does not lead to a dicentric chromatid or and acentric fragment unless the rejoining is incomplete. Also called a quadriradial.

Table 2.2. Cont. Structural chromatid type aberrations: classification and definitions (Kirkland, 1990)

Aberration Type	Diagrams		Aberration Type	Diagrams	
(b) Intrachange, within a chromosome (i) between arms (inter-arm intrachange)	 Asymmetrical (*ctr*)	Centric ring formed	(c) Isochromatid/ chromatid interchange (i/c) giving a triradial (*tr*)	 (*tr* (2 *cen*), i/c)	Dicentric triradial, fragment produced is not an independent aberration
	 Symmetrical (*ct inv*)	Produces an inversion (*inv*) in a chromatid		 (*tr* (1*cen*), i/c)	Monocentric triradial, scored as one aberration
(ii) within an arm (intra-arm intrachange)	 (*ct min*, m)	Minute or interstitial deletion, often remains associated with the chromosome of origin			

2.3.7 Controls

UKEMS guidelines for cytogenetic assays stipulates that a complete test contains (a) a minimum of three doses of test material, (b) positive control (c) solvent control (if necessary) and (d) negative control (Scott *et al.*, 1990). For all experiments seawater was used as the negative control and at least three concentrations of test material were tested. Acetone was used as a solvent control in the experiments containing tributyltin (TBT) and either ethylmethane sulphonate (EMS) or methylmethane sulphonate (MMS) were used as positive controls.

2.4 Biological assays using adult organisms

2.4.1 Haemolymph extraction

Blood cells or haemocytes of marine invertebrates have been extensively used in toxicological studies (Dopp *et al.*, 1996; Mersch *et al.*, 1996; Venier *et al.*, 1997). Figure 2.12. represents typical haemocytes (blood cells) from *M.edulis*.

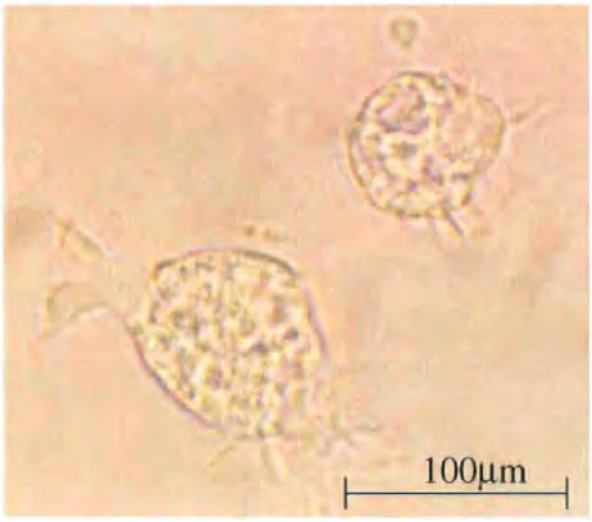
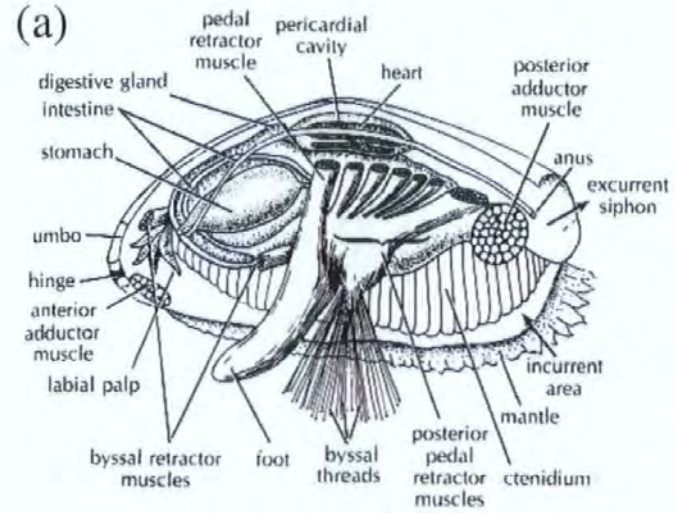


Figure 2.12. Typical live haemocytes of the marine bivalve *M.edulis*.

Adult mussels of approximately the same size (therefore approximately the same age) were removed from their tanks and the shells dried with tissue. Sharp scissors were inserted between the valves and gentle opened in order to gain access to the interior of the mussel. A 26-gauge needle attached to a 1ml syringe was gently introduced into the posterior adductor mussel as shown in figure 2.13.



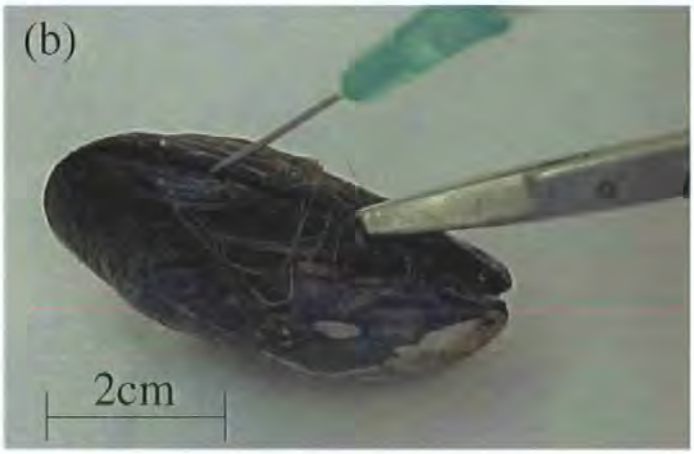


Figure 2.13. (a) diagram of internal morphology of *M.edulis,* (Boyle, 1981)

(b) obtaining a haemolymph sample from the posterior adductor muscle.

Approximately 0.5 ml (approx. 500,000 to 1,000,000 cells) of free circulation haemolymph was then drawn up into the syringe. Only mussels whom valves closed after haemolymph extraction were returned to the aquarium as repeated extraction was possible.

2.4.2 Cell viability

Eosin Y (Sigma, CAS Nº: 15086-94-9) solution was prepared to a final concentration of 2 mg/ml of water. An aliquot of 20 µl of haemolymph was placed onto a microscope slide and 1 µl of Eosin Y solution added. Living cells appeared green in colour

whereas dead cells were red (figure 2.14.). The percentage viability was calculated as living cells / total cells counted. Only cell samples with viability greater than 95% were used in the following assays.

Figure 2.14. *P.dumerilii* coelomocytes stained with Eosin Y (green = alive, red = dead).

2.4.3 Neutral red retention assay (NRR)

The neutral red technique measures the retention of neutral red, a weak base dye, within the lysosomal compartment. The dye is added to isolated live blood cells and permeates into the lysosome in its unprotonated form where it becomes trapped by protonation inducing perturbations such as swelling and enhanced autophagy (Wedderburn *et al.*, 2000). Lysosomal integrity has been utilised in a number of field studies as a biomarker for the consequences of environmental contamination (Regoli, 1992; Lowe *et al.*, 1995; Wedderburn *et al.*, 2000).

Haemolymph was drawn into a syringe containing approx. 0.5 ml of physiological saline (20 mM HEPES, 435 mM Sodium Chloride, 100 mM Magnesium Sulphate, 10 mM

Potassium Chloride, 10 mM Calcium Chloride, pH 7.36). After mixing, 200 µl of the haemolymph solution was pipetted into 2 replicate microplate wells of a Sterilin flat bottom 96 well non-sterile microtitre plate (Fisher Scientific UK). The plate was sealed and left for 45 min until the cells had adhered to the bottom. After the designated incubation time of 45 minutes excess haemolymph solution was thrown away. 200 µl of 0.33% neutral red (in distilled water) was pipetted into each well and left for 3h, after which time the neutral red solution was removed by turning the plate upside down over a sink. The cells were carefully washed three times in physiological saline. Aliquots (200 µl) of 1% acetic acid in 50% ethanol were added to all the wells. The plate was gently shaken and then read at 550 nm using a Molecular Devices optimax tunable microplate reader. The solution was removed and the protein concentration of each well calculated as follows. The amount of neutral red per well is dependent on the number of cells and by calculating the amount of protein the amount of neutral red may be standardised per mg of protein (Pipe *et al.*, 1999). The protein concentration per well was analysed using a bicinchoninic acid protein assay (Pierce Chem. Co. N° 23223). To each well, 200µl of reagent solution (500 µl reagent B + 25 mls reagent A) was added. Blanks were set up containing 200 µl of protein reagent and 10 µl of physiological saline and Bovine serum albumen (Sigma) was used as standards ranging between 0-2 mg/ml at intervals of 0.5 mg/ml. The plate was sealed with a Mylar plate sealer (Dynex technologies Inc.) and incubated at 37°C for 30 min after which the seal was removed and the plate was read at 562 nm as previously described. The results were presented using the Soft Max Pro ©[1998] version 2.4.1 software (Molecular Devices Corp.) as the optical density / mg protein, with the healthier cells retaining more neutral red (Coles *et al.*, 1995, Pipe *et al.*, 1999).

2.4.4 Micronucleus (Mn) assay

Approximately 0.5ml of haemolymph (approx. 500,000 to 1,000,000 cells) was placed on slides that had been pre-coated in 10% poly l-lysine solution that facilitates the

adherence of cells to the slides. Slides were placed into a humidifier for 30mins to allow the cells to adhere to the slide, after this time the excess solution was removed by tilting the slide on a sheet of absorbent tissue and allowing the excess solution to run onto the tissue. The slides were left to air dry and then fixed in methanol GPR (BDH) for 15 mins, followed by staining in 5% Giemsa/buffer solution for 20mins. A few drops of DPX mountant for microscopy (BDH) and a coverslip were placed onto the slides to permit scoring under x100 (oil immersion). Slides were scored blind under x40 and Mn validated under oil immersion. A total of 1000 cells were analysed per mussel. Figure 2.15 represents typical micronuclei observed in haemocytes of *M.edulis* (x100 magnification). The Mn were identified according to the following criteria: (1) diameter smaller than one-third of the main nucleus but greater than one-tenth (2) no contact with nucleus (absence of chromatid bridge) (3) colour and texture resembling the nucleus (4) spherical cytoplasmic inclusions with sharp contour (Countryman and Heddle, 1976). Figure 2.16 demonstrates micronuclei that are not included in scoring.

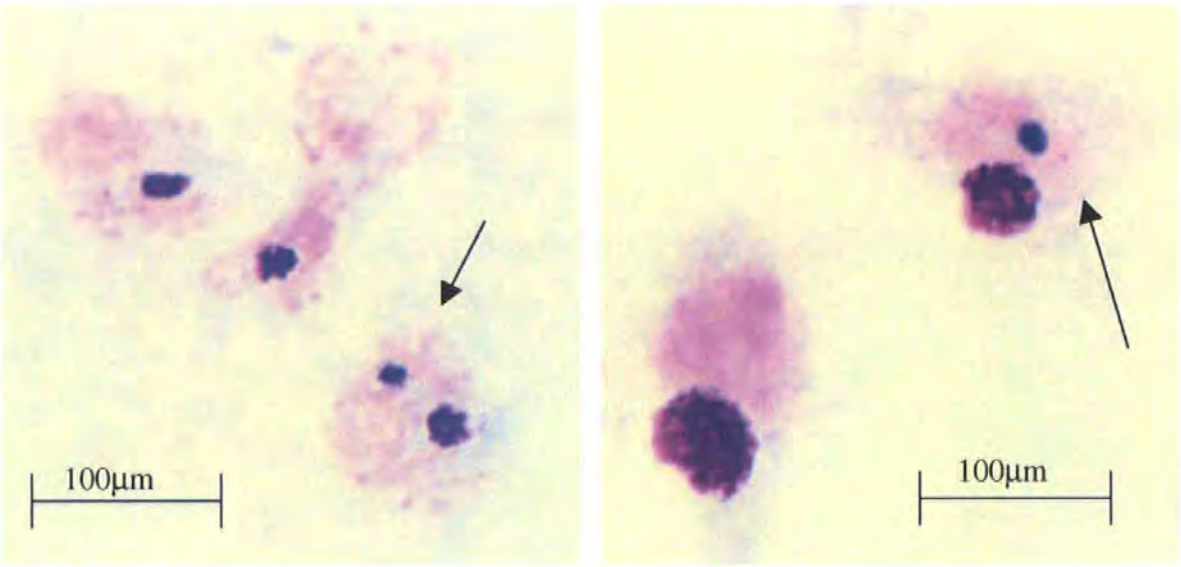


Figure 2.15. Micronuclei (→) in haemocytes of *M.edulis* stained with Giemsa.

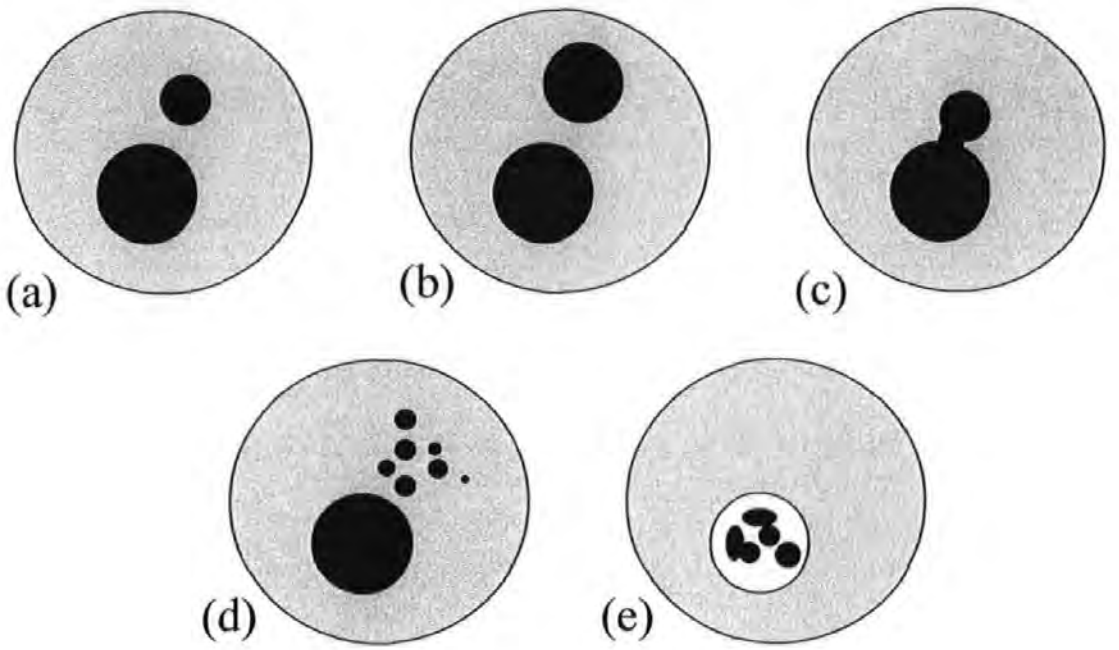


Figure 2.16. Criteria adopted to analyse for structures that are not micronuclei

(a) normal cell containing one nucleus and a micronuclei, (b) binucleate cell in which one of the nuclei is relatively small but has a diameter greater than one-third the diameter of the other nuclei, (c) nuclear blebs that are still attached to the main nucleus (nucleoplasmic bridge), (d) dense stippling in a specific region of the cytoplasm, (e) apoptotic cells where the nucleus has disintegrated (adapted from Fenech, 1996).

2.4.5 Single cell gel electrophoresis (SCGE) or "Comet assay"

2.4.5.1 Comet assay protocol

The comet assay was adapted from a protocol described by Singh *et al.* (1988) with modifications adopted by different authors for use with marine/aquatic organisms (Mitchelmore *et al.*, 1998; Nacci *et al.*, 1996; Steinert *et al.*, 1998). The protocol followed is summarised in figure 2.17.

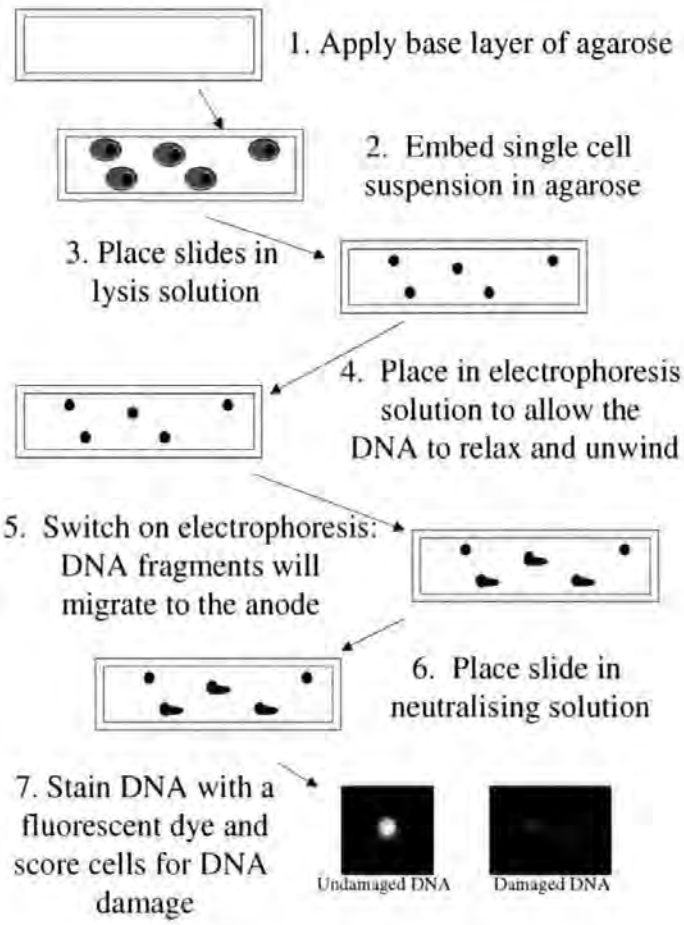


Figure 2.17. Summary of "Comet assay" protocol

Two aliquots containing 85 µl of melted high melting point (HMP) agarose (1% agarose in TAE solution which contains 40 mM Tris and 1 mM EDTA) was pipetted onto a frosted slide. Each drop of HMP agarose was covered with a 22 x 22 mm coverslip and the slide was placed at 4°C for 10 min until the agarose had set after which the coverslips were removed.

100 µl of haemolymph (approx. 100,000 to 200,000 cells) was placed into 1 ml of 4°C physiological saline (20 mM HEPES, 435 mM Sodium Chloride, 100 mM Magnesium Sulphate, 10 mM Potassium Chloride, 10 mM Calcium Chloride, pH 7.36) which were kept on ice. The cell suspension was centrifuged at 200 xg at 4°C for 2-3 min and the supernatant was removed. 85 µl of low melting point (LMP) agarose (1% agarose in Kenny's salt solution which contains 400 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO4, 2 mM

$NaHCO_3$) was pipetted on to the cell pellet. The cell suspension was then carefully pipetted onto the HMP agarose covered frosted slide, sandwiching the cells between two layers of agarose gel. A 22 x 22 mm coverslip was placed onto the LMP agarose and the slides were placed at 4°C for 10 min until the agarose had set. After the gel had set the coverslips were removed and the slides placed into a freshly prepared lysing solution at 4°C for 1h (2.5 M NaCl, 100 mM Na^2-EDTA, 10 mM Tris, 1% Na sarconisate, pH 10.0, immediately prior to use 1% Triton X-100 and 10% DMSO was added to the lysis solution). The lysis and following steps of the comet assay protocol were carried out in the dark and at 4°C to prevent any additional damage to the cells DNA.

The slides were then removed from the lysis solution and placed on a horizontal gel electrophoresis tank. The tank was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM Na^2-EDTA) and the DNA was allowed to unwind for 40 min before electrophoresis, which was carried out at 25 V for 30 min. After electrophoresis the slides were washed three times, for 5 min each time, in neutralising buffer (0.4 M Tris, pH 7.5) to remove alkali and detergents which would interfere with staining. The slides were stained with 20 µl of 5 µg/ml, 4',6-Diamidine-2-phenylindole dihydrochloride (DAPI) (in distilled water) solution and covered with a coverslip. DAPI has maximum absorbency at $\lambda = 340$ nm and an emission maximum at $\lambda = 488$ nm. The slides were viewed under ultraviolet fluorescence light using a Leica DMR microscope (Leica Microsystems Ltd. UK).

2.4.5.2 Scoring of comet assay

A total of 300 randomly chosen cells were scored for each treatment, 50 per replicate organism. Comets were classified as described in figure 2.18.

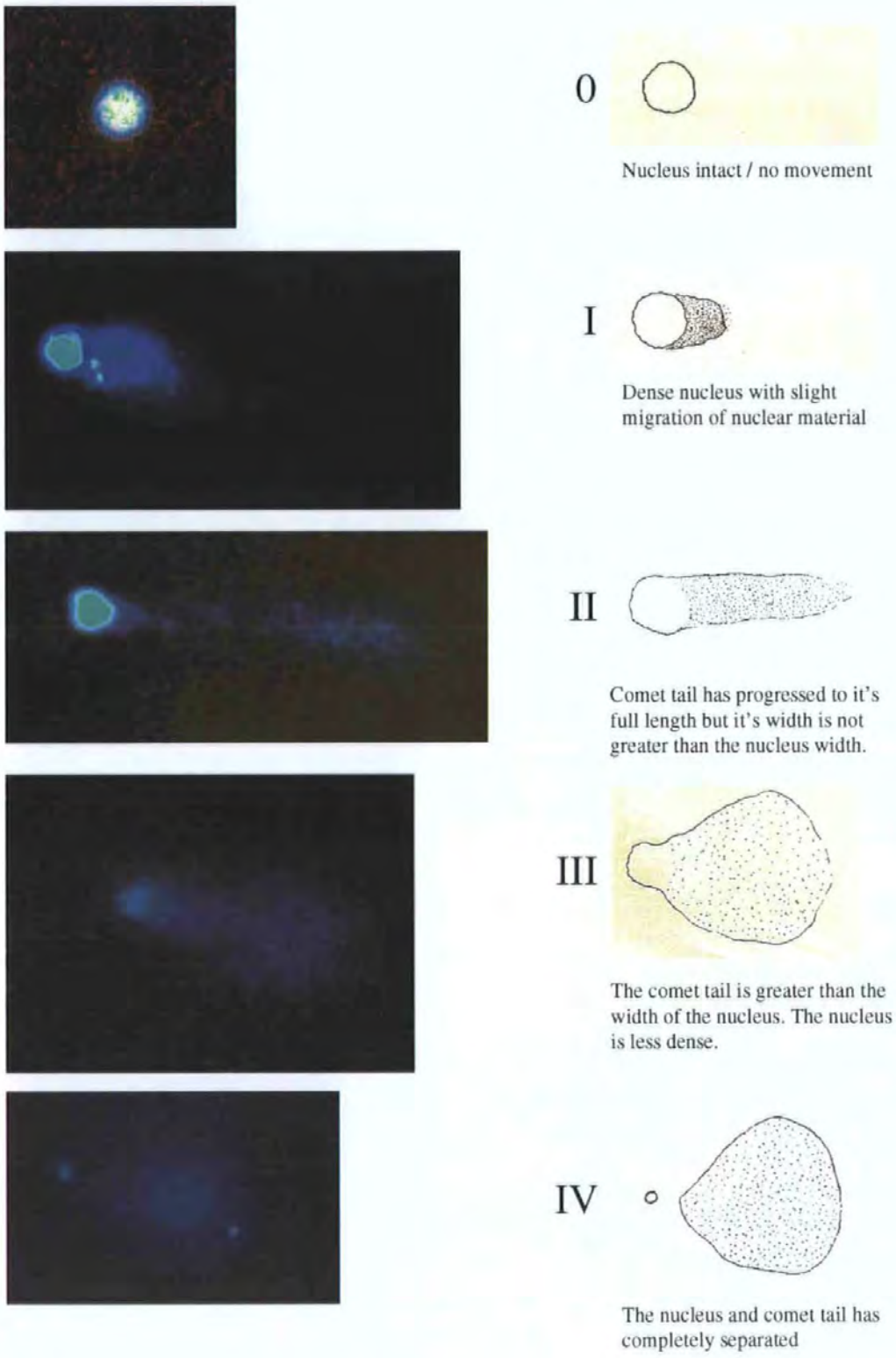


Figure 2.18. Classification of "comet" into 5 classes. (Based on Collins *et al.*, 1997).

In class 0, the nucleus is intact and there is no migratory movement visible. Class 1 comets have a dense nucleus region with obvious but only slight DNA migration. Class 2 comets still process a clear nucleus region but the tail is much more pronounced than in class 1 and it is likely to reach the full length of DNA migration. Class 3 is distinguished by a characteristic pear shape, in which the majority of the DNA material is present in the tail and very little is left in the nucleus head. Class 4 is defined by the complete separation of the comet tail and the nucleus, these cells are also defined as apoptotic cells (Devaux *et al.*, 1997; Olive and Banáth, 1995; Olive *et al.*, 1993). Visual scoring of the comets was validated to correlate with the length of the comet tail using a computerised image analyse package, Image Pro® Plus version 4.1 (Media cybernetics®, USA). Figure 2.19. shows the correlation the visual classification and tail length between 200 randomly chosen comets ($P<0.00005$; $R^2=88.52\%$).

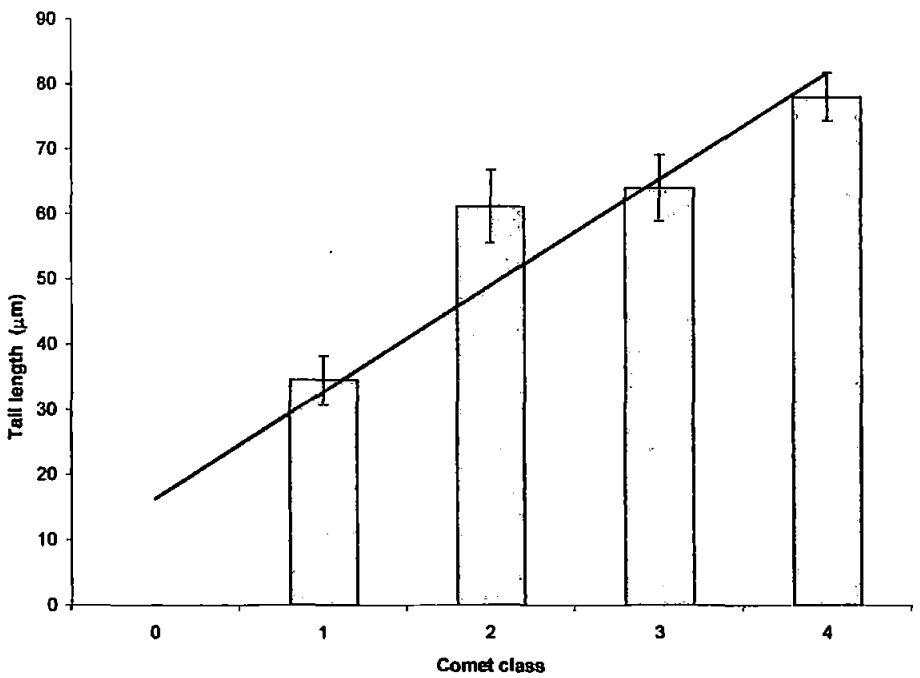


Figure 2.19. A graph showing the correlation between visual (class) classification and tail length ($P=0.00005$; $R^2=88.52\%$).

2.5 Detection of intrinsic mutagenic activity of seawater by Ames test

The *Salmonella* bacteria mutagenicity assay or Ames test was designed by Bruce Ames to identify mutagenic chemicals. When potential mutagens are tested against stains of *Salmonella typhimurium* the appearance of wildtype revertants gives an indication of mutagen activity. The *Salmonella* test was first validated in a study of 300 chemicals and is now widely used as standard in environmental management programmes and product assessments to test for mutagenicity (Maron and Ames, 1983).

Minimal glucose agar were produced containing 1.5% (w/v) agar, 5% (v/v) of 40% glucose, 2% (v/v) 50X VB salts (Vogel-Bonner medium E 50X) (10 mM Magnesium sulphate, 52 mM Citric acid, 287 mM Potassium phosphate, 84 mM Sodium ammonium phosphate). Following autoclaving, 30 ml of minimal glucose agar medium was poured into sterile plastic petri plates and left to set. Top agar containing 0.6% (w/v) agar and 0.5% (w/v) sodium chloride was autoclaved. 10 ml of a sterile solution containing 0.5 mM L-histidine.HCl (Sigma, CAS N^{o}: 70-00-1) and 0.5 mM biotin was added to the molten top agar and mixed by swirling. Cultures of TA1535 and TA1537 *Salmonella typhimurium* (Xenometric, Inc. USA) were removed from liquid nitrogen and placed into a shaker 24h prior to use. *Salmonella* strain TA1535 detects base pair substitution and strain TA1537 detects frameshift mutations (Maron and Ames, 1983). 100 µl of *Salmonella* strain and 100µl of test sample (filtered autoclaved seawater) were added to the molten top agar and then immediately poured onto the set minimal glucose agar. The top agar was left to set for 30 min after which the plates were transferred to an incubator at 37°C for 48h. Blanks were used to correct for the occurrence of any spontaneous reversions and Methlymethane sulphonate (1.0×10^{-3} MMS) was used as a positive control. 10 replicate plates per sample site were scored and the number of revertant colonies recorded after 48h.

2.6 Heavy metal analysis

2.6.1 Total concentration of heavy metals in sediment

Sediment cores were dried in an oven at 60°C for 48h and then sieved (500 μm mesh). 1 g of the fine fraction of sediment was weighted out and placed into a centrifuge tube with 10 ml of 0.25% EDTA (Sigma). The tube was sealed with a bung and shaken vigorously for 2 min, after which the samples were centrifuged at 2000 rpm for 5 min. The supernatant was decanted into 25 ml volumetric flask. The residue of sediment was resuspended in 10 ml of deionised water and recentrifuged. The process was further repeated with 5 ml of water. The supernatant was adjusted to 25 ml and filtered through 0.45 μm filter paper. The solutions were then analysed by Atomic Aborption Spectrophotometry (AAS) using the SpectrAA 600 (Varian, UK) (Devkota, 2001).

2.6.2 Biologically available concentration of heavy metal in sediment

Sediment cores were dried in an oven at 60°C for 48h and then sieved (500 μm mesh). 1 g of the fine fraction of sediment was weighted out and placed into a 100 ml conical flask. 10 ml of 0.5 M Hydrochloric acid was added to the flask. The solution was brought to the boil and then left to simmer for 20 min. After the solution had cooled it was transferred to a centrifuge tube. The solution was centrifuged at 2000 rpm for 5 min. The supernatant was poured into a 50ml volumetric flask. The residue was resuspended in 10 ml of deionised water, the solution was centrifuged and the supernatant poured into the 50 ml flask. This process was further repeated with another 10 ml. The supernatant was adjusted to 50 ml and filtered through 0.45 μm filter paper. The solutions were then analysed by Atomic Aborption Spectrophotometry (AAS) using the SpectrAA 600 (Varian, UK) (Ma, 1982).

2.6.3 Concentration of heavy metals in seawater

The seawater samples were filtered through 0.45 μm filter paper and the samples were placed into brown acid washed glass 1 litre bottles and stored at 4°C. The samples were analysed within a week of collection by Atomic Aborption Spectrophotometry (AAS) using the SpectrAA 600 (Varian, UK) (Buffin, 1999).

2.7 Radioactivity measurements

2.7.1 Liquid Scintillation Counting (LSC)

A liquid scintillation sample consists of three components: radioactive material, a solvent and a fluor (or scintillator) so that the emitted radiations are in intimate contact with the detector. A particle of radiation emitted by the sample material is absorbed in, and its energy transferred to the solvent and then to the phosphor which emits a scintillation of light photons. These photons are absorbed by the photocathode of a photomultiplier tube that converts them into an electronic pulse. The pulse, after simple amplification, is registered as a count corresponding to the emission of the particle or radiation. A configuration of a typical LSC is shown in figure. 2.20. (Valkovic, 2000).

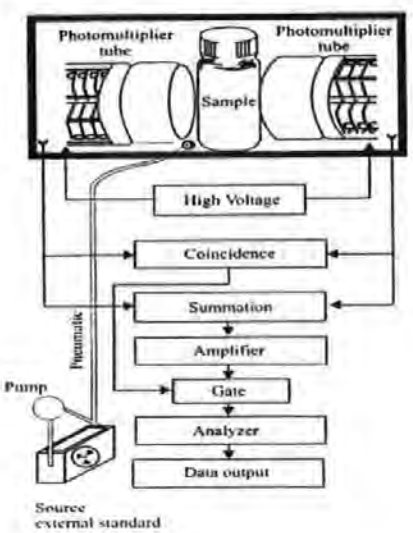


Figure 2.20. Configuration of a typical scintillation counter (Valkovic, 2000).

Pre and post-tritium levels were calculated using 100 µl of test solution in 5 ml of liquid scintillation cocktail, Packard Ultima Gold (Sigma-aldrich Ltd.). Scintillation vials containing the solution were placed into a scintillation counter (Beckman LS6500, USA), and read for 10 min (detection limits range from 0.06 keV-14.5 keV). Readings were displayed as counts per minute (CPM) and then converted to kBq/ml as described in section 4.3.1.

2.7.2 Dose calculations following exposure to radionuclides

The estimated dose to growing embryo-larvae developing in tritiated water (^3H), an β emitting radionuclide, was calculated by a method used by Strand *et al.*, (1977) on fish larvae. The dose equation is as follows:

$$D_\beta = 2.13\varepsilon_\beta C$$

where,

D_β = dose in rads per hour, ε_β = average beta-ray energy in MeV (0.00569), and C = concentration of ^3H in µCi/ml.

These calculations were based on the assumptions that tritium (^3H) was uniformly distributed within the embryo-larvae over the exposure period, and secondly that no concentration of tritium above a water equilibrium level occurred in the embryos.

2.8 Statistical analysis

Statistical analysis was carried out using the statistical software package STATGRAPHICS Plus for Windows 3.1 © from Statistical Graphics Corp. 1994-1997.

The data was plotted and analysed to assess if it was normally distributed and not skewed by analysing the standard deviations between the samples as it is assumed that if

the data is normally distributed the standard deviations would be equal. The variance of the data was checked using Bartlett's test. If the data was normally distributed, one way Analysis of Variance (ANOVA) was carried out on the data to determine if there was a statistical difference between the means of the data set. Multiple range tests then displayed which means were statistically different from each other as calculated using Fisher's least significance (LSD) procedure.

If the data was not normally distributed it was transformed in attempt to standardise the data. Transformation of percentages was carried out by (ASIN(SQRT(data))) (Burgeot *et al.*, 1995). Transformation of values (e.g. Mn frequencies) was carried out by either logging the data (LOG(data)) or by taking the square root (SQRT(data)). After transformation if the data was normally distributed, ANOVA was carried out to analyse variations of the mean. If after transformation the data was still not normally distributed the medians of the data were analysed using the non-parametric Kruskal Wallis test (Sparks, 2000). For the Kruskal Wallis test the data from all levels were combined and ranked in order from smallest to largest. The average rank was then computed for the data at each level.

Linear regression analysis was carried out to test for a relationship between parameters (data sets) and to calculate/predict LC_{50}'s. Logarithmic regression was used for assessing the correlation between dose and the induction of chromosomal aberrations. No restricts were placed on any the regression methods e.g. intercept defined or outliners excluded.

Chapter 3

Validation of developmental, cytotoxic and genotoxic assays using embryo-larvae of two marine invertebrates using the chemicals tributyltin (TBT) and methylmethane sulphonate (MMS)

(Based on papers published in peer-reviewed journals: Appendix 2, 3 and 4)

3.1 Introduction

3.1.1 Validation of techniques

The principle of genotoxic tests is to detect if a compound can interact and damage DNA. Over 200 tests have been developed to detect various genotoxic lesions at different levels of biological organisation. These tests vary in complexity and cover a wide range of cell types and genetic endpoints (Elliott, 1994). Nevertheless, a large proportion of these tests are unsuitable for use with marine organisms as they either focus on the detection of carcinogenicity in mammalian systems or are based on cultured cells. From the remaining tests available, a selection of tests can be chosen that represent the various biochemical-cellular genotoxic endpoints. The framework for this research requires a broad view of the consequences of genotoxic exposure, and ultimately mechanisms and effects from different levels of biological organisation, (DNA up to population), needs to be adopted (Anderson *et al.*, 1994b). In selecting which tests are appropriate, the following criteria may be adopted, *relevance*, *validation*, detection of *genotoxic lesions*, *quantitative sensitivity*, *convenience* and *cost-efficiency* (Gert-Jan de Maagd and Tonkes, 2000). Although the criteria described above were selected for the evaluation of genotoxic assays, its principles can be applied to all aspects of toxicity testing.

The importance of validating genotoxicity tests has long been recognised in human health and safety. Before a test can be routinely used it needs to be assessed to determine whether it detects or predicts, accurately, effects that are recognised as being detrimental to the health of an organism (Clayson *et al.*, 1991). Although this has been adopted as standard practice in human health, it is often overlooked in evaluating genotoxic effects in the natural biota or wild species. *In vitro* and bacterial test systems can be used as screening tools to define intrinsic genotoxicity of a substance however for a definitive ecotoxicological risk assessment the genotoxic expression in ecologically relevant organisms should be considered. By using ecologically relevant organisms the environmentally realistic routes of exposure, the effects of metabolism and DNA repair

efficiency can be taken into account (Jha *et al.*, 2000a). Genotoxicity assays provide the means to evaluate complex industrial effluents and environmental mixtures, fill important data gaps that hinder risk assessments and management, and guide the use of limited resources for the control, elimination, and clean-up of toxic substances. Bioassays evaluation genotoxicity can be an integral tool in the evaluation of complex environmental wastes and effluents (Claxton *et al.*, 1998).

The validation of a test is determined by testing its accuracy, sensitivity and specificity (Gert-Jan de Maagd and Tonkes, 2000). Newly developed tests are often validated by comparing their performance with existing, well-established tests. However, since most of the assays used in marine genotoxicology are adapted from pre-existing, validated mammalian tests the need for validation is not based solely on the test but on the validation of existing tests on new organisms with known mutagens. Therefore the aim of this study was the validation of the developmental stages of two marine invertebrates, *P.dumerilii* and *M.edulis*, and the validation of mammalian based cytotoxic and genotoxic assays (chromosomal aberrations, sister chromatid exchanges and proliferative rate index) using embryo-larvae stages of the invertebrates after exposure to a potential (tributyltin) and reference (methylmethane sulphonate) genotoxin.

3.1.2 Organism selection

The selection of organism depends largely on the hypothesis that needs to be addressed. If the potential genotoxic effects of a pollutant or contaminant were to be assessed using a cytogenetic approach, ideal species would need to possess a suitable karotype or chromosomal complement.

Ideally, for cytogenetic assays, there needs to be a rapid turnover of cells and therefore, for most chromosomal based techniques, the embryo-larvae stage of species is used (Dixon, 1983; Hutchinson *et al.*, 1998; Jha *et al.*, 2000b, Pesch *et al.*, 1981). However, this is often limited to laboratory based exposures. Many vertebrates and

invertebrates have large numbers of very small sized chromosomes and may also have low cell division rates probably due to living in cold temperatures. Therefore, in order to overcome the problem of unsuitable karotype and low cell turnover in adult organisms, other techniques may be used that study DNA damage at molecular or cellular levels, (i.e. DNA strand breaks, micronuclei induction etc.), thus avoiding the analysis of chromosome complements as discussed in chapter 2.

If field based exposures are suspected, then different criteria for species selection need to be adopted. Most importantly is whether, or not, the organisms are sedentary, territorial or inter-tidal, in order to localise the exposure source. It is also relevant to assess the organisms' ecological and commercial importance, to anticipate if the exposure will have population or human health effects (Taylor *et al.*, 1999). For field based experiments, however, it is important that the organism and genotoxic tests have been previously validated under laboratory conditions to verify the sensitivity, reproducibility and reliability.

3.1.3 Dose selection

Genotoxic assays are vital for the screening of possible mutagens and therefore it is important that these assays are robust and in this respect one of the most important protocol aspects is dose selection (Mackay, 1995). Cytotoxic potential of contaminants is considered to be a major confounding factor in evaluating genotoxic effects (Mendelshon *et al.*, 1992; Moore *et al.*, 1992). Dose selection is usually based on environmentally realistic concentrations or those based on previous experiments, which were shown to have an effect. For example, the range of TBT concentrations used in these validation experiments was adopted from earlier work reported on mussels and oyster embryos, as discussed later (Dixon and Prosser, 1986; Alzieu, 1991). However, the test concentrations were modified and selected from the semi-logarithmic series (i.e., 0.31, 0.54, 1.75, 3.11, 5.44, 10.51 $\mu g\ l^{-1}$; w/v etc.) routinely used for aquatic toxicity testing (Doudoroff, 1951).

An alternative to the previous described methods of dose selection is the use of the maximum tolerated dose (MTD). In regulatory mammalian *in vivo* genotoxicity assays, for a test material to be described with confidence as negative, it is necessary to evaluate it at sufficiently high dose level (Mackay, 1995). Determination of MTD is considered to be necessary in regulatory genotoxicological assays due to the toxic potential of chemicals which is considered to have major confounding influence in interpreting results for genotoxicity evaluation (Mackay, 1995). Although there is some discrepancy and ambiguity in defining MTD in mammalian genotoxicity testing, in aquatic genotoxicology, especially with the use of invertebrates, it is easier to determine apparent toxic effects by evaluating various behavioural, physiological, morphological and developmental endpoints following exposure to a contaminant (Hebel *et al.*, 1997; Jha *et al.*, 2000a). This dose level is expected to produce some indication of toxicity either on the target cells for the assay or at the whole animal level in terms of survival (Mackay, 1995).

3.1.4 Reference toxic and genotoxic agents: Tributyltin and methylmethane sulphonate (MMS)

Organotin compounds, particularly tributyltin (TBT) and triphenyltin (TPhT) are widely utilised in the production of biocides, as catalysts for the production of polyurethane foams and in the stabilisation of polyvinyl chloride (Mizuhashi *et al.*, 2000). Amongst the use of biocides, in a variety of consumer and industrial produces, antifouling paints are the most important contributor of organotin compounds to the aquatic environment, where they are known to cause deleterious effects to non-target organisms (WHO, 1980; Alzieu, 1991). These exclusively anthropogenic compounds are considered to be one of the most toxic agents entering the marine environment (Goldberg, 1986).

The environmental effects of the leachate from anti-fouling paints on marine organisms first became apparent in the late 1970s when it was suspected to be the cause of declining oyster production in France and subsequently in different parts of the world

(Alzieu, 1991). This led to the implementation of legislation in Europe and North America to restrict the use of tributyltin based paints on small boats (< 25 m in length) in order to reduce concentrations around marinas and in estuaries. However, TBT from larger vessels, which were excluded from the ban, remains an environmental concern around the world especially around ports where they continue to affect wildlife (Morgan *et al.*, 1998; Takahashi *et al.*, 1998, 1999).

Tributyltin compounds are known to produce a variety of pathological conditions in marine organisms. Documented effects include impairment of growth, development, reproduction, and survival of many marine species (Heard *et al.*, 1989). Of particular concern has been the induction of "imposex" in neogastropods wherein male sex organs, notably a penis and sperm duct (vas deferens), are superimposed onto the female of gonochoristic gastropods. Normal egg-laying is prevented and ultimately results in a population decline (Bryan *et al.*, 1986). This masculization phenomenon is considered to be the best example of disruption of the endocrine functions among invertebrates unequivocally linked to a specific environmental pollutant. This dramatic effect on the reproductive organs is reported to be due to an increased level of testosterone titers that masculinize TBT-exposed females, although the mechanisms by which increased levels of testosterone are produced has not been fully elucidated (Matthiessen and Gibbs, 1998). It has been suggested that organotin compounds act as competitive inhibitors of cytochrome P450-mediated aromatase (Matthiessen and Gibbs, 1998) leading to hormonal imbalance (Lee 1991; Matthiessen and Gibbs, 1998). In this context, hormonal imbalance has been shown to results in embryotoxicity, teratogenicity and carcinogenicity (IARC, 1979).

Methyl methanesulfonate (MMS) (Sigma, CAS Nº: 66-27-3) is a colourless liquid that is also know as dimethyl sulphite, methyl mesylate and methyl ester of methanesulphonic acid. It has a molecular formula of $C_2H_6O_3S$ and a molecular weight of 110.14. It is a confirmed carcinogen with carcinogenic and neoplastigenic data. It is poisonous by ingestion, intraperitoneal, intravenous and subcutaneous routes. The

concentrations used in this study were adopted from earlier studies using embryo-larval stages of a polychaete worm and bivalve mollusc (Jha *et al.*, 1996, 2000a). In view of the documented toxic (TBT) and genotoxic (MMS) effects, the chemicals were selected for this study for validation purposes.

3.2 Methods

Initially, TBTO (obtained from Lancaster Synthesis Ltd., UK) was dissolved in acetone (as it is not soluble in water) to provide a stock solution of 100 mg l^{-1} (97.3 mg l^{-1} as TBT), which was later diluted for use. After initial range finding exposures, embryo-larvae of *P.dumerilii* were exposed to 0.31, 0.54, 1.75, 3.1 µg/L of TBT, whereas *M.edulis* were exposed to 0.54, 1.75, 3.1 and 5.4 µg/L. A single concentration of acetone (0.01% (v/v)) and the reference mutagen, methyl methane sulphonate (MMS: 1 x 10^{-3} M) was used to serve as solvent and positive controls respectively.

The experimental protocol, including the evaluation of developmental, cytotoxic and genotoxic effects, and linking different levels of biological organisation, is summarised in figure 3.1.

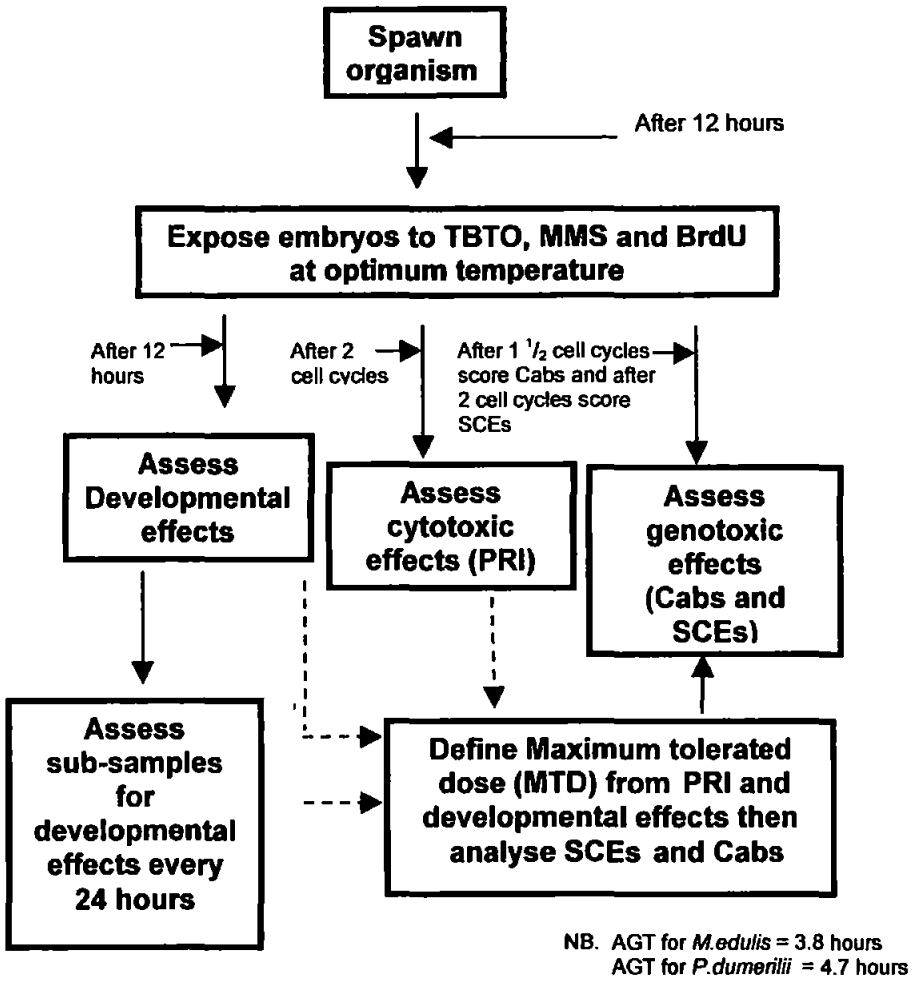


Figure 3.1. The protocol for analyses of developmental, genetic (chromosomal aberrations and sister chromatid exchanges) and cytotoxic (proliferative rate index) effects on *P.dumerilii* and *M.edulis*. AGT = average generation time.

Gametes and subsequently embryo-larval stages of *M.edulis* (collected as adults from Whitsand Bay, Cornwall, UK.), and *P.dumerilii* (cultured in the laboratory), were obtained as discussed in chapter 2 and elsewhere in details (Jha *et al.*, 2000a). Following fertilisation, the developing embryos were allowed to grow (in duplicate) in the incubator (15 ± 1°C for mussels; 20 ± 1°C for worms) for 12 h. The 12 h old embryos were then exposed to different concentrations of TBT for a period of either approximately one and half and two cell cycles, for the analysis of Cabs and SCEs respectively. Using sister chromatid differential (SCD) staining, earlier studies (Jha *et al.*, 2000a) have indicated that

the cell cycle of *M.edulis* at approximately 15°C the average generation time (Ivett and Tice, 1982) of the embryo-larvae cells between 12 and 24h post fertilisation is approximately 3.8h whereas for *P.dumerilii* it is 4.7h at approximately 20°C (Jha *et al.*, 1995). At the end of these exposure periods, sub-samples of the growing embryo-larvae were collected for chromosome preparations and the rest of the samples were allowed to grow for 3 days to evaluate the development and survival of the individual embryo-larvae. A minimum amount of 5-bromodeoxyuridine (BrdU: $1x\ 10^{-5}$ M) dissolved in seawater was used to elucidate SCD staining and analyses of SCEs.

For analysis of TBT concentration a 500ml water sample was extracted with 20 ml of pentane in a separating funnel. The pentane layer was collected and stored in a round bottom flask. The extraction was repeated a further two times and the extracts combined. The pentane in the round bottom flask was then removed by nitrogen blow down (evaporated) and any residue was re-dissolved in 1 ml of the mobile phase. The extract solutions were analysed using inductively coupled plasma-mass spectrometry (ICP-MS), as described elsewhere in details (Hill, 1997). Briefly, the operating conditions for the instrument (VG PlasmaQuad PQ3) were as follows: forward power: 1.7 kW; coolant flow: 15 L/min; Auxiliary flow: 1.0 L/ min; nebuliser flow: 1.05 L/min (containing 4.1 % v/v oxygen). The concentration of the standard used was 2500 ng / ml as TBT.

3.3 Results

3.3.1 Developmental and survival effects

Figure 3.2. represents collectively the developmental and survival effects of TBT exposed embryo-larvae for both of the species.

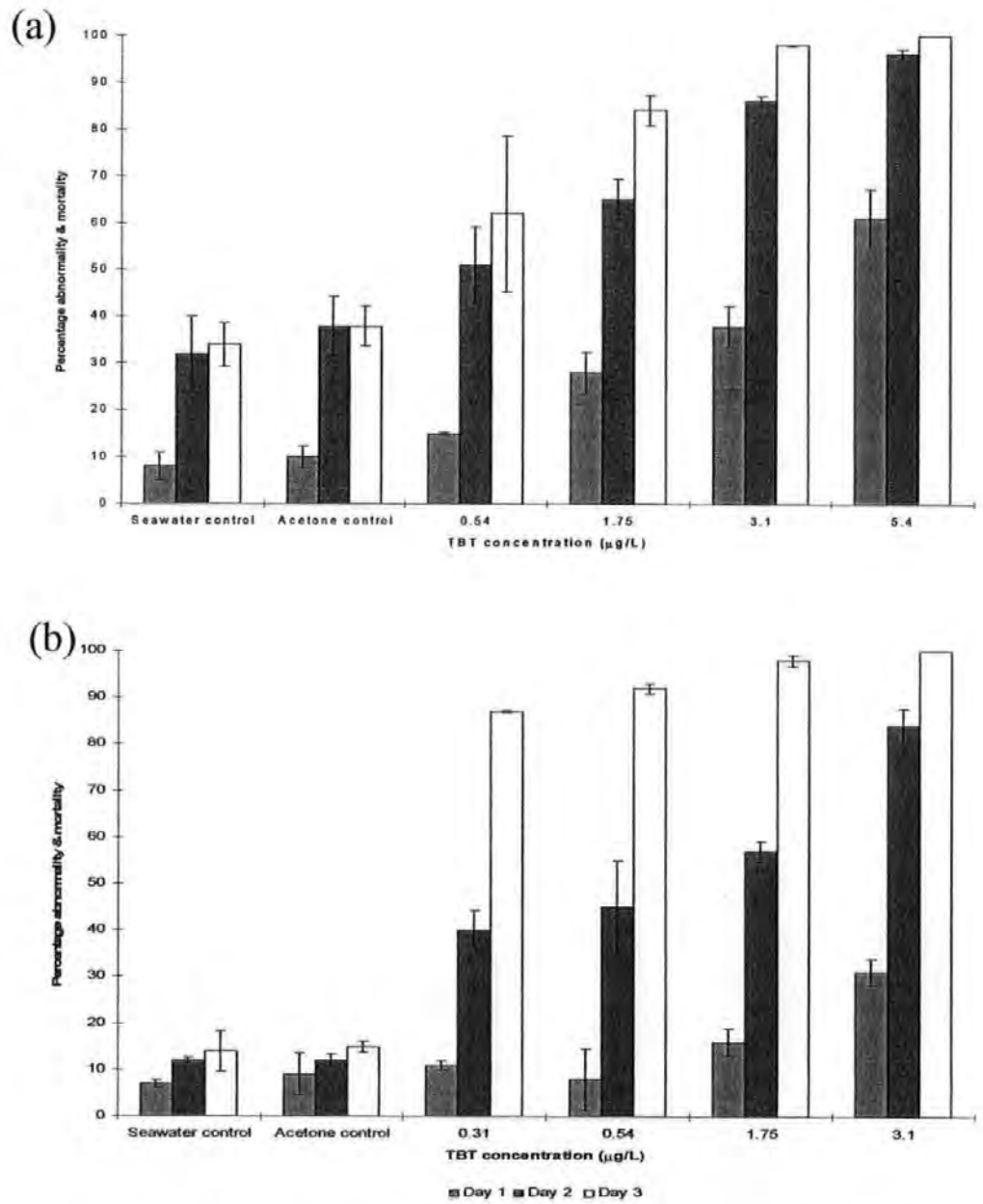


Figure 3.2. The effects of TBT on the development and survival of embryo-larvae of (a) *M.edulis* and (b) *P.dumerilii* (error bars represent standard deviation).

For *P.dumerilii* there was a concentration and time-dependent increase for developmental abnormalities and mortality. Statistical analysis was carried out as described in section 2.8. There was no significant difference, investigated using analysis of variance (ANOVA), between seawater and solvent controls. Whilst significant differences in developmental and survival were observed for all but the lowest (0.31 µg /L) concentrations of TBT ($P < 0.01$). LC_{50} values (lethal concentration for 50% of the population) for 24, 48 and 72 h larvae were found to be 10.06, 2.45 and 1.175 µg /L respectively and the 24h MTD (based on survival and abnormalities) was calculated to be 2.85 µg/L.

The frequency of dead and abnormal *M.edulis* embryos was shown to increase with increasing TBT concentration and observation period. At the highest concentration (5.6 µg/L), following 3 days observation (15 ± 1 °C), 100% of the larvae were either dead or abnormal (distinctly under developed). At higher concentrations, many of the growing embryo-larvae appeared as a shrunken mass of necrotic tissue within the outer membrane. After 3 days, 64 ± 4.20 % of the embryo-larvae in the seawater controls were normal or had reached the typical "D-shaped" stage. LC_{50} values (lethal concentration for 50% of the population) for 24, 48 and 72 h larvae were found to be 4.39, 0.93 and 0.56 µg /L respectively and the 24h MTD (based on survival and abnormalities) was 3.69 µg/L.

From figure 3.2., it is clear that *P.dumerilii* is more sensitive to TBT in terms of abnormality and mortality with over 80% of larvae being abnormal or dead after 72h in all concentrations, in comparison to only 60% at 0.54 µg/L for *M.edulis* larvae. Although, if the LC_{50} values are compared, *M.edulis* appears to be more susceptible to mortality caused by TBT, than *P.dumerilii*. However, in the controls, *M.edulis* showed a higher baseline level of abnormality/mortality than *P.dumerilii*, indicating the differences in brood survival and developmental strategies and this may possibly effect the LC_{50} values. Furthermore if the MTD is compared between species it can be seen that *P.dumerilii* is more sensitive to changes in morphology and behaviour after exposure to TBT than *M.edulis*.

3.3.2 Proliferative Rate Index (PRI)

Cytotoxic effects were assessed by calculating the proliferative rate index (PRI). Figure 3.3. represents the PRI for both *M.edulis* and *P.dumerilii* after exposure to TBT.

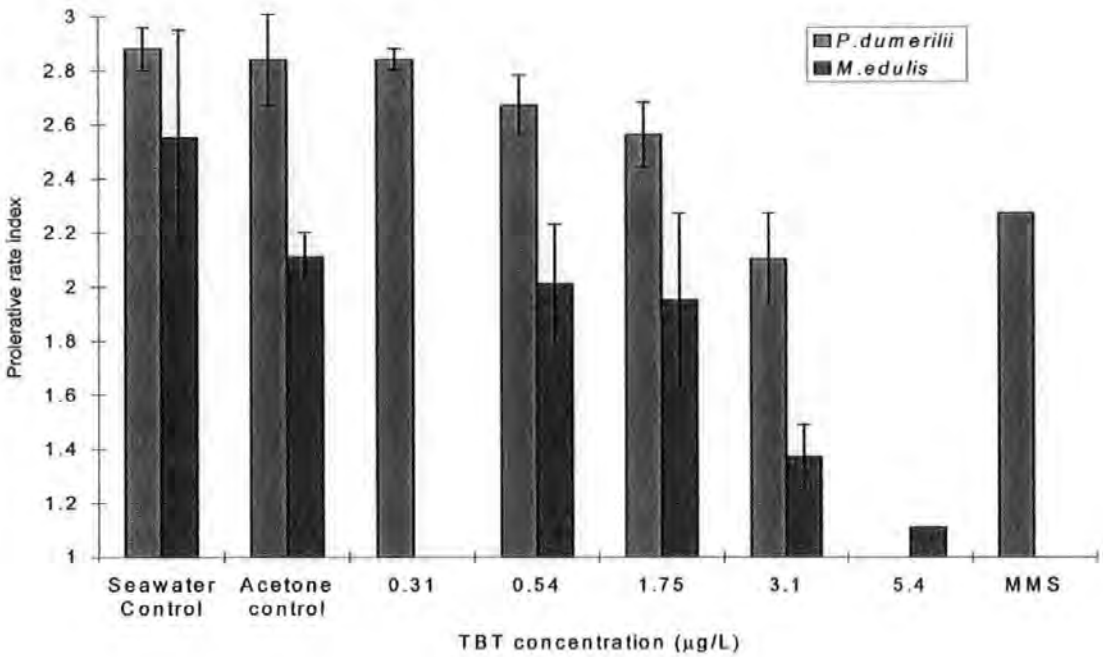


Figure 3.3. The effect of TBT on the proliferative rate index (PRI) for two marine invertebrates (error bars represent standard deviation).

For *P.dumerilii*, there was a clear dose related decrease for PRI values with increasing TBT concentration. However, the PRI value for lowest concentration of TBT (0.31 µg /L) was not significantly different from the controls. The highest concentration of TBT (3.1 µg /L) produced a PRI value of 2.10, this value was even lower than the value obtained for MMS exposure (PRI = 2.27). The PRI results were not normally distributed hence they were analysed using the Kruskal-Wallis non-parametric test, which indicated a statistically significant decrease in the PRI ($P < 0.00005$) as a function of TBT concentrations. The R^2 statistic (co-efficient of determination) for a linear dose-response model showed a value of 80.62%. This indicates that the fitted model explains 80.62% of the variability in PRI. Usually, a value of R^2 of 70% or greater is considered to be a good

fit. In comparison to *P.dumerilii*, the PRI values for *M.edulis* embryo-larvae were significantly lower, suggesting that TBT is more cytotoxic to *M.edulis* than *P.dumerilii*. Although, as with the developmental results, the baseline PRI values in the controls (seawater and solvent), for *M.edulis* larvae, were significantly lower than those for *P.dumerilii*. However, the decrease from the base level PRI (controls) to 3.2µg/L for *P.dumerilii* was less than 1 fold whereas for *M.edulis* it was 2 fold.

3.3.3 Induction or evaluation of Sister Chromatid Exchanges (SCEs)

Figure 3.4. represents the induction of sister chromatid exchanges in *P.dumerilii* and *M.edulis* embryo-larvae after exposure to TBT. The mean baseline frequencies for the induction of SCEs in seawater and solvent controls were found to be extremely low for both species (0.53 ± 0.96 and 0.63 ± 0.98 respectively for *M.edulis* and 0.55 ± 0.24 and 0.42 ± 0.13 respectively for *P.dumerilii*). The phenomenon of low base or spontaneous level SCEs has been recognised in all earlier studies using early life stages (< 24 h old) of marine invertebrates (Harrison and Jones, 1982; Brunetti *et al.*, 1986; Dixon and Prosser, 1986; Jha *et al.*, 1996; 2000a). The low-levels of SCEs observed in the present study was therefore in agreement with the earlier studies. The range of SCEs in the control treatments ranged between 0-4. In contrast, all the treatments showed a very sharp increase for the induction of SCEs.

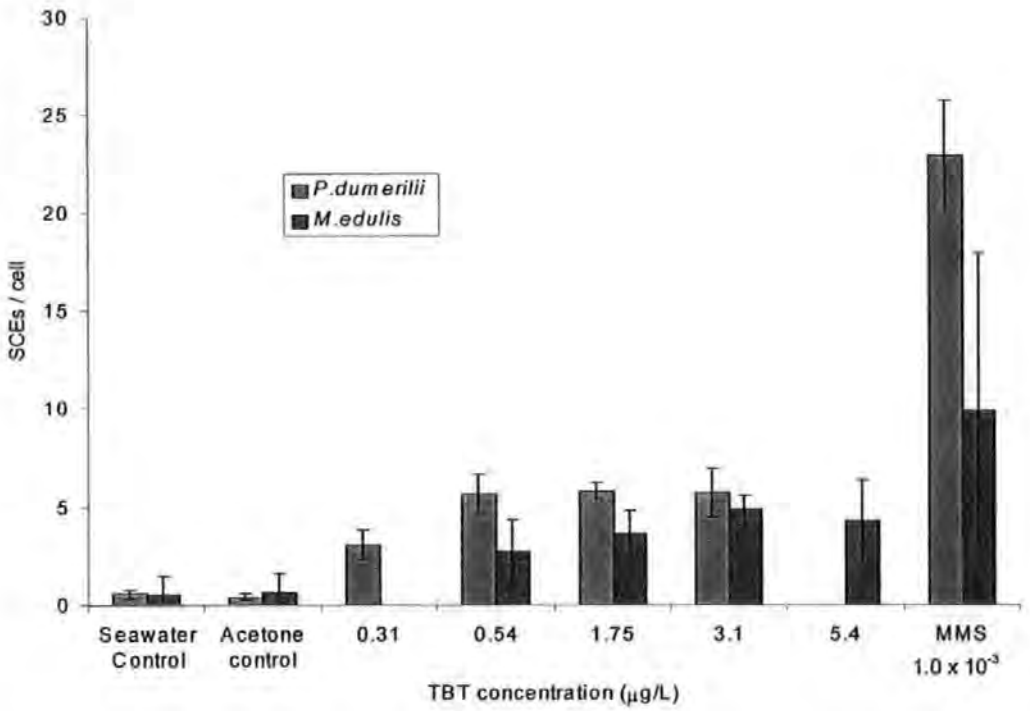


Figure 3.4. Induction of sister chromatid exchanges (SCEs) in two marine invertebrates after exposure to TBT.

For both species, the frequency of SCEs/cell in TBT exposed samples ranged from 0-7, while in MMS (positive control) exposed samples, there was a significant difference between species with *M.edulis* showing a mean value of 10.0 ± 8.05 and *P.dumerilii* observing a mean of 23 ± 2.85 SCEs/cell. The induction of SCEs was analysed using the Kruskal-Wallis test, and was significantly different ($P < 0.00005$; $R^2 = 49.87\%$) for various concentrations compared with controls for *P.dumerilii*. Statistical analyses (ANOVA) of *M.edulis* results also suggested a clear concentration-dependent increase for the induction of SCEs ($p < 0.0001$).

3.3.4 Induction and evaluation of chromosomal aberrations (Cabs)

For Cabs, a dose-related increase in the number of aberrations / cell was found (figure 3.5.) for both species and a clear dose-response relationship was also shown for aberrant metaphases (figure 3.6.).

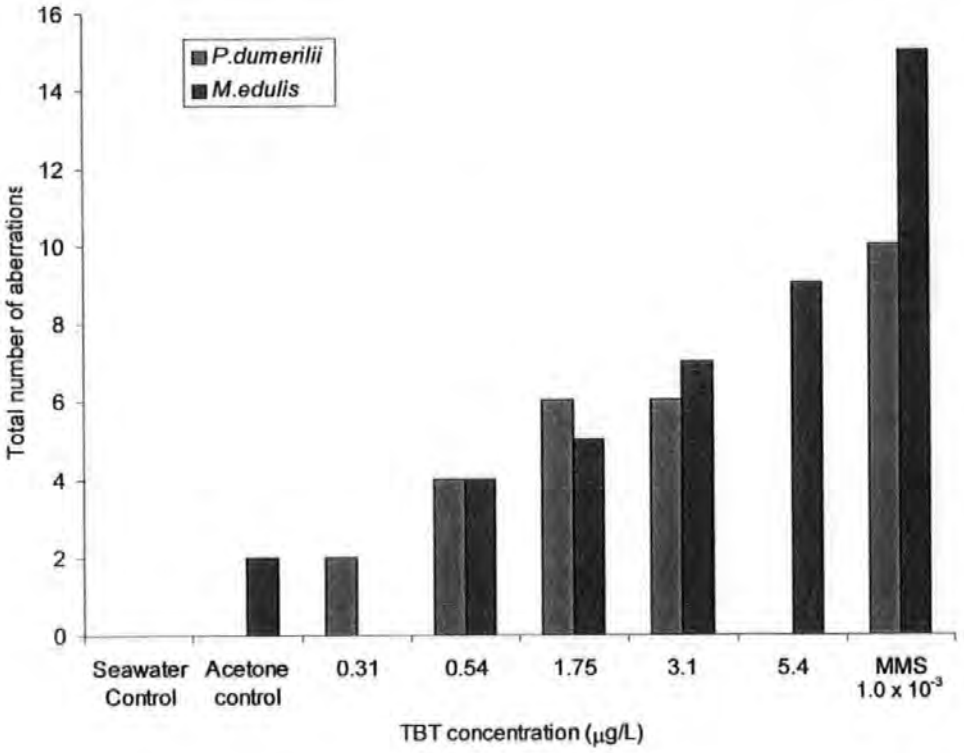


Figure 3.5. The total number of aberrations induced by TBT in two marine invertebrates.

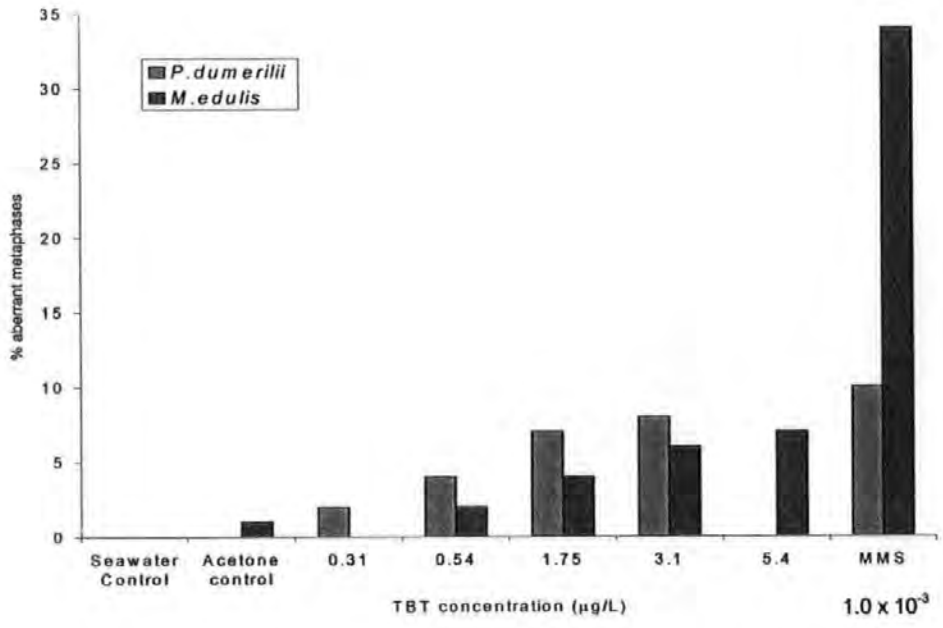


Figure 3.6. The percentage of aberrant metaphases induced by TBT in two marine invertebrates.

The two species showed similar numbers of aberrant cells and total aberrations/cell after exposure to TBT. No aberrations were found in the seawater controls for either species although the solvent control did produce a small percent of aberrations in *M.edulis* embryo-larvae. Statistical analysis of the data revealed no differences between the frequency of total aberrations (chromosome and chromatid type excluding gaps) and aberrant metaphases in seawater and solvent controls, for either species. The statistical analyses (ANOVA) of the results showed a concentration dependent-increase for the induction of total aberrations and aberrant metaphases for *M.edulis* ($P < 0.001$) and for *P.dumerilii* ($P < 0.00005$) between the controls and TBT treatments.

Following TBT treatment, 73.9% aberrations were chromosome and chromatid type breaks (deletions), while 26.1% were chromosome and chromatid type exchanges for *M.edulis*, whereas for *P.dumerilii*, 88.9% were breaks and only 11.1% were exchanges. For the MMS exposed embryo, *M.edulis* showed a larger increase with of aberrant cells (34%) in comparison to *P.dumerilii* that had only 10% aberrant cells. At the higher concentrations of TBT some cells were highly damaged which hindered scoring of total number of aberrations, consequently, the frequency of total aberrations for some treatments are shown to be lower than aberrant metaphases.

The percentage of cells that contained gaps also proved to vary between species with an average of only 37.6% of aberrant cells produced by TBT in *M.edulis* containing gaps whereas for *P.dumerilii* the average was 49.6%. Furthermore the occurrence of gaps in *P.dumerilii* chromosomes appeared to be dose dependent, with 100% of aberrant cells containing gaps in the seawater and acetone controls. A decrease in the number of cells containing gaps occurred with increasing toxicity, 77.8%, 55.6%, 40%, 25% for embryos exposed to 0.31, 0.54, 1.75 and 3.21 μg /L of TBT respectively, suggesting that *P.dumerilii* may be more adapted to repairing aberrations at lower concentrations than *M.edulis*.

Although it may also be possible that the cells were dead as indicated by the effects on mortality and development.

3.3.5 Determination of pre-and post-exposure concentrations of tributyltin

Because of technical limitations (e.g. small size and number of embryo-larvae used in the study), it was not possible to measure the TBT levels in the embryo-larvae as a function of exposure time. Analysis of the seawater suggested a decrease for the concentrations of TBT in post-exposure samples for both species. The overall recovery of the procedure was found to be 90 ± 6% and no TBT was detected in the seawater and solvent controls. Table 3.1. shows the nominal & definitive concentrations (μg l^{-1}) of TBT in seawater before and after exposure of *M.edulis* and *P.dumerilii* embryo-larvae.

Table 3.1. Nominal & definitive concentrations (μg l^{-1}) of TBT in seawater before and after exposure of *M.edulis* and *P.dumerilii* embryo-larvae.

	Mytilus edulis		*Platynereis dumerilii*	
Nominal Concentration	Definitive pre-exposure concentration	Definitive post-exposure concentration	Definitive pre-exposure concentration	Definitive post-exposure concentration
0.31			0.27	0.31
0.54	0.57	0.51	0.41	0.38
1.75	1.43	1.06	1.55	1.2
3.1	2.47	1.56	2.8	2.3
5.4	4.63	2.18		

3.4 Discussion

While determining the maximum tolerated dose (MTD) in terms of developmental toxicity and mortality, the results suggested that the toxicity of TBT increased as a function of concentration and time. In terms of abnormality and mortality *P.dumerilii* appears to be more sensitive to TBT than *M.edulis,* with over 80% of larvae being abnormal or dead after 72h in all concentrations even 0.31 µg/L, in comparison to only 60% at 0.54 µg/L for *M.edulis* larvae. Although, the LC_{50} values show that *M.edulis* is more susceptible to mortality caused by TBT, than *P.dumerilii.* However, the LC_{50} might be affected by the high levels of spontaneous/ natural mortality in control samples of *M.edulis* embryo-larvae. The developmental toxicity results, in general, are in agreement with earlier studies carried out on mussel embryo-larvae (Dixon and Prosser, 1986). If the MTD is compared between species it can be seen that *P.dumerilii* is more sensitive to changes in morphology and behaviour after exposure to TBT than *M.edulis.* In conclusion, in terms of developmental and survival, *P.dumerilii* are more likely to become abnormal after exposure to TBT whereas *M.edulis* are likely to be killed. This is probably due to the differences in the programmed developmental pattern and baseline reproductive output of these phylogenetically different groups of organisms.

In contrast to the developmental toxicity results, for cytotoxicity (PRI), *M.edulis* appeared to be more sensitive than *P.dumerilii* although the basal PRI value (spontaneous level) was also lower in the *M.edulis.* It must be mentioned here that in contrast to mammalian cells growing under *in vitro* conditions, the PRI for embryo-larvae represents an average figure, based on a mixed population of differentiating cells with differing mitotic rates over the exposure period described.

TBT showed a concentration-dependent increase for both the species for the induction of sister chromatid exchanges (SCEs), a cytogenetic biomarker for exposure, *P.dumerilii* being more sensitive than *M.edulis.* For the induction of chromosomal aberrations (Cabs), a cytogenetic biomarker for effects, a concentration dependent increase

was also observed with *P.dumerilii* again appearing to be more sensitive than *M.edulis*, both in terms of total aberrations (excluding gaps) and aberrant metaphases. The results of genotoxicity endpoints therefore unequivocally indicated that TBT is capable of inducing damage to the genetic material of the embryo-larval stages of marine invertebrates, and *P.dumerilii*, a non-target species, is more sensitive compared to *M.edulis*, a target species.

The results also indicate that genotoxic effects are closely linked with development and survival of organisms and that the same concentration-range of a chemical may produce different cytotoxic and genotoxic effects in different species. Species sensitivity may be due to numerous factors such as genetic make-up, reproductive pattern and programmed developmental commitment of the growing embryo-larvae, to mention a few.

From the criteria set for the selection of appropriate tests, the following were suggested, *relevance*, *validation*, detection of *genotoxic lesions*, *quantitative sensitivity*, *convenience* and *cost-efficiency*. It is clear from the use of TBT and MMS, to validate the chosen assays on two different marine invertebrates, that they fulfil the criteria set. In terms of relevance, it is vital to understand the effects of potential toxicants at various levels of organisation in order for us to predict the potential effects at population and ecosystem levels. Validation of the proposed assays was carried out on two different organisms with a reference mutagen and a toxicant (with the potential to have genotoxic effects). Both chemicals produced developmental, cytotoxic and genotoxic effects at various concentrations thus validating the assays. The detection of genotoxic lesions was apparent in the chromosomal aberration assay, thus fulfilling the criteria set for the usefulness of the assay. Sensitivity of the assays was shown between the species used and also as a function of concentration and time. Although the cytogenetic techniques were time consuming the tests were relatively easy to perform, after the appropriate training and were convenient and cost effective. Therefore it can be concluded that the assays (i.e. development/survival, proliferative rate index, sister chromatid exchange and

chromosomal aberrations) would be suitable tests for the evaluation of potential toxicants for the two species studied.

Chapter 4

The genotoxic, cytotoxic and developmental effects of tritium on the embryo-larvae of the marine polychaete *Platynereis dumerilii*

Hypotheses

1. Low doses of radiation, delivered by a reference radionuclide (^{3}H), produce genotoxic, cytotoxic and developmental effects in embryo-larvae of the marine polychaete *Platynereis dumerilii.*

2. Effects produced are related to age of the embryo at the time of exposure and the length of the exposure period.

3. The induced genetic damage is linked to effects at higher orders of biological organisation.

4.1 Introduction

Tritium is an isotope of the element hydrogen (^{3}H). It occurs naturally but it is also manufactured commercially and released as a by-product of nuclear reactions. It has a half-life of 12.26 y, decaying to helium (^{3}He) while emitting a beta particle. The beta particles, while of a low energy (18.6 keV max, 5.7 keV average), have enough energy to produce ionisations (≈0.03 keV per ion pair) and excitations of molecules in their path. The beta particles released during the decay of tritium has a penetration range of <1-6 μm (ICRP, 1984).

Tritium is discharged into the environment in elemental, aqueous or organic forms, with aqueous tritium contributing to the largest levels of discharge (Moghissi *et al.*, 1973). The first large-scale release of anthropogenic tritium into the atmosphere occurred with the detonation of the Ivy- Mike thermonuclear device on the 31st October 1952. Since then tritium, in various chemical forms, has been used in nuclear reactors, reactor fuel element reprocessing and has been produced for both civilian and military use (Mason and Östlund, 1979). Tritium is released from nuclear establishments in relatively large quantities.

However for the population as a whole, discharges from nuclear installations contribute less than 0.1% to the annual average dose of 2.6 millisieverts (mSv) (Department of the Environment, Transport and Regions (DETR), 2000). Some members of the public close to nuclear installations may receive higher doses, through ingestion and external exposure. The highest estimated dose to a small "critical group" of members of the public in the UK is around 0.20 mSv a year, as a result of current and historic discharges from Sellafield (DETR, 2000).

As a result of nuclear establishments around coastal areas, the marine environment is often the ultimate recipient of contamination by a variety of radionuclides. An analysis of historic trends in liquid and aerial radioactive discharges carried out for the period 1979 to 1998 shows that there has been a sustained increases in discharges of tritium and sulphur ^{35}S from the nuclear power sector, and of tritium and carbon (^{14}C) from reprocessing plants (DETR, 2000). Tritium is a naturally occurring radionuclide that forms a small proportion of the hydrogen in water. Nuclear operations measurably enhance the amount of tritium present, although it remains comparatively small (e.g. for a typical nuclear power station, in the region of 7 grams of tritiated water in 200 tonnes of ordinary water a year). Because tritium emits relatively low energy radiation, its radiological significance is usually small, even though the amounts of activity discharged in becquerels may appear large (DETR, 2000).

Although tritium is considered to be one of the least hazardous isotopes to humans, due to low energy, it is ecologically of concern because it is rapidly transported through the environment and is readily taken up by organisms. Tritium in the form of tritiated water can move freely in the biosphere. A significant amount may be incorporated in plant and animal products and therefore it may be ingested by humans not only through drinking water but also through food. When plants take-up tritiated water it may be incorporated into organic matter during the photosynthetic process. Ingestion of this tritiated material by animals may then lead to a tritium content occurring that is higher than expected in organic

molecules which are synthesised by an animal (Kirchmann *et al.*, 1973). Tritium in the form of tritiated water could also be directly bound to carbon in organic molecules through various metabolic pathways, where it is less likely to be exchanged with hydrogen in water molecules. Thus when an organism is kept in or exposed to tritiated water, the specific activity (how radioactive a radioisotope is related to the amount of material) of metabolically incorporated tritium (mainly bound directly to carbon) will at steady state approach the specific activity of tritium in the tritiated water (Kasche *et al.*, 1979). Recently significantly elevated levels of tritium have been detected in seafood collected in the Bristol Channel (McCubbins *et al.*, 2001). In filtered seawater concentrations of approximately 10 Bq kg^{-1} were detected, whereas levels in surface sediment, seaweed (*Fucus vesiculosus*), mussels (*Mytilus edulis*) and flounder (*Platichthys flesus*) were in the order of 6×10^2, 2×10^3, and 10^5 Bq kg^{-1} (dry weight), respectively. The high concentration of tritium found in these materials in comparison to that in seawater was suggested to be due to the presence of organically-labelled tritium compounds in the radiochemical waste, which were biologically available. It is also believed that organisms that consume tritiated food accumulate organically bound tritium (OBT) at a faster rate than an organism exposed only to tritiated water, and may attain a higher rate concentration by bioaccumulation (McCubbins *et al.*, 2001). This highlights the bioaccumulation potential of tritium that may lead to an increase of detrimental effects to organisms higher up the food chain. Relatively high levels of tritium were found in shrimps, in 1999, at Capenhurst, Cheshire (a site used for the enrichment of uranium and dismantling of redundant plant) however this elevation was not detected the following year (RIFE-6, 2001).

Although, generally, most of the tritium taken up is in the form of water and is therefore lost fairly rapidly by the organism, with a fraction of the tritium bound in tissues which may be incorporated into macromolecules such as protein and DNA (Blaylock *et al.*, 1986). Compared to other biological molecules DNA has a long biological half-life time and is transmitted to the daughter cells each time a cell divides and thus it is therefore an

important target for tritium decay (Kasche *et al.*, 1979). The decay of a tritium atom within the nucleus produces a dose of about 170 rad to a sphere of tissue 1μm in diameter, a dose large enough to severely damage the cell if located in a radiosensitive area (Lambert, 1973). Every part of the cell nucleus lies within one tritium beta range of some part of a chromatid and the nucleus contains no sizeable contiguous "insensitive" volume of a radius exceeding the effective range of tritium beta rays (1-2 μm) (Feinendegen and Bond, 1973). It is therefore important that the relative dose from tritium delivered to the cell and its potential detrimental effects on DNA is determined.

Considerable attention in recent years has been paid towards the detection and potential effects of contaminants that may be mutagenic or carcinogenic in marine organisms, and which may also affect human health via the food chain (Jha *et al.*, 1997). A small amount of tritium activity was detected in grass and green crabs from the River Lyner (RIFE-6, 2001). The dose to the most exposed group taking account of consumption of marine foods and occupancy times was estimated to be 0.009 mSv which was less than 1% of the principal dose limit for members of the public of 1 mSv. The radiological significance of this, in common with other defence establishments, continues to be low. However evidence of enhanced tritium levels in potatoes has been observed due to leachate from Drigg transferring to the food (RIFE-6, 2001).

In light of the above mentioned information the aims and objectives of the current study was to investigate if low doses of radiation have genotoxic, cytotoxic and developmental effects in embryo-larvae of the marine polychaete *P.dumerilii* following exposure to a range of concentrations of tritium (a reference radionuclide). It was also aimed to see whether these effects, if any, were related to age of the organism and the period of exposure.

4.2 Methods

4.2.1 Chemicals

Tritiated water (^3H) was obtained from ICN Pharmaceuticals, Inc., UK at a concentration of 3.7 GBq/ml (100 mCi/ml). Upon arrival it was diluted to give 10 ml at a concentration of 0.37 Gbq/ml (10 mCi/ml). DPX mountant for microscopy, Giemsa stain (CAS Nº: 51811-82-6) and Giemsa buffer pH 6.4 were obtained from BDH, Merck Ltd, UK. 5–bromo-2'-deoxyuridine (BrdU) (CAS Nº: 59-14-3), colchicine (CAS Nº 64-86-8) Hoechst 33258 also known as BisBenzimide H 33258 (CAS Nº: 23491-45-4), methyl methanesulfonate (MMS) (CAS Nº: 66-27-3) and all other chemicals were obtained from Sigma-Aldrich Company Ltd., UK.

4.2.2 Exposure scenario 1: Exposure after 16h growth

Figure 4.1 demonstrates the exposure protocol for assessing the development, cytotoxicity (PRI) and genotoxicity (SCEs and Cabs) effect of tritium on *P.dumerilii* embryo-larvae, 16h post-fertilisation.

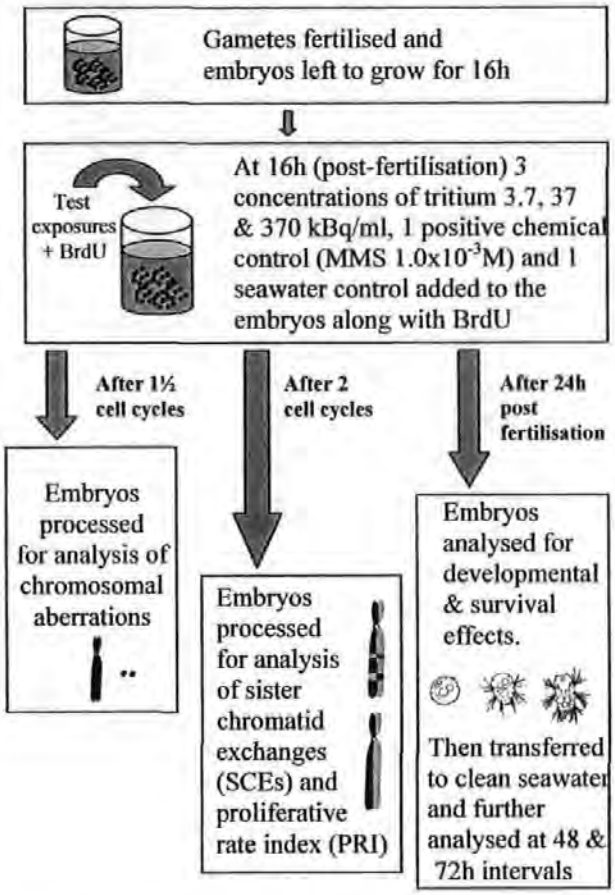


Figure 4.1. Exposure scenario 1. Protocol for the exposure of *P.dumerilii* embryo-larvae to tritium 16h post-fertilisation (1 cell cycle = approx. 3h).

3 sexually mature female worms and 2 males were collected and allowed to spawn naturally as described in section 2.2.1. Approx. 30,000 embryos were produced with a 96.7% fertilisation rate (as described in section 2.2.1). The embryos were allowed to grow for 16h, in incubators, at 20°C ± 1°C. After which they were divided into exposure vessels and exposed to three concentrations of tritium (3.7, 37 & 370 kBq/ml), 1 negative control containing just filtered seawater (10 µm) and 1 positive control containing 1.0 x 10^{-3}M MMS (adopted from previous studies). The final concentration of embryos was approximately 10 embryos/ml in 500 ml. A concentration of 10^{-5} M BrdU was added to all the vessels simultaneously with the tritium and MMS exposure. After 1½ cell cycles (1 cell cycle = 3h), a subset of embryos were placed into a 0.025% (w/v) solution of colchicine and processed for chromosomal aberrations as described in section 2.3.2. A further subset

was removed after 2 cell cycles and processed for analysis of proliferative rate index and sister chromatid exchanges as described in section 2.3.3 and in figure 4.1. When the remaining embryos reached 24h post-fertilisation they were divided into replicates and placed into unexposed clean seawater and analysed for developmental and survival effects as described in section 2.3.1. Further analyses of morphology, behaviour and mortality were assessed at 48 and 72h post-fertilisation.

4.2.3 Exposure scenario 2: Exposure after 1h post-fertilisation

Figure 4.2. demonstrates the exposure protocol for assessing the development, cytotoxicity (PRI) and genotoxicity (SCEs and Cabs) effect of tritium on *P.dumerilii* embryo-larvae, 1h post-fertilisation.

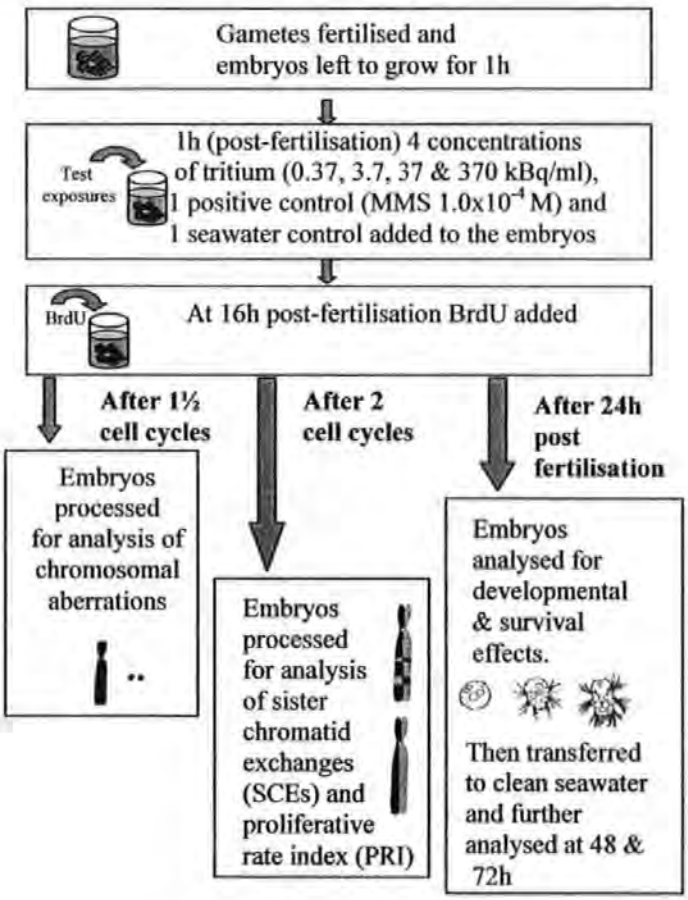


Figure 4.2. Exposure scenario 2. Protocol for the exposure of *P.dumerilii* embryo-larvae to tritium 1h post-fertilisation (1 cell cycle = approx. 3h).

2 sexually mature female worms and 2 males were allowed to spawn naturally in 50 ml of filtered seawater. Approx. 21,000 embryos were produced with a fertilisation rate of 97.1%. Following fertilisation the gametes were allowed to grow for 1h, after which they were divided into exposure vessels and exposed to four concentrations of tritium (0.37, 3.7, 37 & 370 kBq /ml), 1 negative control containing just filtered seawater and 1 positive control containing 1.0×10^{-4} M MMS. The final concentration was approximately 7 embryos/ml in 500 ml. A minimal concentration of BrdU (10^{-5} M) was added to all the vessels at 16h post-fertilisation, after 1½ cell cycles (1 cell cycle = approx. 3h), a subset of embryos were placed into colchicine and processed for chromosomal aberrations as described in section 2.3.2. A further subset was removed after 2 cell cycles and processed for analysis of proliferative rate index and sister chromatid exchanges as described in section 2.3.3. When the remaining embryos reached 24h post-fertilisation they were divided into replicates and placed into unexposed clean seawater and analysed for developmental and survival effects as described in section 2.3.1. Further analyses of morphology, behaviour and mortality were assessed at 48 and 72h post-fertilisation.

4.3 Results

4.3.1 Scintillation counts of tritium

Scintillation counts of tritium concentrations prior to exposure and post-exposure of embryo-larvae were analysed as described in section 2.7.1. Readings were displayed as counts per minute (CPM) and converted to disintegrations per minute (DPM) of the sample by multiplying the CPM by the efficiency factor (1.65) of the counter for the isotope tritium. The total concentration of microcurie per sample was calculated by multiplying the DPM of the sample by the total volume of test sample and dividing by 2,220,000 (the number of DPM/microcurie). This figure was then converted to kBq by multiplying by 37.

The percentage of initial concentration that was removed/lost during exposure of embryo-larvae was calculated by dividing the definitive amount of tritium lost (difference in the concentrations) by the definitive pre-exposure concentration.

Table 4.1. Exposure scenario 1 (16h post-fertilisation): Nominal & definitive concentrations (kBq/ml) of tritium in seawater samples before and after exposure of *P.dumerilii* embryo-larvae.

Nominal concentration of tritium in samples (kBq/ml)	Definitive pre-exposure concentration (kBq/ml)	Definitive post-exposure concentration (kBq/ml)	Difference in concentration (kBq/ml)	Percentage of initial concentration removed or lost
Seawater	0.0049 ± 0.001	0.0040 ± 0.001	0.000	-18
MMS	0.004 ± 0.001	0.004 ± 0.001	0	0
3.7	4.44 ± 0.04	2.61 ± 0.03	1.83	-41.17
37	49.85 ± 0.5	27.59 ± 0.27	22.25	-44.64
370	352.14 ± 2.78	272.62 ± 2.45	79.52	-22.58

Table 4.2. Exposure scenario 2 (1h post-fertilisation): Nominal & definitive concentrations (kBq/ml) of tritium in seawater samples before and after exposure of *P.dumerilii* embryo-larvae.

Nominal concentration (kBq/ml)	Definitive pre-exposure concentration (kBq/ml)	Definitive post-exposure concentration (kBq/ml)	Difference in concentration (kBq/ml)	Percentage of initial concentration removed or lost
Seawater	0.004 ± 0.001	0.005 ± 0.001	0.001	+20
MMS	0.004 ± 0.001	0.005 ± 0.001	0.001	+20
0.37	0.28 ± 0.008	0.25 ± 0.007	0.03	-10.8
3.7	2.73 ± 0.027	2.21 ± 0.022	0.52	-19.15
37	28.69 ± 0.29	22.83 ± 0.23	5.86	-20.42
370	267.08 ± 2.43	240.16 ± 2.31	26.92	-10.08

By comparing table 4.1. and 4.2., it is clear that there was approximately a 2-fold increase in the uptake of tritium from embryo-larvae that are exposed after 16h post-fertilisation in comparison to those exposed after 1h. This may be due to various reasons, such as the younger embryo-larvae were smaller in size or they might not have developed lipid droplets where tritium may become organically bound. For both exposure scenarios the percentage of tritium lost was similar in embryo-larvae exposed to 3.7 and 37 kBq/ml (41-46% and 19-20%). In comparison there is approximately half the percentage uptake in embryo-larvae exposed to 370 kBq/ml when compared to 3.7 and 37 kBq/ml (22 and 10% for embryo-larvae exposed after 16h and 1h post-fertilisation respectively). This might be due to a saturation of tritium uptake at the highest concentration used in these experiments.

4.3.2 Dose received by embryo-larvae

Doses received by the embryo-larvae were calculated following the formulae by Strand *et al.* (1977) as described in section 2.7.2.

Table 4.3. Dose (mGy) received by *P.dumerilii* embryo-larvae during exposure to tritium, 16h post-fertilisation (scenario 1) and 1h post-fertilisation (scenario 2).

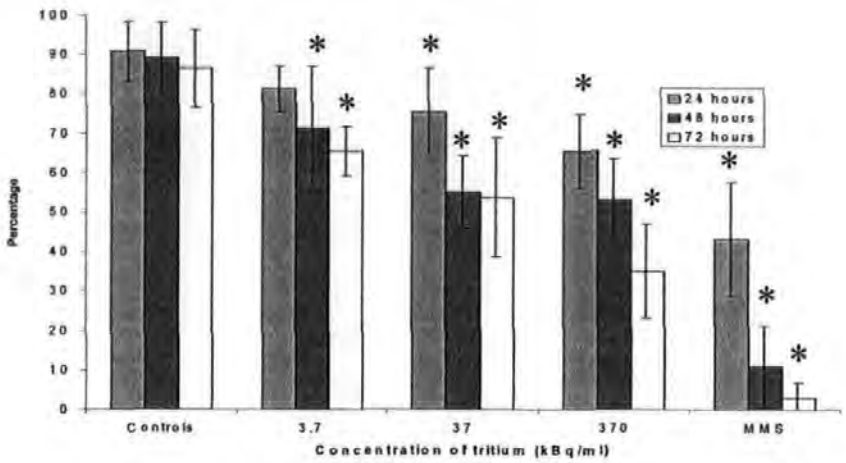
	Scenario 1 16h post-fertilisation				Scenario 2 1h post-fertilisation		
	Cabs	SCEs and PRI	Developmental & survival effects		Cabs	SCEs and PRI	Developmental & survival effects
Exposure period (hr)	4.5	6	8		19.5	21	24
Concentration (kBq/ml)							
370	**5.40**	**7.26**	**9.68**		**23.6**	**25.4**	**29.04**
37	**0.54**	**0.73**	**0.97**		**2.36**	**2.54**	**2.90**
3.7	**0.05**	**0.07**	**0.1**		**0.24**	**0.25**	**0.29**
3.7					**0.02**	**0.02**	**0.03**

Table 4.3. illustrates the variation in dose between the two experiments/exposure scenarios. Embryo-larvae exposed after 1h receive significantly higher doses compared to those exposed after 16h. There is a 3-4-fold increase in the dose received by the 1h-exposed embryo-larvae due to increased exposure time.

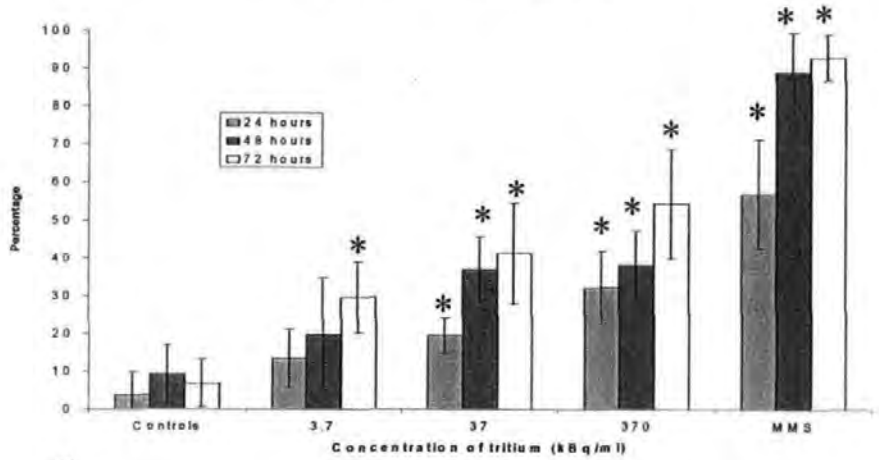
4.3.3 Developmental and survival/mortality effects

4.3.3.1 Exposure scenario 1 (16h post-fertilisation)

(a)



(b)



(c)

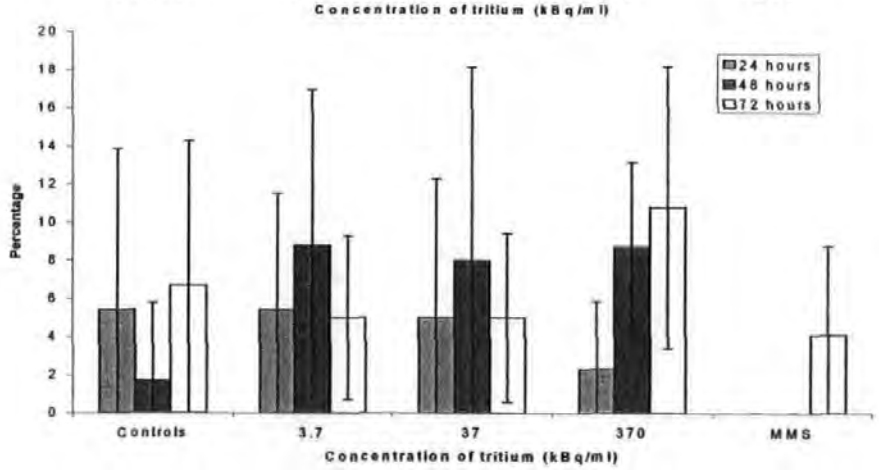


Figure 4.3. Percentage of (a) normal, (b) abnormal, and (c) dead *P.dumerilii* embryo-larvae after exposure to tritium 16h post-fertilisation (exposure scenario 1). Error bars represent standard deviation (* = significantly different from control; $p<0.05$) ($n = 5$).

Figure 4.3. represents the percentage of normal, abnormal and dead *P.dumerilii* embryo-larvae after 24, 48 and 72h post-fertilisation following exposure to tritium and MMS. Statistical analysis was carried out using one way ANOVA unless otherwise stated. There was a dose dependent decrease in the percentage of normal embryo-larvae at 24h (P=0.00005) and only the lowest concentration of 3.7 kBq/ml was not significantly different to the control. No variation between the controls occurs over the three days with the mean percentage of normality ranging between 87-91%. At 48 and 72h all the larvae that were exposed to tritium and MMS are significantly different from the control (P=0.00005 and 0.00005 respectively) with only approximately 30% of the embryo-larvae exposed to 370 kBq/ml of tritium appearing and behaving normally. The dose dependent relationship was most significant at 72h with a R^2 value of 51.95%. In conjunction with the decrease in normality, with increasing time and concentration, there was a corresponding increase in the percentage of abnormal embryo-larvae. There was no significant increase in mortality with increasing concentration of tritium and MMS or with increasing time. The predicted LC_{50} for *P.dumerilii* after exposure to tritium for 72h would be approximately 7.34 Bq/ml, and the dose received would be 2.4 rad or 24 mGy.

4.3.3.2 Exposure scenario 2 (1h post-fertilisation)

Figure 4.4. represents the percentage of normal, abnormal and dead *P.dumerilii* embryo-larvae after 24, 48 and 72h post-fertilisation following exposure to tritium and MMS.

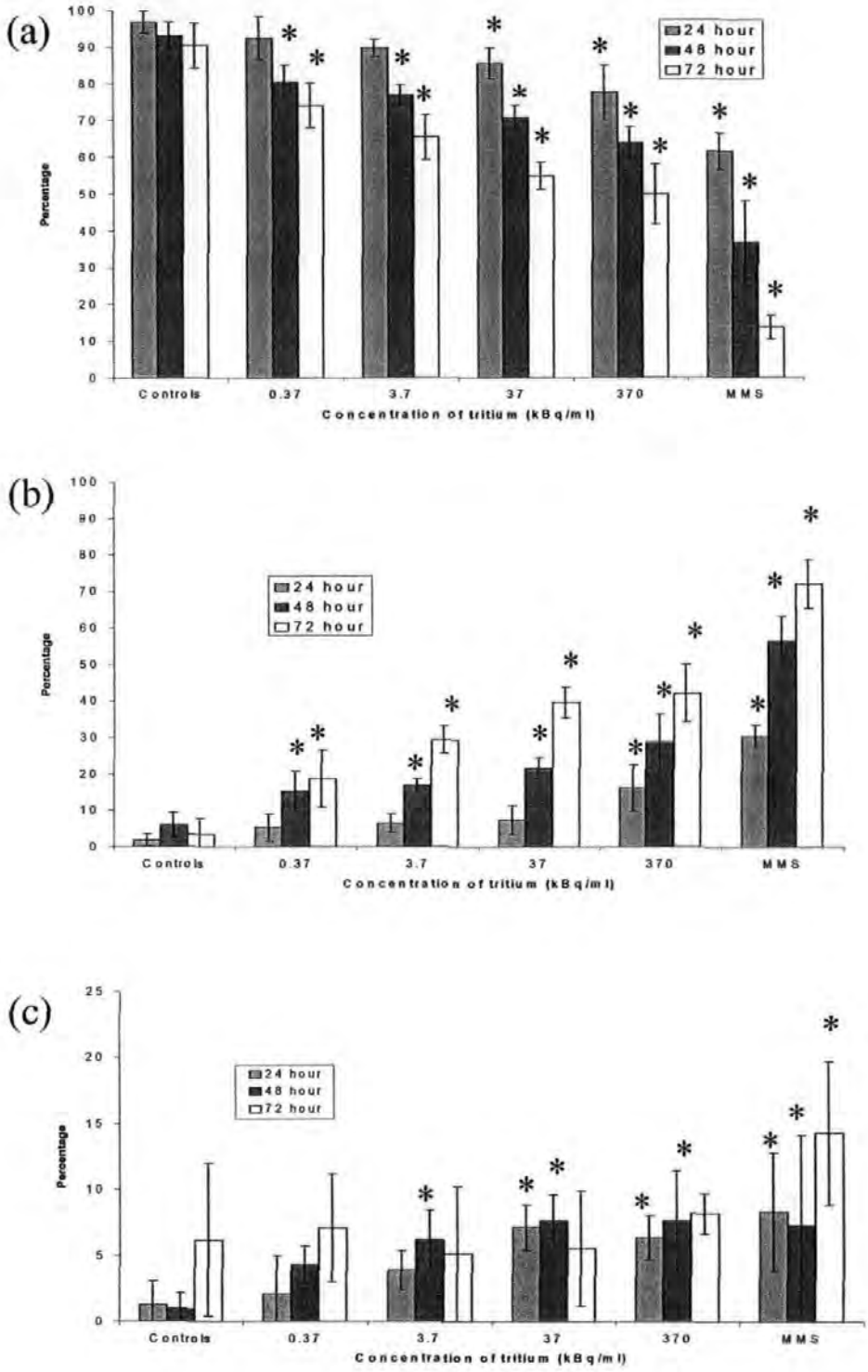


Figure 4.4. Percentage of (a) normal, (b) abnormal, and (c) dead *P.dumerilii* embryo-larvae after exposure to tritium 16h post-fertilisation (exposure scenario 2). Error bars represent standard deviation (* = significantly different from control; $p<0.05$) (n = 5).

There is a dose dependent decrease in the percentage of normal embryo-larvae at 24h (P=0.00005) although only the highest concentration of tritium (370 kBq/ml) and the MMS concentration is significantly different from the control. No variation between the controls occurs over the three days with the mean percentage of normality ranging between 90-95%. At 48 and 72h all the larvae that were exposed to tritium and MMS are significantly different from the control (P=0.00005 and 0.00005 respectively) with only approximately 50% of the embryo-larvae exposed to 370 kBq/ml of tritium appearing and behaving normally. The dose dependent relationship is most significant at 24h with a R^2 value of 48.59% and this then decreases over time. In conjunction with the decrease in normality the percentage of abnormal embryo-larvae increases. There appears to be threshold effects at 72h, with 0.37 and 3.7 kBq/ml being statistically similar and 37 and 370 kBq/ml being also. Due to the relatively low percentage of dead in the controls (mean=1%) there is a significant difference (P=0.0029) between the two highest concentrations of tritium (37 and 370 kBq/ml) and the concentration of MMS. Although at 48h and 72h there is no statistical difference (P=0.063; P=0.36 respectively). The predicted LC_{50} for *P.dumerilii* after exposure to tritium for 72h is 3.11 Bq/ml, and the dose received would be 1.02 rad or 10 mGy.

4.3.3.3 Developmental effects: Comparison between two exposure scenarios

In general, there was little difference for the percentage of normal, abnormal and dead embryo-larvae exposed after 1 or 16h post-fertilisation for the various concentrations over time. However a few discrepancies did occur at 24h and 48h.

4.3.4 Proliferative rate index (PRI)

Figure 4.5. shows the differences in the proliferative rate index between *P.dumerilii* embryo-larvae that were exposed to tritium following 1 and 16h post-fertilisation.

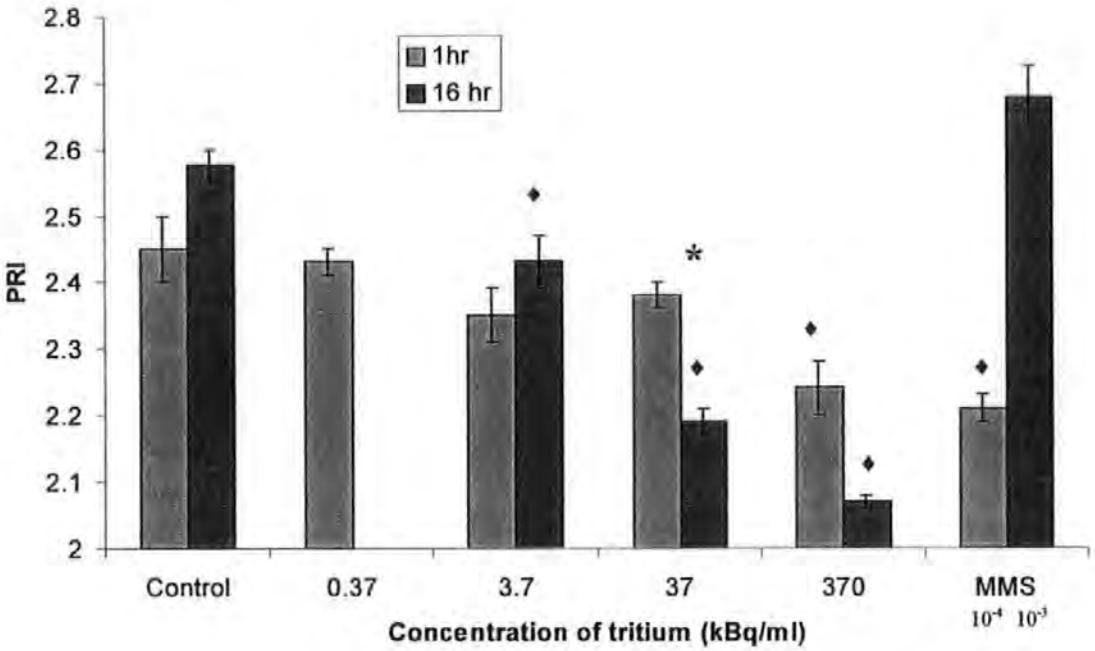


Figure 4.5. The PRI of 1 or 16h post-fertilised *P.dumerilii* embryo-larvae after exposure to tritium. Error bars represent standard deviation (* = significant difference between exposures; ♦=significantly different from control, $p<0.05$) (n = 2).

4.3.4.1 Exposure scenario 1 (16h post-fertilisation)

Figure 4.5. shows a significant reduction in the proliferative rate index for embryo-larvae of *P.dumerilii* after exposure to tritium in comparison with controls (P=0.0001). All three concentrations produced a decrease in the cell proliferation rate that was dose dependent (R^2=59.43%). The maximum reduction occurred in the highest concentration of 370 kBq/ml. The embryo larvae exposed to MMS showed a slight increase in the PRI in comparison to the controls.

4.3.4.2 Exposure scenario 2 (1h post-fertilisation)

Statistical analysis using ANOVA showed that there was a dose dependent decrease in the PRI with increasing doses of tritium (P=0.0108). Although only the highest concentration of 370 kBq/ml and the MMS exposed samples were statistically significantly lower than the controls. Simple regression suggested that the decrease in PRI was related to concentration (R^2=64.46%; P=0.0052).

4.3.4.3 PRI: Comparison between exposure scenarios

Although there was a decrease in the PRI for both exposures with increasing doses there is little difference between the two exposure scenarios. Only the concentration, 37 kBq/ml, produced a statistically significant difference in the PRI between two exposures. The embryo-larvae exposed to MMS were significantly different between exposure scenarios. The embryo-larvae exposed after 1h had a lower cell turnover rate than those exposed after 16h, even though the concentration of MMS used for the 16h exposure was 10 folds greater than at the concentration used for the 1h exposure. This suggests that the time of exposure strongly influenced the cell turnover rate of embryo-larvae after exposure to a genotoxic agent, irrespective of concentration.

4.3.5 Sister chromatid exchanges (SCEs)

Figure 4.6. represents the induction of SCEs after exposure to tritium following 1 and 16h post-fertilisation of embryo-larvae of *P.dumerilii*.

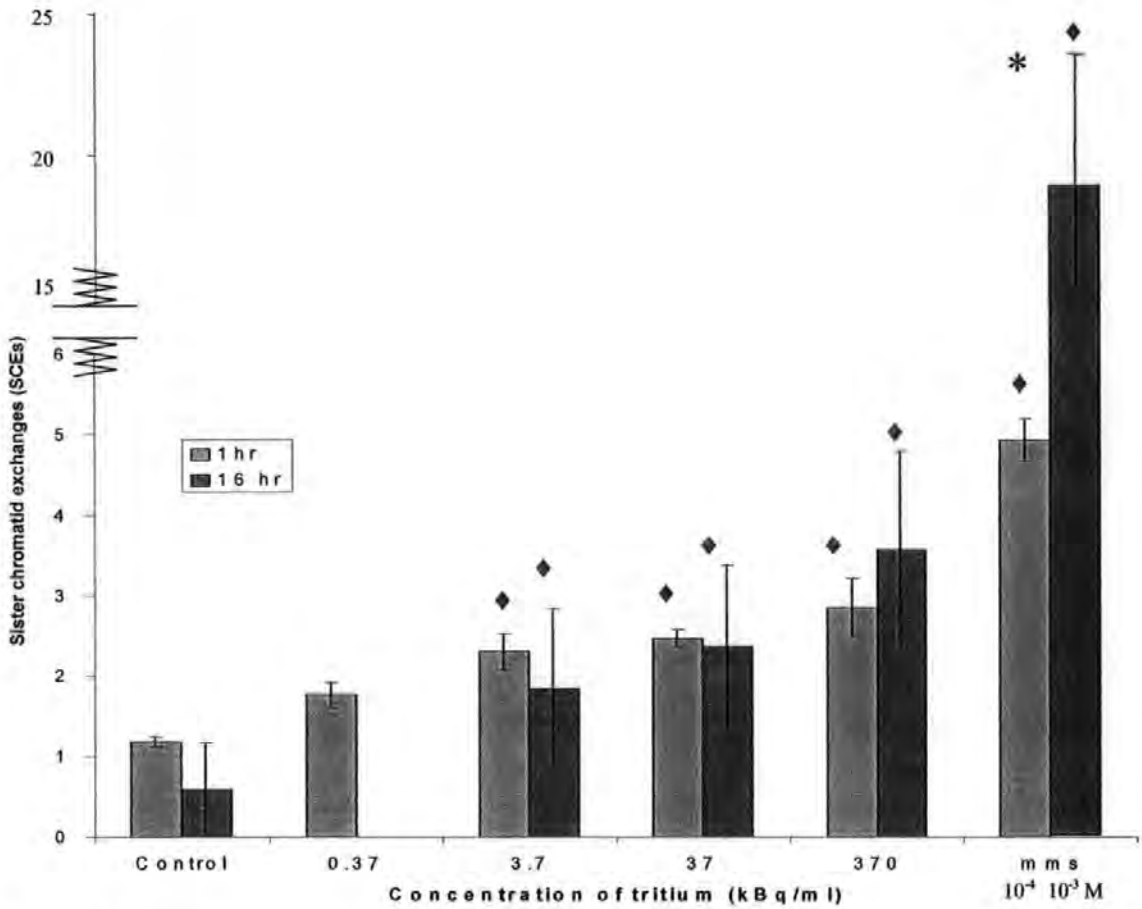


Figure 4.6. The induction of sister chromatid exchanges (SCEs) in *P.dumerilii* embryo-larvae following exposure to tritium after 1 or 16h post-fertilisation. Error bars represent standard deviation (* = significant difference between exposures; ♦ = significantly different from control; $p<0.05$) (n = 2).

4.3.5.1 Exposure scenario 1 (16h post-fertilisation)

The embryo-larvae exposed to MMS had the highest incidence of SCEs with a mean of 18.76 ± 5.8 SCEs per cell. Tritium exposed embryos produced mean SCEs of between 1-4 for the three concentrations and the occurrence of the SCEs was dose dependent (R^2=70.5%). The mean baseline level of SCEs was 0.58 ± 0.84 per cell, so although the levels of SCEs in tritium exposed cells were relatively low, in comparison to the controls the median values were all significantly different (P=0.05) as analysed using the non-parametric Kruskal-Wallis test.

4.3.5.2 Exposure scenario 2 (1h post-fertilisation)

The frequency of SCEs increased with increasing concentration of tritium. Analysis showed that there was a significant difference between some of the concentrations and the controls (P=0.0002). The controls were not significantly different to the lowest concentration (0.37 kBq/ml) but there was a statistical significant difference between the controls and the other three concentrations of tritium and the MMS exposed embryo-larvae. No significant difference occurred between the three highest concentrations of tritium. However MMS produced a higher number of SCEs than the tritium-exposed samples. A dose relationship was observed (P=0.0467) although the relationship between the induction of SCEs and tritium concentration was not strong (R^2=40.84%).

4.3.5.3 SCEs: Comparison between exposure scenarios

There was no significant difference between the two different exposure scenarios for the controls or any of the concentrations of tritium. However there was a statistical significant difference in the induction of SCEs for the embryo-larvae exposed to the MMS (P=0.031). The highest frequency of SCEs occurred after 16h post-fertilisation exposure to 10^{-3} M of MMS. This suggests that the higher the concentration of MMS the greater the induction of SCEs irrespective of length of exposure. This result is probably influenced by the low turnover of cells experienced in embryo-larvae that were exposed only 1h after fertilisation (as indicated in the PRI results, section 4.3.4).

4.3.6 Chromosomal aberrations

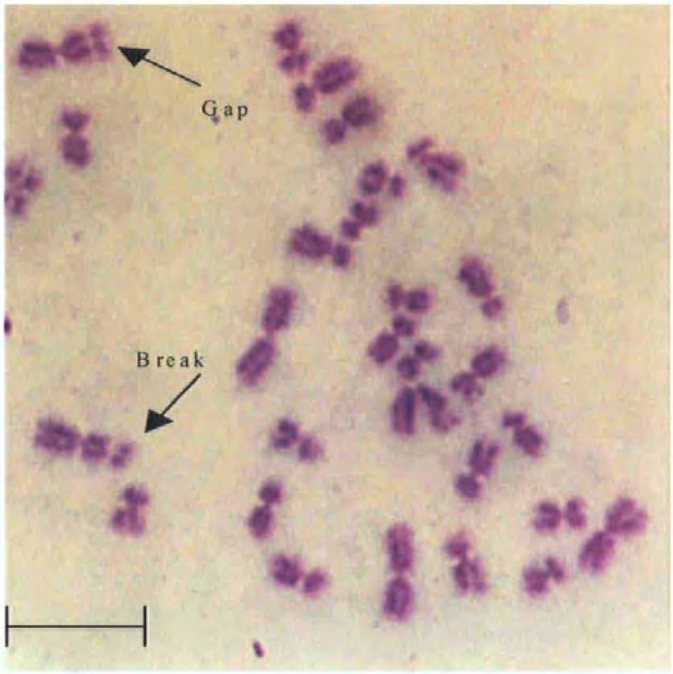


Figure 4.7. Chromosomal aberrations observed in a metaphase spread of *P.dumerilii* (gap = chromosome gap; break = chromosome break). Scale bar = 10μm.

The frequency of embryo-larval cells containing aberrations following exposure to tritium 16h post-fertilisation is shown in figure 4.8a and figure 4.8b represents the frequency of embryo-larval cells containing aberrations following exposure to tritium 1h post-fertilisation.

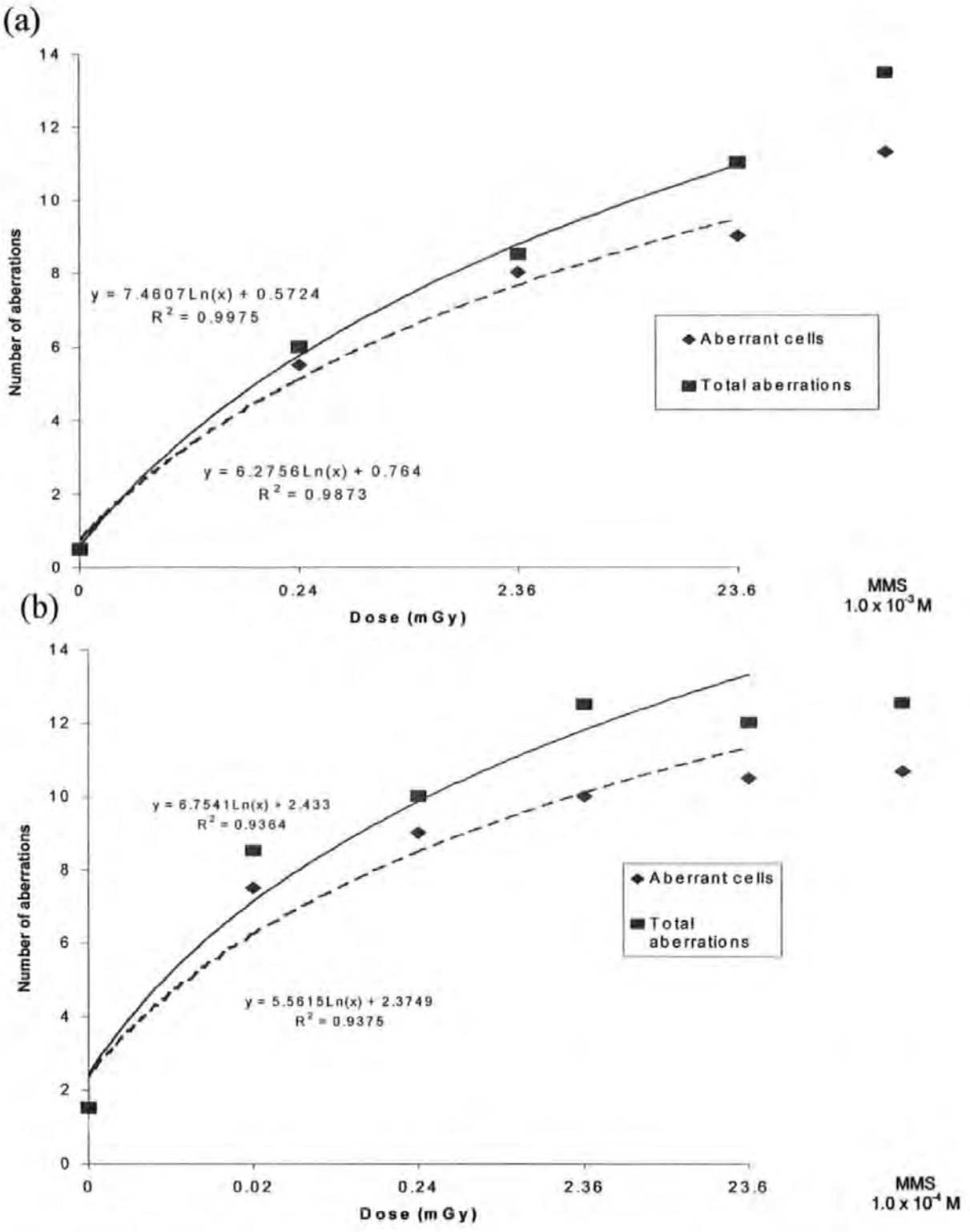


Figure 4.8. Induction of chromosomal aberrations in (a) 16h post-fertilised and (b) 1h post-fertilised *P.dumerilii* embryo-larvae after exposure to tritium. Logarithmic line of best fit (n = 2).

4.3.6.1 Exposure scenario 1 (16h post-fertilisation)

Figure 4.8.a represents the induction of chromosomal aberrations in embryo-larvae of *P.dumerilii* after exposure to tritium 16h post-fertilisation. All three concentrations of tritium produced embryo-larvae with a higher incidence of cells containing aberrations in comparison to the controls (P=0.0002). There is a slight increase in the incidence of aberrant cells with increasing concentrations although the two highest concentrations (37 and 370 kBq/ml) were not significantly different from each other. Embryo-larvae exposed to the reference chemical (MMS) had significantly more cells with aberrations than the controls and the tritium exposed individuals.

Figure 4.8a. also demonstrates the total number of aberrations that occurred in all cells scored after exposure to tritium and MMS. As with the incidence of cells containing aberrations the total number of aberrations observed increased with increasing concentration of tritium producing a clear dose response relationship. All three concentrations of tritium and the MMS exposure produced more aberrations than the controls (P=0.0006). There was no significant difference between the total aberrations scored in the two highest concentrations, 37 and 370 kBq/ml tritium, with the number of aberrations scored in the MMS exposed organisms. The total number of aberrations scored was always higher than the number of cells containing aberrations in all three concentration of tritium, suggesting that sometimes more than one aberration occurred per cell. However only at the highest concentration of tritium (370 kBq/ml), did the number of aberrations significantly increase in comparison to the number of cells with aberrations. This indicates that a similar number of cells are prone to aberrations although the level of damage (number of aberrations per cell) in these cells may increase. In comparison, the reverse is shown in MMS exposed cells where a higher number of cells with aberrations occur in proportion to the total number of aberrations observed. This can be explained by the occurrence of highly damaged cells which contain large numbers of uncountable aberrations that reduces the number of total aberrations scored.

4.3.6.2 Exposure scenario 2 (1h post-fertilisation)

Figure 4.8.b represents the induction of chromosomal aberrations in embryo-larvae of *P.dumerilii* after exposure to tritium 1h post-fertilisation.

There was a statistically significant increase for the induction of both aberrant cells (P=0.0028) and the number of total aberrations (P=0.0358) scored from embryo-larvae that had been exposed to both tritium and MMS in comparison to the controls. However, there was no difference between any of the tritium and MMS exposed samples suggesting a threshold effect for the dose range.

4.3.6.3 Chromosomal aberrations: comparison between exposure scenarios

Figure 4.9. represents the number of aberrant cells in *P.dumerilii* embryo-larvae after exposure to tritium 1 and 16h post-fertilisation. Although there was a slightly higher number of aberrant cells produced in embryo-larvae after exposure from only 1h post-fertilisation in comparison to those exposed after 16h, there was no statistical significant difference between the controls or any of the concentrations of tritium.

Figure 4.10 shows the number of total aberrations scored in *P.dumerilii* embryo-larvae after exposure to tritium 1 and 16h post-fertilisation. There was a statistically significant difference in the number of aberrations formed in the 37 kBq/ml concentration of tritium, but no difference was detected for the controls, the MMS or any of the other concentrations of tritium. The difference between the number of aberrations at the 37 kBq/ml concentration is mainly due to the low variation between the cells scored at 1h post-fertilisation. Although it may also be due to the low PRI expressed at the 16h exposure, thus resulting in fewer dividing cells and therefore fewer aberrations being expressed.

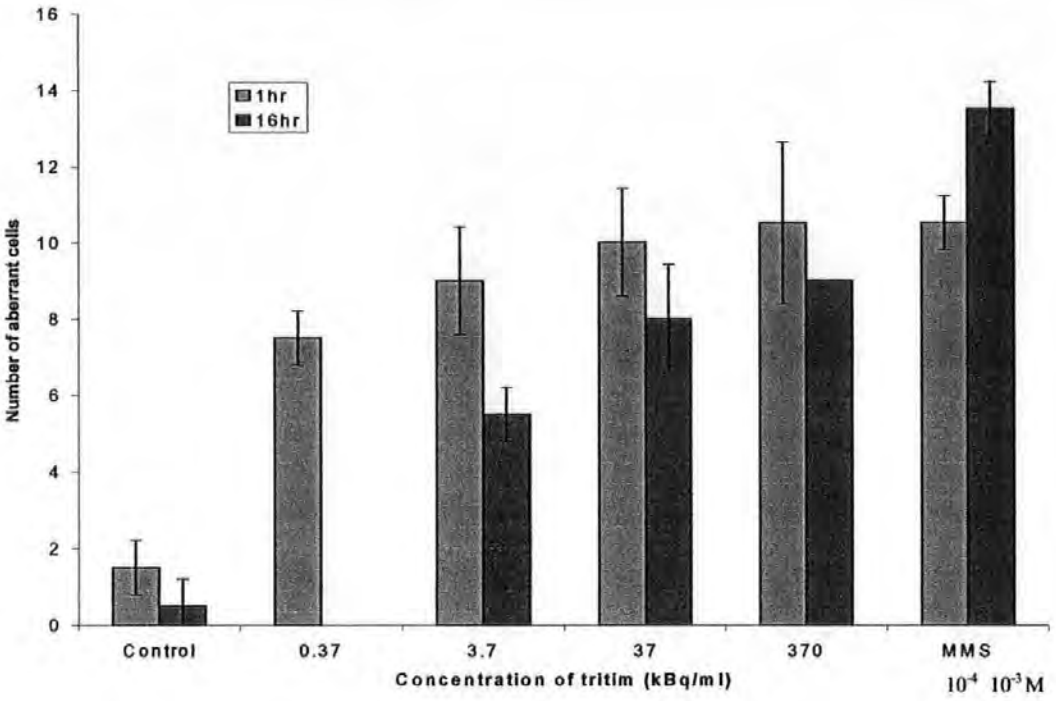


Figure 4.9. Number of aberrant cells in *P.dumerilii* embryo-larvae after exposure to tritium following 1 and 16h post-fertilisation. Error bars represents standard deviation (n = 2).

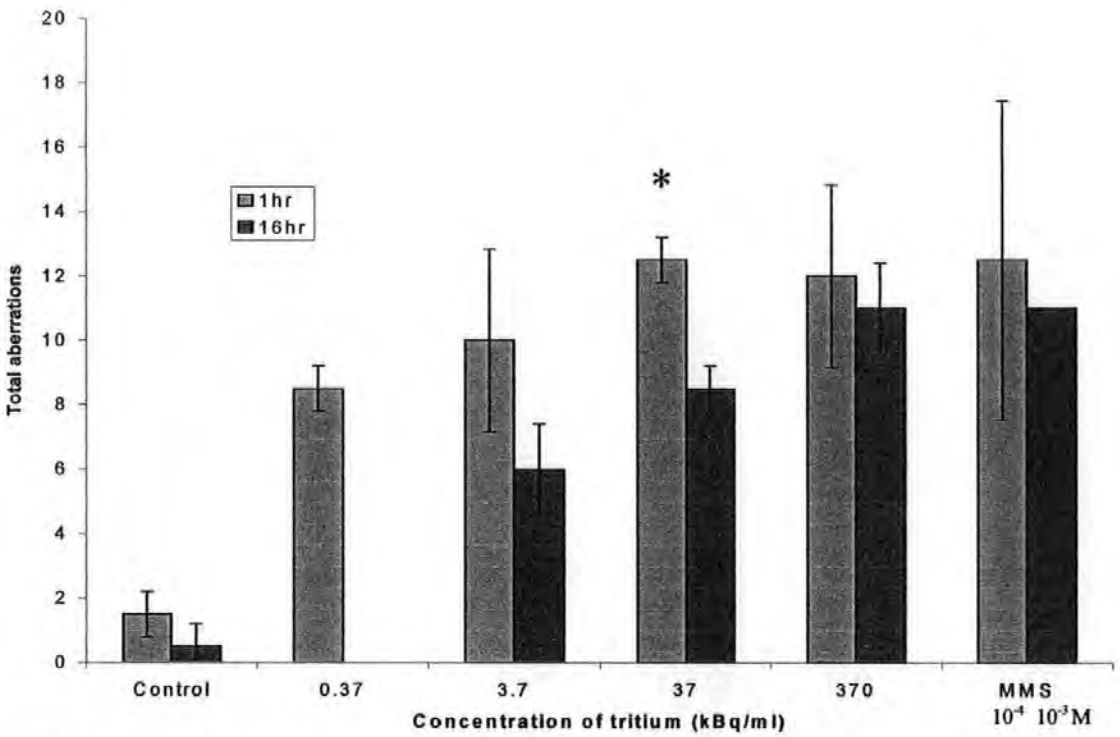


Figure 4.10. Number of total aberrations in *P.dumerilii* embryo-larvae following exposure to tritium 1 and 16h post-fertilisation. Error bars represent standard deviation (* = significant difference between scenarios; $p<0.05$) (n = 2).

4.3.7 Summary of results

For both exposure scenarios, an increase in abnormality consequently resulted in a decrease in the percentage of normality, with no statistical increase in mortality over the sampling period (i.e. 72h). All the cytogenetic and cytotoxic assays produced strong differences between exposed and control samples. There appeared to be a threshold response in the induction of aberrations after exposure to tritium with all the concentrations producing similar results although a dose response was more prevalent for the induction of SCEs and alterations in PRI. Although there is a 3-4-fold increase in the dose received by the 1h-exposed embryo-larvae there was little difference between the two different exposure scenarios and the developmental, cytotoxic and genotoxic endpoints examined.

4.4 Discussion

Several factors (physical, biological and chemical) can modify the levels at which lethal effects are observed following acute exposure to ionizing radiation. These can include, the life stage irradiated, the time period of observation after exposure, the species studied, the intensity of ionisation of the radiation source, and other factors such as dose rate, temperature, and salinity (Anderson and Harrison, 1990b). Lethal responses ranging between 2.1 and 566 Gray (Gy) have been reported after exposure of a selection of adult invertebrates such as crabs, copepods, annelids, gastropods (Anderson and Harrison, 1990a). Whereas embryo mortality, in invertebrates, has been observed below 10 Gy and in fish species between 0.16-0.9 Gy. The levels varied greatly depending on the time period of observation, the species, and the exact embryonic life stage. During this study, 72h LC_{50}'s of 0.01 and 0.024 Gy were observed for *P.dumerilii* that were exposed after 16h and 1h respectively, which is considerably lower than that observed for adult invertebrates, although little work has been carried out on invertebrate embryos. The highest detrimental effects that have been demonstrated in both mammals and aquatic organisms in experiments when irradiation occurred immediately after fertilisation

(Anderson and Harrison, 1990a). They state that, depending on the developmental pattern of an organism, an impact to one cell of a two-celled embryo is more likely to have a detrimental effect on development than would an impact on one cell of a multi-thousand celled embryo. Furthermore, the irradiation of cells immediately after fertilisation may also encompass the sensitive period of second mitosis. The embryonic stages of fish was shown to exhibit mortality at lower doses than any other life stage studied (Woodhead, 1984), although limited studies have been carried out on invertebrates to emphasis this point (Anderson and Harrison, 1990b). During the present study there was no significant increase in mortality of the embryo-larvae due to exposure to tritium at either exposure periods (i.e. 1h and 16h post-fertilisation). Although as the levels of abnormality increased over time it is likely that these abnormal organisms will not be able to survive and thus potentially there may be a delay in mortality due to radiation exposure. However no attempt was made to irradiate later life stages during the current study.

Anderson and Harrison (1990a) suggested that the low effect levels observed (following exposure to external irradiation source of ^{137}Cs) for *Neanthes arenaceodentata* was due to the fact that the species is a terminal spawner, as is *P.dumerilii,* where the females spawn once and then die. They therefore speculate that under normal circumstances the worms are less likely to exhibit asynchronous oocyte development that might result in an increase in abnormal development and lead to higher levels of mortality. In comparison lizards are thought to be as sensitive to irradiation as primates because they both exhibit low fecundity (Anderson and Harrison, 1990a). Woodhead (1977) claims that as well as low fecundity, slow generation times and gamete sychroncy are also factors that make a species susceptible to greater damage due to higher accumulation of doses to the gametes.

It has also been shown that the type of radiation used may also influence its effect on organisms. β-rays from tritiated water have been shown to be more effective than gamma rays (^{90}Sr, ^{90}Y (in solution), ^{60}Co and X-rays (external source)) in inducing

chromosomal aberrations in the blastula stage of *Oryzias* (freshwater teleost) eggs, therefore possibly leading to an increase in abnormality and mortality (Suyama, *et al.*, 1981). Moskalev *et al.* (1973) also found that tritium appeared to be more effective than gamma-radiation in mortality in rats, although the tritium was administered internally in the form of injections whereas the gamma irradiation was an external source of ^{137}Cs. The relatively greater biological effectiveness of tritium appeared to be due to the fact that it developed 10 to 30 times greater ionisation density per unit of tissue volume compared to X- or gamma radiation (Moskalev *et al.*, 1973)

Following chronic irradiation (0.005 Gy/d of ^{60}Co) of *Neanthes arenaceodentata* embryos for entire life cycle, Harrison and Anderson (1988) found that the percentage of normal polychaete worms was significantly reduced. The dose (0.005 Gy/d) received by *Neanthes* was similar to that predicted for *P.dumerilii* embryo-larvae (0.00003-0.029 mGy/d). Although the *Neanthes* polychaete worms were exposed to ionising radiation for a longer period (an entire life cycle) than during the present experiment (a few hours). This suggests that either *P.dumerilii* may be more sensitive to acute dose of radiation than other polychaetes or as previously stated the RBE of tritium is higher than gamma-radiation. Knowles and Greenwood (1997) found that chronic ^{137}Cs γ-irradiated worms (*Ophryotrocha diadema*) exposed to a dose of 7.4 mGy/h^{-1} (for entire life cycle) had significant reductions in the production of eggs but the survival of the worms was not affected. In comparison worms exposed to the same absorbed dose of β-radiation from tritiated water had significant reductions in the survival of eggs to larvae but no difference in the production of eggs. The concentration of tritium that caused an increase in mortality in *Ophryotrocha diadema* was 2.24 MBq/ml whereas during the current study there was no significant increase in mortality at the highest concentration (370 kBq/ml or 0.37 MBq/ml) for the sampling period (i.e. 72h).

High radiosensitivities for β-radiation, from tritiated water, has been reported in larvae of sea urchins suggesting that the relative biological effectiveness (RBE) of β-

radiation, compared with X or γ-radiation for such organism could be very high. This could lead to an underestimation of the risk of tritium (Akita and Shiraja, cited in IAEA, 1976). Abbott and Mix (1979) also observed high radiosensitivity of tritiated water on the development of the goose barnacle *Pollicipes polymerus*. A molting index (based on molting stages in crustacean) was used to evaluate the effects of tritiated water (HTO) on normal development and effects were observed at concentrations as low as $7x10^{-6}$ μCi/ml (0.003 kBq/ml). They reported that the loss of early (embryonic) cells could not often be compensated for as in vertebrates, and hence cellular damage in a population of embryos was more likely to be detectable. The experiments carried out by Abbott and Mix (1979) showed that development was affected at very low concentrations of tritium however we only observed developmental effects *in P.dumerilii* at $1x10^{-3}$ μCi/ml (0.37 kBq/ml) of tritium although only after 48h. However no attempts were made during the current study to assess the developmental effects at lower concentrations. Effects on survival were seen after 32 days in the larval goose barnacles when they were reared in seawater containing only 5.55×10^{-4} μCi/ml of tritiated water (20 Bq/ml, estimated dose rate ≈ 0.07 μGy/h). During the current experiment there was no statistical difference in the percentage of mortality between the control and the embryo-larvae that were exposed to tritium by the end of the experiment (72h) and thus no comparison between species can be made. Furthermore the goose barnacles were exposed for 30+days and it is difficult to predict the long-term effects of tritium exposure on *P.dumerilii* after a relatively acute exposure period (see chapter 9).

In genetic ecotoxicology it is essential to link cellular and genotoxic effects with detrimental developmental consequences. Veatch and Okada (1969) showed, in mouse leukemic cells (L5178Y) exposed to thymidine, that one tritium "absorption event" represented 0.3 rad and produced about one single strand break/cell (G_1 stage) and 0.1 double strand breaks. Hence only one in ten involved cells will retain residual injury due to the induction of the double strand breaks which are involved in the production of

chromosomal aberrations (Natarajan and Obe, 1978; 1984). Chromosomal aberrations induced in juvenile polychaetes (*Neanthes arenaceodentata*) occurred at the same doses at which fecundity was reduced in adults, thus indicating that molecular damage might be a good indicator of effects at cellular and organismal levels (Anderson and Harrison, 1990b). Colvin and Everts (1973) have investigated chromosome damage in Chinese hamsters resulting from the percutaneous absorption of tritium activity from luminous compounds. Hamsters were exposed to 5 mg of luminous compound with approximate activities of 100 μCi and 1000 μCi. After exposure for 7 days there was a significant increase in the percentage of aberrant cells from kidney and lung tissue from 0.8-0.9% for the controls to 5.4-7.7% (approx. 7-8.5-fold increase). The majority of aberrations that occurred were chromatid breaks indicating that the majority of the damage occurred during the G_2 phase of the cell cycle. The occurrence of G_2 phase aberrations indicates that the cell is still passing through the cell cycle (as suggested by the lack of complete first stage cells from the PRI results). Therefore the radiation appears not to affect the synthesis of DNA (S phase) which does not usually proceed if there is any DNA damage. Sister chromatid exchanges are believed to be an S-phase dependent phenomenon. Ionising radiations (especially those with a low LET) are considered to be poor indicators of SCEs (Perry and Evans, 1975). However a dose dependent increase for the induction of SCEs in this study suggests that ^3H either gets incorporated into the DNA molecules (either bases or sugars) or in the enzymes involved in the replication or repair processes. This complements the PRI results, which suggest a concentration dependent delay in cell cycle progression. If the cell cycle does not proceed this may suggest that the G_2 phase of the cell cycle is sensitive to radiation induced DNA damage or that radiation has damaged the cell checkpoint mechanism at DNA synthesis. Some exposed cells still appear to be able to complete metaphase as the PRI suggested that most cells were in their second cell cycle. However at this stage the cells exposed to tritium were spending more time in the cell cycle compared to control cells. Cells may have been in an early apoptotic state and therefore would not

pass through the cell cycle (Wang *et al.*, 1999a) or they may have been in a radiation induced cytoprotective response, where the cell favours survival and may alter cell cycle regulation (Schmidt-Ullrich *et al.*, 2000). Bateman and Chandley (1962) reported that after exposure of mice to tritiated thymidine (activity 200 μc./ml) only about 1% of transmutations detected produced a dominant lethal mutation that in turn might roughly be equated to a chromosome break. It could not be concluded from the study whether the transmutations detected failed to produce a chromosome break due to the absence of initial damage or whether it was due to the high rate of recovery (repair or restriction). Suyama *et al.* (1981) investigated the effects of tritiated water on the early development of fish (*Oryzia*) eggs. They found that the frequencies of chromosome bridges increased significantly when the eggs were treated with solutions of concentrations higher than 0.51 Ci/l (510 μCi/ml or 18870 kBq/ml) although no significant effects was detected in terms of hatchability and frequency of abnormal larvae up to the concentration of 1 Ci/l (1 mCi/ml). It is of particular interest that the induction of aberrant cells was initiated at a lower concentration than the manifestation of abnormality, although the concentrations that did initiate such effects were fifty times greater than those used during the present experiment.

In conclusion, tritium has been shown to be detrimental to the development of embryo-larvae of *P.dumerilii* resulting in an increase in abnormality. Furthermore, tritium increased the induction of chromosomal aberrations and sister chromatid exchanges, in exposed embryo-larvae; thus indicating that tritium has genotoxic potential. Tritium also produced cytotoxic effects in embryo-larvae resulting in a decrease in the cell proliferation rate. Therefore genetic damage induced at a molecular level may be linked to effects on development and mortality. Low doses of radiation does have genotoxic, cytotoxic and developmental effects of embryo-larvae of the marine polychaete *Platynereis dumerilii*, although the effect is not related to the age of the embryo when the exposure occurred and the exposure period thus hypothesises 2 can be rejected.

While the concentrations of tritium used in the present study, which were selected on an earlier study (Abbott and Mix, 1979), could be considered to be not environmentally realistic, the doses produced by these concentrations may be considered as extremely low. This emphasises the need to assess many different types of organisms at various life stages in order to assess the effects of radiation on the ecosystem.

Chapter 5

The genotoxic, cytotoxic and developmental effects of tritium on the embryo-larvae of the marine mollusc *Mytilus edulis*

Hypotheses

1. Low doses of radiation, delivered by a reference radionuclide (tritium), produces genotoxic, cytotoxic and developmental effects in embryo-larvae of the marine mollusc *Mytilus edulis*.

2. Observed effects are related to age of embryo at the time of exposure and the period of exposure.

3. Observed effects are not related to the age of the embryo but are influenced by the length of exposure.

5.1 Introduction

The world's inventory of tritium arises from both natural and artificial sources. The global inventory of naturally occurring tritium is about 1.3×10^{18} Bq and is formed at a rate of approximately 70 PBq per year (Higgins *et al.*, 1996). The principal sources of man-made tritium are electrical power generating reactors, reactor producing nuclear materials for defence purposes, atmospheric tests of nuclear weapons, heavy water plants, nuclear reactors designed specifically to produce tritium, tritium separation plants, and tritium handling operations (Crowson, 1973). The global contribution from the nuclear fission fuel cycle amounts to about 8.6 PBq per year but similar or larger amounts are released annually during the disposal of consumer products. Therefore releases from all industrial activity add up to approximately 25% of the amount of tritium produced naturally in the atmosphere and can lead to regional anomalies (Higgins *et al.*, 1996). Concern over the exposure of the public to tritium has recently been raised in Britain (Harrison and Stather, 1998). The suggestions are not that exposures have been underestimated but that doses and risks from intakes of tritium are greater than those calculated by the International Commission on Radiological Protection (ICRP). Tritium in the form of tritiated water can

move freely in the biosphere and may be ingested by man not only through drinking water but also by food, especially from fish and shellfish (Kirchmann *et al.*, 1973). Immensely high levels of radioactive tritium have been found in shellfish from the Severn estuary (Wallis, 2000). Readings of 47,000 Bq/kg were recorded in comparison to water and sediment levels of 10 to 100 Bq/kg.

In continuation with the experiments carried out on the marine worm, *P.dumerilii* (chapter 4), the aim of this study was to assess the effects of tritium (a reference radionuclide) on the embryo-larvae of the marine mussel, *M.edulis* (Mollusca: Bivalvia). As previously mentioned, *M.edulis* has been used widely for monitoring the bioaccumulation, ecotoxicological and genotoxicological effects of marine contaminants (Harrison and Jones, 1982; Dixon and Prosser, 1986; Brunetti *et al.*, 1986; Jha *et al.*, 2000a). Assays based on embryo-larvae stages of this specie have shown it to be a reproducible and sensitive model for the detection of mutagens present in seawater (Jha *et al.*, 2000a; 2000b). In contrast to the sexually mature adult animals, the embryo-larval stages of aquatic organisms are considered to be several orders of magnitude more sensitive in terms of toxicological injury (Jha *et al.*, 2000a). Furthermore, due to their small size they are particularly susceptible to a rapid, uniform distribution of contaminants, within all of the cells, throughout the organism. The advantages of using bivalve embryos as an acute lethal test are that the exposure times are generally short (24-48h), the biological endpoints are easily determined (e.g. shelled larvae) and they are tolerant to a range of salinity (<15-35‰). They also have moderate sensitivity, the cost of organisms are low and bivalve species are commercially and ecologically important (His *et al.*, 1999). Despite being a popular organism for ecotoxicological work, and the growing scientific and public concern over the presence of tritium in the environment (Environment Agency, 2001), no serious attempts have been made to evaluate the toxicological impact of tritium on this or other organisms. Therefore the aim of the present study is to assess the effects of low doses of radiation on genotoxic, cytotoxic and development endpoints in embryo-

larvae of the marine mollusc *Mytilus edulis*. Furthermore if there is an effect on this biological endpoints are they related to age of embryo at the time of exposure and the length/period of exposure? This will provide a useful indication as to the potential effects of tritium discharge at various periods of embryo-larvae development and will provide useful information as to the sensitivity of the various embryo-larvae life stages.

5.2 Methods

Figure 5.1. demonstrates the general exposure protocol used for assessing the development, cytotoxicity (PRI) and genotoxicity (SCEs and Cabs) effect of tritium on *M.edulis* embryo-larvae.

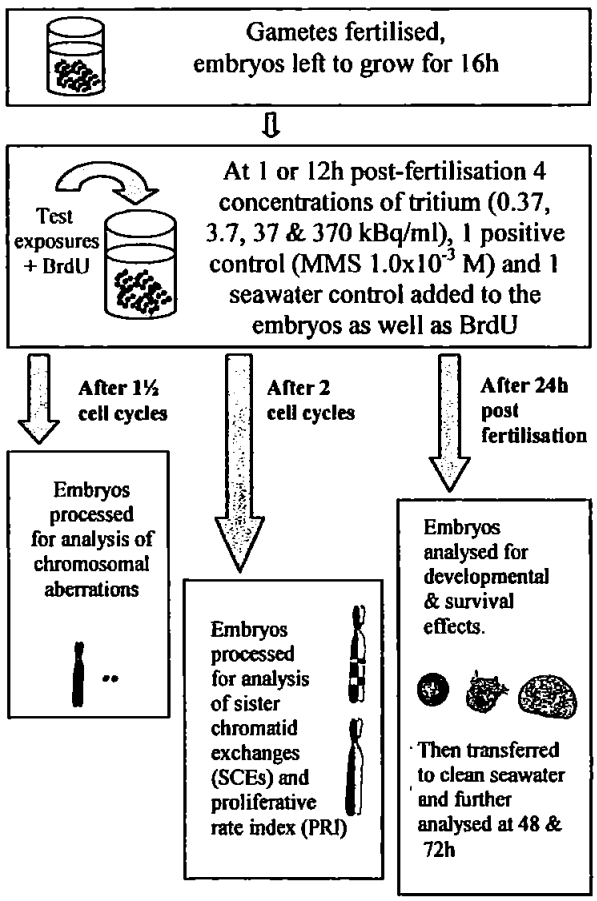


Figure 5.1. Experimental protocol for the exposure of *M.edulis* to tritium 1 or 12h post-fertilisation period (1 cell cycle = 3.8).

Figure 5.2. represents the exposure period used for the three different exposure scenarios. Experiment 1 was carried out as per standard exposure protocols. Experiment 2 was conducted to examine the effects of tritium in a more realistic exposure scenario, where embryo-larvae would be exposed continuously immediately after fertilisation. Experiment 3 was performed in response to the first two experiments in order to compare the effects of time of exposure and length of exposure.

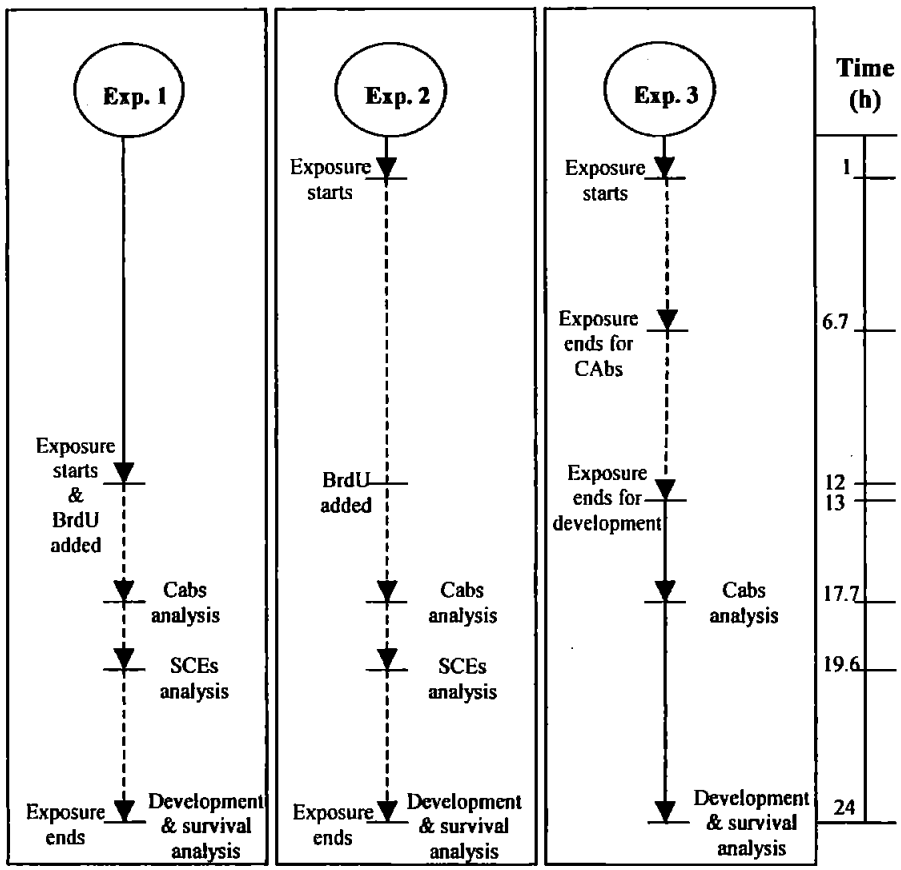


Figure 5.2. Exposure scenarios for exposure of *M.edulis* embryo larvae to tritium (- - - - - represents exposure period, 1½ cell cycles = 5.7h, 2 cell cycles = 7.6h).

5.2.1 Chemicals

All the chemicals were obtained as mentioned in section 4.2.1.

5.2.2 Exposure scenario 1: Exposure after 12h growth

Experiment 1 was conducted using eggs from 1 female mussel and sperms collected from 2 males, spawning was carried out as described in chapter 2. Approx. 60,000 embryos were produced with a 96% fertilisation rate (calculated as described in section 2.2.2); thus the density of embryos was approximately 10 embryos per ml. The embryos were allowed to grow for 12h in incubators at 15°C ± 1°C as described in section 2.2.2, after which they were divided into exposure vessels and exposed to four concentrations of tritium (0.37,3.7, 37 & 370 kBq/ml), 1 negative control containing just filtered seawater and 1 positive control containing 1.0×10^{-3} M MMS (final volume of 500 ml). A minimal concentration of 10^{-5} M BrdU was added to all the vessels simultaneously with the tritium and MMS exposure. After 1½ cell cycles (1 cell cycle = 3.8h), a subset of embryos were placed into colchicine and processed for chromosomal aberrations as described in section 2.3.2. A further subset was removed after 2 cell cycles and processed for analysis of proliferative rate index and sister chromatid exchanges as described in section 2.3.3. When the remaining embryos reached 24h post-fertilisation they were divided into replicates and placed into unexposed clean seawater (500 ml) and analysed for developmental and survival effects as described in section 2.3.1. Further analyses of morphology, behaviour and mortality were assessed at 48 and 72h post-fertilisation.

5.2.3 Exposure scenario 2: Exposure after 1h growth

In experiment 2, the eggs from 2 female mussels and sperm from 1 male were collected and processed as in section 2.2.2. Approx. 520,000 embryos were produced with a 92% fertilisation rate. The embryo density was then adjusted to approximately 30 embryos per ml. The embryos were allowed to grow for 1h, after which they were divided into exposure vessels and exposed to four concentrations of tritium (0.37, 3.7, 37 & 370 kBq /ml), 1 negative control containing just filtered seawater and 1 positive control containing 1.0×10^{-3} M MMS (final volume of 500ml). A minimal concentration of 10^{-5} M

BrdU was added to all the vessels at 12h post-fertilisation. After 1½ cell cycles (1 cell cycle = 3.8h) a subset of embryos were placed into colchicine and processed for the analysis of chromosomal aberrations as described in section 2.3.2. A further subset was removed after 2 cell cycles and processed for analysis of proliferative rate index and sister chromatid exchanges as described in section 2.3.3. When the remaining embryos reached 24h post-fertilisation they were divided into replicates and placed into unexposed clean seawater (500 ml) and analysed for developmental and survival effects as described in section 2.3.1. Further analyses of morphology, behaviour and mortality were assessed at 48 and 72h post-fertilisation.

5.2.4 Exposure scenario 3: Exposure after 1h growth for 1½ cell cycles

In experiment 3, the eggs from 3 female mussels and sperm from 2 males were collected and processed as in section 2.2.2. Approx. 510,000 embryos were produced with a 94.1% fertilisation rate. The embryo density was then adjusted to approximately 30 embryos per ml. The embryos were allowed to grow for 1h, after which they were divided into exposure vessels and exposed to four concentrations of tritium (0.37, 3.7, 37 & 370 kBq /ml), 1 negative control containing just filtered seawater and 1 positive control containing 1.0×10^{-3} M MMS (final volume of 500 ml). After 1½ cell cycles (1 cell cycle = 3.8h) a subset of embryos were placed into clean seawater and at 17.7h post-fertilisation the embryo-larvae were processed for chromosomal aberrations as described in section 2.3.2. The remaining subset of embryo-larvae were transferred from the tritiated water and placed into clean seawater (500 ml) after 12h of exposure. At 24, 48 and 72h post-fertilisation the embryo-larvae were analysed for morphological and behavioural abnormalities and survival effects as described in section 2.3.1.

5.3 Results

5.3.1 Scintillation counts of tritium

Tables 5.1., 5.2., and 5.3. describes the different levels of tritium in the different test vessels for the three different exposure scenarios.

Table 5.1. Exposure scenario 1: Nominal & definitive concentrations (kBq/ml) of tritium in seawater before and after exposure of *M.edulis* embryo-larvae (12h post-fertilisation: 12h exposure).

Nominal Concentration (kBq/ml)	Definitive pre-exposure concentration	Definitive post-exposure concentration	Difference in concentration (kBq/ml)	Percentage of initial concentration removed
Seawater (SW)	0.0035 ± 0.001	0.0025 ± 0.001	0.001	-28.57
SW + MMS	0.0026 ± 0.001	0.0058 ± 0.001	0.0026	+ 200
0.37	0.323 ± 0.02	0.260 ± 0.02	0.063	-19.5
3.7	3.24 ± 0.08	1.92 ± 0.05	1.32	-40.7
37	29.13 ± 0.19	25.93 ± 0.17	3.20	-10.99
370	299.74 ± 0.63	245.41 ± 0.52	54.33	-18.13

Table 5.2. Exposure scenario 2: Nominal & definitive concentrations (kBq/ml) of tritium in seawater before and after exposure of *M.edulis* embryo-larvae (1h post-fertilisation: 23h exposure).

Nominal Concentration (kBq/ml)	Definitive pre-exposure concentration	Definitive post-exposure concentration	Difference in concentration (kBq/ml)	Percentage of initial concentration removed
Seawater (SW)	0.0032 ± 0.001	0.0026 ± 0.001	0.0006	-18.75
SW + MMS	0.0031 ± 0.001	0.0023 ± 0.001	0.0008	-25.8
0.37	0.496 ± 0.01	0.361± 0.008	0.135	-27.2
3.7	2.46 ± 0.025	2.32 ± 0.023	0.14	-5.7
37	30.20 ± 0.30	22.61 ± 0.22	7.59	-25.13
370	284.18 ± 2.67	220.20 ± 1.96	63.98	-22.51

Table 5.3. Exposure scenario 3: Nominal & definitive concentrations (kBq/ml) of tritium in seawater before and after exposure of *M.edulis* embryo-larvae (1h post-fertilisation: 12h exposure).

Nominal Concentration (kBq/ml)	Definitive pre-exposure concentration	Definitive post-exposure concentration	Difference in concentration (kBq/ml)	Percentage of initial concentration removed
Seawater (SW)	0.0022 ± 0.001	0.0057 ± 0.001	0.0035	+ 159.09
SW + MMS	0.0034 ± 0.001	0.0028 ± 0.001	0.0006	-17.65
0.37	0.233 ± 0.05	0.005 ± 0.001	0.228	-97.85
3.7	2.61 ± 0.05	0.55 ± 0.01	2.06	-78.92
37	46.09 ± 0.82	21.27 ± 0.40	24.82	-53.85
370	312.52 ± 2.16	229.38 ± 1.84	83.14	-26.60

For scenarios 1 and 2 there is little variation in the percentage of tritium lost during the experiment between the various concentrations. No trend in the percentage of tritium lost was observed with increasing concentrations for either scenario 1 or 2. A decrease in the percentage of tritium was however observed in scenario 3 with the lowest concentration losing 97.85 % of the initial tritium in the water and subsequent concentrations (3.7, 37 and 370 kBq/ml) losing 78.92, 53.85, 26.6% respectively. More tritium was lost in scenario 3, where the embryo-larvae was exposed after 1h post-fertilisation for 12h than was lost for scenarios 1 or 2.

5.3.2 Dose received by embryo-larvae

Doses received by the embryo-larvae were calculated following the formulae by Strand *et al.* (1977) as described in section 2.7.2.

5.3.2.1 Dose receive by embryo-larvae

Table 5.4. Dose (mGy) received by *M.edulis* embryo-larvae during exposure to tritium, 12h post-fertilisation: 12h exposure (scenario 1), 1h post-fertilisation: 23h exposure (scenario 2), and 1h post-fertilisation: 12h exposure (scenario 3).

	Scenario 1 (12h post-fertilisation for 1½ cell cycles for cabs, 2 cell cycles for SCEs and PRI and 12h for developmental)			Scenario 2 (1h post-fertilisation continuously)			Scenario 3 (1h post-fertilisation for 1½ cell cycles for cabs and 12h for developmental)	
	Cabs	SCEs and PRI	Developmental & survival effects	Cabs	SCEs and PRI	Developmental & survival effects	Cabs	Developmental & survival effects
Exposure period (hr)	5.7	7.6	12	16.7	18.6	23	5.7	12
Concentration (kBq/ml)								
370	**6.9**	**9.2**	**14.52**	**21.42**	**23.72**	**27.83**	**6.9**	**14.52**
37	**0.69**	**0.92**	**1.45**	**2.14**	**2.37**	**2.78**	**0.69**	**1.45**
3.7	**0.07**	**0.092**	**0.14**	**0.21**	**0.24**	**0.28**	**0.07**	**0.14**
0.37	**0.007**	**0.009**	**0.01**	**0.02**	**0.02**	**0.03**	**0.007**	**0.01**

By comparing the values in table 5.4. the variation in dose between the three experiments can be made. The embryo-larvae that were exposed for 1½ cell cycles (chromosome aberrations) and 12h (developmental analysis) at either 12h post-fertilisation (scenario 1) or 1h post-fertilisation (scenario 3) both received the same dose of radiation from tritium. Therefore comparisons could be made between exposure scenarios 1 and 3 as to the effect of radiation at different life stages. Table 5.4. indicates a 2-3-fold increase in the dose received by 1h post-fertilised embryo-larvae that were exposed continuously as the time of exposure has increased. Therefore a comparison maybe made between exposure scenarios 2 and 3 in an attempt to compare the effect of different doses of radiation on embryo-larvae (1h post-fertilisation).

5.3.3 Developmental and survival/mortality effects

5.3.3.1 Exposure scenario 1 (12h post-fertilisation, exposure period 12h)

Figure 5.3. represents the percentage of normal, abnormal and dead *M.edulis* embryo-larvae after 24, 48 and 72h post-fertilisation following exposure to tritium and MMS. There is a dose dependent decrease in the percentage of normal embryo-larvae at 24h ($P=0.00005$; $R^2=65.24$ %) however the lowest concentration (0.37 kBq/ml) and the MMS exposed embryos were not significantly different to the control. By 48 and 72h all the larvae that were exposed to tritium were significantly different from the control ($P=0.00005$ and 0.00005 respectively) with under 50% of the embryo-larvae exposed to 3.7, 37 and 370 kBq/ml of tritium appearing and behaving normally by 72h. In conjunction with the decrease in normality, with increasing concentration, the percentage of abnormal embryo-larvae increased at 24 and 48h ($P=0.00005$ for both times). However, at 72h the number of abnormal embryo-larvae had decreased and levelled in all concentrations although 3.7 and 370 kBq/ml were still significantly different from the control ($P=0.0191$). The decrease in abnormality at 72h was due to a 4-5-fold increase in mortality in all concentrations. A significant dose dependent increase in mortality with increasing concentration of tritium and MMS occurred with increasing exposure time. The predicted LC_{50} for *M.edulis* after exposure to tritium for 72h is 6.51 kBq/ml, and the dose received was calculated to be 0.002 rad or 0.02 mGy.

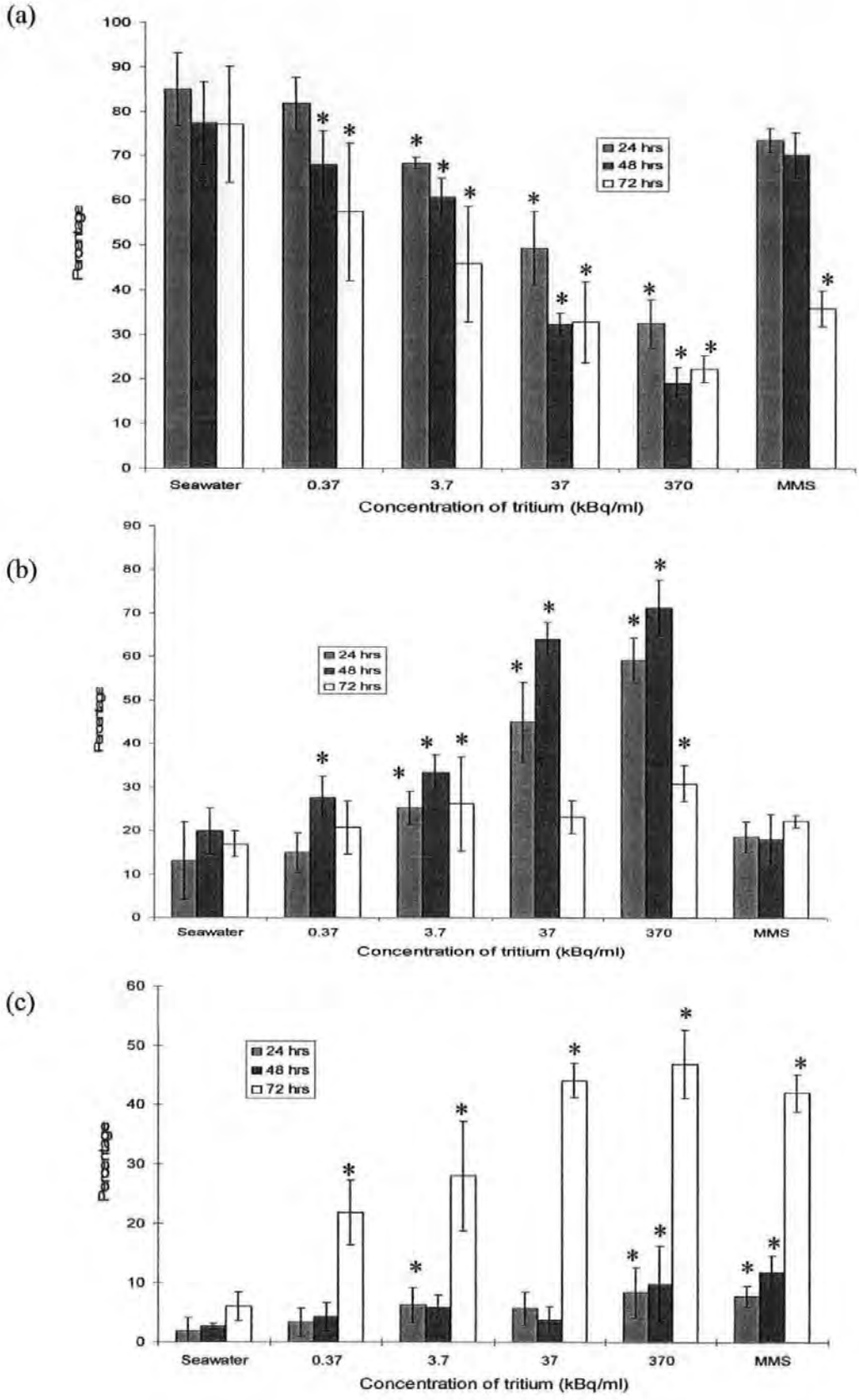


Figure 5.3. Percentage of (a) normal, (b) abnormal, and (c) dead *M.edulis* embryo-larvae after exposure 12h post-fertilisation for 12h exposure period. Error bars represent standard deviation. (* = significantly different from control, $P<0.05$) (n = 5).

5.3.3.2 Exposure scenario 2 (1h post-fertilisation, exposure period = 23h)

Figure 5.4. represents the percentage of normal, abnormal and dead *M.edulis* embryo-larvae after 24, 48 and 72h post-fertilisation following exposure to tritium and MMS after 1h post-fertilisation for approximately 23h. A dose dependent decrease in the percentage of normal embryo-larvae occurred at 24, 48 and 72h (P=0.00005 for all times) with all the concentrations of tritium and the MMS concentration being significantly different from the control. At 48h there were no normal embryo-larvae in the two highest concentrations (37 and 370 kBq/ml) and the MMS exposed samples. By 72h only the lowest concentration of 0.37 kBq/ml contained a small percent of normal embryo-larvae and all the other concentrations were devoid of normality. The decrease in normality at 24h (with increasing concentration) was related to an increase in the percentage of abnormal embryo-larvae (P=0.0001). At 48h all the concentrations of tritium except the lowest one (0.37 kBq/ml) were significantly different from the control (P=0.0281). At 72h post-fertilisation only the highest concentration of tritium (370 kBq/ml) and the MMS exposed samples were significantly different from the control (P=0.00005). The percentage of dead in the controls did not vary greatly over the three days with approximately 10% of the embryos dying. At 24h there was a significant difference (P=0.00005) between all but the lowest concentration of tritium and the control and by 48h there was statistical difference (P=0.00005) between all the concentrations of tritium and the MMS concentration and the control. At 72h there was a large increase in mortality in embryo-larvae exposed to tritium and MMS with all the concentrations of tritium and the MMS concentration being significantly different from the control (P=0.00005). Regression coefficient indicates a moderately strong dose relationship (R^2=42.69%). 100% mortality was exhibited in tritium and the MMS concentration at 72h. The predicted LC_{50} for *M.edulis* after exposure to tritium for 72h was not possible. The predicted 48h LC_{50} was 0.267 Bq/ml, and the dose received would be 0.09 rad or 0.9 mGy.

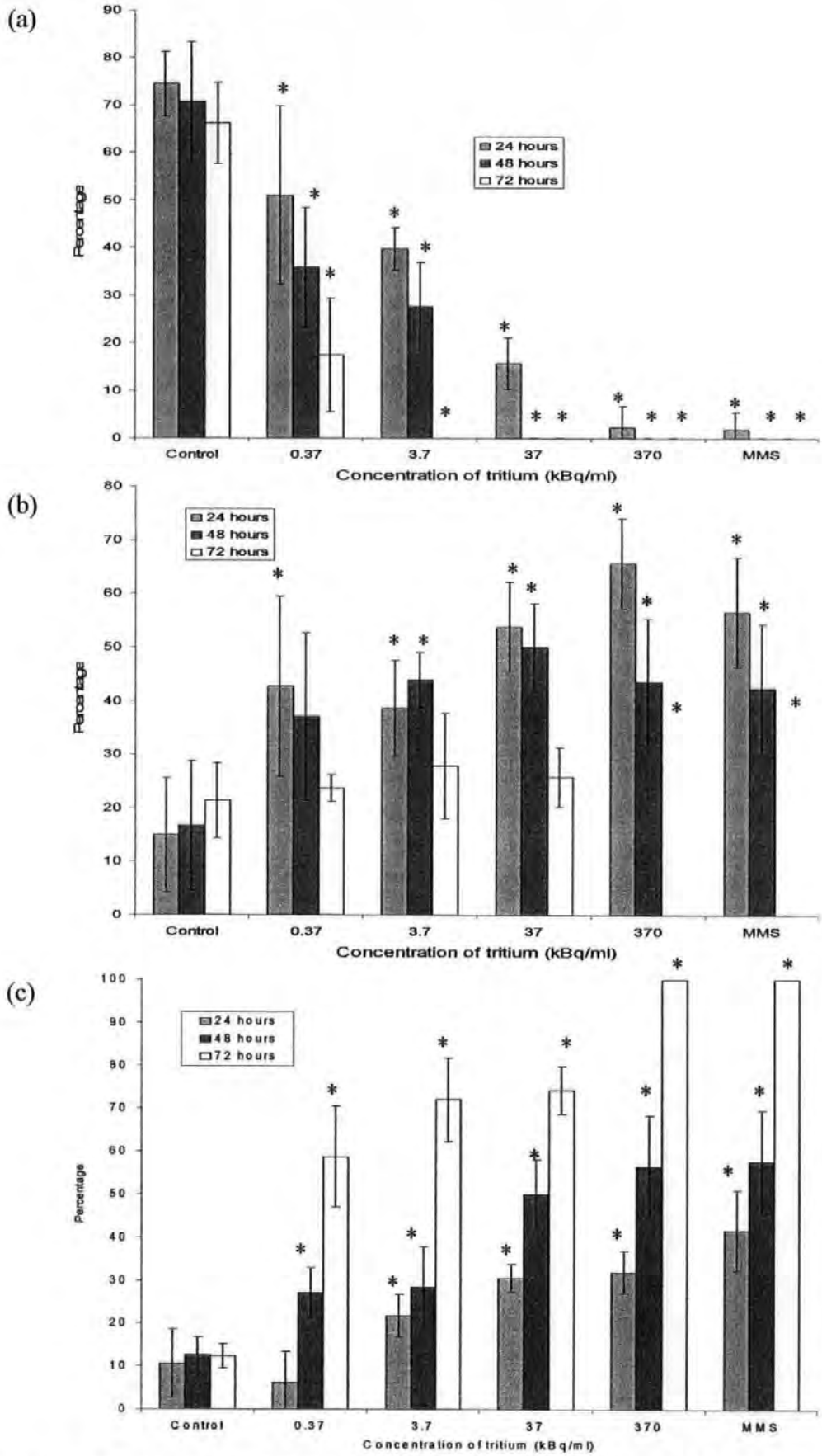


Figure 5.4. Percentage of (a) normal, (b) abnormal, and (c) dead *M.edulis* embryo-larvae after exposure 1h post-fertilisation for 23h exposure period. Error bars represent standard deviation. (* = significantly different from control, $P<0.05$) (n = 5).

5.3.3.3 Exposure scenario 3 (1h post-fertilisation, exposure period 12h)

Figure 5.5. represents the percentage of normal *M.edulis* embryo-larvae after 24, 48 and 72h post-fertilisation following exposure scenario 3 which represents, exposure to tritium after 1h post-fertilisation for an exposure time of 12h. At 24h the lowest concentration of 0.37 kBq/ml were not significantly different to the control, although overall there was a dose dependent decrease in the percentage of normal embryo-larvae ($P=0.0009$; $R^2=19.45$ %). At 48 and 72h all the larvae that were exposed to tritium were significantly different from the control ($P=0.00005$ and 0.00005 respectively) with under 20% of the embryo-larvae exposed to 37 and 370 kBq/ml of tritium appearing and behaving normally. In conjunction with the decrease in normality, with increasing concentration and time, there was a significant increase in the percentage of abnormal embryo-larvae at 24, 48 and 72h ($P=0.00037$, 0.00005 and 0.00005 respectively). However at 72h there is a slight decrease in the number of abnormal embryo-larvae probably due to an increase in mortality. There was no statistical significant difference in mortality between concentrations at 24h after analysis with ANOVA ($P=0.2686$) but by 48h a significant difference in mortality between the controls and the tritium exposed embryo-larvae was observed ($P=0.027$). Only the lowest concentration (0.37 kBq/ml) was statistically similar to the controls. There was no statistical difference in the percentage of dead embryo-larvae between the three highest concentrations of tritium (3.7, 37 and 370 kBq/ml). At 72h there was a 2-3 fold increase in mortality in the two highest concentrations of tritium and only these two were statistically different from the controls ($P=0.007$). A regression co-efficient of $R^2=62.47\%$ indicated a strong dose relationship between the percentage of dead embryo-larvae and concentration. The predicted LC_{50} for *M.edulis* after exposure to tritium for 72h would be approximately 672.2 kBq/ml, and the dose received would be 0.22 rad or 2.2 mGy.

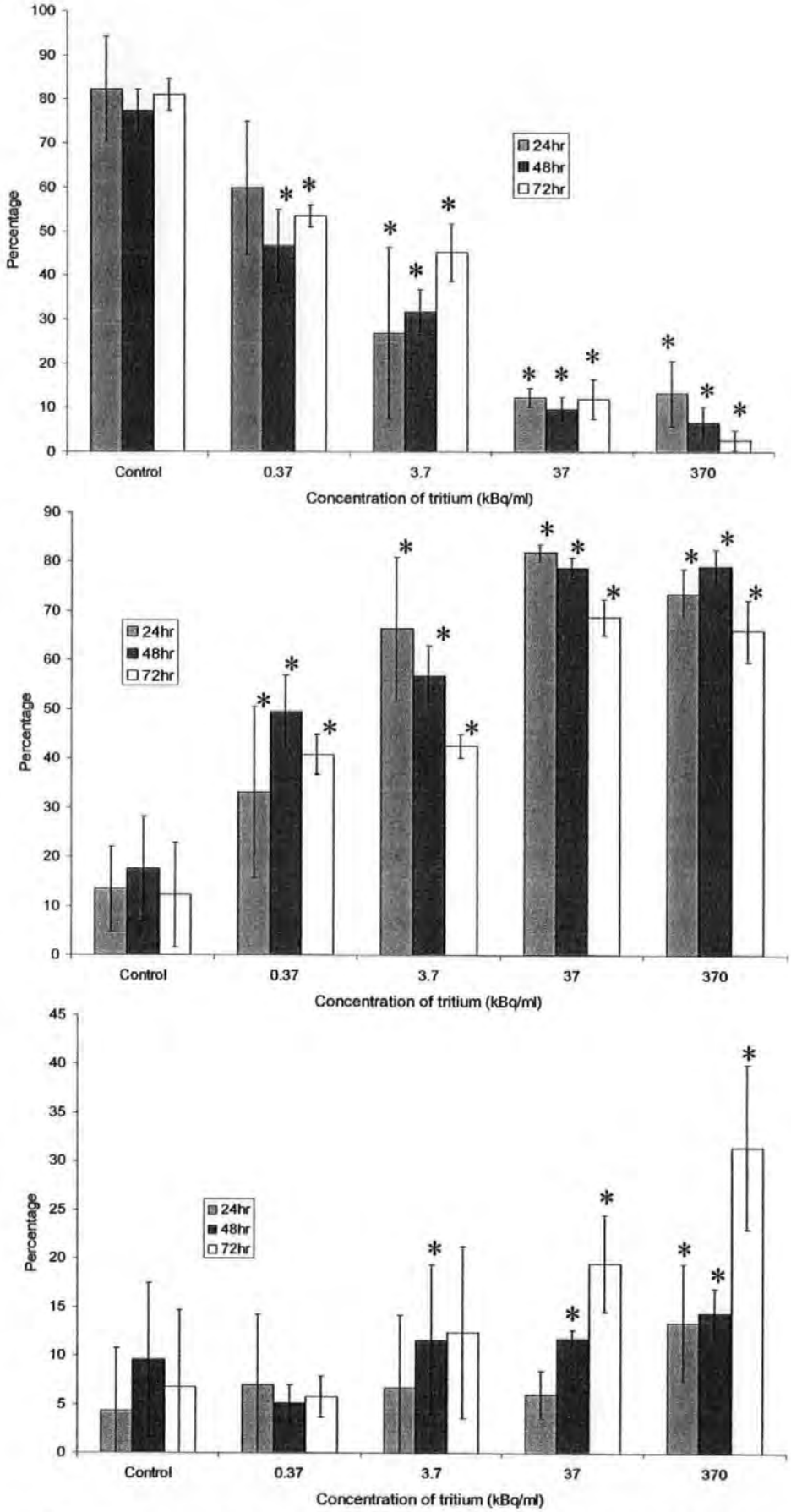


Figure 5.5. Percentage of (a) normal, (b) abnormal, and (c) dead *M.edulis* embryo-larvae after exposure 1h post-fertilisation for 12h exposure period. Error bars represent standard deviation. (* = significantly different from control, $P<0.05$) (n = 5).

5.3.3.4 Comparison between exposures

Figure 5.6. represents the percentage of dead *M.edulis* embryo-larvae at 72h following exposure to tritium. Experiment 2, where the embryo-larvae were exposed after 1h post-fertilisation for a period of 23h showed the highest levels of mortality. Experiment 1, where the embryo-larvae were exposed 12h-post-fertilisation for a period of 12h experienced higher levels of mortality than embryo-larvae exposed also for 12h but were exposed 1h after fertilisation. This might indicate that the embryo-larvae exposed at an earlier life stage might be able to repair damage to a greater extent than if the exposure occurred at a later stage. At 72h the levels of mortality in the controls of experiment 2 were significantly higher than the controls of experiments 1 and 3, although the levels of mortality were not elevated above the baseline levels.

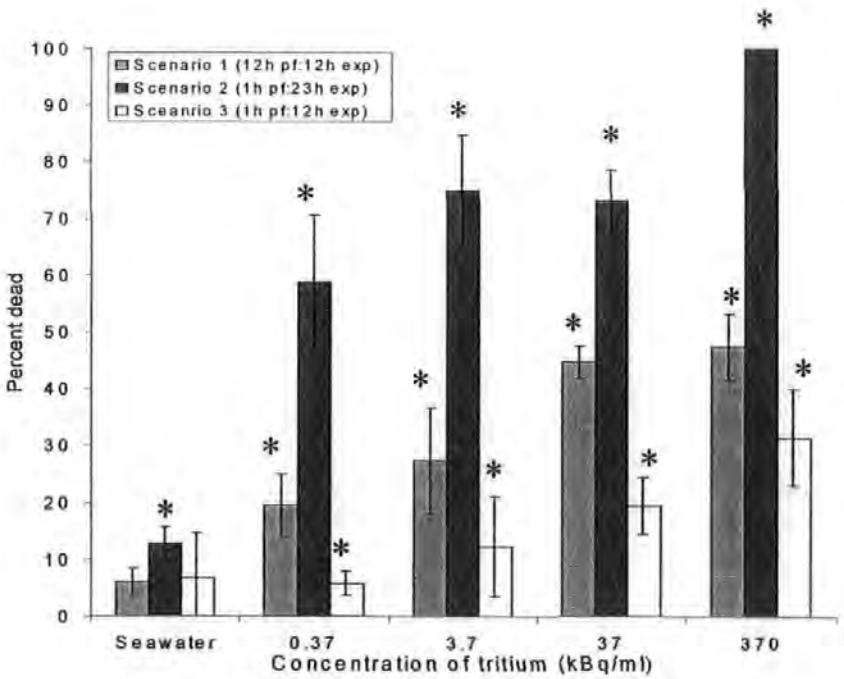


Figure 5.6. Percentage of dead *M.edulis* embryo-larvae after 72h following exposure to tritium (* = significantly different between scenarios $P<0.05$) (pf = post-fertilisation: exp = exposure) (n = 5).

5.3.4 Proliferative rate index

Figure 5.7. shows the proliferative rate index of embryo-larvae of *M.edulis* after exposure to tritium in comparison with controls.

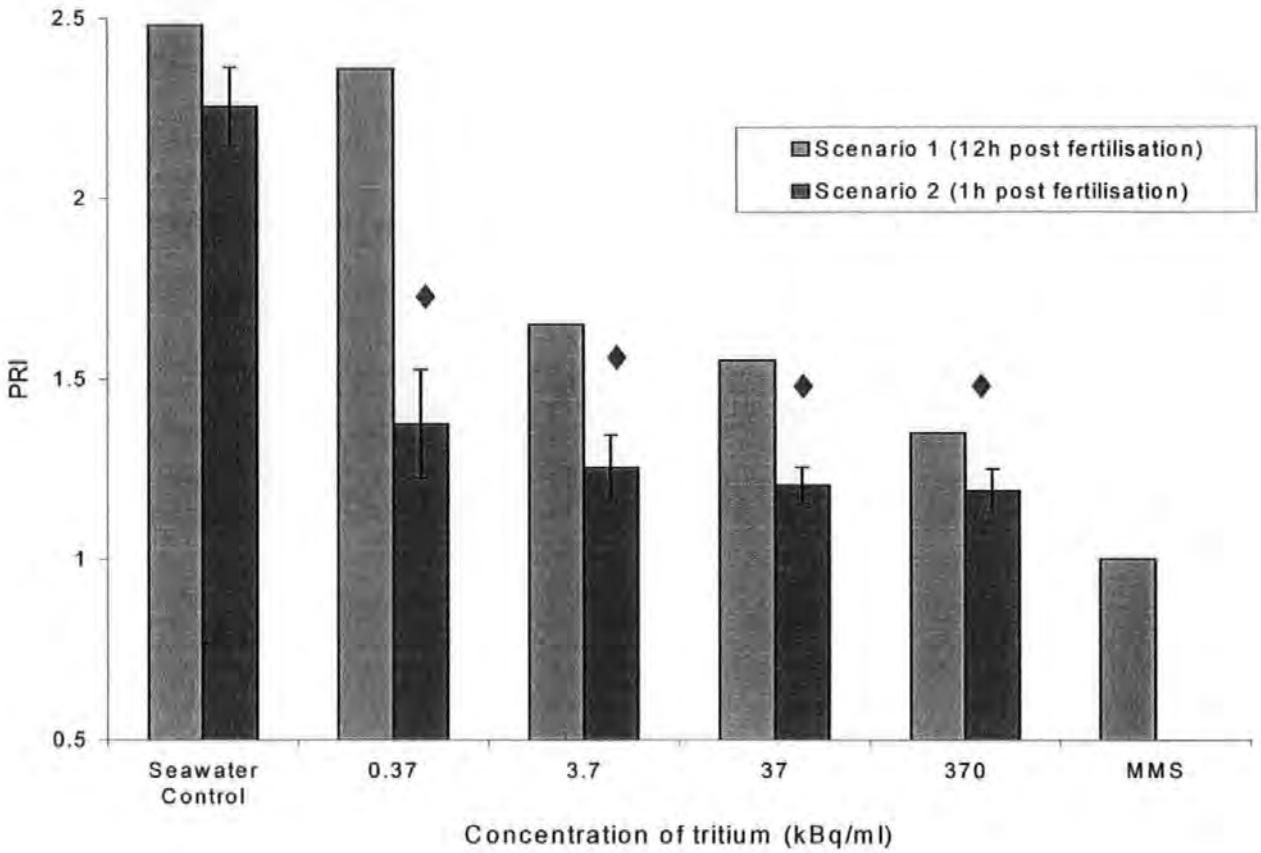


Figure 5.7. Proliferative rate index in *M.edulis* embryo-larvae after exposure to tritium following 1 or 12h post-fertilisation. Error bars represent standard error. (♦ = significantly different from control $P<0.05$) (n = 2).

5.3.4.1 Exposure scenario 1 (12h post-fertilisation)

There appears to be a threshold level of difference at 3.7 kBq/ml and the other higher concentrations of tritium, although the lowest concentration of 0.37 kBq/ml is similar to the control. There is not a statistically significant relationship between the PRI and the concentrations ($P=0.2511$) however, there is a relationship between the variables with the PRI decreasing with increasing concentrations ($R^2=40.14$ %). However due to the lack of replicates this data might not be conclusive.

5.3.4.2 Exposure scenario 2 (1h post-fertilisation)

Statistical analysis of the results using ANOVA show that there is a dose dependent decrease in the PRI with increasing doses of tritium (P=0.001). All four concentrations of tritium were statistically significantly lower than the controls. However there was no significant difference between the various concentrations of tritium except for the 0.37 kBq/ml and the 370 kBq/ml. No chromosomes were present in the MMS exposed embryo-larvae and therefore the analysis of proliferation rate index could not be carried out. The regression coefficient indicates a relatively weak relationship between concentration and PRI probably due to a threshold effect (R^2=13.55 %).

5.3.4.3 Comparison between scenarios

Due to a lack of replicates in exposure scenario 1, a statistical comparison between exposure scenarios 1 and 2 could not be attempted. In general there appears to be little variation between the controls, however in the exposed embryo-larvae cells there appears to be a large decrease in the PRI in exposure scenario 2 (1h post fertilised embryo-larvae exposed for 18.6h). The variation between exposure scenarios appeared greatest at the lowest concentration and then decreased as the PRI in exposure scenario 1 was reduced to similar levels as scenario 2.

5.3.5 Sister chromatid exchanges (SCEs)

The induction of sister chromatid exchanges in the embryo-larvae of *M.edulis* is shown in figure 5.8.

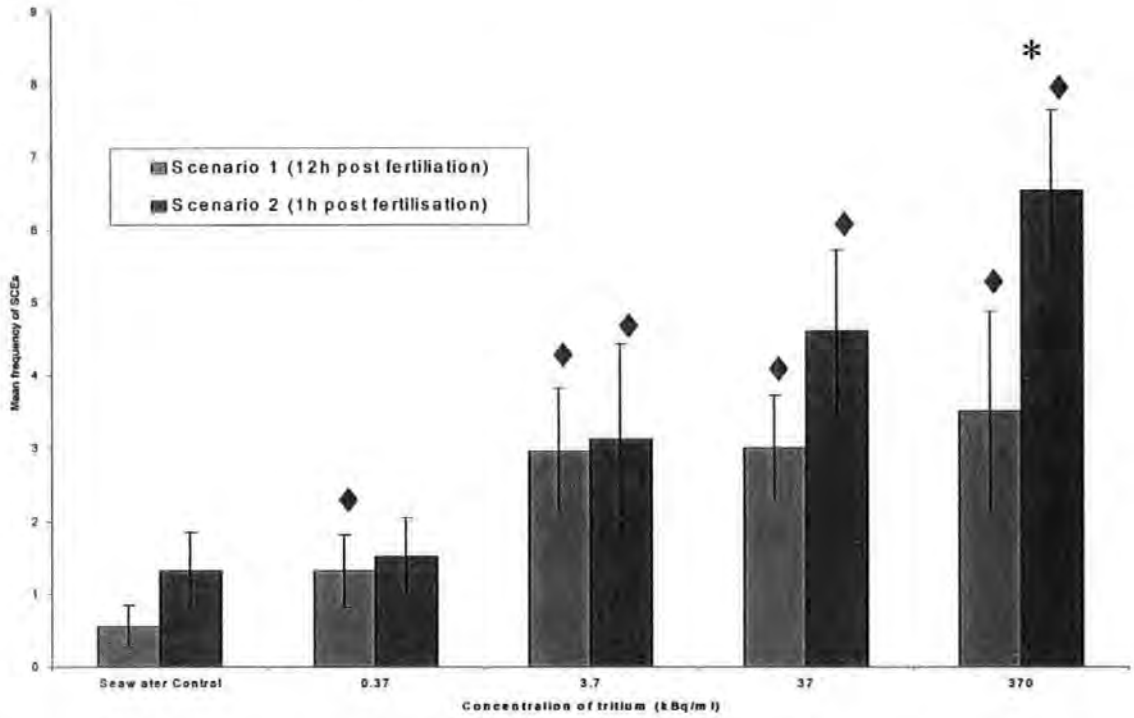


Figure 5.8. The induction of sister chromatid exchanges (SCEs) in *M.edulis* embryo-larvae following exposure to tritium after 1 or 12h post-fertilisation. Error bars represent standard error (* = significant difference between exposures; ♦= significantly different from control, $P<0.05$) (n = 2).

5.3.5.1 Exposure scenario 1 (12h post-fertilisation)

The embryo-larvae exposed to MMS had all only divided once in the presence of BrdU and therefore did not exhibit differential staining and could not be scored for SCEs. Tritium exposed embryos produced mean SCEs of between 1.32 ± 1.2 and 3.5 ± 2.2 for all four concentrations which were statistically significantly different from the controls ($P=0.00005$). However the relationship between induction of SCEs and concentration was not dose dependent with a regression coefficient of only $R^2=7.42\%$. The mean baseline level of SCEs in the controls was approximately 0.56 ± 0.71 SCEs per cell.

5.3.5.2 Exposure scenario 2 (1h post-fertilisation)

The frequency of SCEs increased with increasing concentration of tritium. Statistical analysis carried out using Kruskal Wallis test for non-parametric data showed that there was a significant difference between the controls and the samples treated with tritium (P=0.005). The controls were not significantly different to the lowest concentration (0.37 kBq/ml) but there was a statistical significant difference between the controls and the other three concentrations of tritium. There was also a difference between the three highest concentrations of tritium that had statistically higher levels of SCEs than the controls, however due to the low numbers of SCEs produced the dose relationship, as shown by simple regression analysis, was not strong (R^2=28.58%). As for the PRI analysis (section 5.3.4.2) there were no chromosomes present in the MMS exposed embryo-larvae and therefore the induction of SCEs could not be examined.

5.3.5.3 Comparison between exposures

There was no significant difference in the mean frequencies of SCEs between embryo-larvae exposed after 12h for 7.6h (scenario 1) and embryo-larvae exposed after 1h for 18.6h (scenario 2) in the controls, 0.37, 3.7 and 37 kBq/ml. There was a significant increase in the mean frequency of SCEs in embryo-larvae exposed to 370 kBq/ml in scenario 2 compared to scenario 1 after analysis using the non-parametric Kruskal-Wallis test (P=0.0028).

5.3.6 Chromosomal aberrations (Cabs)

Figure 5.9. provides examples of chromosomal aberrations observed in metaphase spreads of *M.edulis*.

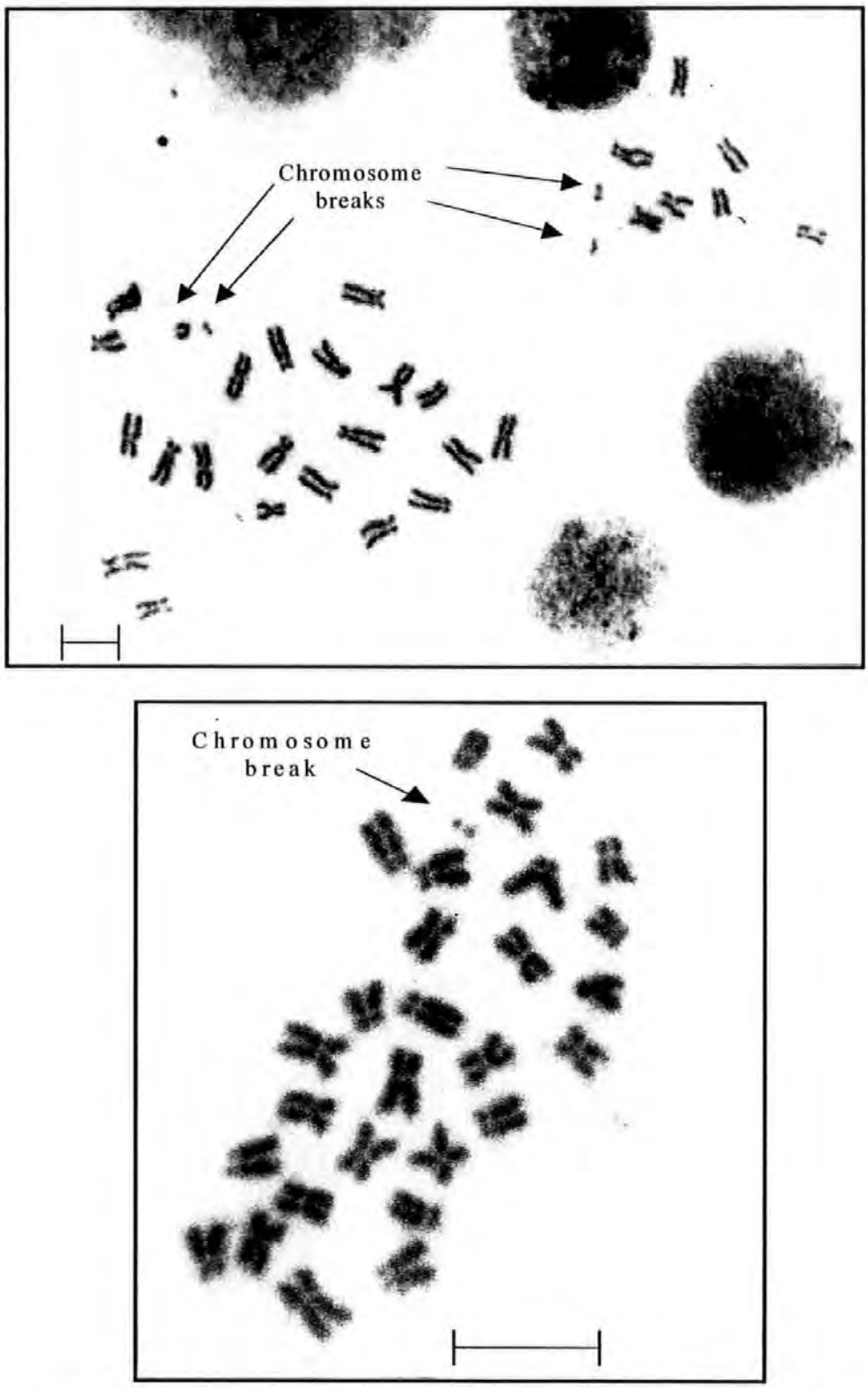


Figure 5.9. Chromosomal aberrations observed in a metaphase spread of *M.edulis* (2n = 28 chromosomes) (scale bar = 10μm).

5.3.6.1 Exposure scenario 1 (12h post-fertilisation)

Figure 5.10. represents the induction of chromosomal aberrations in embryo-larvae of *M.edulis* after exposure to tritium 12h post-fertilisation for 5.7h.

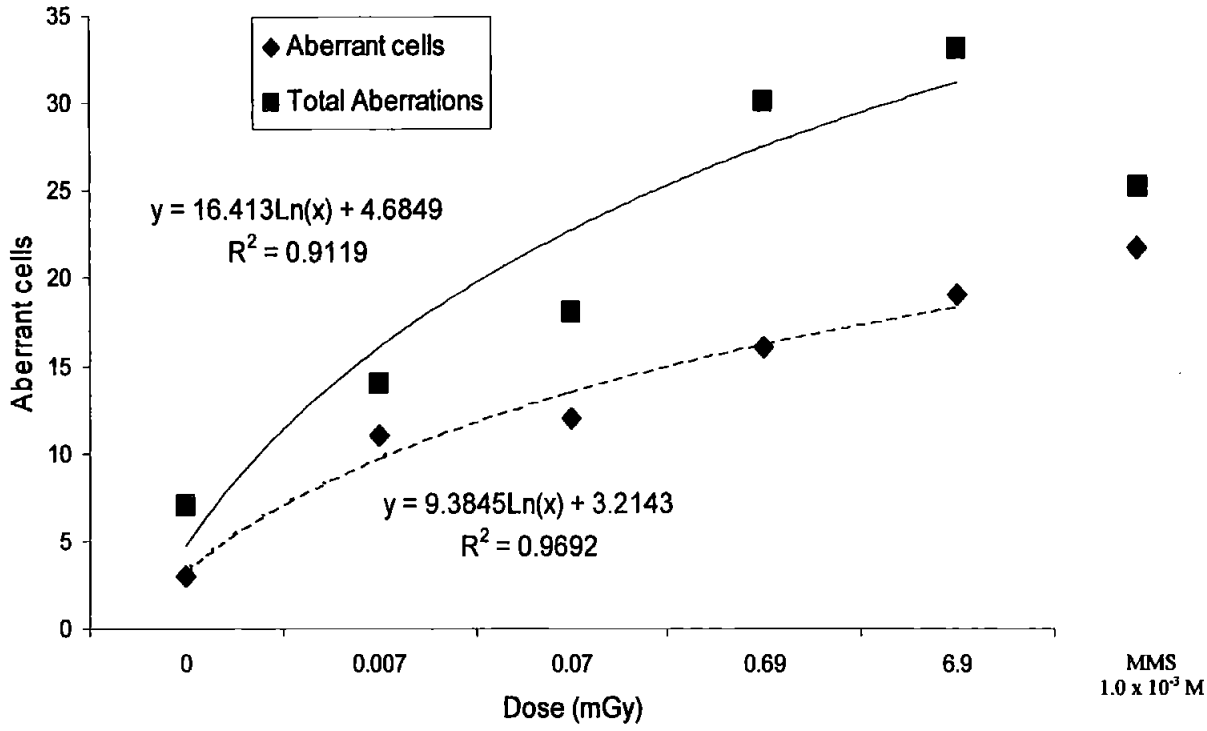


Figure 5.10. Induction of chromosomal aberrations in *M.edulis* embryo-larvae after exposure to tritium after 12h of fertilisation for 5.7h (exposure scenario 1) Logarithmic line of best fit (n = 2).

Embryo-larvae exposed to the chemical MMS had significantly more cells with aberrations than the controls, and the number of cells containing aberrations was slightly greater than in embryo-larvae exposed to tritium. However, the number of aberrations were lower in the MMS exposed embryo-larvae than the two highest concentrations of tritium (37 and 370 kBq/ml) indicating that tritium caused more damage per cell than MMS. There were more chromatid aberrations than chromosome type aberrations in all of the exposed cells. The percentage of aberrations that were chromatid type aberrations ranged from 78-96% for all the concentrations.

5.3.6.2 Exposure scenario 2 (1h post-fertilisation)

Figure 5.11. represents the induction of chromosomal aberrations in embryo-larvae of *M.edulis* after exposure to tritium 1h post-fertilisation for 16.7h.

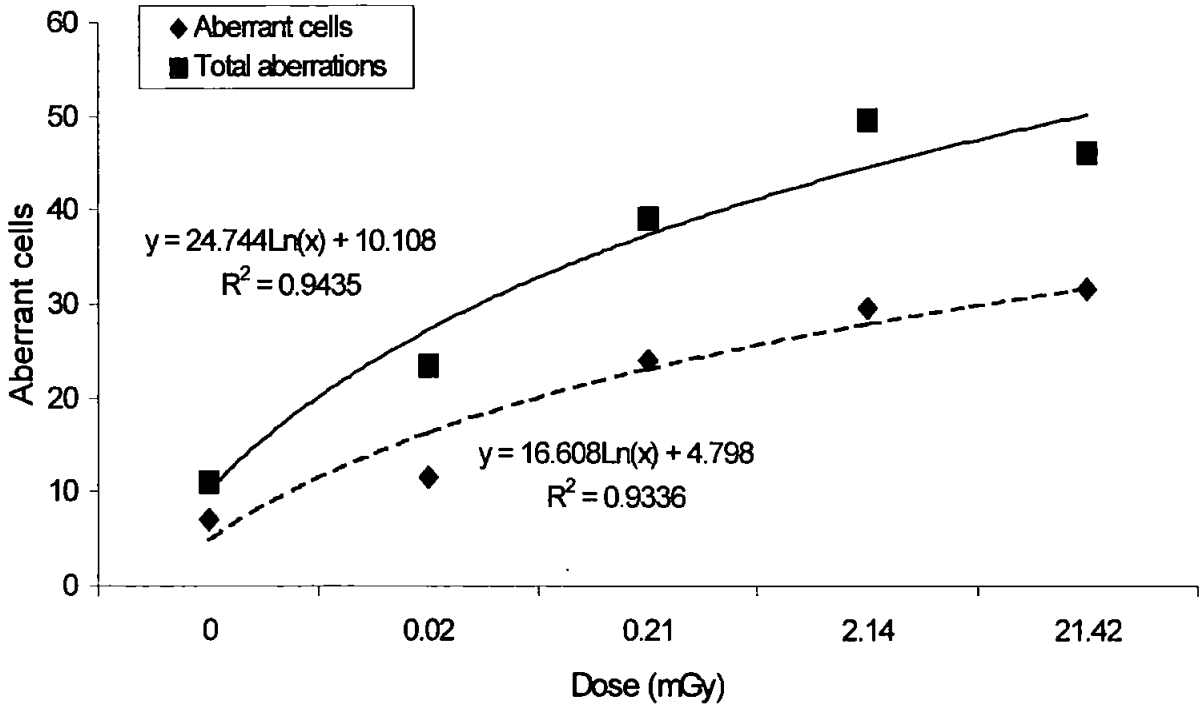


Figure 5.11. Induction of chromosomal aberrations in *M.edulis* embryo-larvae after exposure to tritium after 1h of fertilisation for 16.7h (exposure scenario 2). Logarithmic line of best fit (n = 2).

There was a statistically significant increase in the induction of both aberrant cells (P=0.0002) and the number of total aberrations (P=0.0022) scored from embryo-larvae that had been exposed to tritium in comparison to the controls. However, there was no difference between the three highest concentrations of tritium. The baseline level of aberrations in the controls was approximately 7 ± 1.4 aberrant cells containing 11 ± 4.2 aberrations in total. In the controls, 31.8% of aberrations observed were chromatid type aberrations. In the cells exposed to tritium the number of aberrations ranged from 45.2-67.9%, there was no trend in the ratio of chromosome and chromatid aberrations. No

chromosome exchanges were observed in the control cells, in contrast 2.5 ± 0.5 exchanges were observed per 100 cells in tritium exposed samples.

5.3.6.3 Exposure scenario 3 (1h post-fertilisation for 1½ cell cycles)

The number of chromosomal aberrations induced in embryo-larvae of *M.edulis* after exposure to tritium and MMS, 1h post-fertilisation for 5.7 (1½ cell cycles) is shown in figure 5.12.

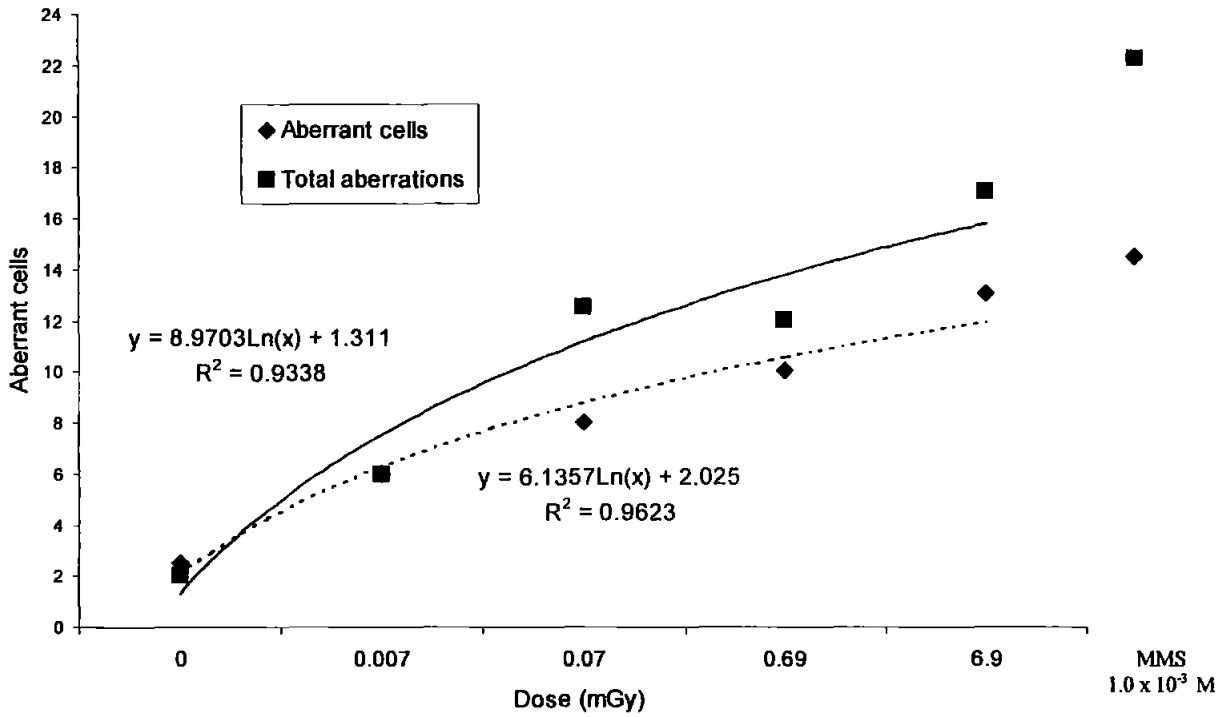


Figure 5.12. Induction of chromosomal aberrations in embryo-larvae of *M.edulis* after exposure to tritium after 1h fertilisation for 1½ cell cycles (5.7h) (exposure scenario 3). Logarithmic line of best fit (n = 2).

All the concentrations of tritium produced embryo-larvae with a higher incidence of total aberrations and cells containing aberrations in comparison to the controls (P=0.0001 and P=0.0018 respectively). Embryo-larvae that were exposed to the chemical MMS also appeared to have significantly more cells with aberrations than the controls. For most of the concentrations there was equal numbers of chromosome and chromatid type aberrations, although there was slightly more chromosome type aberrations in the tritium-

exposed samples. In the controls, 25% of aberrations observed were chromatid type aberrations. In the cells exposed to tritium the number of aberrations ranged from 41.7-52.2% whereas the MMS exposed cells contained only 23.4% chromatid aberrations.

5.3.6.4 Comparison between scenarios

Figure 5.13. shows the frequency of total aberrations scored in *M.edulis* embryo-larvae after exposure to tritium 1h post-fertilisation, for either 5.7 or 17.7h, and 12h post-fertilisation for 5.7h.

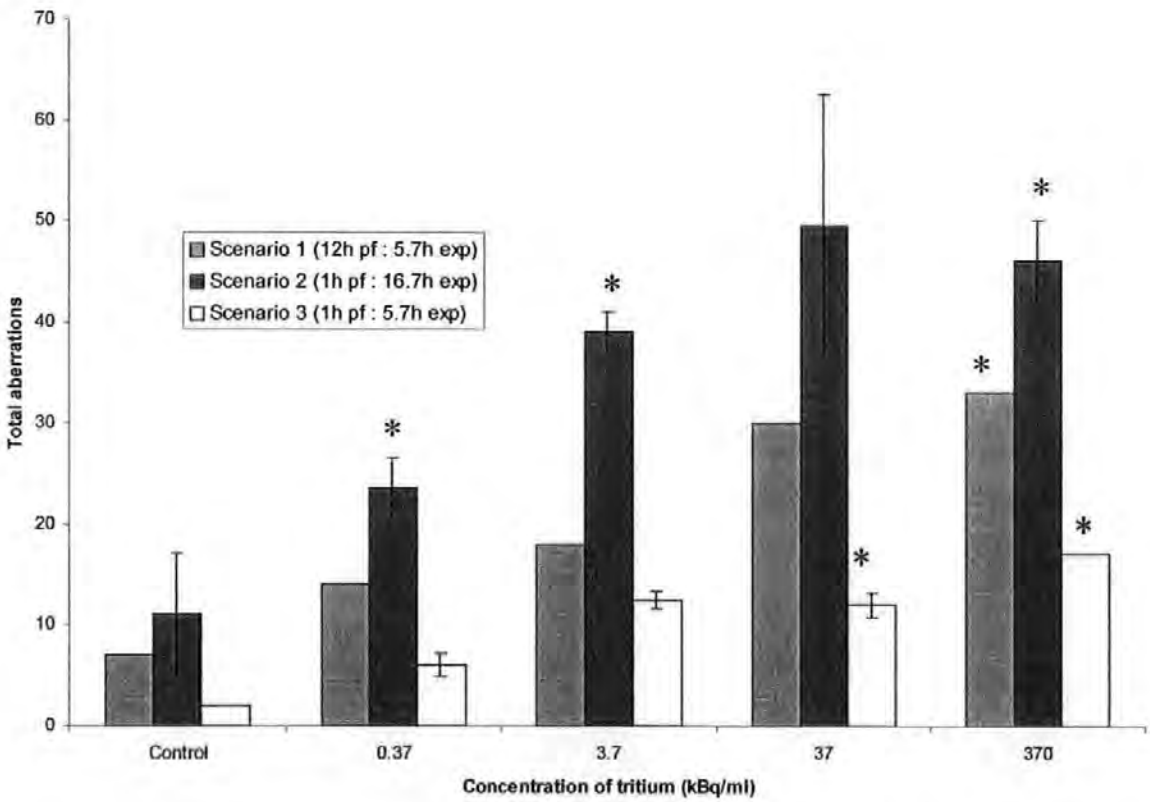


Figure 5.13. Total number of aberrations in *M.edulis* embryo-larvae after exposure to tritium. Error bars represents standard deviation (* = significant difference between scenarios, $P<0.05$) (pf = post-fertilisation : exp = exposure) (n = 2).

There was a statistically significant difference in the number of aberrations formed in the embryo-larvae exposed to 0.37 and 37 kBq/ml of tritium 1h post-fertilisation for a period of 17.7h. No difference in the number of aberrations was detected at these concentrations for embryo-larvae exposed for 5.7h at either 1 or 12h post-fertilisation. There was a difference between the number of aberrations at the 37 kBq/ml concentration between experiments, 1 and 2, and experiment 3. This suggests that the levels of aberrations in experiment 1 (12h-post-fertilisation for 5.7h) had increased and were more similar to those in experiment 2 than experiment 3. In the highest concentration (370 kBq/ml) there was a statistically significant difference between all the number of aberrations in all the experiments. The large increase in number of aberrations between experiments 1 and 3, where the embryo-larvae were exposed at different life stages for the same period of exposure, also shows the same trend as the increase in mortality. Therefore it can be suggested that the induction of aberrations, analysis in embryo-larvae aged 17.7h, resulted in an increase in mortality of the larvae a few days later.

Figure 5.14. represents the number of aberrant cells in *M.edulis* embryo-larvae after exposure to tritium 1h post-fertilisation, for either 5.7 or 16.7h, and 12h post-fertilisation for 5.7h.

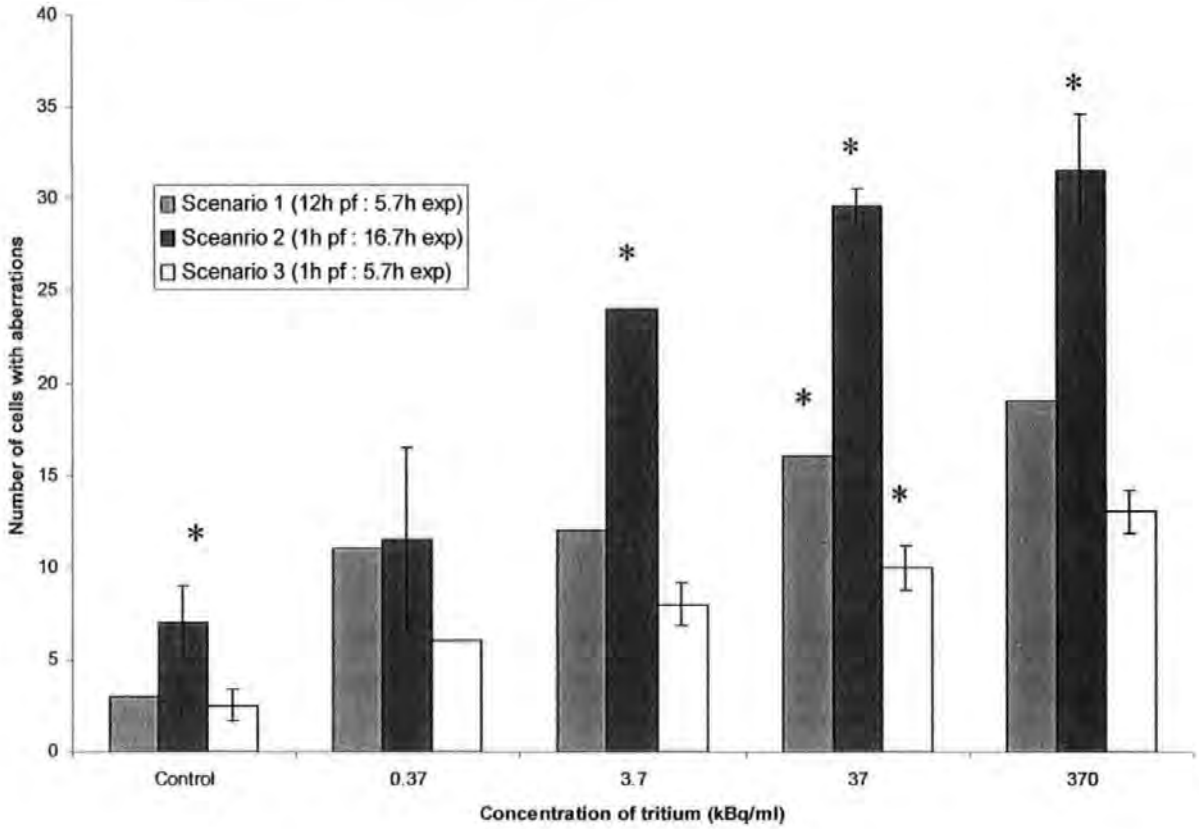


Figure 5.14. Number of cells with aberrations in *M.edulis* embryo-larvae after exposure to tritium. Error bars represents standard deviation (* = significant difference between scenarios, $P<0.05$) (pf = post-fertilisation : exp = exposure) (n = 2).

There was no difference in the number of embryo-larval cells containing aberrations in the lowest concentration (0.37 kBq/ml). Although there is a higher number of aberrant cells produced in embryo-larvae after exposure from only 1h post-fertilisation for 17.7h for both the 3.7 and 370 kBq/ml concentrations of tritium. There is a statistical difference in all experiments after exposure to 37 kBq/ml which does not seem to display a relationship between mortality or the total numbers of aberrations, although this might have been due to varying levels of heavily damaged cells experienced during the experiments.

5.4 Discussion

Tritium significantly increased mortality and cytogenetic (sister chromatid exchanges and chromosomal aberration) and cytotoxic (proliferative rate index) parameters in *M.edulis* embryo-larvae. There appeared to be no difference in the induction of aberrations caused by tritium after exposure of 1h and 12h post fertilised embryo-larvae for a period of 1½ cell cycles. However there was a statistical difference in the percentage of mortality between these two exposure scenarios. Embryo-larvae exposed after 12h experienced a higher percentage of mortality than those exposed after 1h. This might indicate that the embryo-larvae exposed at the earlier stage of life had more time to be able to repair potential genetic damage (e.g. DNA strand breaks) and hence produced larvae with a reduced mortality rate. Therefore although the age of the organism did not seem to affect the degree of chromosomal damage it did influence the overall development of the larvae. There was both a developmental and genotoxic (chromosomal aberrations) difference between those embryo-larvae exposed after 1h for either a short or long period. This indicates that the length of exposure and thus the dose also affects the potential toxicity of tritium. Goose barnacles (*Pollicipes polymerus)* exposed for approximately 30 days were used to evaluate the effects of HTO on normal development (moulting index) (Abbott and Mix, 1979). Effects were observed at concentrations as low as $7x10^{-6}$ μCi/ml. Abbott and Mix (1979) supported the linear theory as seen in the present study and not the threshold theory of radiation effects after exposure of goose barnacles to tritium. During the current experiments there was a statistical difference in the percentage of mortality between the control and the embryo-larvae that were exposed to tritium by the end of the experiment. Although the highest levels of mortality were observed after exposure of tritium to embryo-larvae 1h post-fertilisation for an exposure period of 23h, significantly elevated levels of mortality were observed at the lowest concentration of 370 Bq/ml (1 x 10^{-3} μCi/ml, estimated dose rate ≈ 1.21 μGy/h) for all experiments. Similar results were reported by Abbott and Mix (1979) who observed effects on survival in the larval goose

barnacles when they were reared in seawater containing only 5.55 x 10^{-4} μCi/ml of tritiated water (20Bq/ml, estimated dose rate ≈ 0.07μGy/h). Nelson (1971) showed that tritium affected the abnormality of larvae of the Pacific oyster, *Crassostrea gigas*. A statistically significant increase in the number of abnormal larvae were observed at concentrations of 10^{-3} Ci/l (approximately 1 rad), which is within the range of the current experiment 0.0029-2.9 rads. However they found no radiation effects on the rate of development or survival. The effects of tritiated water were tested on the brine shrimp, *Artemia salina*, a known radioresistant animal (Higuchi *et al.*, 1980). The growth of the shrimp was not affected by 3.5 mCi/ml (129.5 MBq/ml) tritiated water. Although the life span was reduced by rearing the shrimps in tritiated water with a concentration of 0.5 mCi/ml (18.5 MBq/ml) which is approximately 50-5000 times greater than the concentrations used during the present experiment.

Knowles and Greenwood (1997) found that the development and survival of eggs to larvae of the polychaete worm, *Ophryotrocha diadema*, was statistically reduced when exposed to 2.24 MBq/ml (7.3 mGy) of tritiated water but was not affected by similar doses of γ-radiation (^{137}Cs). Thus it is possible that the RBE for β-radiation is greater than γ-radiation, for some aquatic invertebrates, although this is not the case in the majority of mammalian data (Straume and Carsten, 1993). This, however, supports the high radiosensitivities observed during the current study. In general tritium is believed to be less harmful than gamma rays due to their low penetration potential particularly in terrestrial environments. The uptake of tritium is increased in aquatic organisms that are continuously exposed via their habitat. In addition the potential harmful effects of tritium is increased if it is ingested by the organism as its penetration distance is reduced and it can affect vital organs. Therefore tritium is not considered as much as a threat to mammalians as gamma radiation unless it is accidentally ingested. The concentrations causing significant levels of mortality shown by *Ophryotrocha diadema* were 6-600 times greater than during the present study although no attempt was made to investigate the effects on survival at lower

concentrations and therefore it can not be ruled out that mortality effects in *Ophryotrocha diadema* may be observed at similar concentrations used in the present study. Adult worms were exposed to tritiated water prior to their egg laying, these eggs were then irradiated until they reached maturity where the reproductive output of these adults were quantified. The irradiation of adults and thus eggs prior to laying may also reduce the effects of irradiation due to a phenomenon termed adaptive response to ionising radiation (Wojcik and Shadley, 2000). The result of numerous studies indicate that cells can become refractory to the detrimental effects of ionising radiation when previously exposed to a low "adapting dose". Olivieri *et al.* (1984) found that by pretreating human lymphocytes *in vitro* with low concentrations of ^{3}H-thymidine the cells were then protected from the effect of subsequent high X-ray dose. It is suggested that the induced radioresistance may be due to the induction of DNA repair systems that efficiently protect the adapted cells from the effects of a subsequent, high challenging dose (Wojcik and Shadley, 2000). It may be possible that the eggs that contain elevated levels of lipids and therefore less water may receive a lower dose of irradiation than the adult tissue and subsequently after laying where the cells would have developed and are also exposed directly to the tritiated water. While there is much evidence that an "adapting dose" can trigger some protecting mechanisms within a cell, it appears that there is no single, universal mechanism of the adaptive response that is valid for all cell types, irradiation conditions and various organisms (Wojcik and Shadley, 2000). A further explanation as to the increased radiosensitivity of *M.edulis* compared to the worm *Ophryotrocha diadema* is the differences in reproductive strategies. *M.edulis* release planktotrophic larvae in which very large numbers of small eggs are produced whereas *Ophryotrocha diadema* lays a few large eggs in transparent sacs and these develop into larvae within the sacs (lecithotrophic larvae). Therefore the large worm eggs contain more energy in the form of yolk and are therefore more adaptive to survival than the small eggs of the mussel that contains very little yolks. Thus *Ophryotocha diadema* larvae have a greater energy source and a longer period of

development within protective sacs and therefore maybe more resistant than *M.edulis* eggs. The sacs may also reduce the amount of damaging radiation that the eggs and larvae might receive due to the increase in the penetration distance that the radiation must travel.

In contrast to the sexually mature adult animals, the embryo-larval stages of aquatic organisms are considered to be several orders of magnitude more sensitive in terms of toxicological injury (Jha *et al.*, 2000a). The beta rays of tritium have a maximum range in water of approximately 6μm and an average range of 0.8 of 1μm. With spheres smaller than the "standard cellular nucleus" an increasing amount of radiation escapes from the nucleus. In comparison, an increasing amount of radiation is retained within the nucleus in larger spheres (cells) and for spherical cells of 12 μm in diameter nearly all of the energy will be deposited within the cell (Cronkite *et al.*, 1973). The size of *M.edulis* embryos is approximately 40-50 μm and therefore radiation is likely to be retained in their cells. The incorporation of tritium into genetic material has caused considerable apprehension as a result of the short range of the tritium beta particle and the possible effects of transmutation (Cronkite *et al.*, 1973). Hill and Johnson (1993) also state that since the range of the beta particles is small compared to the dimension of a cell the radiation hazard will depend on the size and shape of the cells of interest and upon the length of time the radionuclide remains there.

Straume and Carsten (1993) reported a statistically significant increase for the incidence of sister chromatid exchanges on bone marrow cells of mice maintained on 111 kBq/ml of ingested HTO for 81-216 d. During the present study elevated levels of sister chromatid exchanges was detectable between 0.37-370 kBq/ml in comparison to the controls. Ionising radiation is believed to be a poor inducer of SCEs (Perry and Evans, 1975). However during this study the beta emitting radionuclide tritium was shown to produce elevated frequencies of SCEs in comparison to controls. There was no significant difference between the two exposure scenarios at the lower concentrations (0.37-37 kBq/ml), only at 370 kBq/ml were the induction of SCEs significantly elevated in embryo-

larvae exposed following 1h fertilisation and for a longer exposure period. As well as containing an elevated frequency of SCEs tritium exposed embryo-larvae exposed after 1h of fertilisation also experienced a reduction in the proliferation rate of the cells. As SCEs are an S dependent phenomenon, the levels of SCEs may have been higher if the cells were still actively proliferating (most were in stage 1). The reduction of the cell cycle is also indicated in the percentage of chromatid and chromosomal type aberrations present in metaphase spreads of embryo cells. Chromatid type aberrations indicate that DNA damage was induced in the G_2 phase of the cell cycle and chromosomal type aberrations are due to DNA damage occurring the G_1 phase. In exposure scenario 1, the proliferation rate was between 1.35-2.36 and thus the cells were still actively dividing and therefore they had more chromatid type aberrations (between 78-96%). In comparison the tritium exposed cells in scenario 2 had a PRI of between 1.19 and 1.375 which indicated that the cells were not actively dividing and therefore the percentage of chromatid type aberrations was lower (between 45.2-67.9%). Suyama *et al.* (1981) found that the frequency of chromosome aberrations (chromosome bridges) increased significantly when eggs of the freshwater teleost, *Oryzias latipes*, were treated with concentrations higher than 19 rad of tritiated water. This is slightly higher than the dose that induced significantly elevated levels of aberrations in the embryo-larvae of *M.edulis* (0.007-0.7 rads). However, Strand *et al.* (1977) found that the primary immune response of rainbow trout was affected above 1.0 μCi/ml (37 kBq/ml) which is similar to the present study and a decrease in immune systems of early life stages may lead to an increase in mortality.

Under natural conditions the degree of exposure to tritium or any other radionuclides may also be greater in sediment feeders. Marine mussels are intertidal filter feeding animals that are capable of filtrating large amounts of water daily and consequently they might accumulate radionuclides present in micro-organisms (bacteria, protozoa, microalgae) and in suspended particulate matter (Bonotto, *et al.*, 1983). Rodgers (1986) found that the steady state specific activity of organically bound ^3H (OBT) was

significantly affected by the intake of tritium incorporated as food in comparison to that taken in as tritiated water. The steady state specific activity of organically bound ^{3}H of trout maintained in tritiated water but fed non-tritiated diets was approximately 20% of that of the ambient water, whereas for trout fed on a diet containing tritium the steady state specific activities of organically bound tritium were in the order of 80% of the diet. Adams *et al.* (1979) reported an average increase of 1.8 times the level of tritium in bottom sediments than in water. It was suggested that high levels of OBT in filter feeding shellfish from the Bristol Channel was due to the primary and major accumulation of tritium arising at the base of the food chain, via micro-organisms (Wallis, 2000).

From the above information it is clear that tritium may cause damage to small sized cells and organisms due to the penetration depth of the beta rays. However in light of the current experiments it may be concluded that *M.edulis* embryo-larvae are extremely radiosensitive to the beta emitting radionuclide tritium, in terms of cytotoxic, genotoxic and developmental endpoints. In natural conditions, organisms that survive an initial dose of radiation might be more radioresistant if further exposure to ionising radiation due to an "adaptive response" however, adult mussels will be more likely to experience a higher degree of exposure to radiation due to filter feeding. Analysis of the different exposure scenarios suggests that the age of organisms at the time of exposure does not influence the degree of damage caused to individuals, probably due to the similar small size of the organisms and hence the penetration of the beta rays at the various life stages. The length of exposure and hence the dose received by the organisms did increase the induction of chromosomal aberrations and subsequently lead to an increase in mortality. In conclusions if embryo-larvae are exposed to tritium at early life stages for a few hours there would lead to a 20-50% increase in mortality (at the concentrations investigated) however if the time period of exposure is doubled then the mortality is also doubled to 60-100%.

For risk assessment, data from the present study indicates that it is vital that the period of exposure be kept to a minimum. This may be accomplished by releasing tritium

into large volumes of water where it will be dispersed rapidly, for example at times of the year when there are high tides or fast movements of water.

Chapter 6

Relative sensitivity of embryo-larvae of marine invertebrates following exposure to a cocktail of radioactive effluent

Hypotheses

1. Does radioactive effluent, containing a cocktail of radionuclides, cause developmental, genotoxic and cytotoxic effects in embryo-larvae of the marine mollusc *Mytilus edulis* and the polychaete worm *Platynereis dumerilii.*

2. There is a difference in sensitivity between the two species after exposure to either radioactive effluent or tritium.

6.1 Introduction

Nuclear power production, nuclear fuel reprocessing, research activities and defence facilities all contribute to the release of radionuclides into the aquatic environment (Department of the Environment, Transport and the Regions (DETR), 2000; Valkovic, 2000). In the UK analysis of the trends of liquid discharge has been carried out for a period 1979 to 1998, for different sectors as represented in figure 6.1.

Nuclear power currently accounts for 27% of the UK's electricity production (DETR, 2000). Currently the UK has 35 nuclear reactors used in the production of energy. These consist of 20 Magnox type reactors (Government-owned through British Nuclear Fuels Limited), 14 Advanced Gas Cooled Reactors and 1 Pressurised Water Reactor (all owned by British Energy). All sites except two are situated on the coast and hence are important contributors to marine radioactive pollution through the discharge of their liquid wastes into the sea (Marine Conservation Society website, 2002). The Sellafield nuclear power and reprocessing plant is the main source of marine radioactive pollution in the UK and the North Sea.

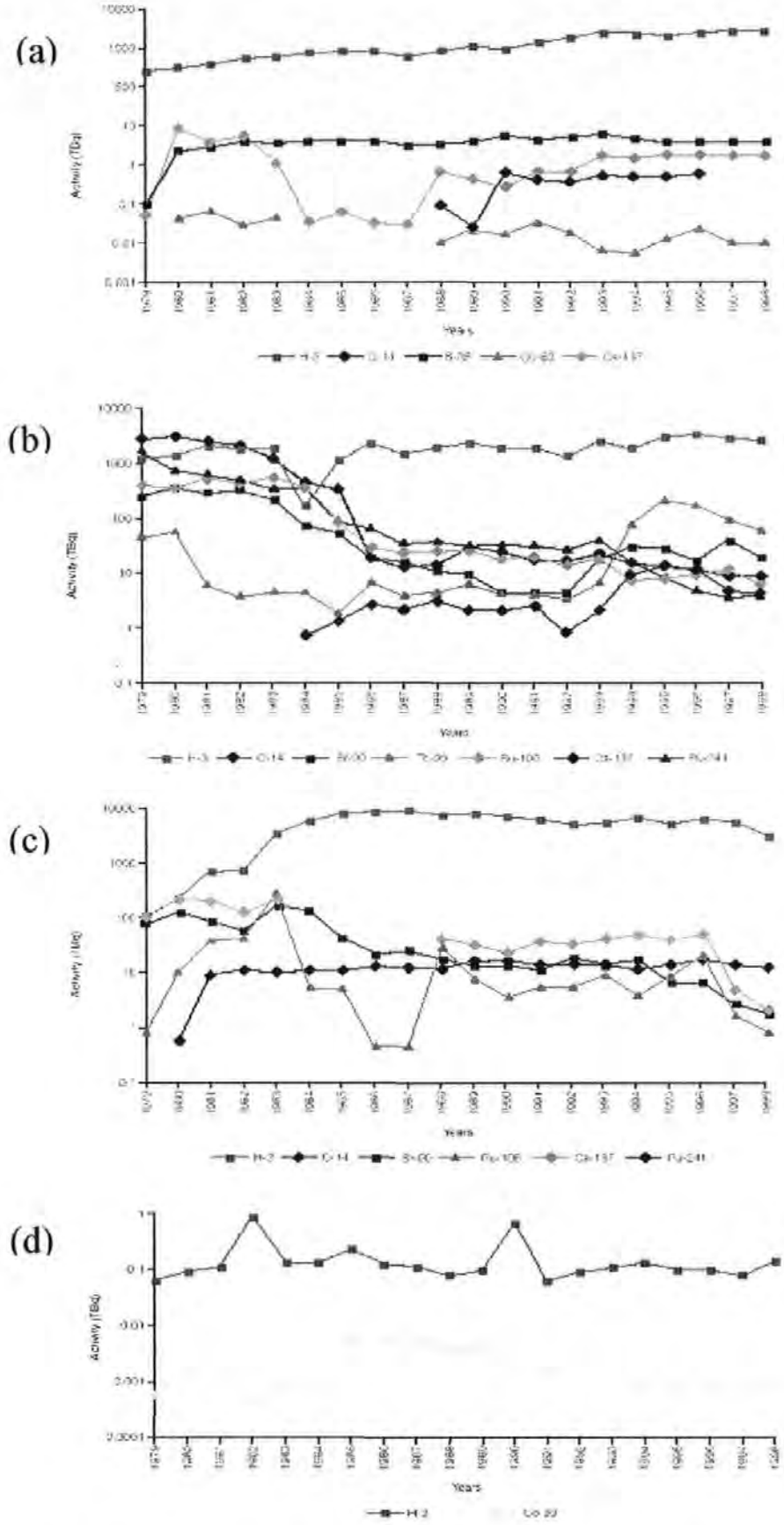


Figure 6.1. Liquid discharges from nuclear sectors in the UK between 1979 and 1998. (a) nuclear energy production, (b) nuclear fuel reprocessing (c) research sector and (d) defence sector (reproduced from DETR, 2000).

The level of radioactive contamination in the sediments of the New Biggin and Muncaster estuaries south of Sellafield was found to be comparable to Chernobyl levels. Historic radioactivity from Sellafield can be detected around most of the coastal area of the UK. However since the 1970s discharges from Sellafield have reduced over 100-fold and are much less radiologically significant (Environment Agency, 1998). Discharges from Sellafield were partly, if not entirely, the cause of the demise of the colony of black-headed gulls on the Driggs dunes in the Ravenglass Estuary, (Woodhead, 1986). The discharges of the radionuclide technetium-99 have increased since 1994 and although it is of minimal radiological significance, this radionuclide has been found to accumulate in lobsters. Environmental studies of the Irish Sea have shown that a substantial proportion of certain radionuclides present in the discharges from Sellafield were accumulating in fine sediments and thus native benthic fauna were being subjected to enhanced radiation exposure (Environment Agency, 1998).

The other major sources of radioactive pollution in UK waters are the installations at Dounreay in Scotland (due to end reprocessing by 2006) and La Hague situated in France. Analysis of sediment and seawater at the end of the La Hague nuclear waste discharge pipe indicated high levels of radionuclides, in some cases the environment itself was classified as radioactive waste. Tables 6.1. and 6.2. represent the radioactivity detected in sediment and seawater at La Hague. The classifications of radioactive waste vary within Oslo and Paris Conventions Strategy (OSPAR) countries. Under UK classifications the levels of each of the radionuclides ^{60}Co, ^{106}Ru, ^{125}Sb and ^{137}Cs in sediment samples would qualify the seabed itself for treatment as low-level radioactive waste (LLW) (UNSCEAR, 1977). The levels of tritium (^{3}H) in the seawater sample would classify the seawater as intermediate-level waste (ILW) under UK classifications, and the levels of ^{137}Cs would classify it as low-level waste (LLW). High level waste is highly radioactive waste that is produced when spent nuclear fuel is reprocessed to recover uranium and plutonium. It is self heating and long lived and thus requires cooling. Over 95% of the activity in

radioactive waste arising from the nuclear power industry are high level, but it has a very small volume. Intermediate level waste is sufficiently radioactive to require shielding during its handling and transportation. Such wastes may include resins from reactor operations or solidified chemical sludges. They are currently stored at nuclear sites. Low level waste consists of lightly contaminated materials such as paper, gloves, rags, glass and is disposed of by burial at approved sites, principally at the surface repository at Drigg in Cumbria (Valkovic, 2000).

Table 6.1. Radioactivity detected in a small stone near the discharge pipe from La Hague, France (UNSCEAR, 1977).

Radionuclide	**Activity (Bq/kg)**
Am-241	1 300
Ba-140	3 700
Co-60	146100
Eu-154	380
Mn-54	1 700
Sb-125	3 600

Table 6.2. Radioactivity detected in one of the seawater samples near the discharge pipe from La Hague, France analysed by the Hamburg Department of Environment (UNSCEAR, 1977).

Radionuclide	Activity (Bq/l)
H-3	160 000 000
Be-7	206
Na-22	11
K-40	5
Mn-54	39
Co-60	503
Ru-106	1 150
Sb-125	146
Cs-134	1 890
Cs-137	23 900
Eu-154	37
Am-241	182

In addition to the discharge of radioactive waste into the sea a further contributor to the release of radionuclides are from submarine building, refitting and/or maintenance activities. At the end of the Cold War, in 1989, there were over 400 nuclear-powered submarines operational or being built. Some 250 of these submarines have now been scrapped and some on order cancelled, due to weapons reduction programs. Russia and USA had over one hundred each, with UK and France less than twenty each and China six. The total number of existing nuclear powered submarines is estimated to be about 160 (Gates, 1990). In the UK, work has been carried out on nuclear submarines at Devonport, (Plymouth, Devon) since the 1970s. Refuelling refits started in 1979 and since then eleven Swiftsure and Trafalgar class submarine refits have been completed in the purpose built Submarine Refit Complex (SRC). In these submarines nuclear fission is used in pressurised water reactors to provide energy to power the submarines. Nuclear subs and aircraft carriers are powered by nuclear reactors that use similar technology to the

pressurised water reactors used in commercial power plants. The reactor produces heat to generate steam to drive a steam turbine. The turbine in a ship directly drives the propellers, as well as electrical generators. The two major differences between commercial reactors and reactors in nuclear ships are, the reactor in a nuclear ship is smaller and the reactor in a nuclear ship uses highly enriched fuel to allow it to deliver a large amount of energy from a smaller reactor (how stuff works website, 2002).

Uranium in fuel modules is used for this process and when they are consumed the modules need to be replaced. Before any refuelling of a nuclear submarine can take place the primary circuit is decontaminated and connected to shore-based services which support and monitor the reactor whilst the submarine is being refuelled in dry dock. Decontamination of the reactor compartment results in the production of liquid radioactive waste that is treated to reduce the radioactivity, prior to being disposed into the marine environment. In the UK, the modules of used uranium are removed from the reactor one at a time after which they are transferred to a used fuel facility to await transfer in heavily shielded transport containers to BNFL, Sellafield (Valkovic, 2000).

All radioactive waste disposals from Ministry of Defence (MOD) premises are made in agreement with the Environment Agency (EA) in England. These agreements conform to the same standards of control as those imposed upon other operators of nuclear plants and are based on the recommendations of the International Commission on Radiological Protection (ICRP) and interpreted in the UK by the National Radiological Protection Board (NRPB) in accordance with Government policy objectives (Cowling *et al.*, 1996). Small quantities of radioactive liquid wastes arising from submarine building, refitting and/or maintenance activities are discharged from establishments at Devonport, Faslane, Rosyth and Barrow in the United Kingdom. Following contractorisation and subsequent privatisation of the former Royal Dockyards, radioactive discharges and corresponding authorisations at Devonport and Rosyth are now formally the responsibility

of Devonport Royal Dockyard Ltd. (DML) and Babcock Rosyth Defence Ltd. (BRDL) respectively (Cowling *et al.*, 1996).

The radionuclides present in the radioactive discharges from DML include ^{3}H, ^{63}Ni, ^{55}Fe, ^{14}C and ^{60}Co. These radionuclides are classified as activation products. Activation products are nuclides formed through transformation of stable isotopes into radioactive isotopes after intense bombardment with fission products. Radioactivity is thus induced through neutron bombardment or other types of radiation in reactor vessel components and corrosion products that were stable before the reactor vessel went critical. Important activation products include carbon-14 and tritium (^{3}H) as well as activation products derived from activated stainless steel and carbon steel, activated sludge, corrosion deposits and concrete, and contaminated building products e.g. ^{55}Fe, ^{54}Mn, ^{65}Zn, ^{58}Co and ^{60}Co (Valkovic, 2000).

Tritium (^{3}H) is a radionuclide that is naturally occurring in the environment but is also artificially produced, usually in nuclear reactors as a by-product of the irradiation of lithium, ($^{6}Li + n \rightarrow {}^{3}H + {}^{4}He.$). It is a beta emitter with a half-life of 12.346y and maximum beta energy of 0.018610 MeV. Carbon-14 is also a radionuclide that is artificially produced by the neutron activation of ^{14}N, but also occurs naturally, whose principle decay mode is via emission of beta particles. It has a half-life of 5730y and a maximum energy of 0.155 MeV. Carbon-14 is produced in nuclear reactors as a result of absorption of neutrons by nitrogen, carbon, or oxygen present as components of air, coolant, moderator, structural materials, fuel, or impurities. Iron-55 is an activation product that decays due to electron capture and has a half-life of 2.68y. Nickle-63 is also an activation product although its principal decay mode is by the discharge of a beta particle; it has a half-life of 100y and a maximum energy of 0.067 MeV. Cobalt-60 is an activation product that decays by the release of a beta particle although it has a half-life of only 5.2719y and a maximum energy of 0.31788 MeV. With the exception of tritium, the beta emitters also have gamma emissions.

The disposal of radioactive waste is a problem that arises at all establishments that produce nuclear material. Waste management of radioactive material is based on limiting the degree of exposure to humans and despite the fact that large amounts of radionuclides are discharged into marine and coastal ecosystems little information exists on their potential effects to aquatic organisms. Therefore in light of the information presented above the aim of the current study was to investigate the effects of a realistic cocktail of radionuclides produced from a nuclear establishment on the embryo-larvae of two marine invertebrates, *M.edulis* and *P.dumerilii*. It is also aimed to examine if there is any difference between the sensitivity of the two species after exposure to radioactive effluent or tritium (species comparison between results of chapters 4 and 5).

6.2 Materials and methods

6.2.1 Source of radioactive waste and its chemical composition

A sample of radioactive liquid waste was obtained from Devonport Royal Dockyard Ltd. (DML) on the 9th of August 2000. The sample contained a cocktail of radionuclides that was produced during the refitting and refuelling of a nuclear submarine. The sample obtained was prior to its release and subsequent dilution in the environment. Table 6.3. shows the specific activity of the radionuclides present in the sample of radioactive effluent.

Table 6.3. Specific activity of radionuclides in the sample of radioactive waste (*).

	Nuclide	Specific activity MBq/m^3	Limit of detection MBq/m^3
Liquid scintillation	^{3}H	3.9×10^{2}	3.4×10^{-2}
Other nuclides	^{63}Ni	2.3×10^{-2}	1.6×10^{-2}
	^{55}Fe	8.4×10^{-3}	1.7×10^{-3}
	^{14}C	1.5×10^{0}	2.7×10^{-2}
Gamma-ray spectroscopy	^{60}Co	5.6×10^{-1}	4.2×10^{-3}
pH	*6.6*		
Conductivity	*650 µS/cm*		

* (Analysis of the sample was carried out by DML)

Due to regulatory limitations, i.e. the constraints of transporting radioactive material, all experiments were carried out in the MODIX laboratory on the site of DML. The sample of radioactive effluent was diluted so that the embryo-larvae were exposed to 1.8, 3.2, 5.6 and 18% solutions. The dilutions were based on a semi-logarithmic scale routinely used in ecotoxicology testing. Table 6.4. shows the dose from tritium received by the embryo-larvae after exposure to the radioactive material. Little information is available for the calculation of dose received by embryo-larvae stages of marine organisms for the other radionuclides present in the radioactive effluent.

Table 6.4. Specific activity of tritium in diluted samples obtained from DML.

Dilution %	Amount of radioactive waste (final volume adjusted to 500mls with seawater)	Specific activity of tritium in radioactive waste	Total activity in diluted solutions	Specific activity in diluted solutions
18	*90 ml*	*0.39 kBq/ml*	*35.10 kBq*	*0.0702 kBq/ml*
5.6	*28 ml*	*0.39 kBq/ml*	*10.92 kBq*	*0.0218 kBq/ml*
3.2	*16 ml*	*0.39 kBq/ml*	*6.24 kBq*	*0.0125 kBq/ml*
1.8	*9 ml*	*0.39 kBq/ml*	*3.51 kBq*	*0.0070 kBq/ml*

6.2.2 Exposure Scenario

Figure 6.2. describes the exposure protocol for assessing the developmental, cytotoxic (PRI) and genotoxic (SCEs and Cabs) effect of radioactive waste on *P.dumerilii* and *M.edulis* embryo-larvae, 16h post-fertilisation.

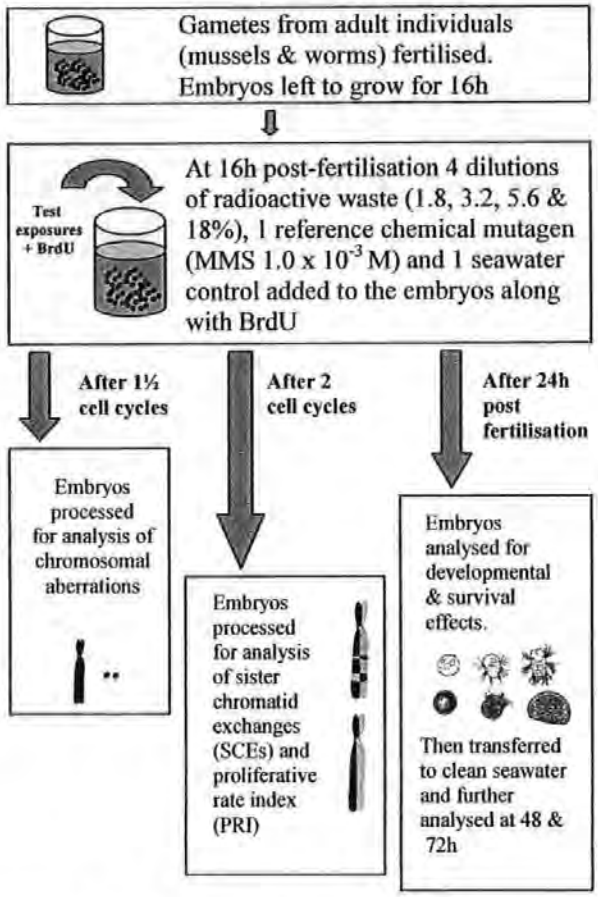


Figure 6.2. Experimental protocol for the exposure of *P.dumerilii* and *M.edulis* to a cocktail of radioactive waste 16h post-fertilisation.

For the worms 3 sexually mature females and 2 males were collected and allowed to spawn naturally in 50 ml of filtered seawater. Approx. 33,000 embryos were produced with a 97 % fertilisation rate. The embryos (5-6 per ml) were allowed to grow for 16h, after which they were divided into duplicate exposure vessels and exposed to four dilutions of a cocktail of radionuclides (1.8, 3.2, 5.6 and 18%), 1 negative control containing just filtered seawater and 1 reference chemical mutagen (1.0×10^{-3} M MMS). A minimal concentration of 10^{-5} M BrdU was added to all the vessels simultaneously with the radionuclide and MMS exposure (final volume equalled 500 ml). Due to constraints in experimental conditions no cooled incubators were available, therefore in order to minimise problems, due to elevated temperatures, the exposure vessels were kept in a cooled water bath and maintained at 20°C ± 1°C. Due to the slight elevation in temperature

above the normal (15°C) for *M.edulis* the cell cycle would be faster than the normal 3.8h. Therefore in an attempt to allow for the elevation in temperature and for comparison between species, the cell cycle for *M.edulis* and *P.dumerilii* was both taken to be 3h. After 1½ cell cycles (1 cell cycle = 3h), a subset of embryos were placed into colchicine and processed for chromosomal aberrations as described in section 2.3.6. A further subset was removed after 2 cell cycles and processed for the analysis of proliferative rate index and sister chromatid exchanges as described in section 2.3.3. When the remaining embryos reached 24h post-fertilisation they were placed into clean seawater and analysed for developmental and survival effects as described in section 2.3.1. Further analyses of morphology, behaviour and mortality were assessed at 48 and 72h post-fertilisation.

For mussels, 1 female and 3 males were allowed to spawn naturally in 250 ml of filtered seawater. Approx. 244,000 embryos were produced with a 92% fertilisation rate. The gametes were fertilised and allowed to grow for 16h, after which they were divided into duplicate exposure vessels at a final concentration of 30 embryo-larvae per ml. They were then exposed to four dilutions of a cocktail of radionuclides (1.8, 3.2, 5.6, 18%), 1 negative control containing just filtered seawater and 1 reference chemical mutagen containing MMS (1.0×10^{-3} M). A minimal concentration of 10^{-5} M BrdU was also added to all the vessels at 16h post-fertilisation, after 1½ cell cycles (1 cell cycle ≈ 3h), a subset of embryos were placed into colchicine and processed for chromosomal aberrations as described in section 2.3.6. A further subset was removed after 2 cell cycles and processed for analysis of proliferative rate index and sister chromatid exchanges (SCEs) as described in section 2.3.3. When the remaining embryos reached 24h post-fertilisation they were placed into unexposed clean seawater and analysed for developmental and survival effects as described in section 2.3.1. Further analyses of morphology, behaviour and mortality were assessed at 48 and 72h post-fertilisation.

6.3 Results

6.3.1 Relative species sensitivity following exposure of radioactive waste

6.3.1.1 Developmental and mortality effects

Figure 6.3.a, b and c respectively shows the percentage of normal, abnormal and dead embryo-larvae of *M.edulis* and *P.dumerilii* after exposure to a series of dilutions of radioactive waste and a concentration of MMS after 72h post-fertilisation.

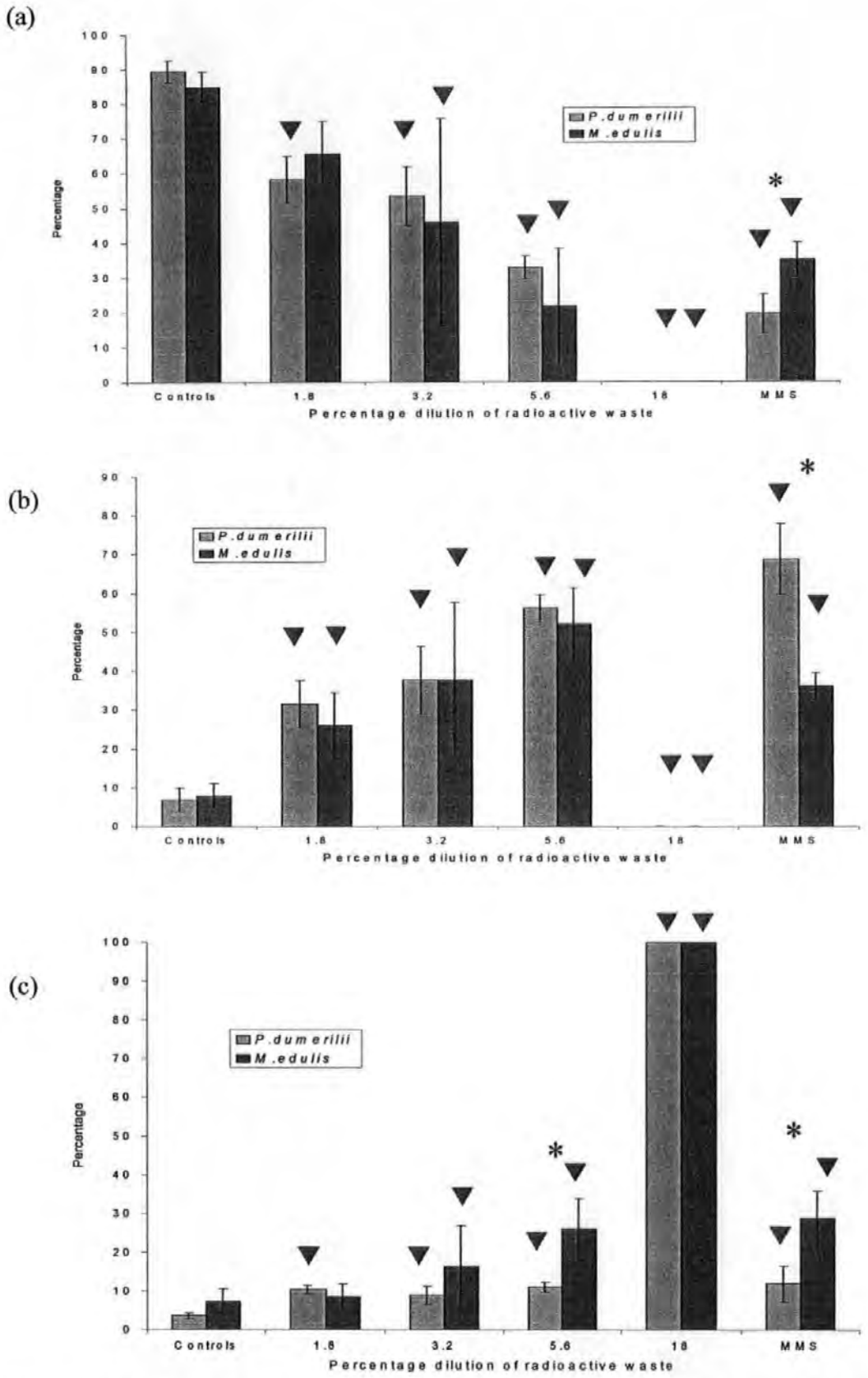


Figure 6.3. Comparison between two species: The percentage of (a) normal, (b), abnormal and (c) dead embryo-larvae at 72h following exposure to radioactive effluent (▼ = significantly different from controls, * = significantly different between species, $P<0.05$) (Error bars represents standard deviation , n = 6).

By 72h post-fertilisation the percentage of normal *P.dumerilii* embryo-larvae decreased with increasing amounts of radioactive effluent and the MMS concentration, all were significantly different from the controls (P=0.00005). Regression analysis suggests a relationship between the percentage of normal *P.dumerilii* and the percentage of radioactive effluent (R^2=82.91%). For *M.edulis* the lowest dilution of radioactive waste was not statistically different from the control although the other dilutions and the MMS exposed embryo-larvae were significantly less normal (P=0.002; R^2=65.85%).There was no difference in the percentage of normality between the two species after exposure to the dilutions of radioactive waste although there was a statistical decrease in the percentage of normal *P.dumerilii* after exposure to MMS (P=0.001) in comparison to *M.edulis*.

In conjunction with the decrease in normality (figure 6.3.a) with increasing percentage of radioactive waste there was an increase in the percentage of abnormal embryo-larvae. Figure 6.3.b shows the percentage of abnormal embryo-larvae of *M.edulis* and *P.dumerilii.* There was a statistically significant increase in the percentage of abnormal *P.dumerilii* and *M.edulis* with increasing concentrations (P=0.00005 and P=0.0017 respectively). There was a relatively weak dose relationship between abnormality and percentage of dilution for both species, as shown with the regression coefficient (R^2=15.05 % and R^2=14.35 % respectively). As with the incidence of normality there was no difference in abnormality between the two species after exposure to the radioactive effluent but there were more abnormal *P.dumerilii* in the MMS exposed samples than *M.edulis* (P=0.0014).

There were no living *P.dumerilii* or *M.edulis* embryo-larvae in the 18% dilution of radioactive waste at any time period (figure not shown). There was a significant difference between the percentage of dead *P.dumerilii* and *M.edulis* in the controls compared to the dilutions of radioactive waste and for the MMS concentration at 72h (P=0.005 and P=0.00005 respectively). Only for *M.edulis* was the percentage of mortality statistically similar to the controls in the lowest percentage of radioactive effluent (1.8%). There was a

strong dose relationship between the dilutions and the increase in mortality (R^2=94.36% and R^2=96.0% respectively). Figure 6.3.c shows the percentage of dead embryo-larvae of *M.edulis* and *P.dumerilii.* There was a statistical difference between the two species at the 5.6% dilution of radioactive waste (P=0.019) with a larger increase in mortality in *M.edulis* than in *P.dumerilii*. As for the percentage of normality and abnormality there is a difference between species after exposure to MMS. MMS increased the levels of mortality in *M.edulis* in comparison to the *P.dumerilii* in which MMS increased abnormality.

6.3.1.2 Proliferative rate index (PRI)

Figure 6.4. represents the proliferative rate index for *M.edulis* and *P.dumerilii* embryo-larvae after exposure to a series of dilutions of radioactive waste and a concentration of MMS.

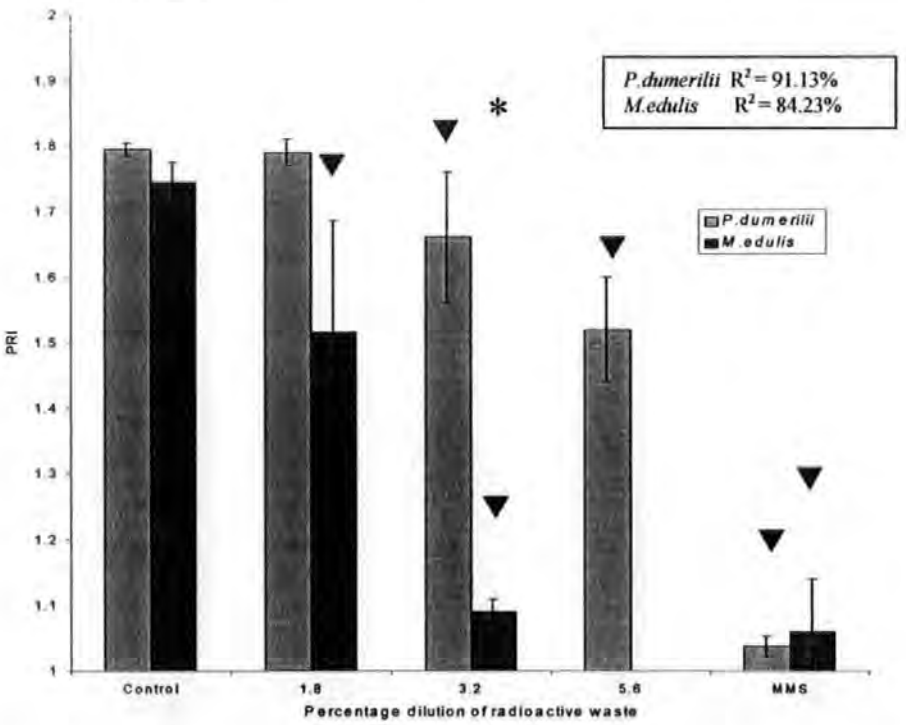


Figure 6.4. Proliferative rate index of embryo-larvae exposed to radioactive effluent (▼ = significantly different from control, * = significant difference between species, P<0.05) (Error bars represents standard deviation , n = 2).

For *M.edulis* there were no visible chromosomes/metaphases in the 5.6% dilution and therefore no PRI could be calculated. There was a significant decrease in the PRI at 1.8 and 3.2% (P=0.0013; R^2=91.13%) with the 3.2% dilution almost experiencing no cells that were actively dividing. For *P.dumerilii* there was a statistical difference in the PRI between the exposed embryo-larvae and the control (P=0.00005; R^2=84.23%). However the lowest dilution factor (1.8%), was not significantly different from the control. The MMS concentration produced similar results between species and the PRI ranged between 1.02-1.1. There was no statistical difference in the PRI of the control embryo-larvae between both species. In general *M.edulis* experienced a lower PRI after exposure to the radioactive waste than *P.dumerilii* and there was a statistical difference (P=0.0079) at the 3.2% dilution between the two species.

6.3.1.3 Sister chromatid exchanges (SCEs)

Figure 6.5. represents the incidence of sister chromatid exchanges after exposure of embryo-larvae to a series of dilutions of radioactive waste and a concentration of MMS.

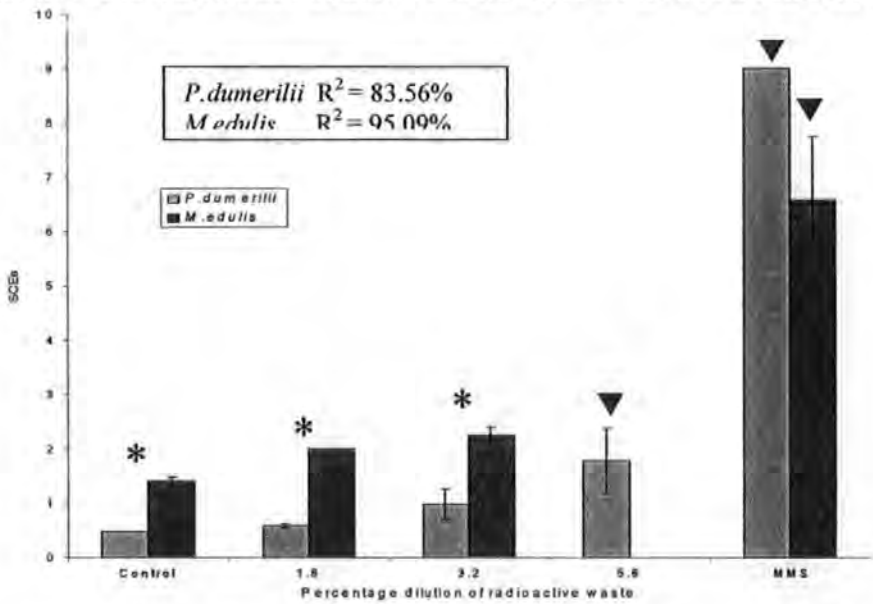


Figure 6.5. Induction of sister chromatid exchanges in embryo-larvae after exposure to a cocktail of radionuclides (▼ = significantly different from control, * = significant difference between species, P<0.05) (Error bars represents standard deviation , n = 2).

There is a statistically significant difference between the control and the embryo-larvae exposed to the radioactive waste and MMS for *P.dumerilii* (P=0.00005) although only the highest dilution factor of 5.6% and the MMS were significantly different from the control. For *M.edulis* there was a statistical difference between the control and the MMS exposed embryo-larvae (P=0.0001) although the embryo-larvae exposed to the radioactive waste did not have significantly elevated levels of sister chromatid exchanges compared to the control. There were no visible chromosomes/metaphases in the 5.6% dilution for *M.edulis* and therefore the number of SCEs could not be calculated. There was a significant difference in the number of sister chromatid exchanges between the two species for the controls and the 1.8 and 3.2% dilutions of radioactive waste. The levels of sister chromatid exchanges were extremely low in the controls and the radioactive waste exposed embryo-larvae and the difference between species reflects the difference in baseline limits. Unlike the PRI there is a stronger dose relationship, between SCEs and concentration, in *M.edulis* (R^2=95.09%) compared to *P.dumerilii* (R^2=83.56%).

6.3.1.4 Aberrant cells and Total aberrations

Figure 6.6. represents the number of cells containing aberrations for *M.edulis* and *P.dumerilii* embryo-larvae after exposure to a series of dilutions of radioactive waste and a concentration of MMS (1.0×10^{-3} M) and figure 6.7 represents the total number of aberrations.

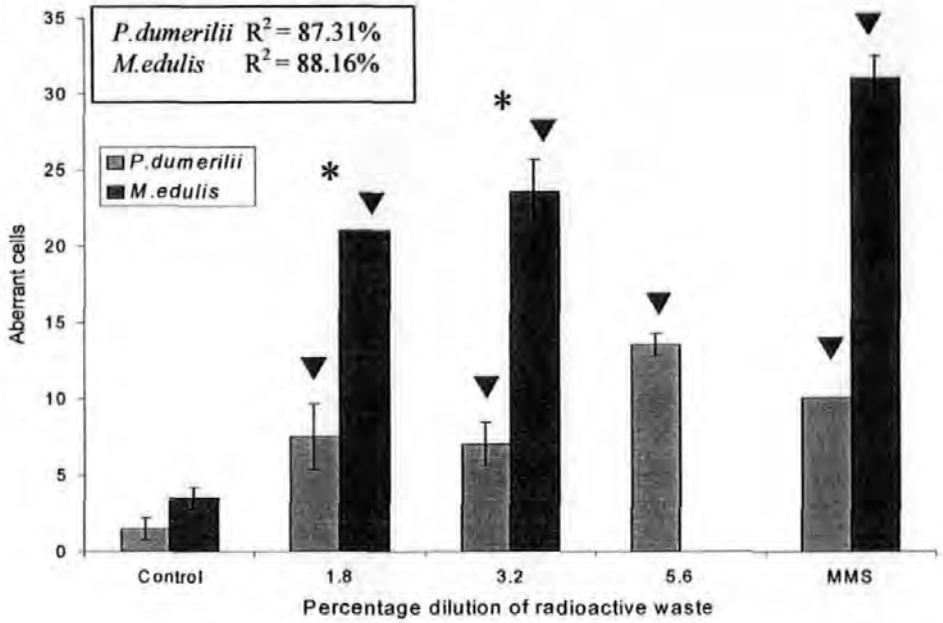


Figure 6.6. Number of aberrant cells in embryo-larvae after exposure to radioactive effluent (▼ = significantly different from control, * = significant difference between species, P<0.05) (Error bars represents standard deviation , n = 2).

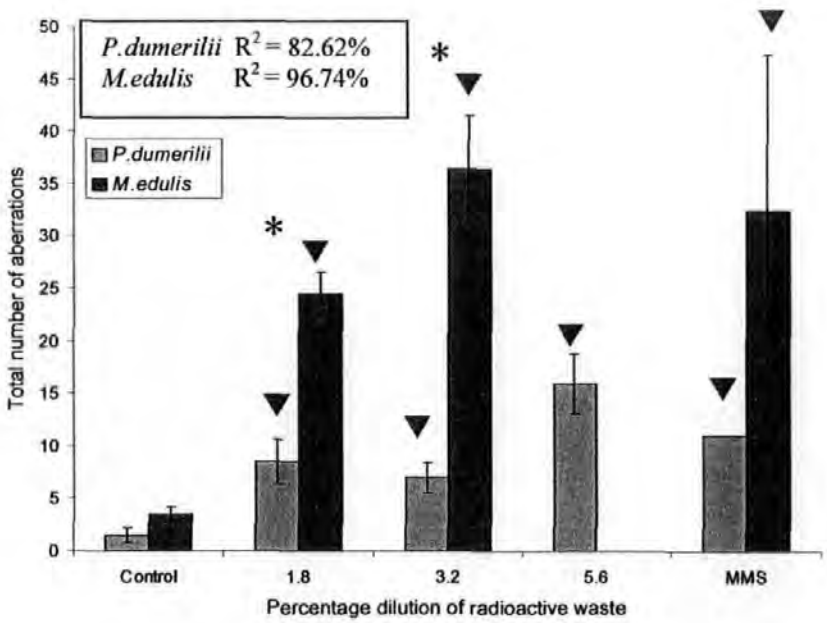


Figure 6.7. Total number of aberrations in embryo-larvae after exposure to radioactive effluent (▼ = significantly different from controls, * = significant difference between species, P<0.05) (Error bars represents standard deviation , n = 2).

There were significantly elevated levels of cells containing aberrations for both species after exposure to radioactive waste and MMS (P=0.0065; P=0.0001) for worms and mussels respectively. There was not a statistical difference between the controls for both species although there was a difference at the 1.8 and 3.2% dilutions, with *M.edulis* experiencing over a 2-fold increase in cells containing aberrations in comparison to *P.dumerilii* embryo-larvae. There were no visible chromosomes in the 5.6% dilution for *M.edulis* embryo-larvae and therefore the number of cells containing aberrations could not be calculated. There is a similar strong dose relationship coefficient for both species (R^2=88.16%, R^2=87.31%; *M.edulis* and *P.dumerilii* respectively).

There was no statistical difference between the controls for both species. Both species showed significantly higher numbers of total aberrations in the embryo-larvae exposed to the radioactive waste and the concentration of MMS when compared to the controls (P=0.01; P=0.046) for worms and mussels respectively. As with the number of cells containing aberrations there is a statistical difference between species exposed to the 1.8 and 3.2% dilutions of radioactive waste with *M.edulis* embryo-larvae having more total aberrations than *P.dumerilii*. There were no visible chromosomes in the 5.6% dilution for *M.edulis* embryo-larvae and therefore the total number of aberrations could not be calculated. There is a stronger dose relationship, between the total number of aberrations and concentration, in *M.edulis* embryo-larvae (R^2=96.74%) compared to *P.dumerilii* (R^2=82.62%).

6.3.1.5 Comparison among developmental/mortality, cytotoxic and genotoxic effects following exposure to radioactive waste

Regression analysis between the percentage of abnormal and dead embryo-larvae and the induction of PRI, SCEs and Cabs was carried out in an attempt to link the effects at molecular and cellular levels with changes at the individual level.

Table 6.5. Comparison between developmental/mortality, cytotoxic and genotoxic effects in *P.dumerilii* embryo-larvae following exposure to radioactive waste

	% of 72h abnormal larvae		% of 72h dead larvae	
	P value	**R^2**	**P value**	**R^2**
PRI	0.1230	76.91%	0.4081	35.04%
SCEs	0.1079	79.58%	0.3703	39.66%
Aberrant cells	***0.0206***	***95.91%***	0.1023	80.59%
Total aberrations	***0.0426***	***91.65%***	0.1262	76.36%

Table 6.6. Comparison between developmental/mortality, cytotoxic and genotoxic effects in *M.edulis* embryo-larvae following exposure to radioactive waste

	% of 72h abnormal larvae		% of 72h dead larvae	
	P value	**R^2**	**P value**	**R^2**
PRI	0.1858	91.71%	0.5687	39.29%
SCEs	0.0769	98.54%	0.3059	78.63%
Aberrant cells	0.1827	91.99%	0.2002	90.43%
Total aberrations	***0.0219***	***99.88%***	0.3609	71.16%

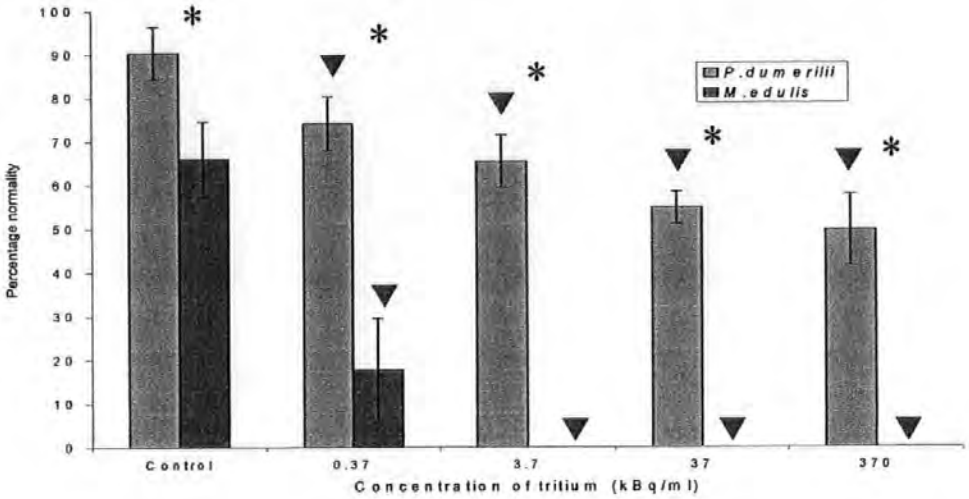
For *P.dumerilii* embryo-larvae there is a relationship between the percentage of abnormal larvae at 72h with the induction of cells containing aberrations and the total number of aberrations scored (table 6.5). Similarly for *M.edulis* the effects of radioactive waste on embryo-larvae showed that the percentage of abnormal larvae at 72h was related to the total number of aberrations produced (table 6.6). Therefore exposure to radioactive liquid waste as described above induces an increase in the chromosomal aberrations that could result in an increase in mortality.

6.3.2 Relative species sensitivity of tritium in *P.dumerilii* and *M.edulis* embryo-larvae

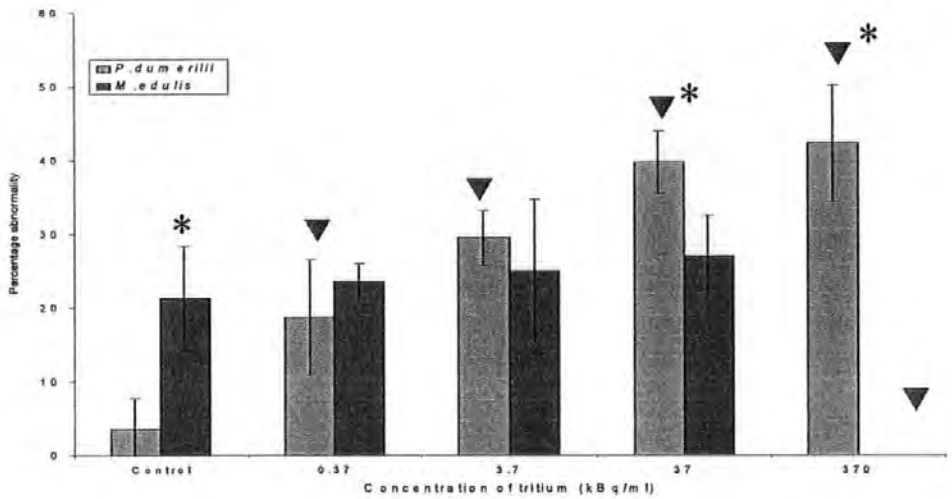
6.3.2.1 Developmental and mortality effects: comparison between species

Figure 6.8.a, b and c respectively shows the percentage of normal, abnormal and dead 72h embryo-larvae of *M.edulis* and *P.dumerilii* after exposure to four concentrations of tritium for 23h, 1h post-fertilisation.

(a)



(b)



(c)

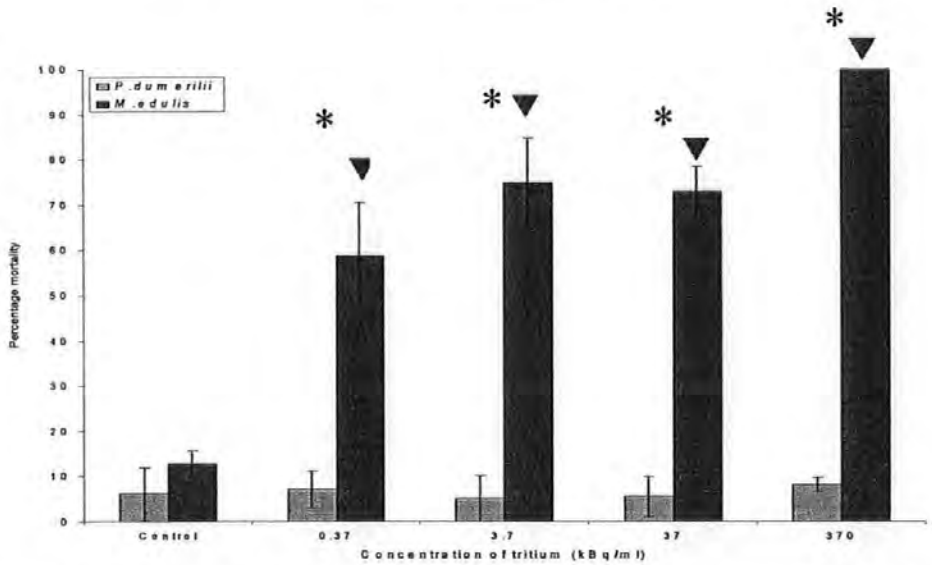


Figure 6.8. Comparison between species: The percentage of (a) normal, (b) abnormal and (c) dead embryo-larvae 72h post-fertilisation after exposure to tritium for 23h (▼ = significantly different from control, * = significance difference between species, $P<0.05$) (Error bars represents standard deviation , n = 5).

There was a difference in the percentage of normality between the two species after exposure to tritium. All concentrations produce significantly lower percentages of normal embryo-larvae in comparison to the controls. There were no normal *M.edulis* after exposure to concentrations 3.7, 37 and 370 kBq/ml. There was a statistical difference between the two species for every concentration including the controls.

Figure 6.8.b, represents the percentage of abnormal 72h embryo-larvae of *M.edulis* and *P.dumerilii* after exposure to four concentrations of tritium for 23h, 1h post-fertilisation. As with the incidence of normality there was a difference in abnormality between the two species after exposure to tritium but there was more abnormal *P.dumerilii* than *M.edulis*. There was no difference in abnormality between the *M.edulis* exposed to tritium in comparison to the control whereas there is a statistically significant elevation of abnormality in *P.dumerilii* after exposure to tritium.

There was a statistical difference between the two species for the embryo-larvae exposed to tritium with approximately a 5-10-fold increase in mortality in *M.edulis* than in *P.dumerilii*. Figure 6.8.c represents the percentage of dead 72h embryo-larvae of *M.edulis* and *P.dumerilii.* While there was a significant difference for abnormality there was no difference in mortality for *P.dumerilii* after exposure to tritium compared to the control. Whereas, there was a sharp increase in mortality for *M.edulis* after exposure to all concentrations of tritium for the sampling time (72h).

6.3.2.2 Proliferative rate index (PRI)

Figure 6.9. represents the proliferative rate index in embryo-larvae of *M.edulis* and *P.dumerilii* after exposure to tritium 1h post-fertilisation.

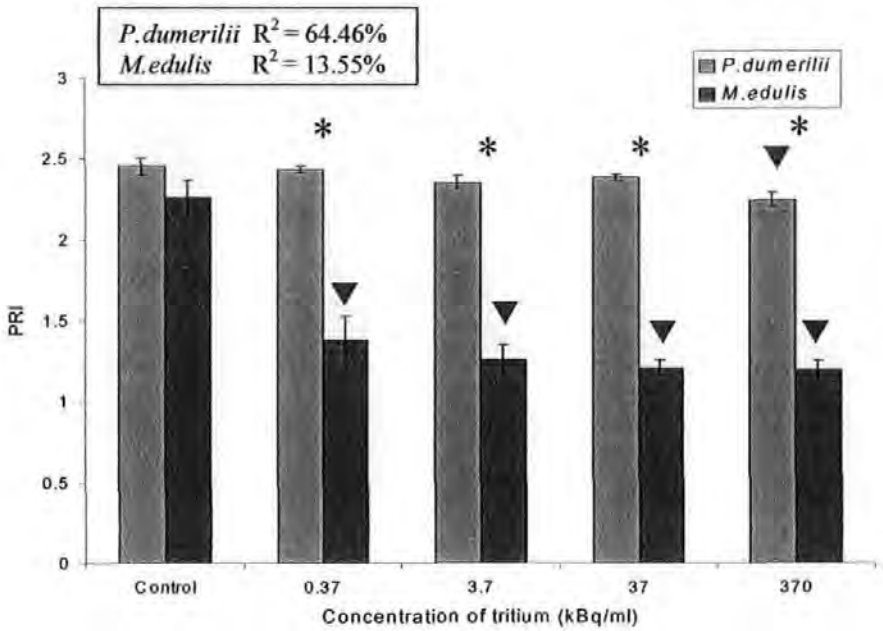


Figure 6.9. Proliferative rate index of embryo-larvae following exposure to tritium continuously 1h post-fertilisation (▼ = significantly different from control, * = significance difference between species, $P<0.05$) (Error bars represents standard deviation , n = 2).

Only the *P.dumerilii* exposed to 370 kBq/ml produced a statistically lower PRI in comparison to the control, whereas *M.edulis* exposed to all four concentrations of tritium resulted in a decrease in the PRI. There was not a statistical difference in the controls for the two species, although there was for all the samples exposed to tritium. There was a stronger dose relationship of PRI with concentration in *P.dumerilii* (R^2=64.46%) compared to *M.edulis* (R^2=13.55%).

6.3.2.3 Sister chromatid exchanges (SCEs)

Figure 6.10. shows the incidence of SCEs in embryo-larvae of *M.edulis* and *P.dumerilii* after exposure to tritium 1h post-fertilisation.

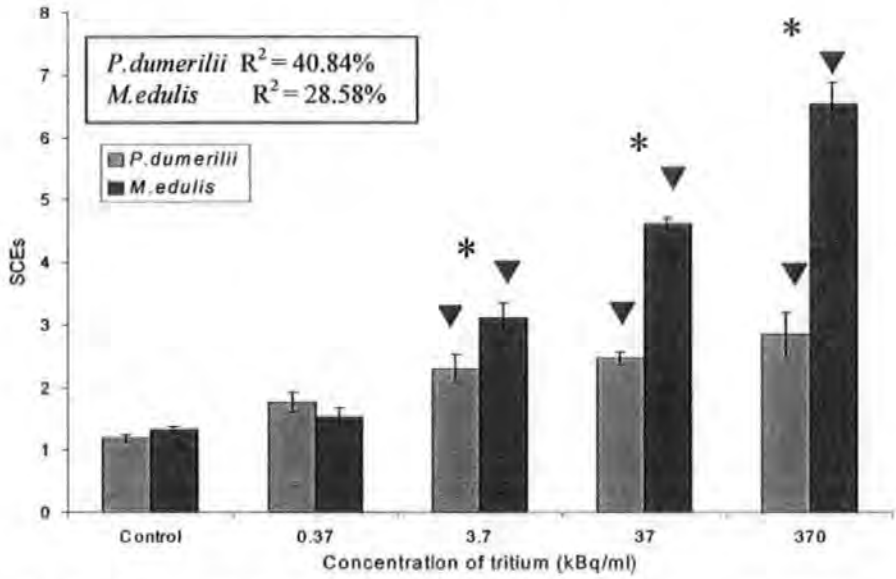


Figure 6.10. Induction of sister chromatid exchanges following exposure of 1h old embryo-larvae to tritium continuously (▼ = significantly different from control, * = significance difference between species. $P<0.05$) (Error bars represents standard deviation , n = 2).

There was a significant increase in SCEs for both species after exposure to 3.7 kBq/ml or greater. However *M.edulis* experienced a greater induction of SCEs at the higher concentrations and therefore there was a statistical difference between species for the 3.7, 37 and 370 kBq/ml concentrations. As with the PRI there was a stronger dose relationship, between SCEs and concentration, in *P.dumerilii* (R^2=40.84%) compared to *M.edulis* (R^2=28.58%).

6.3.2.4 Aberrant cells and Total aberrations

Figure 6.11. represents the induction of cells containing aberrations in embryo-larvae of *M.edulis* and *P.dumerilii* after exposure to tritium 1h post-fertilisation and figure 6.12 represents the total number of aberrations.

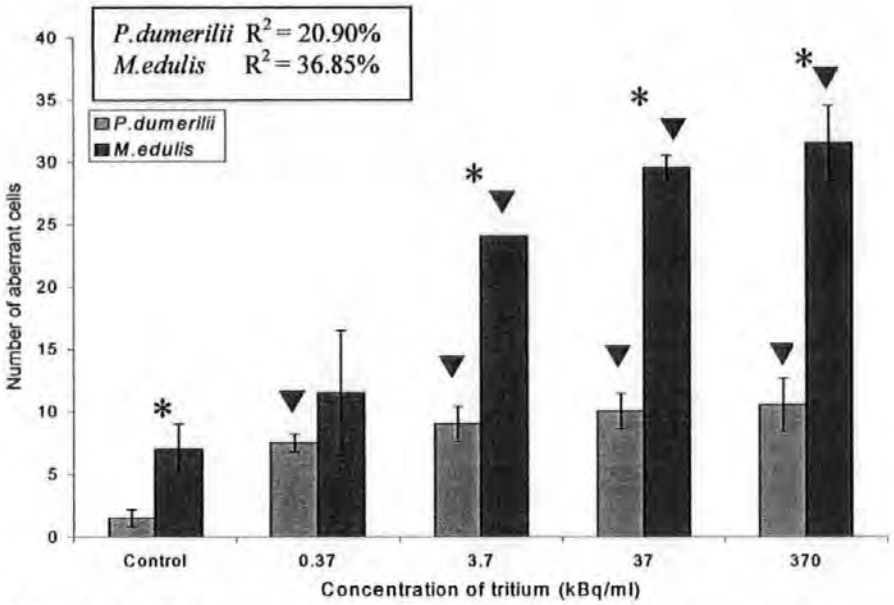


Figure 6.11. Induction of aberrant cells in embryo-larvae following exposure to tritium continuously 1h post-fertilisation (▼ = significantly different from control, * = significance difference between species, P<0.05) (Error bars represents standard deviation , n = 2).

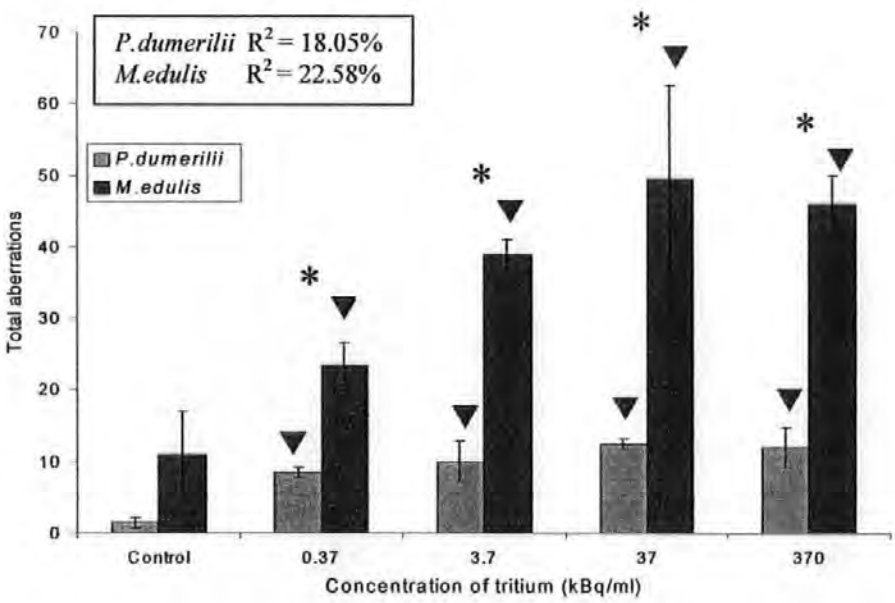


Figure 6.12. Number of total aberrations in embryo-larvae following exposure to tritium continuously 1h post-fertilisation (▼ = significantly different from control, * = significance difference between species, P<0.05) (Error bars represents standard deviation , n = 2).

The *P.dumerilii* embryo-larvae exposed to all concentrations of tritium produced elevated numbers of cells that contained aberrations than the control. In comparison *M.edulis* experienced an elevated incidence of aberrant cells with concentration 3.7 kBq/ml or greater. There was a statistical difference between species at 3.7 kBq/ml or greater of tritium with *M.edulis* showing a 2.5-3-fold increase in aberrant cells than *P.dumerilii*. Unlike the PRI and SCEs, there was a stronger dose relationship, between aberrant cells and concentration, in *M.edulis* (R^2=36.85%) compared to *P.dumerilii* (R^2=20.90%), although both are moderately weak relationships.

For both species, the embryo-larvae that were exposed to tritium contained elevated numbers of total aberrations when compared with the control. There was a statistical difference between species at all concentrations of tritium although *M.edulis* showed a 2.5-5-fold increase in the number of aberrations than *P.dumerilii*. Unlike the PRI and SCEs, there was a stronger dose relationship, between number of aberrations and concentration, in *M.edulis* (R^2=22.58%) compared to *P.dumerilii* (R^2=18.05%), although both were weak relationships.

6.3.2.5 Comparison among developmental/mortality, cytotoxic and genotoxic effects following exposure to tritium

Regression analysis between the percentage of abnormal and dead embryo-larvae and the induction of PRI, SCEs and Cabs was carried out in an attempt to link the effects at molecular and cellular levels with changes at the individual level.

Table 6.7. Comparison between developmental/mortality, cytotoxic and genotoxic effects in *P.dumerilii* embryo-larvae following exposure to tritium

	% of 72h abnormal larvae		% of 72h dead larvae	
	P value	**R^2**	**P value**	**R^2**
PRI	0.1158	61.6%	0.4403	20.78%
SCEs	***0.004***	***95.4%***	0.7634	3.49%
Aberrant cells	***0.0136***	***90.1%***	0.8655	1.12%
Total aberrations	***0.008***	***93.0%***	0.8405	1.58%

Table 6.8. Comparison between developmental/mortality, cytotoxic and genotoxic effects in *M.edulis* embryo-larvae following exposure to tritium

	% of 72h abnormal larvae		% of 72h dead larvae	
	P value	**R^2**	**P value**	**R^2**
PRI	0.8129	2.17%	***0.0197***	***87.4%***
SCEs	0.2058	46.3%	0.0679	72.3%
Aberrant cells	0.5208	14.9%	***0.0174***	***80.1%***
Total aberrations	0.5414	13.6%	***0.0402***	***88.4%***

For *P.dumerilii* there was a relationship between the percentage of abnormal larvae at 72h with the induction of cells containing aberrations, the total number of aberrations scored and the incidence of sister chromatid exchanges (see table 6.7). This suggests that tritium was genotoxic to *P.dumerilii* embryo-larvae and the consequences of the genotoxicity are manifested as alterations in the normal morphology and behaviour of the organism. In comparison the effects of tritium on *M.edulis* showed that the percentage of dead larvae at 72h was related to the induction of cells containing aberrations, the total

number of aberrations produced and the reduction of the proliferative rate index (see table 6.8). Therefore tritium was cytotoxic and genotoxic to *M.edulis* embryo-larvae and a reduction in the PRI and the induction of chromosomal aberrations resulted in an increase in mortality.

6.3.3 Summary of results

6.3.3.1 Species sensitivity after exposure to radioactive effluent

P.dumerilii and *M.edulis* exposed to a cocktail of radioactive waste showed an increase in the percentage of abnormality with increasing concentrations of radionuclides. There was a slight increase in mortality for both species although this was not above the expected baseline levels. The PRI was reduced in both species although *M.edulis* experienced a more dramatic reduction than *P.dumerilii*. The elevation of SCEs above base levels was not generally observed in either species. The frequency of chromosomal aberrations were significantly higher in both species after exposure to the radioactive waste, although as for the PRI, *M.edulis* experienced a greater induction of aberrations than *P.dumerilii*. For both species, induction of chromosomal aberrations was related to an increase in abnormal development. Therefore the concentration of radionuclides in the radioactive waste provided causes an increase in chromosomal aberrations in embryo-larvae of *M.edulis* and *P.dumerilii* and this resulted in an increase in the percentage of abnormality.

6.3.3.2 Species sensitivity after exposure to tritium

Exposure to tritium 1h post-fertilisation for approximately 23h resulted in an increase in the percentage of abnormal *P.dumerilii*, although for *M.edulis* the exposure to tritium resulted in an increase in mortality. The proliferative rate index was generally not reduced in for *P.dumerilii* after exposure to tritium but there was a sharp threshold decrease in PRI for *M.edulis* at every concentration. The incidence of sister chromatid

exchanges was elevated for both species at 3.7 kBq/ml or higher although the number of SCEs in *M.edulis* was higher than for *P.dumerilii*. Similarly the induction of chromosomal aberrations was elevated following tritium exposure in both species, with *M.edulis* generally producing higher number of aberrant cells and total number of aberrations. Tritium was cytotoxic and genotoxic at the concentrations tested and there was a significant relationship between the percentage of dead embryo-larvae and the reduction of the PRI and the induction of chromosomal aberrations for *M.edulis*. In comparison tritium was genotoxic to *P.dumerilii*, as it resulted in an increase in SCEs and chromosomal aberrations. Furthermore there was a significant relationship between the genotoxic effects of tritium and an increase in the percentage of abnormality.

6.4 Discussion

Between 1946-1993 a total of 85 PBq of radioactive waste was dumped in the worlds oceans, 45 PBq into the Atlantic, 1.4 PBq into the Pacific and 38 PBq into the Arctic (Povinec *et al.*, 2000). The most important part of the beta-activity of these dumped wastes was ^{3}H and ^{14}C. The process giving rise to tritium discharged from Devonport is one in which water in the primary cooling circuit of the nuclear reactor in a nuclear submarine is activated by capturing neutrons originating from the nuclear reaction in the reactor core. Therefore the tritium present is likely to be in the form of tritiated water. There is the possibility of a small percentage of OBT (up to 1%) in the effluent. Seawater sampled and analysed for tritium close to the vicinity of DML's discharge point in the Hamoaze has been conducted by the Environment Agency and results showed activity concentrations ranging from <10 - 311 Bq/l, with an average of 26 Bq/l (Environment Agency, 2001).

The concentrations and hence the dose rates of tritium from the cocktail of radioactive waste were approximately 1000+ times less than those used when tritium was exposed in isolation, as the predicted dose from a cocktail of radionuclides which contains

β and γ-radiation would be expected to be significantly greater. However Woodhead (1984) claims that as the absorption coefficient of γ-radiation is so low it is generally assumed that γ-ray emission from internal radionuclides would not make a significant contribution to the exposure of organisms that are spherical in shape and approximately 50 μm in diameter. During the current experiment the source of the gamma radiation was external and therefore this might have accounted for the increase in activity. Although the embryo-larvae were small in size and due to the wavelength of gamma irradiation it might be expected that the embryo-larvae would not be affected by the irradiation as indicated by Woodhead (1984). However during the current study the embryo-larvae that were exposed to the 18% dilution of radioactive waste which contained 0.07 kBq/ml of tritium produced 100% mortality. In comparison embryo-larvae exposed to 0.37 kBq/ml of tritium in isolation produced approximate mortality of 6 and 20% for *P.dumerilii* and *M.edulis* respectively, therefore it is clear that the combination of radionuclides or one of the other radionuclides in isolation is responsible for the increase in mortality. Furthermore there was no difference between species after exposure to the radioactive waste whereas for tritium *M.edulis* was more sensitive than *P.dumerilii*. This may suggest that species sensitivity might only become apparent at low levels of radiation exposure and that a uniform effect is observed at high levels or due to other radionuclides present in the radioactive waste. The detrimental radiation effects of tritiated seawater (^{3}H), in isolation, has been shown to affect the normal developmental of goose barnacle embryos, *Pollicipes polymerus*, under laboratory conditions (Abbott and Mix, 1979). In addition exposure to complex mixtures of radionuclides have been shown to cause detrimental effects. Fallout radioactivity extracted from rainwater after weapons test in the Pacific in 1954 were found to have a damaging effect on the development of eggs of zebra fish, however no information was obtained as to the radionuclide composition and the chemical form of the residue (Hibiya and Yagi, 1956). Cooley (1973) reported a reduction in the frequency of egg capsule production in a natural population of the aquatic snail *Physa heterostropha*

after exposure to chronic low-level radiation (^{125}Sb, ^{106}Ru, ^{90}Sr, ^{65}Zn, ^{60}Co), indicating that as well as altering the normal development of organisms, fecundity may also be affected by radiation. Laboratory studies on the effect of ^{60}Co on the same snail species showed that fecundity was reduced significantly by 10 rads/hr and would probably lead to extinction of the population in two generations (Cooley and Miller, 1971). Mortality in adult organisms due to exposure of radionuclides generally occurred at high accumulative doses. A review by Woodhead (1984) stated that the mortality of freshwater snails *Physa heterostropha* was only significantly affected when accumulative doses of ^{60}Co reached 28 krad. At 10 rad h^{-1}, marine clams *Mercenaria mercenaria* and scallops *Argopecten irradians* were also found to be resistant to ^{60}Co at annual doses of 8-8.8krad (0.9-1.0 rad h^{-1}). Blue crabs *Callinectes sapidus* was unaffected by continuous irradiation by ^{60}Co until after 50d at which time the accumulative dose was 33krad (Engel, 1967).

The induction of aberrations was the parameter that produced the most significant results after exposure to radiation in comparison to the controls for both species. Suyama *et al.* (1980) also found that tritium, in isolation, induced chromosomal aberrations when he examined anaphase translocation bridges in embryos of the plaice *Limanda yokohamae*. A significant increase over the controls was detected in the group exposed to 379 Bq/L (0.379 Bq/ml) but not at 3.79 Bq/L (0.00379 Bq/ml). This is similar to the current experiment where chromosomal aberrations were induced in embryo-larvae of *M.edulis* and *P.dumerilii* after exposed to 1.8% dilution of radioactive waste that contained 7.02Bq/ml. Suyama *et al.* (1981) found that β-rays from tritium were more effective than other types of radiation (^{90}Sr and ^{90}Y) in inducing chromosome aberrations in *Oryzias* eggs. Several studies have suggested that different radionuclides may interact synergistically or additively to produce genetic damage in humans (Holmberg and Jonasson, 1974; Holmberg and Strausmanis, 1983) and plants (Bubryak *et al.*, 1992). Higher mutation rates have been observed in barley pollen from combined alpha, beta and gamma irradiation due to radionuclide contamination compared to similar doses from an

external gamma source (^{60}Co) in isolation (Bubryak *et al.*, 1992). In the natural environment, catfish from cooling ponds (near Chernobyl) contaminated with radionuclides were found to exhibit greater genetic damage (in the form of single strand breaks) than catfish from control pools (Sugg *et al.*, 1996). During the current study the induction of chromosomal aberrations was related to the percentage of abnormal *P.dumerilii* and *M.edulis* produced by 72h. Chromosomal aberrations have previously been linked to abnormality and mortality (Anderson *et al.*, 1990; Evans, 1996).

Of additional interest in the assessment of radiation risk is the potential for bioaccumulation. Radionuclides behave the same as other chemicals in the same column of the periodic table of elements for example radionuclides such as ^{45}Ca, ^{90}Sr, ^{140}Ba, ^{226}Ra and ^{45}Ca behave like calcium and ^{40}K, ^{86}Rb and ^{137}Cs behave like potassium (Kennish, 1997). Consequently, if the element is able to bioaccumulate in biota then so will the radionuclide. If radionuclides accumulate in the food chain then the dose received by the human population might also increase. Furthermore if the radionuclide is capable of inducing damage as shown in this study, then the human population will be at greater risk. Studies have shown that internal deposition of radionuclides in radiation workers caused chromosomal aberrations (Bradom *et al.*, 1979). A study of Sr transfer in the trophic food chain undertaken in dams on the Douro river (Portugal) showed that Sr was not accumulated from plankton to fish species, but there was a link between levels of Sr in the water and those found in fish and plankton (Carraça, 1990). There was a strong seasonal variation in levels of Sr in water and in the different levels of the food chain with minimum values occurring in the rainy season when the water flow rate was highest an a clear dilution effect was noted. Radionuclides (^{137}Cs and ^{40}K) in harbour porpoises, *Phocoena phocoena*, showed that there were elevated concentrations of ^{137}Cs in porpoises originating from the Irish Sea compared to porpoises form the Celtic Sea, the Atlantic Ocean and the North Sea (Berrow *et al.*, 1998). For airborne radionuclides the most critical food chain is the lichen-caribou-human food chain, as lichens are the main winter forage for caribou,

which in turn are a main dietary staple for many northern Canadians (Thomas and Gates, 1999). Thus airborne radionuclides, particularly ^{137}CS, ^{210}Pb and ^{210}Po are transferred efficiently through this simple food chain to people elevating their radiological dose. In addition radionuclides have been shown to accumulate in the fruiting bodies of mushrooms, Rb-83, Zn-65 and Mn-54 were shown to be accumulated by 10-fold (Ban-Nai *et al.*, 1997).

During the current experiment the induction of chromosomal aberrations was statistically increased in *M.edulis* compared to *P.dumerilii* and whilst it might be due to the fact that *M.edulis* was more sensitive to radiation damage there is a possibility that it might be because they are were exposed to a higher temperature than normal. The effect of temperature on the induction of chromosomal aberrations has been investigated (Bajerska and Liniecki, 1969). Elevated temperatures (heat shock) has been shown to cause an increase in nuclear anomalies (micronucleus assay), chromosomal aberrations and DNA damage (comet assay) in the goldfish (*Carassius auratus*) (Anitha *et al.*, 2000). Furthermore temperature reduction, hypothermia, has also been shown to induce micronucleus in mouse bone marrow cells possibly due to disturbance of the mitotic apparatus (Asanami and Shimono, 1997). In addition temperature was shown to effect the protective capabilities of caffeine on radiation-induced chromosomal aberrations in human lymphocytes (Stoilov *et al.*, 1994.)

Seasonal changes in temperature might also affect the uptake and effects of radionuclides. Seasonal variation was also detected in ^{137}Cs concentrations in the mussel *Mytilus galloprovincialis* and in water concentrations from the river Rhône in France (Charmasson *et al.*, 1999). Although the variation in the mussels was believed to be linked to the reproductive cycle of the organism and not environmental factors.

In comparison to the similar effects produced after exposure to radioactive waste, *M.edulis* appeared to be more sensitive than *P.dumerilii* in terms of PRI, SCEs, Cabs and mortality after exposure to tritium. The variation between species in tritium exposed

embryo-larvae might be due to their reproductive strategies. As previously mentioned, by Jha *et al.* (2000c), several factors could account for the variation in sensitivity such as genetic make up, reproductive pattern and programmed developmental commitment of the growing embryo-larvae.

In conclusion, the effect of radioactive waste at the concentrations used during the present study were shown to induce chromosomal aberrations in both, *M.edulis* and *P.dumerilii*, which resulted in an increase in abnormality of embryo-larvae. However the dilution factors of the radioactive waste may not have been realistic. It is also worth mentioning that natural sources annually release 2 to 3 orders of magnitude more radioactive substances into the atmosphere than all manmade sources taken together (Jaworowski, 2002). In addition studies on the measurements of the naturally occurring radionuclide ^{210}Po and anthropogenic ^{137}Cs in seawater and biota (fish and shellfish) suggest ^{210}Po contributed most of the dose to man and that ^{137}Cs produced a negligible dose (Aarkrog *et al.*, 1997).

Chapter 7

The validation of the single cell gel electrophoresis or "comet" and micronucleus assays using haemocytes of *Mytilus edulis.*

Hypotheses

1. DNA damage can be detected in blood cells (haemocytes) of adult mussels (*Mytilus edulis*) after exposure to a genotoxic agent

2. A correlation will exist between the induced damage expressed at both cellular and cytogenetical levels.

3. Radiation will induce a genotoxic effect at molecular and cellular levels in haemocytes of adult *Mytilus edulis*.

7.1 Introduction

The effects of pollution on marine organisms are often difficult to detect as all are not observed immediately, but may take years before they are realised, such as cancer or congenital diseases (Kadhim, 1990). Because effects at higher levels of organisation such as growth, development, and survival are initiated at the molecular and cellular levels attempts have been made to identify useful biomarkers at these levels (Steinert, 1999). In an attempt to extrapolate the potential effects of carcinogens and mutagens, DNA damage detection at lower levels of biological organisation (e.g. DNA) is assessed and, if possible, linked with the manifestation at higher levels. In this study DNA damage will be assessed at molecular or biochemical level using the single cell gel electrophoresis (SCGE) or "comet assay" and at cellular level using the micronucleus assay (Mn).

Both the "comet" and micronucleus assays have been successfully used in haemocytes of *Mytilus edulis* (Galloway *et al.*, 2002; Livingstone *et al.*, 2000; Rank, 1999; Shaw *et al.*, 2000; Wrisburg and Rhemrev, 1992). However there has been no attempt to perform these two assays concurrently on the same individuals. A limited number of experiments using human lymphocytes have used the comet and micronucleus assays simultaneously although few have endeavoured to correlate both assays. He *et al.* (2000), showed that both the comet and micronucleus assays could be used to monitor X-ray radiation induced genetic damage in humans. A good correlation was observed between

the comet and micronucleus assays, with the comet assay being more sensitive overall. Tafazoli and Kirsch-Volders (1996), also suggested that the comet assay might be a more suitable and sensitive screening method than the micronucleus test, although they did imply that this might be due to the assays detecting different endpoints, however no correlation between them was attempted. One aim of the current study was to validate the use of haemocytes of *M.edulis* for these two parameters using a reference genotoxic agent, ethyl methanesulfonate (EMS) and to assess if there was any correlation between the two assays.

EMS (Sigma, CAS N^{o}: 62-50-0) is a monofunctional ethylating agent that has been found to be mutagenic in a wide variety of genetic test systems from viruses to mammals (Sega, 1984). EMS owes it biological reactivity to its ethyl group that can be transferred to a variety of cellular, nucleophilic sites. For EMS, this transfer of the ethyl group can be accomplished either through an SN_1 (substitution, nucleophilic, unimolecular) or an SN_2 (substitution, nucleophilic, bimolecular) mechanism. While ethylation of DNA occurs principally at nitrogen positions in the bases, because of the partial SN_1 character of the reaction, EMS is also able to produce significant levels of alkylation of oxygen such as the O^6 of guanine and in the DNA phosphate groups. Genetic data obtained using micro-organisms suggests that EMS may produce both GC to AT and AT to GC transition mutations, base pair insertions or deletions, as well as more extensive intragenic deletions. In higher organism, EMS has also been shown to break chromosomes, although the mechanisms are not well understood (Sega, 1984).

Once the assays were validated, a further aim of this study was to assess the effects of a reference radionuclide, tritium, on the production of single strand DNA breaks and micronucleus formation in adult *M.edulis*. As previously mentioned tritium is one of the major radionuclides released by nuclear facilities. It is a beta emitting radionuclide, which can either directly or indirectly (via reactive oxygen species or free radicals) alter DNA. The physio-chemical interaction of ionising radiation with cellular DNA have been shown

to produce a variety of primary lesions, such as single-strand breaks (SSB), double-strand breaks (DSB), DNA-DNA and DNA-protein crosslinks, and damage to purine and pyrimidine bases (Vijayalaxmi *et al.*, 1992).

As mentioned earlier no work has been carried out investigating the use of the comet and micronucleus assays concurrently in bivalve mussels. Given the ecological and economic importance and wide use of bivalve molluscs in ecotoxicological studies, the aim of the current study was to validate the two assays (comet and micronucleus) in the same individual organism using EMS, a reference mutagen. The techniques will then be used to assess the effects of tritium as limited studies have been carried out on the effects of ionising radiation on adult *M.edulis*.

7.2. Methods and materials

Figure 7.1. briefly describes the experimental protocol adopted for the analysis of DNA damage by EMS and tritium. A detailed description of the method is included in sections 7.2.1 and 7.2.2.

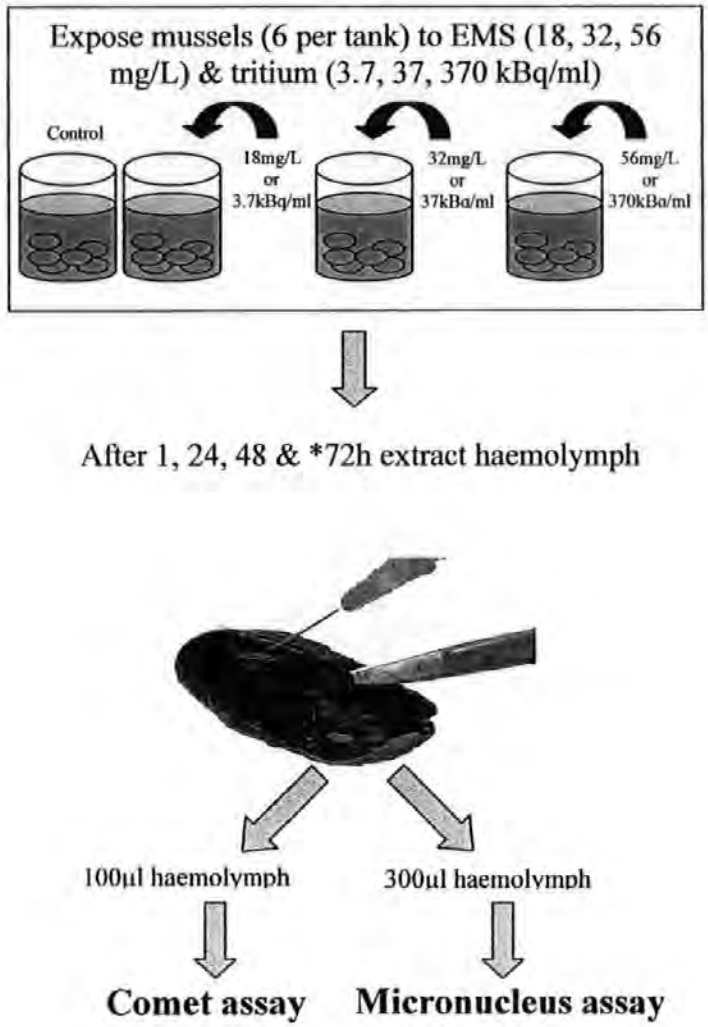


Figure 7.1. Exposure protocol for the analysis of DNA damage in haemocytes of adult *M.edulis* after exposure to EMS and tritium. (* tritium exposure only).

7.2.1 Experiment using EMS

Clean acid washed beakers were filled with 1litre of filtered (10 µm) seawater and placed in an incubator at 15°C ± 1 °C for 24h. A glass pipette was attached to an airline and a steady flow of air was introduced into each test vessel. 6 adult mussels (shell length 5 cm ± 1 cm) were placed into each test vessel and left to acclimatise for approx. 48h. Three concentrations of EMS (18, 32 and 56 mg/L) were added to the test vessels. The concentration of EMS was selected on the basis of earlier work carried out by Dopp *et al.*, (1996) and Belpaeme *et al.*, (1998). Approx. 0.5 ml of haemolymph was extracted from

each mussel, after 1, 24, and 48h as described in section 2.4.1. From the extracted sample, 100 µl of haemolymph was added into 1ml of physiological saline and processed for comet analysis as described in detail in section 2.4.5 and 300 µl of haemolymph was placed onto a poly-*l*-lysine slide for analysis of micronuclei (section 2.4.4). Cell viability was assessed using Eosin Y, as described in section 2.4.2. The salinity, pH, temperature and dissolved oxygen of the seawater pre and post-exposure periods were also recorded.

7.2.2 Experiment using tritium

Clean acid washed beakers were filled with 1litre of filtered (10 µm) seawater and placed in an incubator at 15°C ± 1 °C for 24h. A glass pipette was attached to an airline and a slow stream of air was introduced into each test vessel. 6 adult mussels (shell length 5 cm ± 1 cm) were placed into each beaker and left to acclimatise for approx. 48h. Figure 7.1 describes the protocol undertaken for analysis of DNA damage by tritium. Three concentrations of tritium (3.7, 37 & 370 kBq/ml) were added to the test vessels. For each mussel approx. 0.5 ml of haemolymph was extracted after 1, 24, 48 and 72h as described in section 2.4.1. From the extracted sample, 100 µl of haemolymph was placed into 1ml of physiological saline and processed for comet analysis (section 2.4.5) and 300 µl of haemolymph was placed onto a poly-*l*-lysine slide for analysis of micronuclei (section 2.4.4). Cell viability was assessed using Eosin Y, as described in section 2.4.2. The salinity, pH, temperature and dissolved oxygen of the seawater pre and post-exposure periods were also recorded.

7.3 Results

7.3.1 Cell viability

The cell viability of haemocytes (using Eosin Y staining) following exposure to EMS and tritium is presented in table 7.1.

Table 7.1. Average cell viability of haemocytes from *M.edulis* (± standard deviation).

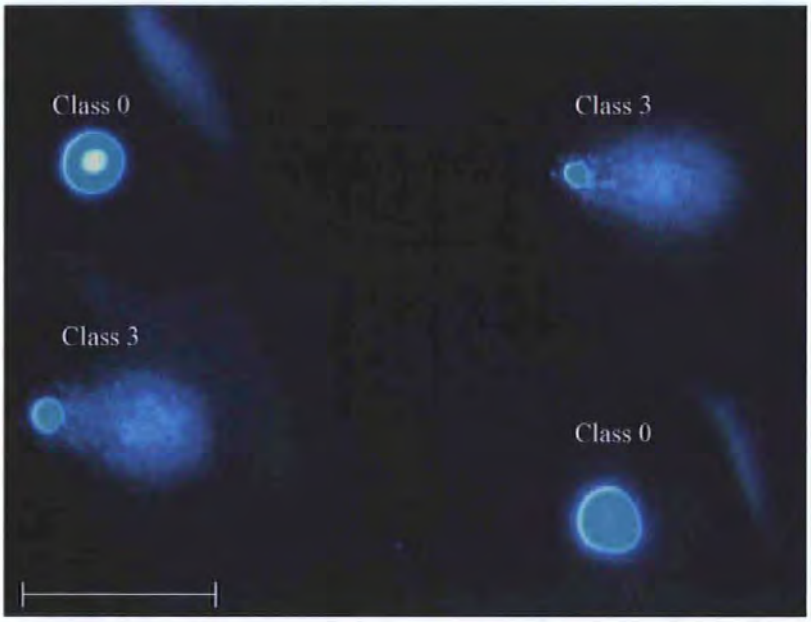
EMS (mg/L)					**Tritium (kBq/ml)**					
Time	*1h*	*24h*	*48h*		*Time*	*0h*	*1h*	*24h*	*48h*	*72h*
Control	**98.7 ± 1.47**	**97.6 ± 1.48**	**97.3 ± 1.18**		Control	**98.1 ± 1.18**	**98.5 ± 0.96**	**98.5 ± 1.17**	**98.8 ± 1.11**	**96.9 ± 1.20**
18	**97.7 ± 0.8**	**97.8 ± 1.57**	**97.7 ± 1.41**		3.7	**98.8 ± 1.53**	**98.5 ± 0.96**	**98.2 ± 1.46**	**98.3 ± 0.57**	**97.3 ± 1.60**
32	**98.8 ± 1.28**	**97.8 ± 0.68**	**98.2 ± 1.18**		37	**98.7 ± 1.55**	**98.0 ± 1.27**	**98.4 ± 1.28**	**98.3 ± 1.26**	**97.8 ± 0.66**
56	**97.5 ± 1.78**	**98.0 ± 1.36**	**97.2 ± 1.43**		370	**98.6 ± 1.05**	**97.3 ± 1.07**	**98.4 ± 1.42**	**98.5 ± 1.33**	**98.5 ± 1.19**

No statistical difference in the cell viability between any concentration or over time for EMS ($P = 0.5129$) and tritium ($P = 0.5989$) was observed (ANOVA, $n = 6$).

7.3.2 Exposure to EMS

7.3.2.1 Induction of single DNA strand breaks (comet assay)

Figure 7.2. illustrates examples of "comets" scored under UV fluorescence after staining with DAPI as described in section 2.4.5.1.



(b)

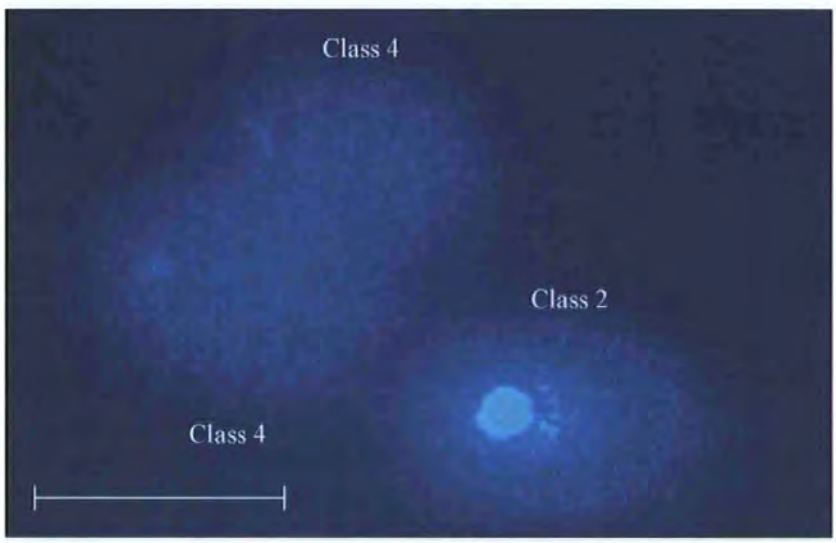


Figure 7.2. Examples of "comets" observed in haemocytes of *M.edulis* following exposure to reference genotoxins (x 20 magnification), (a) two undamaged cells with no tail migration (class 0) and two cells with comet tails (class 3), (b) bottom cell with slight migration (class 2) and two cells with no material left in the nucleus (class 4, also classified as apoptotic cells). (Scale bar = 75 μm).

An arbitrary scale, as described by Collins *et al.*, (1997), was used to quantify the level of DNA damage detected by the comet assay. In brief, 50 randomly selected comets were classified as described in section 2.4.5.2. A value was assigned to each comet with class 0=0, class 1=1, class 2=2, class 3=3 and class 4=4, therefore for each sample a value of 0-200 was produced. The visual classes correspond very roughly to 20% intervals of increasing DNA damaged as assessed using computer analysis (Collins *et al.*, 1997). Figure 7.3. shows the arbitrary scale of DNA damage in haemocytes after exposure of adult *M.edulis* to EMS for 1, 24 and 48h.

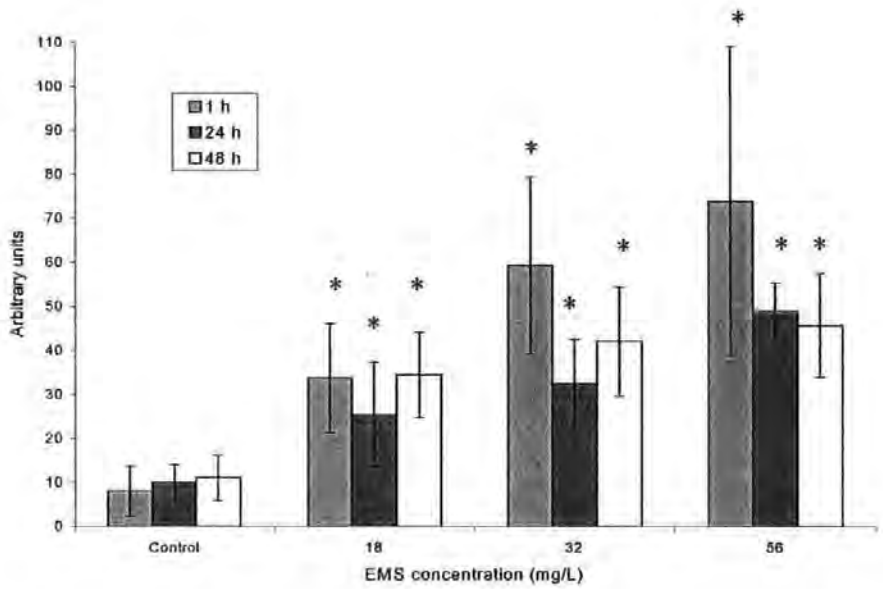


Figure 7.3. Induction of DNA damage in *M.edulis* haemocytes as quantified by the comet assay after exposure to EMS. Error bars represent 2 x standard error, n = 6. (* = statistically different from control, $P<0.05$)

Following 1h exposure there was a statistically significant difference in the amount of DNA damage, as shown by the comet assay, between the controls and all three concentrations (ANOVA, P=0.0001). There was a clear dose response with mussels exposed to 18 mg/L containing significantly less DNA damage than those exposed to 32 mg/L and also between 32 mg/L and 56 mg/L. Regression analysis also showed there was a dose response with a R^2 value of 50.94% (P=0.0001). After 24 h exposure the degree of DNA damage decreased, however all three concentrations were still significantly different

from the controls (P=0.0001). Although the level of damage was less at 24h than at 1h the dose response, as shown by regression, was more pronounced with a R^2 value of 67.23% (P=0.00005). Haemocytes collected from unexposed mussels after 48h exposure were again significantly different from all three EMS concentrations. However there was no statistical difference between the three concentrations, with all three having a mean arbitrary value that ranged from 34-45. At concentrations 18 and 32 mg/L there was a slight increase in the amount of DNA damage from 24h to 48h, this might be due to an increase in the level of stress caused by the removal of haemocytes or due to a microbial infection. One of the six mussels in concentration 32 mg/L did not produce any haemolymph and a further two (out of the six) contained no cells for comet analysis, thus reducing the level of standard error for this concentration and also possibly altering the mean arbitrary level. The largest degree of standard error occurred after 1h exposure and increased with increasing concentration suggesting that individual mussels have a large response variability when exposed to high doses of toxicants although after time the responses between mussels becomes more uniform (the error bars are reduced).

7.3.2.2 Induction of micronuclei

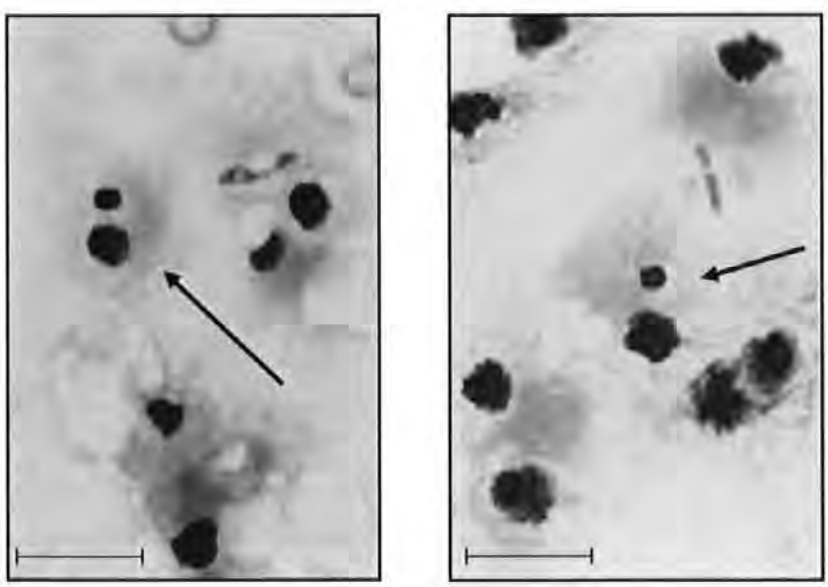


Figure 7.4. Micronuclei (arrows) in *M.edulis* haemocytes stained with 10% Giemsa (scale bar = 100 μm).

Figure 7.4 shows examples of micronuclei observed in haemocytes of mussels and figure 7.5 shows the induction of micronuclei in haemocytes from *M.edulis* following exposure to EMS for 1, 24 and 48h.

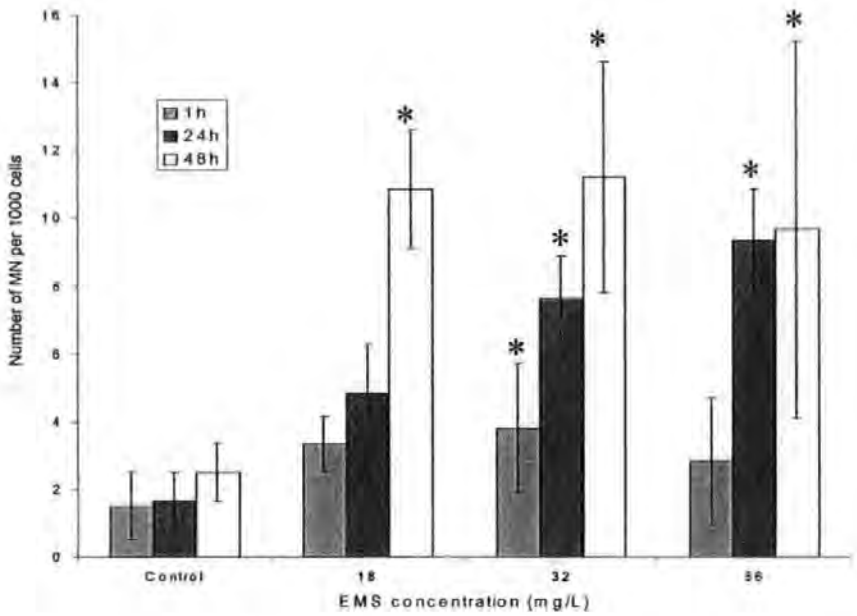


Figure 7.5. Induction of micronuclei in *M.edulis* haemocytes after exposure to EMS. Error bars represent 2 x standard error (n = 6). (* = statistically different from controls using ANOVA, $P<0.05$).

Following 1h exposure there was no statistical significant difference between any of the concentrations ($P=0.1658$). After 24h the two highest concentrations (32 and 56 mg/L) had significantly higher levels of micronuclei than the controls. There was a significant difference between the medians of each concentrations analysed using the Kruskal Wallis non-parameter test ($P=0.002$). In comparison with 1h levels of micronuclei there was an increase in the induction of micronuclei in all three concentrations, the two highest increased by approx. 2- and 4-fold respectively. There was also a dose dependent increase in the number of micronuclei with increasing concentration, although there was no difference between 18 and 32 mg/L and 32 and 56 mg/L. A moderately strong dose relationship was demonstrated using regression analysis, with a R^2 value of 50.13%

(P=0.0002). After 48h exposure with EMS there was still a significant difference between the medians of the controls and all three concentrations, although there was not a difference between the concentrations themselves (P=0.003). The highest concentration (56 mg/L) did not increase at all from 24 to 48h although there was larger inter-individual variation at 48h, this might be an indication of maximum levels of DNA damage that might occur per cell division. In comparison to 56 mg/L, the lowest concentration (18 mg/L) increased by approx. 2-fold from 24 to 48h and produced a similar number of micronuclei per 1000 cells as the higher concentrations. Again this might demonstrate a threshold level of effect as at higher concentrations but for longer exposure periods, although no attempt was made during the present study to test this theory. As there is no dose response at 48h the regression analysis showed a R^2 value of only 26.11% (P=0.0127). Overall there was a dose dependent increase in micronuclei with increasing concentrations and with increasing exposure time.

7.3.3 Exposure to tritium

7.3.3.1 Tritium concentration

Table 7.2. Concentration of tritium (kBq/ml and percentage) in water samples at periods of haemolymph extraction.

Concentration		Time	0h	1h	24h	48h	72h
Seawater control	Nominal amount (kBq/ml)		0.034 ± 0.006	0.014 ± 0.003	0.045 ± 0.002	0.015 ± 0.001	0.006 ± 0.001
	Amount lost (kBq/ml)		-0.0	-0.02	+0.01	-0.02	-0.03
	% remaining		100	41.2	132.3	44.1	17.6
3.7 kBq/ml	Nominal amount (kBq/ml)		**3.03** ± 0.03	**2.74** ± 0.027	**2.73** ± 0.027	**2.48** ± 0.025	**2.75** ± 0.028
	Amount lost (kBq/ml)		**-0.0**	**-0.29**	**-0.3**	**-0.55**	**-0.28**
	% remaining		**100**	**90.4**	**90.1**	**81.8**	**90.7**
37 kBq/ml	Nominal amount (kBq/ml)		29.59 ± 0.28	27.95 ± 0.28	27.91 ± 0.28	25.04 ± 0.25	27.16 ± 0.27
	Amount lost (kBq/ml)		-0.0	-2.0	-1.68	-4.55	-2.43
	% remaining		100	94.4	94.3	84.6	91.8
370 kBq/ml	Nominal amount (kBq/ml)		**296.5** ± 2.55	**277.5** ± 2.39	**271.4** ± 2.42	**265.8** ± 2.39	**258.5** ± 2.35
	Amount lost (kBq/ml)		**-0.0**	**-19.0**	**-25.1**	**-30.7**	**-38**
	% remaining		**100**	**93.6**	**91.6**	**89.6**	**87.2**

Table 7.2. demonstrates the concentration of tritium in water samples before and after exposure. There was a large variability in the levels of tritium in the controls however this was due to the relatively low levels detected in unexposed seawater. There was a general decrease in the levels of tritium in the water after a period of 72h. However this was not related to the amount of tritium present at the start of the exposure with all the samples containing between 87.2-91.8% of the original tritium added remaining after exposure. Although the amount of tritium taken up was dependent on the amount of tritium

in the sample (between 0.28-38 kBq/ml). This may have implications in an environmentally realistic situation as the mussels will take up tritium at a regular rate but the amount of tritium that is removed from the water is dependent on the amount of tritium present to begin with.

7.3.3.2 Induction of single DNA strand breaks (comet assay)

Figure 7.6 shows the arbitrary scale of DNA damage in haemocytes after exposure of adult *M.edulis* to tritium for 1, 24, 48 and 72h.

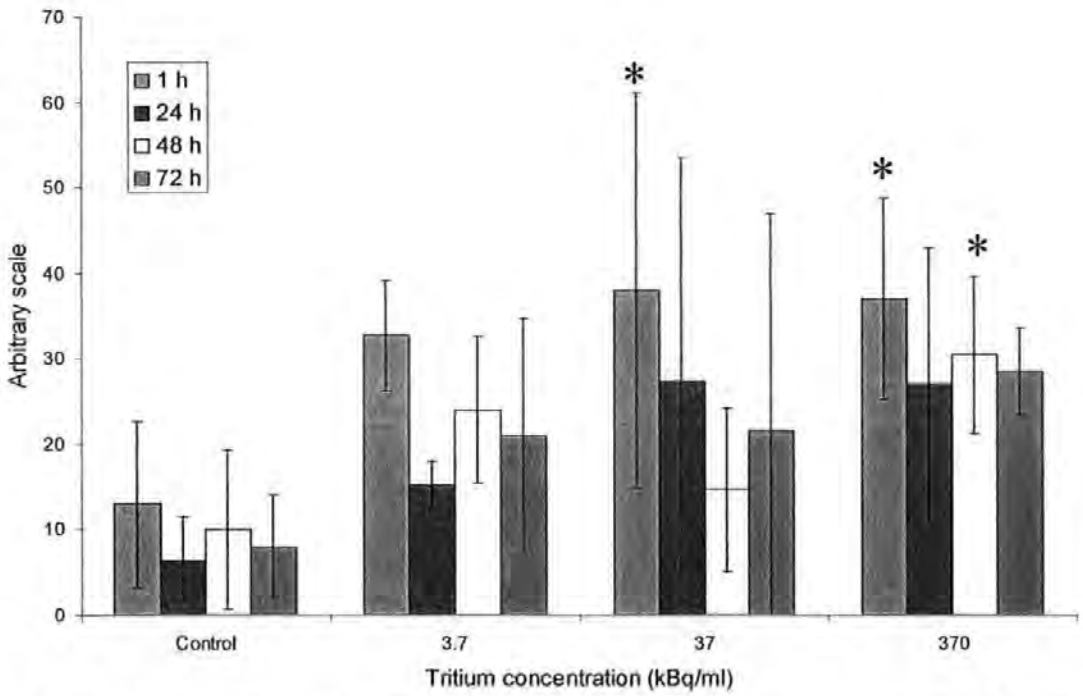


Figure 7.6. Induction of DNA damage in *M.edulis* haemocytes as quantified by the comet assay after exposure to tritium. Error bars represent 2 x standard error (n = 6). (* = statistically different from controls using ANOVA, $P<0.05$).

Pre-exposed mussels were analysed for baseline levels of DNA damage and all the concentrations were similar before the addition of the tritium ($P=0.7367$). Following 1h exposure to tritium the data was not normally distributed and was transformed using square root, then analysed using ANOVA. Haemocytes from unexposed mussels had significantly less DNA damage than the three concentrations of tritium although there was no dose

response for tritium exposed mussels with all three concentrations being statistically similar to each other. This was validated using regression analysis which showed that there was no dose response (P=0.254; R^2=5.86 %). Following 24h exposure the level of DNA damage decreased in comparison with 1h samples. The Kruskal Wallis non-parametric test was used to analyse the degree of DNA damage after 24h exposure, it showed that there was no statistical difference between the controls and any of the three concentrations of tritium (P=0.111). At 48h there was a difference between concentrations with the controls and 37 kBq/ml being significantly different from 370 kBq/ml. The 370 kBq/ml concentration had a slight increase in the amount of DNA damage in comparison to a large decrease at 37 kBq/ml and this might explain the variability between samples. The variation in samples at 37 kBq/ml was very large in all time exposures except at 48h and thus might cause a discrepancy at the other exposure times. At 72h the data was transformed (logged) and ANOVA showed that there was no statistical difference between concentrations although the controls and 370 kBq/ml were still different (P=0.1356).

7.3.3.3 Induction of micronuclei

The frequency of micronuclei prior to exposure ranged from 0-4‰ (mean 1.54 ± 0.63). No concentrations were significantly different from each other before exposure (P=0.135). Figure 7.7 shows the frequency of micronuclei in haemocytes from *M.edulis* after exposure to tritium as a function of time.

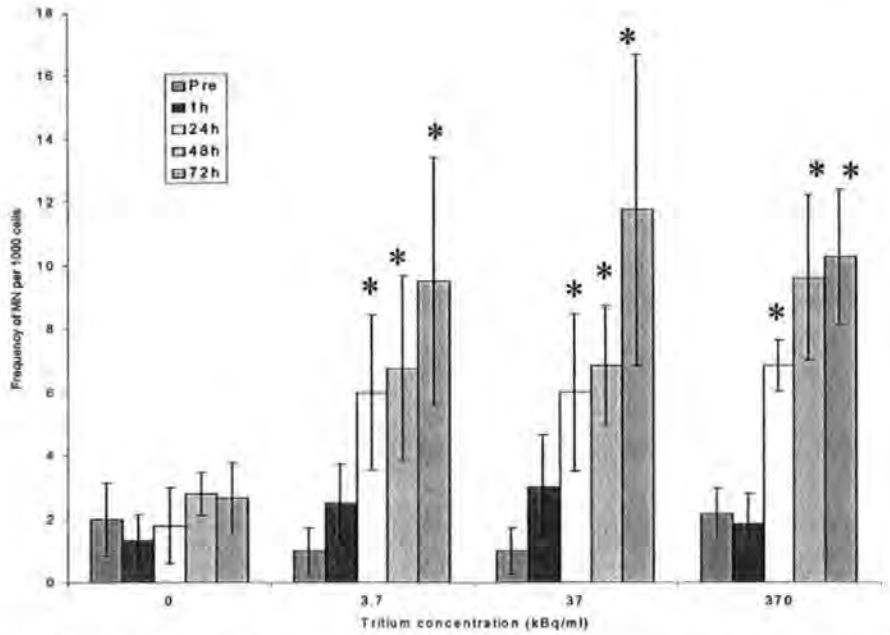


Figure 7.7. Induction of micronuclei in *M.edulis* haemocytes after exposure to tritium. Error bars represent 2 x standard error (n =6). (* = statistically different from controls using ANOVA, P<0.05)

After 1h exposure the level of DNA damage was not significantly different between treatments (P=0.2513). The induction of micronuclei was first detected following 24h exposure. A statistically significant difference between all three concentrations of tritium and the controls was detected using ANOVA (P=0.01). The controls contained between 0-4 (mean 2.12 ± 0.61) micronuclei and tritium concentrations contained between 0-12 micronuclei with mean values of 6 ± 3.0, 6 ± 3.03, 6.8 ± 0.98 for concentrations 3.7, 37 and 370 kBq/ml respectively. A threshold level of micronuclei was observed in the three concentrations of tritium and no significant difference was observed between the different concentrations. Regression analysis confirmed this lack of dose response with a low R^2 value of 13.54% (P=0.092). The number of micronuclei continued to increase with time and at 48h the number of micronuclei ranged from 2-14‰ with means of 6.75 ± 3.59, 6.83 ± 2.31, 9.6 ± 3.21 for concentrations 3.7, 37 and 370 kBq/ml respectively. As at 24h, there was a statistically significant difference, as assessed using ANOVA, in the number of micronuclei in control haemocytes and those exposed to tritium (P=0.0074). However the

number of micronuclei in the three different concentrations were not significantly different from each other. At 72h exposure there was a large increase in variation between tritium exposed samples, the mean values ranged from 9.5 ± 4.8, 11.75 ± 6.02, 10.25 ± 2.63 for concentrations 3.7, 37 and 370 kBq/ml respectively, and the number of micronuclei per 1000 cells ranged from 2-20. ANOVA showed that there was still a statistical significant difference between the control and the exposed samples (P=0.0089). However there was no statistical difference between tritium concentrations and no dose response (P=0.2280). Overall there was a difference in the number of micronuclei in haemocytes from the control and tritium exposed mussels. In the exposed samples the induction of micronuclei was time dependent but not dose dependent.

7.3.3.4 Analysis of correlation between the comet and micronucleus assays

After exposure to EMS the maximum induction of DNA damage as assessed using the comet assay was observed after 1h for all concentrations. After exposure to 56 mg/L the level of damage (single strand breaks) decreased over time. For mussels exposed to 18 mg/L there was no variation in the amount of DNA damage after 1h and 48h although the levels did slightly decrease at 24h. In comparison micronuclei formation was detected following 24h and the level of damage increased with time. The induction of micronuclei is dependent on the cell replication and hence was only detected after 24h and may be produced from single strand breaks that occurred prior to cell replication. Figure 7.8.a indicates the correlation between the arbitrary comet assay score (after 1h) and the number of micronuclei per 1000 cells (after 24h) in individual mussels. The correlation was made between the two parameters at the stated periods of exposures due to the influence of cell turnover rates. DNA damage detected in individuals by the comet and micronucleus assays was correlated using simple regression to determine if a relationship occurs at different biological levels of organisation (DNA single strand breaks and chromosomal aberrations). There was no relationship when comparing levels of DNA damage after 1h using the

comet assay and the formation of micronuclei at 24h (P=0.0593; R^2=15.91%). Whilst comparing 1h comet values with the number of MN formed at 48h (figure not shown) did show a statistically significant relationship (P=0.0066; R^2=30.18%), possibly suggesting that the cell cycle range of *M.edulis* haemocytes might be greater than 24h. However a significant relationship (P=0.01; R^2=28.74%) also occurred when linking 24h comet values and 48h micronucleus formation.

For *M.edulis* exposed to tritium there was no correlation between the amount of DNA damage in haemocytes observed using the comet assay after 1h and the induction of micronuclei at 24, (figure 7.8.b) 48 and 72h (P=0.08, 0.159, 0.1171 respectively). No correlation was observed between the 24h comet and 48 and 72h micronuclei (P=0.06 and 0.32). There was also no relationship between the induction of micronuclei at 72h and the occurrence of comets at 48h (P=0.1268, R^2=13.95%).

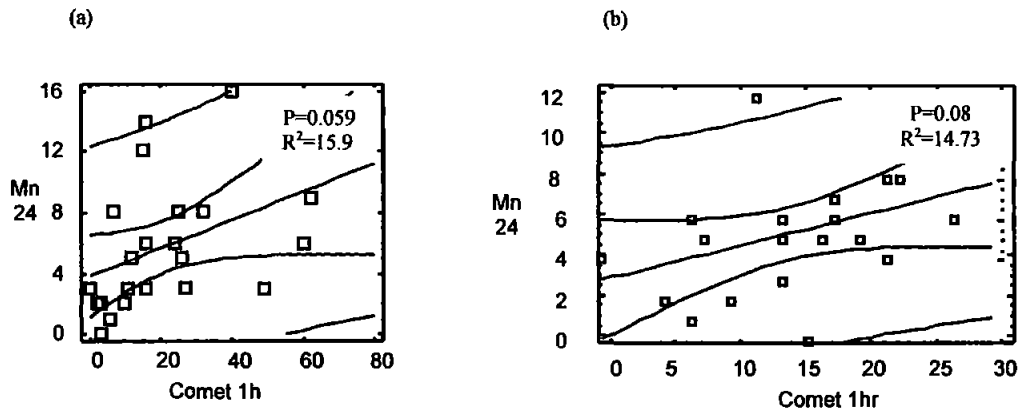


Figure 7.8. Regression relationship of ssDNA breaks (comet assay) formed after 1h and the induction of micronuclei after 24h in haemocytes of *M.edulis* after exposure to (a) EMS, (b) tritium.

7.3.4 Comparison between EMS and tritium results

Figure 7.9. shows the results of the comet assay after 1h for both EMS and tritium and figure 7.10. represents the frequency of micronuclei in *M.edulis* haemocytes after 24h.

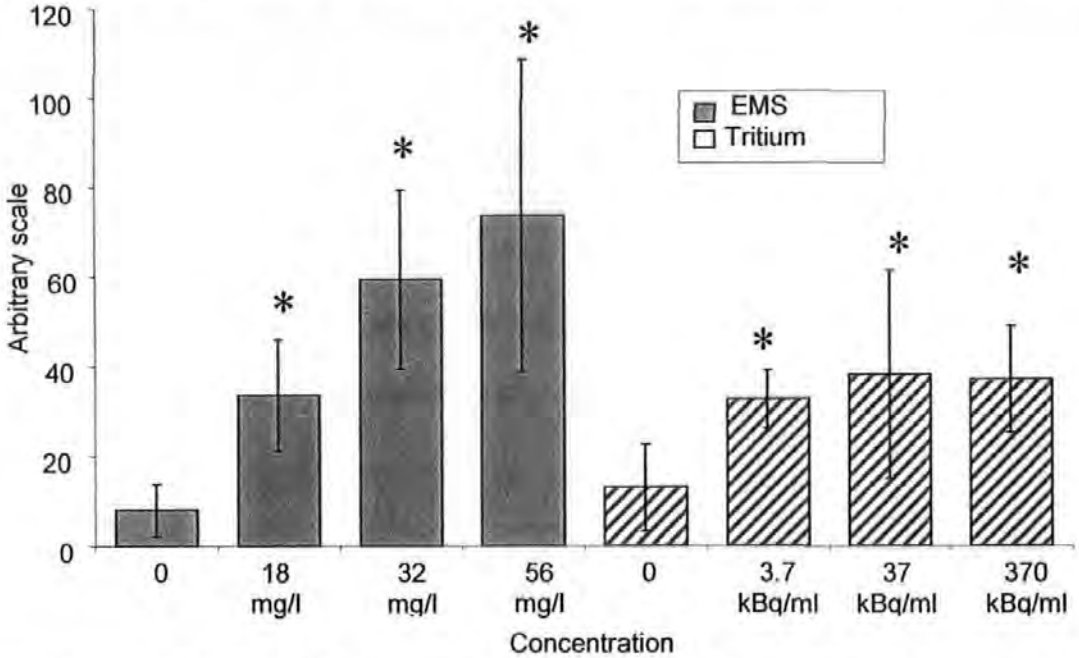


Figure 7.9. Induction of DNA damage in *M.edulis* haemocytes after 1h as quantified by the comet assay. Error bars represent 2 x standard error (n = 6). (* = significantly different from control using ANOVA, P<0.05).

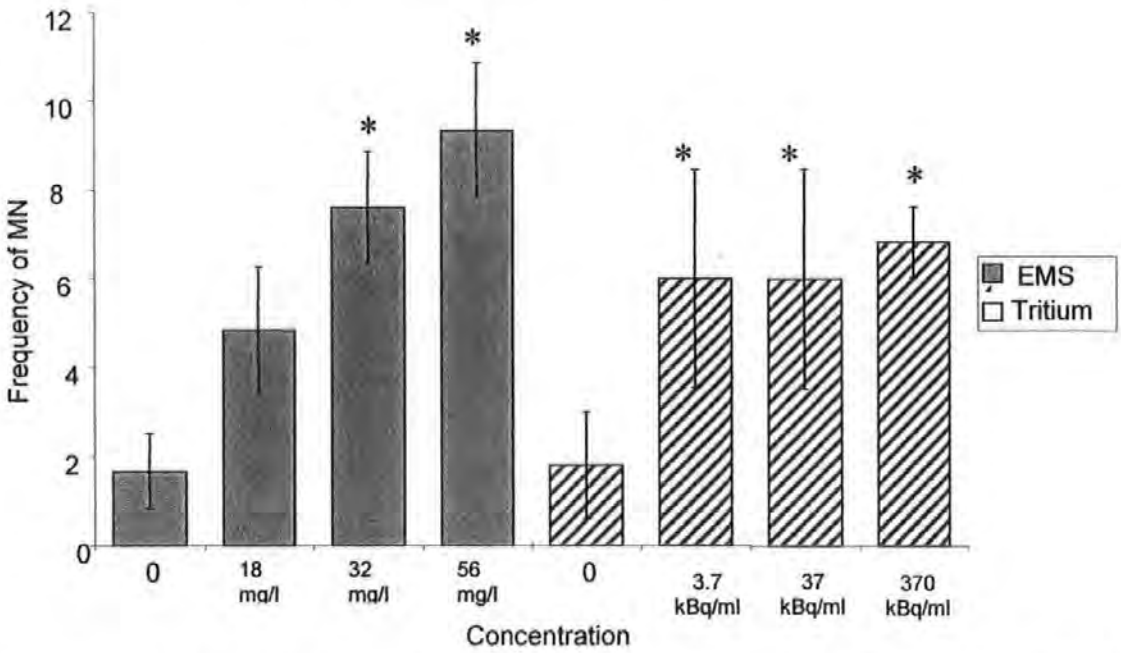


Figure 7.10. Induction of micronuclei in *M.edulis* haemocytes after 24h exposure to EMS and tritium. Error bars represent 2 x standard error (n = 6). (* = significantly different from control using ANOVA, P<0.05).

The maximum amount of DNA damage observed with the comet assay occurred after 1h for both EMS and tritium. Although all three concentrations in both exposures were significantly different from the controls it was clear that EMS produced more DNA damage in comparison to tritium. EMS exposed mussels had significantly more damage than tritium exposed mussels and the level of damage increased with increasing concentration (mean value of 33.7 ± 15.15, 59.2 ± 32.63 and 73.7 ± 42.86 for concentrations 18, 32 and 56 mg/L respectively). Regression analysis also suggests a moderately dose relationship for the induction of single strand DNA breaks (P=0.0001, R^2=50.94%). In comparison, tritium exposed mussels, although significantly different from the controls were not significantly different with increasing dose (mean values of 32.7 ± 7.87, 38.0 ± 28.28 and 37.0 ± 14.35 for concentrations 3.7, 37 and 370 kBq/ml). Analysis of regression confirms no relationship between increasing dose and induction of single strand DNA breaks (R^2 value 5.86%; P=0.2541). In conclusion, EMS produced a dose dependent increase for DNA damage in the comet assay, whereas tritium produced a threshold level of damage in all three concentrations.

The occurrence of micronuclei in haemocytes of mussels exposed to EMS and tritium were both observed only after 24h and subsequently increased over time. Figure 7.10. shows the induction of micronuclei in *M.edulis* haemocytes after exposure to EMS and tritium for 24h. The frequency of Mn induced by the lowest concentration of EMS (18 mg/L) was not significantly different from the control, although the two higher concentrations (32 and 56 mg/L) were. A dose dependent increase for the frequency of micronuclei occurred although 32 and 56 mg/L were not significantly different from each other. Regression analysis shows that there was a moderately strong relationship between concentration and the number of micronuclei (P=0.0002, R^2=50.13%). The tritium exposed mussels showed a significant increase for the induction of micronuclei in comparison with unexposed controls although the levels of micronuclei in the exposed samples were not significantly different between concentrations suggesting a threshold response. The tritium

exposed mussels produced less micronuclei per 1000 cells (mean values of 6 ± 3.0, 6 ± 3.03, 6.8 ± 0.98 for concentrations 3.7, 37 and 370 kBq/ml), than EMS exposed mussels (mean value of 4.8 ± 1.75, 7.6 ± 1.55 and 9.3 ± 1.86 for concentrations 18, 32 and 56 mg/L respectively). Although, variation among the samples were higher in tritium exposed mussels.

7.3.5 Comparison between the correlation of both assays after exposure to EMS and tritium

Table 7.3. Correlation between the results of the comet and Mn assays between individual mussels (n = 24). (Figures in bold represent statistically relationship between assays, * = $p<0.05$ and ** = $p<0.01$).

EMS					**Tritium**			
Comet	*MN*	*P value*	R^2 *value*		*Comet*	*MN*	*P value*	R^2 *value*
1h	24h	0.0593	15.91		1h	24h	0.08	14.73
1h	**48h**	**0.0066****	**30.18**		1h	48h	0.159	11.27
					1h	72h	0.1171	15.56
24h	**48h**	**0.0101***	**28.74**		24h	48h	0.06	19.27
					24h	72h	0.32	6.46
					48h	72h	0.126	13.95

Table 7.3. shows the correlation of comet assay values and formation of micronuclei. The correlation was calculated using simple regression analysis between the results of the comet assay and the micronucleus assay for each individual mussels. This principle was based on the assumption that the DNA damage observed using the comet assay is not dependent on cell cycle kinetics whereas micronucleus formation is. In other words, any

ssDNA breaks observed in haemocytes using comet assay might develop into structural chromosomal alterations and hence micronuclei following cell division (figure 7.11.).

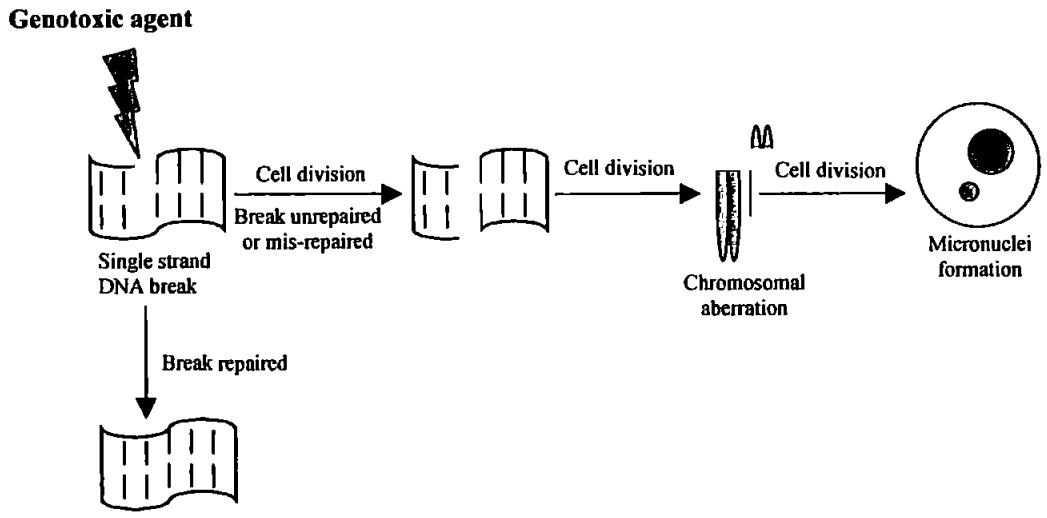


Figure 7.11. Aneugenic effects leading to the formation of Mn which involves actions at microtubule level during cell division.

As the highest amount of DNA damage was observed after 1h in comet analysis it would be reasonable to assume that this would lead to a large number of micronuclei in the subsequent cell replication (24h). There was however no relationship between either EMS or tritium induced damage at these times. There was a significant relationship between the DNA damage detected via the comet assay observed after 24h exposure to EMS and the induction of micronuclei at 48h, ($P=0.01$; $R^2= 28.74\%$). However the strongest relationship occurred between the formation of micronuclei at 48h and the levels of DNA damage after 1h as assessed using the comet assay as shown in table 7.2. This might indicate that the cell cycle time for haemocyte production of *M.edulis* is greater than 24h but it is more likely that EMS inhibits cell cycle progression thus delaying the manifestation of micronuclei.

7.4 Discussion

Studies using mammalian (including human) cells have shown EMS to produce a positive effect when assessed using the comet assay (Belpaeme *et al.*, 1996, Henderson *et*

al., 1998, Leroy *et al.*, 1996), which confirms the observation made in the present study using haemocytes from mussels. In general, the level of DNA damage (single strand DNA breaks) decreased over the time periods examined (24h periods). This suggests that either the cell turnover rate is higher or that DNA is repaired in 24h time periods. Cells from *Mytilus* have been shown to have a cell cycle of 24-30h. The proliferation kinetics of gill cells from the mussel (*Mytilus galloprovincialis*) were tested using sister chromatid differentiation (SCD) staining and the duration of the cell cycle was shown to be 24-30h (Martínez-Expósito *et al.*, 1994). During the present study this was also confirmed by the induction of micronuclei after exposure to EMS for 24h. Furthermore a time related increase in the induction of micronuclei was observed after exposure to EMS over 24 and 48h. Belpaeme *et al.* (1998) reported a clear time related response in blood and gill cells of flatfish that were exposed to 50mg/L EMS for 3 or 7 days. As well as a time dependent increase in the induction of micronuclei there was also a dose related increase. The results found during the present study supports a study by Dopp *et al.* (1996) who reported a dose-related increase in the number of Mn in haemocytes of clams with increasing concentration of EMS. Stopper and Müller (1997) also showed that EMS produced a low percentage of kinetochore positive micronuclei, which suggests that EMS is a clastogen rather than an aneugen. EMS is a compound that is widely used in biological test systems and its mode of action has been extensively investigated (Jaylet *et al.*, 1986) and reviewed in literature (Sega, 1984).

Prior to exposure of *M.edulis* to tritium the frequency of spontaneous micronuclei were comparable to other findings (Wrisberg and Rhemrev, 1992) where baseline frequencies were reported to be in the range of 0.9-1.7‰. During the present study the radionuclide, tritium was found to induce single strand DNA breaks at the two highest concentrations (37 and 370 kBq/ml) and produce elevated frequencies of micronuclei in all three concentrations. No work has been carried out on the induction of micronuclei due to irradiation by tritium although other radionuclides have been reported to induce DNA

damage. Micronuclei were induced in human lymphoblastoid cells after exposure to ^{60}Co and there was a linear dose response. During the present study there was no dose relationship following 72h exposure and thus a threshold effect might be suggested. In the natural environment, Ilyinskikh *et al.* (1998) reported an increase in the frequency of micronucleated erythrocytes in Pike blood as a function of radiocesium concentration in the body. This increase was also found to be age related. In general, younger fish had a higher frequency of MN in comparison to older fish (10+ y). This was suggested to be linked with the biological half-life of the radionuclide and not due to elimination efficiency mechanisms in younger fish. Age has also been shown to influence the DNA integrity and metabolism of *M.edulis* cells (Accomando *et al.*, 1999). They found that the number of single strand breaks in haemocytes increased with age in adult *M.edulis*. During the present study all the *M.edulis* were collected from the same location and were a similar size and thus age, and therefore the induction of single strand breaks due to the age of the organism can be ruled out. However it is worth mentioning that the increase of DNA damage in older individuals of *M.edulis* might be more prevalent with the addition of a further stressers such as radionuclides or chemical contamination. In addition, as mentioned in previous chapters environmental factors, such as temperature and salinity may also act additively or synergistically with radiation to enhance the degree of damage. Seasonal changes in the reproductive state of *M.edulis* has also been shown to induce varying levels of DNA strand breaks (Shaw *et al.*, 2000). During the present study we have shown that high levels of the radionuclide tritium induced single strand breaks, thus in *in situ* situations seasonal changes as reported by Shaw *et al.* (2000) in conjunction with tritium may cause elevated levels of DNA damage as measured by single strand breaks. However as previously mentioned single strand breaks are repairable and only the mis-repaired or unrepaired single strand breaks may produce chromosomal aberrations and hence micronuclei. No elevation in the number of micronuclei were detected in bank voles (*Clethrionomys glareolus*) exposed to fallout from Chernobyl in comparison to unexposed

voles (Rodgers and Baker, 2000). This may possibly be due to increased radioresistance of the exposed animals.

Tritium exposed *M.edulis* were shown to have a significant increase in the level of DNA damage in their haemocytes in comparison to the controls. However there was no difference between concentrations of tritium possibly indicating a threshold level of DNA damage. It is commonly accepted that risk assessments of genotoxic chemicals are based on linear extrapolations methods, however there is substantial evidence that some chemicals may be genotoxic only at high doses or under specific conditions in genotoxicity assays, but are genotoxically inert at concentrations within the range of human exposure levels (Henderson *et al.*, 2000). There are a variety of possible mechanisms of thresholded genotoxicity, including disruption of cell division and chromosome segregation, inhibition of DNA synthesis, overloading of oxidative defence mechanisms, metabolism or plasma binding capacity, disturbances of metal homeostasis, cytotoxicity and physiological perturbations in *in vivo* assays (Henderson *et al.*, 2000).

During the present study the comet assay was able to detect damage at lower concentrations of EMS than the micronucleus test. He *et al.* (2000) also found that the comet assay was more sensitive than Mn in detecting dose related exposure of X-rays in human lymphocytes. Furthermore during the present experiments the comet assay was able to detect DNA damage in the shortest time (1h) in comparison to the micronucleus test that took a minimum of 24h due to it requiring cell replication to occur for the manifestation of micronuclei. Therefore the comet assay offers considerable advantages over other cytogenetic methods for the detection of DNA damage, (i.e. Cabs, SCEs and Mn) because the cells need not be mitotically active (Pavlica *et al.*, 2001). Although one main advantage of the micronucleus test over other cytogenetic methods for the detection of DNA damage, i.e. Cabs and SCEs, is that it can be applied to any proliferating cell population regardless of its karyotype.

As the maximum level of DNA damage was detected after 1h exposure using the comet assay it should be reasonable to assume that the induction of micronuclei might result as a consequence of these single strand breaks. The relationship between DNA breakage in interphase cells during which exposure to the mutagen occurred and the expression of micronuclei in the following interphase is dependent on different factors such as cell cycle stage, repair capacities and the type of mutagen (Fenech, 1996; Tafazoli and Volders, 1996). During the present study, for EMS, the most significant relationship occurs between DNA damage via comet analysis at 1h and micronuclei generation at 48h. This suggests that the ssDNA breaks occurring after 1h might be responsible for the induction of micronuclei. Although as previously mentioned the cell cycle of *M.edulis* gill cells was reported as being between 24-30h. Therefore if, during the present study, Mn are produced from single strand breaks after 48h it suggests that either the cell cycle of haemocytes is longer than 24-30h or that EMS is inhibiting the cell cycle. As the incidence of micronuclei was first observed following 24h exposure it would therefore seem that the cell cycle is similar to that reported for gill cells. Therefore we can suggest that EMS might be inhibiting or reducing the cell cycle. In previous chapters it has been shown that MMS an alkylating agent similar to EMS was able to reduce the proliferation rate index of *M.edulis* embryo-larval cells and this supports the theory proposed.

No relationship occurred between the levels of DNA damage as assessed by the comet assay after 1h exposure to EMS and the formation of micronuclei following 24h exposure. A number of reasons might explain this. Initial levels of damage (i.e. occurring after 1hour) could have been detected and repaired. Alternatively following longer exposure (48h) of EMS the levels of damage might have increased due to either an accumulation of toxicant in the organism or via inhibition of repair mechanisms possibly due to disruption or deregulation of cell cycle checkpoints. The cell cycle is co-ordinated by external and internal chemical signals, any disruption of these signals might lead to DNA damage being unrepaired (Snustad *et al.*, 1997). Normally damaged DNA induces a

signal to the cell for DNA repair and the damage-induced signal restricts the entry of the cell into the S-phase. If the cell checkpoint is unregulated the cell with damaged DNA moves directly into the S phase and so the damaged DNA is replicated which may lead to further mutations and possibly tumors (Snustad *et al.*, 1997). Mustonen *et al.* (1999), suggested a strong G_2/M arrest after exposure of Raji cells (mammalian) to gamma radiation. They have suggested that the arrest in the cell cycle was for DNA repair. Studies have revealed that radiation-induced single-strand breaks are repaired more rapidly if the cells are in the G_1 phase of the cell cycle in comparison to those in the S phase (Olive and Banáth, 1995). Although in comparison Olive *et al.* (1991) found that S phase DNA appeared to be 3 times less sensitive to X-ray damage than DNA from other phases of the cell cycle. However this discrepancy might be due to a difference in the radiation or cell types.

Single strand breaks as detected via the comet assay are likely to be repaired and it is only when this damage is unrepaired that chromosomal aberrations might occur. Double strand DNA breaks, which are not detected using the alkaline comet assay, are less common than single strand breaks and are more likely to be responsible for the induction of micronuclei than single strand breaks. DNA double strand break has been shown to be the most important lesion, which leads to chromosomal alterations which in turn, produces Mn. When X-ray irradiated cells were post-treated with *Neurospora endonuclease*, a treatment which converts induced single strand breaks to double strand breaks there was an increase in the frequency of chromosomal aberrations (Natarajan and Obe, 1978). As well as double strand breaks other DNA lesions induced by ionising radiation such as base damage may also lead to chromosomal aberrations under DNA repair deficient conditions (Palitti, 1998). Exposure to EMS, during the current experiment, produced micronuclei which were possibly formed from chromosomal aberrations caused by alkylation of the DNA.

There was little correlation between the comet assay and micronucleus formation in tritium exposed mussels, although the levels of micronuclei were similar as in mussels exposed to EMS, the comet assay values were significantly lower. This suggests that, during this study, other types of DNA damage apart from single strand breaks may be responsible for the induction of micronuclei as under alkaline conditions the comet assay only detects single strand DNA breaks. Moreover, double strand breaks have often been cited as the critical lesions produced by ionising radiation (Natarajan and Obe, 1978), and if unrepaired, or incorrectly repaired, double strand breaks can lead to chromosome damage and cell death (Fairbairn *et al.*, 1995). In comparison to the present study, He *et al.* (2000) did find a correlation between DNA strand breaks (using the comet assay) and Mn induction, following exposure of human lymphocytes to X-rays. They suggested that chromosome abnormalities were a direct consequence and manifestation of damage at the DNA level. X-rays are external sources of gamma irradiation which have been proven to induce single strand breaks (Oliveri *et al.*, 1990; Peak and Peak, 1991) as to has the beta emitting tritium (Takakura *et al.*, 1986) therefore the difference between the two experiments may be the level of single strand breaks that occurred. A correlation occurred between X-ray induced single strand breaks and Mn probably due to ratio of single strand breaks in comparison to other forms of DNA lesions. Whereas in the present study DNA lesions other than single strand breaks must have been the primary initiator of Mn formation.

In addition to the likelihood of cell repair mechanisms being altered by EMS it has also been shown that stage of the cell cycle when damage occurs may also influence the severity of the damage as well as the speed of occurrence. The disturbance of the spindle formation has also been shown to lead to the early appearance of Mn in comparison if damage was induced in the G_1 phase of the cell cycle Mn would appear much later (Stopper and Müller, 1997). Experiments have shown that the cell cycle stage at which DNA damage is induced plays a critical role in the fixation of primary DNA lesions

(Andreoli *et al.*, 1999). They found that DNA lesions induced by hydrogen peroxide and MMS were efficiently removed soon after mitogen stimulation (G_0-G_1) and in particular residual damage was barely detectable by the comet assay by late G_1 phase. As a result of the removal only a few primary lesions were available for fixation through misreplication in the forthcoming S phase and hence micronucleated cells were not observed. Only when DNA damage persisted to a large extent up to the first round of DNA replication were micronuclei effectively induced, although most likely as a result of double strand breaks originating from the replication of damaged templates (Fenech and Nerville, 1992).

In conclusion, from the present study it was proven that comet and micronucleus assays could be simultaneously carried out on haemocytes extracted from the same mussels over a period of time. This allowed for the analysis of DNA damage to be carried out at molecular and cellular levels and provided a key to how DNA damage might progress through different levels of biological organisation. Using the reference genotoxin, EMS, it was demonstrated that there was a dose dependent increase in DNA damage with increasing dose and that there was a relationship between single strand breaks observed using the comet assay and the manifestation of chromosomal abnormalities and hence the induction of micronuclei. Following validation of the simultaneous use of the assays and subsequent correlation between molecular and biochemical levels of biological organisation the techniques were used to assess the effects of ionising radiation, tritium, on adult *M.edulis*. Tritium was shown to induce elevated number of micronuclei in the haemocytes of the marine bivalve *M.edulis* but also produced a slight increase in the induction of single strand breaks. However there was no correlation between the single strand DNA breaks produced and the subsequent formation of micronuclei. This suggests that tritium is genotoxic to adult *M.edulis* but the micronuclei produced are not a result of single strand DNA breaks.

Chapter 8

An *in situ* assessment of cytotoxic and genotoxic effects in deployed *M.edulis* in the Tamar estuary

Hypothesis

The effects of environmentally relevant radiation can be detected *in situ* using a suite of biomarkers in adult *Mytilus edulis* deployed in an area of discharged radioactive waste.

8.1 Introduction

DNA is considered to be the primary target for the action of ionising radiation, however few studies have investigated DNA damage in relation to radionuclides in the environment (Meyers-Schöne *et al.*, 1993; Sugg *et al.*, 1995; Theodorakis and Shugart, 1997). Studies that examine genetic damage in natural situations provide useful information about the potential biological hazards associated with such toxic agents and the amelioration or exacerbation of effects via ecological pathways (Sugg *et al.*, 1996). It is widely accepted that marine mussels represent one of the most useful biological indicators of marine pollution. As mentioned earlier they are particularly suitable for investigating the biological impact of pollutants, as they are sessile, intertidal, euryhaline organisms with a wide geographical distribution along the coasts and estuaries at different latitudes. In addition due to their filter feeding habit, they are able to accumulate a wide range of chemical compounds, from the surrounding seawater, in their tissues (Viarengo and Canesi, 1991).

The Naval Dockyard at Devonport, Plymouth, was established in the 1690s and has been developed over a number of years and now covers 4 kms of shoreline (Hiscock & Moore, 1986). Although the number of naval vessels at Devonport has declined, their size has increased. With this increase in size, the vessels have a greater draft and hence there maybe greater disturbance of the seabed, and coastal and estuarine areas. During the late 1980s closed sewage systems were developed, which reduced raw discharges from naval vessels into the environment. Nuclear submarines were introduced in the late 1960s. A

Submarine Refit Complex was built between 1970 and 1980 on the north-western corner of the Keyham extension near Weston Mill Lake (Duffy *et al.*, 1994). In February 2002, Devonport Royal Dockyard Limited (DML) was granted a new authorisation which includes an increase to the limits for discharge of tritium to Hamoaze to 700 GBq per year (Environment Agency, 2001). DML is also authorised to discharge liquid radioactive waste to the sewer with a limit of 4 MBq/ m^3. 97.7% of the liquid discharge of radionuclides produced by the dockyard passes through the Camels Head sewage treatment works then onto the Ernesettle treatment plant where it is converted into treated sewage sludge or "cake" which is finally spread onto agricultural land (Environment Agency, 2001). Only 2.3% of the liquid waste passes through the Dockyards sewage system to be released into the Tamar (Environment Agency, 2001). Water quality is hoped to improve around Plymouth's coastal area with the development of a new sewage treatment works. Part of the scheme involved the construction of a tunnel from Devonport, via Millbay and the city centre to Cattedown treatment works. The discharge of crude sewage from the 23 outfalls will be halted and the treated waste water will be discharged through the existing West Hoe outfall where the sewage undergoes full preliminary, primary and biological secondary treatment and disinfection using ultraviolet light (Hiscock & Moore, 1986).

Bearing in mind the historic and current shipping activities, including the discharge of radioactive substances and domestic waste (which may contain genotoxic agents) in the estuary, and in light of the proposed increase in radioactive discharge, a field survey was carried out to assess the impact of radioactive discharge on adult *M.edulis*, in this area. The sites used for the deployment of the mussels were situated on the East and West banks of the Tamar estuary (figure 8.2.), with the main potential pollution sources being the city of Plymouth and the Royal Naval base of Devonport (Lindsay and Bell, 1997). The Tamar estuary (OS grid ref. SX 245000, 55000) is situated in Southwest England and meets the sea in Plymouth Sound. The Tamar is the largest estuary that empties into Plymouth

Sound. It is tidal for approximately 30 kms and the rivers Lynher and Tavy flow into it (Hiscock and Moore, 1986).

Using a suite of biomarkers the aim of the present study was to investigate the potential effects of radiation on the health of adult *M.edulis* which were transplanted for 3 months at 8 sites located to the North and South of the Devonport Royal dockyard, along the Tamar estuary. The biomarkers used in this study were (a) the neutral red retention assay to measure the effect on lysosomal stability, (b) the comet assay to detect DNA single strand breaks and (c) the micronucleus assay to detect chromosomal abnormalities or spindle dysfunction. In addition the mutagenic activity of the water and sediment samples were evaluated using the Ames *Salmonella* mutagenicity test. In addition sediment and water samples were collected and analysed for heavy metal and radionuclide concentration in order to correlate the observed biological effects with the environmental chemistry obtained for each site.

8.2 Material and methods

8.2.1 Selection of sites

Figure 8.1., is a map of the Tamar estuary, UK, and figure 8.2. shows the 8 study sites at which the mussels were deployed (transplanted) and from where water and sediment samples were also collected.

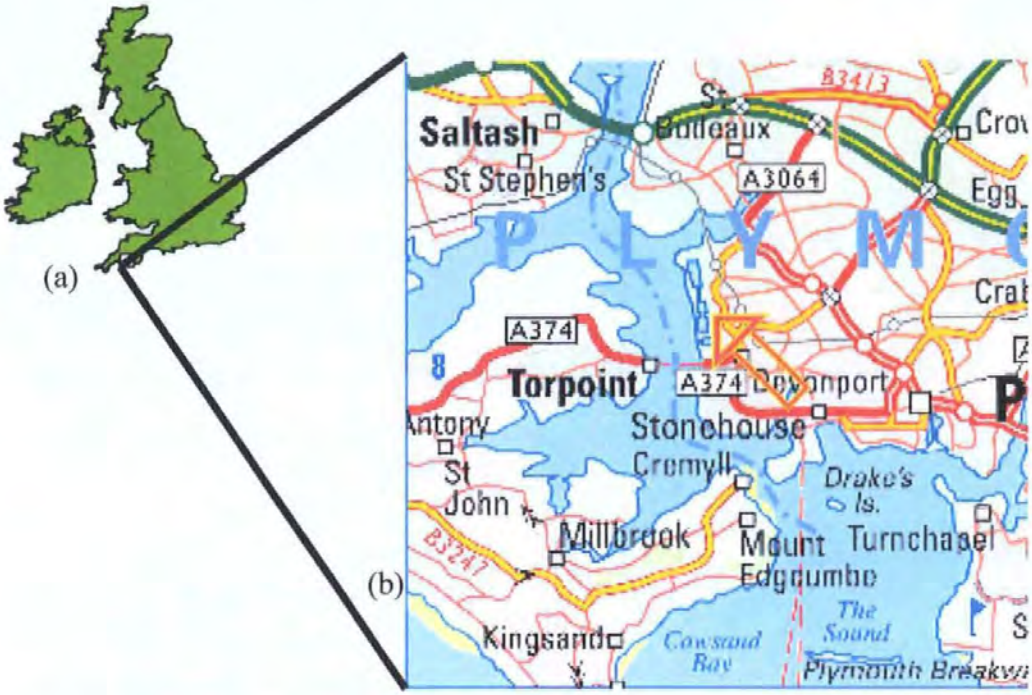


Figure 8.1. (a) Map of the United Kingdom. (b) city of Plymouth and the river Tamar (10km x 10km) (OS grid reference 245000, 55000). Devonport Royal Dockyard and their sewage discharge pipe are located as the blue structures on the map indicated by the orange arrow.

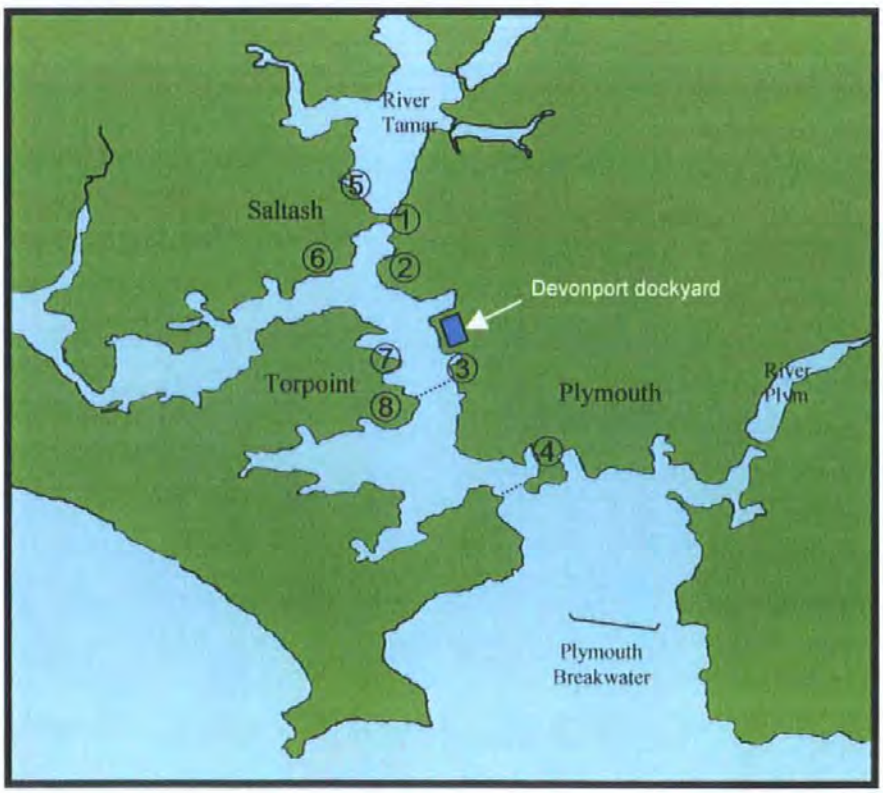


Figure 8.2. Map indicating sites sampled for water and sediments and mussels deployed. Devonport dockyard indicated in blue. (Scale 7.35 x 8.5 miles) (----- indicates ferry crossings).

The study or sample sites chosen are regularly (4 times a year) monitored by DML for radionuclides in the sediment and water and well as in seaweed and mussels (not required at all sites). As indicated in figure 8.2. sites 1-4 were all located on the East (Devon) side of the Tamar whilst sites 5-8 were situated on the West (Cornwall) side of the estuary. Site 1 (OS grid reference: 243715E, 58755N) was situated underneath the Tamar bridge. Site 2 (OS grid reference: 243713E, 58538N) was at the far end of the same shoreline as site 1. Site 3 (OS grid reference: 244970E, 55302N) was situated at a beach in the Stonehouse area close to the jetty where the Cremyll ferry docks. The Devonport Royal Dockyard (DML) is situated between sites 2 and 3 and hence is the area of discharge of radioactive waste. Site 4 (OS grid reference: 246245E, 53855N) was situated near the Torpoint ferry, on a slipway off Ferry road. Site 5 was located underneath the Tamar bridge (OS grid reference: 243220E, 58877N). Site 6 was located on Wearde Quay where the River Lynher meets the Tamar (OS grid reference: 242530E, 57660N). Site 6 was at Thanckes Lake situated near the town of Torpoint (OS grid reference: 243563E, 55760N). Finally site 8 was situated near the Torpoint ferry on a beach off Marine Drive (OS grid reference: 243855E, 54670N). In general sites 1, 2, 5 and 6 were all situated upstream of the point of radioactive discharge and sites 3, 4, 7 and 8 were all situated down stream.

8.2.2 Collection and deployment of mussels at study sites

Adult mussels, *M.edulis,* were collected from the tidal zone, Whitsand Bay, Cornwall in October, 2000. Mussels approx. 50mm in length were removed from the rocks and immediately transported to the aquarium where they were placed into 10μM filtered natural seawater at a constant temperature of 15 $\pm$ 1°C. *M.edulis* was fed Liquifry® (Interpret, Surrey, UK) twice a week (see section 2.1.1 for details of Liquifry®). The mussels were left for 2 weeks in controlled conditions for the mussels to depurate (release any toxicants e.g. heavy metals). Haemolymph from mussels that had been acclimatised in

the aquarium for a period of two weeks at 15°C (fed twice a week) were used as controls in the neutral red, comet assay and micronucleus test. After a period of depuration in laboratory conditions the mussels were placed into nylon mesh sacks (mesh holes measuring approximately 0.5cm^2) (figure 8.3.). Mussels were then deployed to all sites, at low tide, over two consecutive days during October 2000.

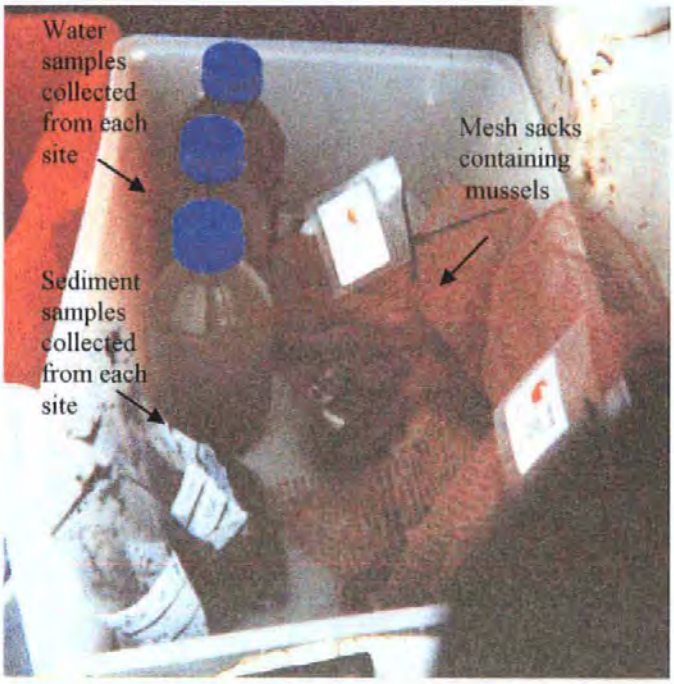


Figure 8.3. A box containing sediment, water and mussels samples collected from the study sites.

A large stone was placed into each sack to prevent them being washed away and also to provide a substrate for attachment of the mussels in order to prevent high stress levels due to tidal actions. Each sack was fastened securely with plastic cable ties and labelled with waterproof material (figure 8.3.). Two sacks per site (20 *M.edulis* in total) were deployed at each site. The sacks were attached to various objects (ladders, jetties, etc.) as near to the low tide line as possible so that they would be emerged in water at high tide.

After 3 months the sacks of *M.edulis* were collected over two consecutive days at the beginning of January 2001. The sacks containing the *M.edulis* were placed into cool boxes

(to avoid stress due to increased temperature) and directly returned to the laboratory where blood from all the mussels was collected immediately. No *M.edulis* were alive at site 8 due to the sacks being covered in silt. Only 1 sack of mussels was found at site 6 and this contained only 4 *M.edulis* (probably due to predation). Native *M.edulis* were therefore collected at site 6 in order to compare native and deployed mussels from this site.

8.2.3 Collection and storage of sediment and water samples

Water samples were collected from each site in 1L acid washed bottles and taken back to the laboratory were they were processed within a week for heavy metal analysis (see section 2.3.7 for details). Surface (5 - 10cm depth) sediment samples were collected from each site and placed into polythene bags after which they were taken back to the laboratory and processed within a week for heavy metal analysis (see section 2.3.7 for details). Water samples were filtered (0.45μm) and preserved in the dark at 4°C until they were used in the Ames test, within a week of collection (see section 2.5 for methods).

8.2.4 Detection of total and bio-available metals

The sediment was processed for analysis of heavy metals as described in sections 2.6.1 and 2.6.2. The water samples were processed for the detection of metals as described in section 2.6.3

8.2.5 Detection of radioactivity in sediment, water and biota

Levels of radioactivity were measured in sediment and native mussels (between 50 - 100 per site) from two of the sites (1 and 6) by Dstl Radiological Protection Services (DRPS) laboratories employed by Devonport Royal Dockyard Ltd. (DML). Tritium concentrations in seawater samples collected from each site were measured as described in section 2.7.1.

8.2.6 Analysis of *M.edulis*

As previously mentioned following 3 months of *in situ* exposure the mussels were collected and immediately transported to the laboratory where haemolymph was collected as described in section 2.4.1. Figure 8.4. describes the use of haemolymph samples for the different biomarkers studied. 10 mussels per sites were used, 5 from each replicate sack (when available). The haemolymph was then analysed for viability using Eosin Y (see section 2.4.2), lysosomal stability using the neutral red assay (see section 2.4.3), single strand DNA breaks using the comet assay (see section 2.4.5) and chromosomal and genomic damage using the micronucleus test (see section 2.4.4).

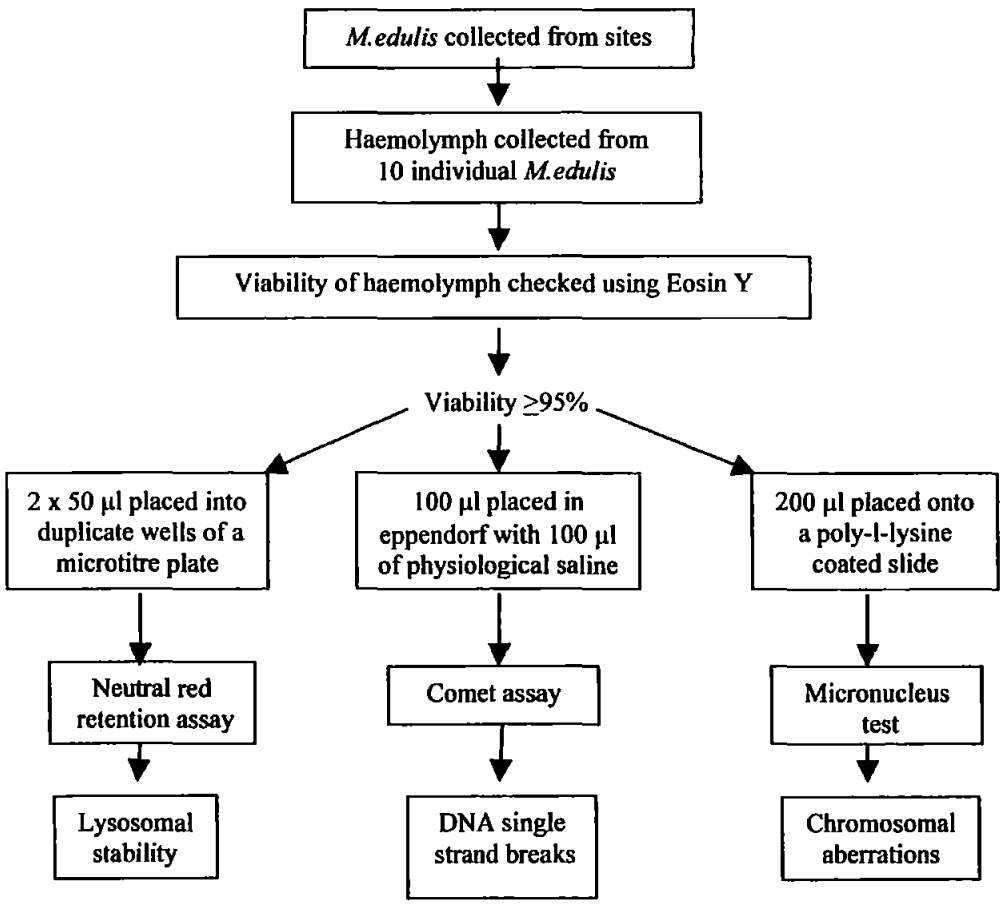


Figure 8.4. Schematic diagram illustrating the use of haemolymph collected from *M.edulis* for the study of different "biomarkers".

8.3 Results

Tables 8.1. - 8.5. represent the heavy metal and radionuclide contents in sediment and water samples collected in October 2000 at different study sites. Table 8.6. provides water quality parameters for these sites.

8.3.1 Heavy metal analysis

Table 8.1. represents the concentration of biologically available heavy metals present at 8 sites along the Tamar estuary. Biologically available heavy metals represents the form or speciation of that metal which allows it to be biologically available for uptake by biota.

Table 8.1. The concentration of biologically available heavy metals in sediment samples collected from the different sites in the Tamar estuary ($\mu g\ g^{-1}$). (ND = not detectable). DL = detection limit.

Sites	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Sn
1	11.95	0.375	0.115	21.11	258	68.25	1.523	35.98	10.9
2	16.3	0.475	1.608	18.27	173.35	61.75	0.948	38.32	19.78
3	11.72	0.525	0.68	14.25	120.35	110	1.588	52.25	12.85
4	5.7	0.375	ND	29.55	37.23	37.25	1.375	23.92	22.6
5	17.18	0.25	ND	10.99	65.43	124	1.213	94.15	25.4
6	31.75	0.525	ND	19.37	308.83	33.75	1.168	51.85	94.98
7	41.95	0.45	ND	0.66	127.7	31.13	0.948	31.95	52.93
8	50.25	0.45	ND	29.9	228.48	37.05	1.925	110.93	60.48
DL	0.11	0.0007	0.005	0.002	0.006	0.002	0.005	0.015	0.031

There was little variation between the levels of arsenic (As) on the East side of the Tamar but on the West side there was a clear trend of increase from sites 5 - 8 for this metal. Chromium (Cr) was present in large amounts at sites adjacent to the dockyard on the East of the estuary (sites 2 and 3). The levels of copper (Cu) were highest at sites 4 and 8, which are mainly influenced by the city and water from Plymouth Sound. There was a site

dependent decrease in the levels of iron (Fe) from sites 1 - 4 on the East side of the estuary however the concentrations were also high at sites 6 and 8. There was a general decrease in the concentration of lead (Pb) from sites 5-7 however at site 8 the levels increased dramatically. The concentration of tin (Sn) was very uniform between sites 1-5 however at sites 6,7 and 8 a 2-5-fold increase was found.

Table 8.2. represents the total concentrations ($\mu g\ g^{-1}$) of heavy metals in sediments from the Tamar estuary, these concentrations reflect the total amount of heavy metals present irrespective of if they are biologically available to organisms.

Table 8.2. The total concentration of heavy metals in sediments from the Tamar estuary ($\mu g\ g^{-1}$), (ND = not detectable), DL = detection limit.

Sites	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Sn
1	87.95	0.8	12.87	122.1	472.5	330	17.88	114	193.15
2	104.85	0.8	13.32	186.7	12290	348	22.94	246.5	189.7
3	90.65	1.95	11.6	162.5	12215	704.5	22.43	214.5	26.2
4	77.2	2.65	ND	2.5	7.87	164	7.30	12.3	37.6
5	116.95	1.6	46.95	10.5	45880	1205	37.3	973.5	130.45
6	199	1.1	12.32	131.0	18320	222.5	28.2	115.5	106.25
7	205.7	1	7.25	79.4	12085	226	19.55	164.8	141.45
8	204.9	1.5	17.57	7.5	21075	386	30.38	502.5	214.65
DL	0.11	0.0007	0.005	0.002	0.006	0.002	0.005	0.015	0.031

The concentration of arsenic (As) was very uniformed between sites 1 - 5 however at sites 6, 7 and 8 there was a 2-fold increase, which was similar to the trend observed for concentrations of biologically available tin (Sn). There was a gradual increase in the concentration of cadmium (Cd) between site 1 - 4. Unlike the biologically available concentrations of chromium (Cr), however, total concentrations of chromium were present at all sites (except 4) but the levels were very similar except at site 5 where the

concentration increased by 4-5-fold. A large concentration of iron (Fe) was present at site 5 and the concentration tended to decrease from sites 6-8. Elevated levels of tin (Sn) were found at sites 1 and 2, although it decreased at sites 3 and 4. Sites 5 - 8 show a gradual increase with site 8 containing the highest concentration for this metal.

In general, the trends (per site) for biologically available concentrations of arsenic (As), manganese (Mn), nickel (Ni) and lead (Pb) followed the same trends per site as the total concentrations of heavy metals in the sediment. Whereas for all other metals there was no general relationship between the total concentration and that which was biologically available.

Table 8.3. The total concentration of heavy metals in water samples collected from the Tamar estuary ($\mu g\ l^{-1}$). Environmental Quality Standard (EQS) of heavy metals in estuarine and marine water obtained from Environment Agency (Council of European Communities, 1976) (ND = not detectable). DL = detection limits.

Sites	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Sn
1	81.4	1.825	ND	1.115	4.78	0.7	4.59	6.65	28.22
2	99.98	1.675	ND	0.87	4.28	0.725	4.53	5.075	23.3
3	103.4	0.25	ND	0.35	0.79	0.125	1.52	1.025	17.88
4	95.48	1.525	ND	0.805	0.431	0.425	4.67	5.575	26.48
5	ND	2.45	ND	1.035	5.75	0.725	5.55	7.4	33.23
6	ND	2.45	ND	1.028	5.85	0.65	3.77	7.7	42.28
7	ND	1.35	ND	0.705	3.34	0.475	3.27	5.125	49.08
8	ND	0.65	ND	0.495	2.12	0.425	1.96	3.1	33.08
DL	0.11	0.0007	0.005	0.002	0.006	0.002	0.005	0.015	0.031
EQS	*25*	*2.5*	*5*	*5*	*1000*	*30*	*15*	*10*	*10*

There was no difference for the concentration of arsenic in water samples from the East side of the Tamar Estuary however no arsenic could be detected in water samples from the West side (table 8.3.). This suggests that the movement of arsenic in water is

limited and that the potential source of arsenic is situated on the Plymouth side of the estuary. The highest concentrations of cadmium were found in water from sites 5 and 6 and the concentrations then decreased down the estuary from sites 7 to 8. Copper (Cu) showed a clear decrease in concentration in water. The highest concentrations were found at the top of the estuary (sites 1 and 5) and the concentrations tended to decrease down the estuary from site 2 - 3 and sites 6 - 8. Iron (Fe) also showed a similar trend to copper with sites up the estuary from the dockyard (sites 1, 2, 5 and 6) containing large concentrations of iron compared to those down the estuary containing significantly less. Manganese (Mn), nickel (Ni) and lead (Pb) showed similar trends with a gradual decrease in concentrations from sites 5-8 and a large decrease in concentrations at site 3. The concentrations of tin (Sn) in water samples showed elevated concentrations on the east side of the estuary compared to the west and an increase in concentration from sites 5 - 7. There appeared to be a relationship, for the 8 sites, between the concentration of tin in water samples and the total concentration of tin in sediment samples. Every listed dangerous substance has a concentration limit called an Environmental Quality Standard (EQS). The EQS is usually an upper concentration that must not be exceeded in any controlled watercourse in England and Wales, although it may vary for fresh, estuarine or coastal waters. The substance (e.g. heavy metal) is not believed to be detrimental to aquatic life at any concentration below its EQS limit. From table 8.3. arsenic concentrations at sites 1-4 exceed the EQS value by approximately 4-fold. Furthermore the concentrations of tin (Sn) in all sites of the Tamar estuary exceed the EQS value of 10$\mu g\ l^{-1}$ by approximately 2-5-fold.

8.3.2 Radionuclide analysis

Table 8.4. shows the data obtained by DML staff. There was no apparent trend either for a site dependent increase or decrease of radionuclide concentration. Although in general, site 4 appeared to have significantly less radionuclides than the other sites. Table

8.5. represents data obtained by DML, which shows the amount of organically bound tritium (OBT), and carbon-14 present in the tissues of mussels collected from sites 1 and 6.

Table 8.4. Radioactivity concentration (Bq/kg)(dry weight) in sediment. Data obtained from DML. (ND = not detected). (Uncertainties of 2 standard deviations based on counting statistics shown in brackets). (October 2000).

Site	^{7}Be	^{40}K	^{60}Co	^{137}Cs	^{208}Tl	^{212}Pb	^{214}Pb	^{214}Bi	^{226}Ra	^{228}Ac
1	ND	1040 (90)	ND	3(1)	15(2)	40(4)	20(3)	20(3)	20(3)	40(8)
2	ND	1040 (90)	ND	3(1)	15(2)	40(4)	20(3)	20(3)	20(3)	40(8)
4	ND	620 (60)	ND	3(1)	9(2)	25(3)	20(3)	15(3)	15(3)	30(7)
6	ND	800 (70)	ND	2(1)	10(2)	25(3)	20(2)	20(4)	20(4)	30(7)
8	ND	770 (70)	ND	ND	ND	30(3)	15(3)	ND	ND	ND

Table 8.5. Concentration of tritium and carbon-14 in *M.edulis* tissue (Bq/kg) (wet weight). Data provided by DML. (Uncertainties of 2 standard deviations based on counting statistics shown in brackets). (October 2000).

Site	Tritium (OBT)	Carbon-14
1	1400 (470)	590 (240)
6	1380 (380)	480 (210)

N.B. The results acquired during October 2000 were obtained during the development of analysis techniques for OBT and carbon-14. Results since October 2000 have been significantly lower, typically limits of detection.

Table 8.6. Concentration of tritium (Bq/ml) in water samples taken from the different sample sites (5 replicates).

	1	2	3	4	5	6	7	8	Aquarium water sample
Conc. Bq/ml	0.058 ± 0.008	0.056 ± 0.009	0.058 ± 0.007	0.058 ± 0.005	0.059 ± 0.004	0.056 ± 0.006	0.059 ± 0.005	0.053 ± 0.005	0.062 ± 0.010

There was no variation between the concentration of tritium detected in water samples amongst the sites and that of a control sample of seawater obtained from the aquarium (see table 8.6.).

8.3.3 Standard water quality parameters

Table 8.7. represents the water parameters measured at each site. The pH, temperature and salinity of the water samples all showed a gradient dependent increase in direction from sites 1 to 4 and 5 to 8. The sites at the top end of the estuary received a greater input of freshwater and therefore the salinity, pH and temperature were lower compared to sites at the mouth of the estuary that had more input from the sea.

Table 8.7. Environmental water standards analysed at each site (once per site in October 2000).

Site	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
pH	7.41	7.35	7.43	7.49	7.41	7.48	7.54	7.56
Temperature (°C)	12.1	11.9	12.2	12.3	11.8	12.0	12.0	12.2
Salinity (‰)	15	15	20	25	14	22	14	27

8.3.4 Ames *Salmonella* mutagenicity test

The Ames *Salmonella* mutagenicity test using seawater samples from the 8 sites suggested that there was no statistical significant difference between any of the sites and the controls after incubation with strain TA1537 of *Salmonella typhimurium* (P=0.4598) (figure not shown). This indicated that no frame-shift mutations had been induced. There was however a significant difference (ANOVA) between samples after exposure to strain TA1535 of *Salmonella typhimurium* (P=0.00005). Figure 8.5. represents the number of reversions induced by seawater from various sites in the Tamar estuary after exposure to the *Salmonella* strain TA1535.

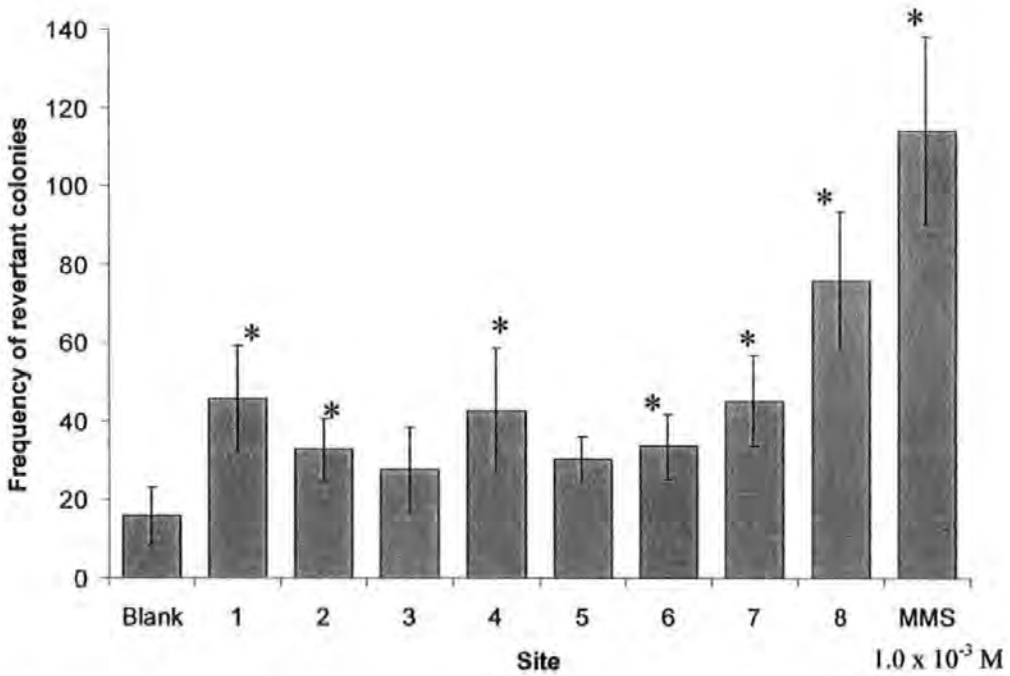


Figure 8.5. Evaluation of mutagenic potential of water samples from the Tamar estuary as determined by the Ames test, *Salmonella* strain TA1535. (* = significantly different from control using ANOVA, $p<0.05$) (Error bars represent 2 x standard error between 10 replicate plates).

Using *Salmonella* strain TA1535 there were significantly higher numbers of wildtype revertants in sites 1, 2, 4, 6, 7 and 8 indicating that mutagen activity was present in the water that was capable of inducing base pair substitutions. Sites 1, 2, 4, 6, 7 and 8 all

indicated mutagen activity statistically higher than the levels in the controls (10μm filtered seawater obtained from the aquarium supply). There appeared to be a increasing trend between sites 5 - 8, with site 5 containing no elevated levels of mutagenic activity and sites 6, 7 and 8 showed an increase in levels of mutagenic activity as the sample sites move seaward (downstream of the dockyard).

8.3.5 Analysis of haemocytes from deployed *M.edulis*

No *M.edulis* were alive at site 8 due to the sacks being covered in silt. Only 1 sack of mussels was found at site 6 and this contained only 4 *M.edulis* (probably due to predation). Native *M.edulis* were therefore collected at site 6 in order to compare native and deployed mussels from this site.

8.3.5.1 Cell viability

Cell viability was analysed using Eosin Y, immediately after haemolymph samples were extracted from *M.edulis* as described in section 2.3.2. Percentage viability ranged between 97.2-99.4% and thus all samples analysed were deemed acceptable for use in further tests.

8.3.5.2 Neutral red

Figure 8.6. represents the results of the neutral red retention assay from *M.edulis* deployed in the Tamar estuary. There was a statistically significant difference between samples (ANOVA, P=0.0003), with deployed *M.edulis* from sites 1 and native *M.edulis* from site 6 containing significantly less neutral red/mg of protein than the other sites.

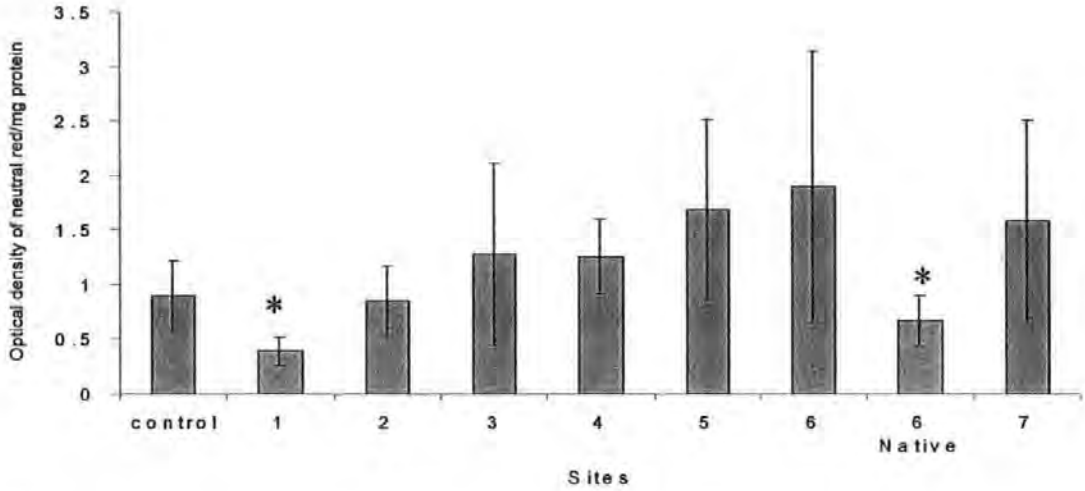


Figure 8.6. The neutral red retained by haemocytes of *M.edulis* deployed at different sites of the Tamar estuary. (* = significantly different from control using ANOVA, $p<0.05$). Error bars represent 2 x standard error between 10 mussels (except site 6 where only 4 mussels were obtained).

8.3.5.3 Comet assay

Figure 8.7. represents the results of comet assay from mussels deployed at different sites along the Tamar estuary.

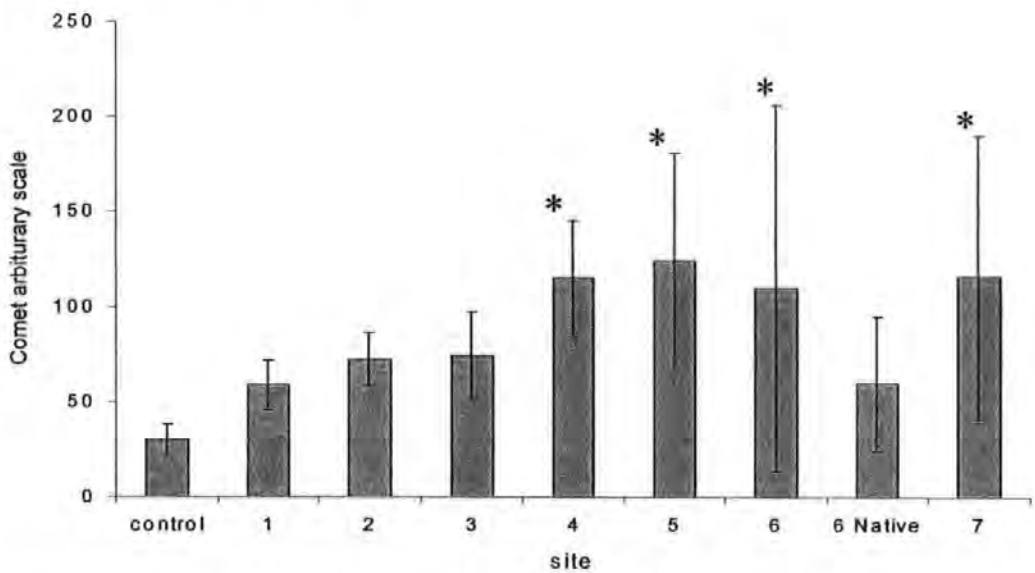


Figure 8.7. Comet assay in haemocytes of *M.edulis* following transplantation for 3 months in the Tamar estuary (* = significant difference from control using ANOVA, $p<0.05$) Error bars represent 2 x standard error (100 cells per 10 individuals per site (except site 6 where only 4 mussels were obtained).

There was a statistically significant difference in the incidence of DNA strand breaks between the sites when compared to the controls (P=0.00057). There was a significant elevation in the degree of damage in *M.edulis* deployed at sites 4, 5, 6 and 7. Therefore there appears to be an increase in agents that cause single strand DNA breaks on the west side of the Tamar estuary, this might be due to flow dynamics of the estuary or differences in sediment characteristics that results in an alteration in the retention capability of genotoxic material.

8.3.5.4 Micronucleus assay

Figure 8.8. shows the results of micronucleus assay from mussels deployed in the Tamar estuary.

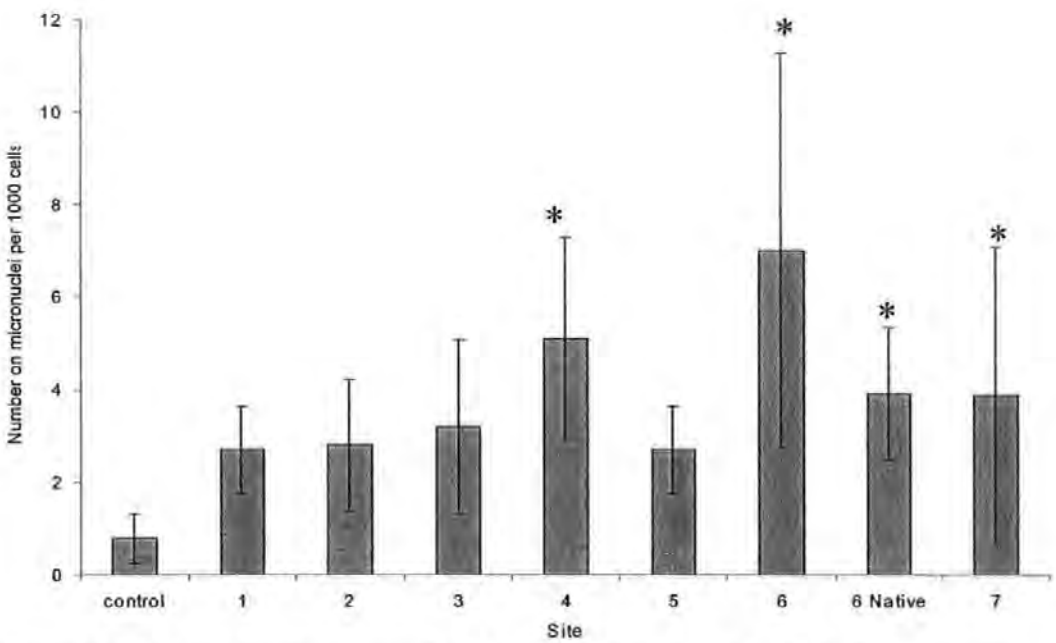


Figure 8.8. Induction of micronuclei in haemocytes from *M.edulis* deployed at different sites along the Tamar estuary for 3 months (* = significant difference from control using ANOVA, $p<0.05$). Error bars represent 2 x standard error between 10 mussels (except site 6 where only 4 mussels were obtained).

There was a statistically significant difference for the incidence of micronuclei between the sites (ANOVA, P=0.016). A significant elevation was observed in the degree of damage in *M.edulis* deployed at sites 4, 6 and 7 and in the native *M.edulis* from site 6. There appeared to be a general decrease in the induction of micronuclei in *M.edulis* between sites 6 to 7, with the incidence of micronuclei decreasing down stream.

8.3.6 Correlation between biological parameters and metal and radionuclide concentration

Correlation between biological parameters (i.e. frequency of revertants in the Ames *Salmonella* mutagenicity test, neutral red retention, comet and micronucleus assays) and environmental variables was carried out using multivariate analyses, using PRIMER 5 (Plymouth Routines in Multivariate Ecological Research) a suite of computer programmes developed at the Plymouth Marine Laboratory, UK (Clarke and Warwick, 1994).

Cluster analyses finds a natural grouping of samples such that those samples within a group are more similar to each other, generally, than samples in different groups. Hierarchical methods of clustering allow samples to be grouped and the groups themselves form clusters at lower levels of similarity. Similarity matrices were constructed using "Bray-Curtis" similarity measure, a data transformation that reduces the contributions to similarity by elevated levels of biological results and therefore increases the importance of the lower values of biological endpoints. The results of the hierarchical clustering are represented by a dendrogram (nearest neighbour method) (figure 8.9.), this shows the similarity of the 7 sites (not including site 8 for which no mussels were collected) in terms of the mutagenic activity in water samples and cytotoxic stress (NRR), single strand breaks (comet) and micronuclei (Mn) induction.

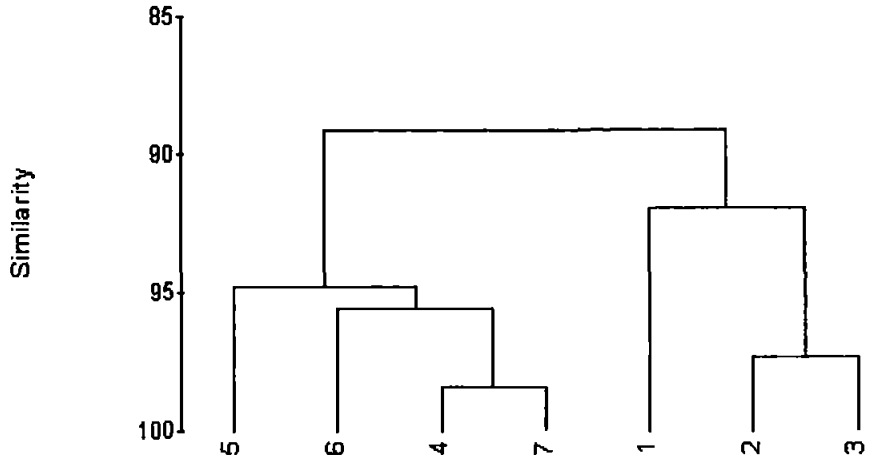


Figure 8.9. Dendrogram representing the similarity between the sites on the Tamar estuary as analysed using biological data.

There appears to be two distinct groups, with sites 1, 2 and 3 being in one group and also on the East side of the estuary and sites 4, 5, 6 and 7 are grouped in the second group and being on the West side of the Tamar (except site 4). Sites 4 and 7 are most similar to each other with an almost 99% similarity co-efficient. Figure 8.10. represents a multi-dimensional scaling (MDS) ordination of samples. It is a technique that "maps" the samples, in which the placement of the samples reflects the similarity of the biological parameters (Clarke and Warwick, 1994). The nearer the points representing the sites the more similar they are in the level of biological effects.

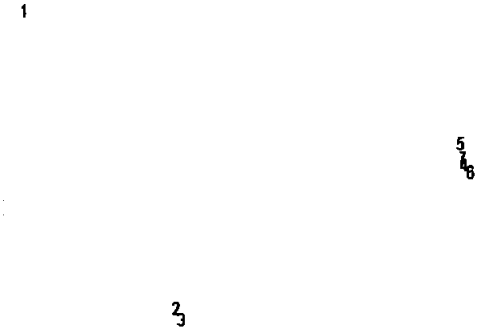


Figure 8.10. A MDS ordination of sites on the Tamar estuary.

From figure 8.10. site 1 appears to be unlike any of the other sites, this might be due to the fact that it was the only site where deployed M.*edulis* exhibited a lower neutral red optical density than the controls. Sites 2 and 3 appear to produce similar effects in the deployed mussels and sites 4, 5, 6 and 7 also appear to produce very similar trends.

The relationship between the multivariate community structure of the biological parameters and environmental variables were examined using a BIOENV procedure. This calculates rank correlations between a similarity matrix derived from site data and matrices derived from various subsets of environmental variables, thereby defining combinations of variables that "best explain" the biological effects observed (Somerfield *et al.*, 1994). Table 8.8. represents a summary of the correlation between environmental data and the biological effects observed.

Table 8.8. Results of the BIOENV showing which environmental variables best explains the trends in biological parameters.

	Environmental variables	**R value**
Ames test	Cd (water)	0.408
	Fe (water)	0.353
	Pb (water)	0.346
Neutral red assay	As (water)	0.220
	Sn (total in sediment)	0.212
Comet assay	As (water)	0.286
	Cu (total in sediment)	0.175
Micronuclei test	Salinity	0.511
	Sn (available in sediment)	0.468
	As (available in sediment)	0.376
All parameters combined	As (water)	0.238
	Mn (available in sediment)	0.214

The environmental factor that best explained the degree of mutagenic activity in the water from the 8 sites was cadmium (Cd) with a relationship co-efficient of R^2=40.8%. The degree of immunological damage in deployed *M.edulis* from the 7 sites as assessed using the neutral red assay was most explained by the amount of arsenic in the water (R^2=22.0%) as was the induction of single strand breaks as detected using the comet assay (R^2=28.6%). Variations in salinity of the water was the variable that best described the induction of micronuclei followed by a strong relationship between the occurrence of available tin in the sediment (R^2=51.1% and 46.8% respectively). Although overall the concentration of arsenic in the water samples best explains the biological effects observed using a combination of all 4 endpoints.

8.3.7 Summary of sample sites for observed biological effects

At site 1, there was a slight elevation in the mutagenic activity as detected by the Ames test. However no indication of genetic damage in the deployed *M.edulis* was detected from this site and there was even a reduction in the optical density of neutral red per mg of protein indicating that the lysosomes of the mussel blood cells were significantly less healthy than that of the controls. A slight increase in mutagenic activity was detected at site 2 although this did not effect immune or genetic endpoints in the deployed mussels. No mutagenic activity was observed at site 3 as assessed using the Ames test and no cytotoxic or genotoxic effects were noted in the deployed *M.edulis*. Site 4 showed elevated levels of mutagenic activity and this was reflected as an increase in single strand breaks and micronuclei in the deployed *M.edulis*, although there was no damage to the lysosomes. No recorded increase in mutagenic activity was detected as measured using the Ames test at site 5 but there was an increase in the frequency of single strand breaks in the deployed mussels at this site but there was no increase in the occurrence of micronuclei. At site 6 the mutagenic activity, as detected using the Ames test, was significantly increased. Deployed *M.edulis* at site 6 showed an increase in the elevation of the single strand breaks and

micronuclei and a elevated optical density of neutral red, although this was not statistically higher than the controls. The native *M.edulis* at site 6 showed a decrease in the optical density of neutral red indicating that they were significantly more stressed and that there immune system was unhealthy than the controls. Furthermore, the native *M.edulis* did not experience an increase in the incidence of single strand breaks which might reflect the overall health of the organisms which will aid in the repair of the DNA damage. Although the native *M.edulis* did exhibit an elevation in the frequency of micronuclei which reflects the mutagenic activity of the site. The water sampled from site 7 showed an elevated level of mutagenic activity as detected by the Ames test. *M.edulis* deployed at this site exhibited an elevated frequency of micronuclei, however there were no increase in the incidence of single strand breaks or damage to the lysosomes. Site 8 showed the highest levels of mutagenic activity, as detected by the Ames test, with approximately a 4-fold increase in the number of reversions produced. There were no *M.edulis* alive at this site due to increased coverage by sediments although the mortality might also be due to the high levels of mutagenic activity.

8.4 Discussion

There was no correlation between the Ames test, the neutral red and comet assays and the incidence of radionuclides. However, there was a weak correlation between Pb-214, Bi-214 and Ra-226 with the induction of micronuclei (R^2=22.3, 18.9 and 18.9% respectively). Although it is important to mention that in laboratory studies with rainbow trout (*Oncorhynchus mykiss*) it was reported that heavy metals such as cadmium and zinc, even at relatively low concentrations reduced the accumulation of radionuclides (^{134}Cs, ^{110m}Ag and ^{57}Co) by the fish (Ausseil *et al.*, 2002). In additional, following field studies in the Lot River (France) they found that caged fish exposed to metals (1.5 µg Cd/L and 152 µg Zn/L) had much lower bioaccumulation of radionuclides than those fish caged at a reference site (<0.05µg Cd/L and 68 µg Zn/L). One possible explanation as to the

reduction in bioaccumulation of the radionuclides was the reduction of respiratory gill activity in the fish due to heavy metal toxicity. The reduction in respiratory rate would thus result in a diminished volume of water being filtered by the gills which in turn would result in a reduction in the quantity of radionuclides coming in contact with the gills. In addition they also speculated that cadmium and zinc might modify the permeability of the gill epithelium to the radionuclides. However during the present study the levels of radioactivity detected in sediment, water and *M.edulis* tissue from the Tamar estuary was extremely low and furthermore, it is difficult to comment on the effects of radionuclides as determined by concentration as there are no Environmental Quality Standards (EQS) for radionuclides. The Environment Agency is responsible for determining the quantity of radionuclide that an establishment may release and this is determined by the area of discharge, discharge rates, presence of other radionuclides and possible exposure routes to humans (Environment Agency, 2001). Following consideration of many factors the dose rate of exposure is determined and this limits the concentration of radionuclides released.

During the present study the biggest influence was observed for the induction of micronuclei due to the presence of heavy metals, in particular arsenic and tin (R value >0.468). In addition, when present, the concentrations of both of these heavy metals exceeded their respective recommended EQS values. Although it is worth noting that during the present study no attempt was made to investigate the concentrations of potential sources of persistent organic pollutants (POPs). These are toxic substances composed of organic (carbon-based) chemical compounds and mixtures and they include industrial chemicals like PCBs and pesticides like DDT. Therefore the occurrence of POPs might be responsible, either individually or due to interaction with other compounds, for some of the biological results observed.

Arsenic concentration in water samples from the Tamar estuary was the environmental factor that best explained the effect on the optical density of neutral red, the induction of single strand breaks and was generally responsible for the combined trends

observed for all of the biological parameters. Although no work has been carried out on the effects of arsenic on cells analysed by the neutral red retention assay, arsenic has been reported to induce damage to cell membranes. Arsenic exposure has been shown to induced changes in the function of cell membranes from cultured brain cells (Chattopadhyay *et al.*, 2002). Furthermore, cell membrane damage was induced in the keratinocyte derived cell line, SCL II, after exposure to Lewisite a highly toxic arsenic compound (Kehe *et al.*, 2001). In addition, arsenic has been shown to induce cellular responses in species of *Mytilus*. Sodium arsenite was shown to have induced p-gp (P-glycoprotein multidrug transporter protein) activity as a general cellular stress response in the gill tissues of the mussel *Mytilus californianus* (Eufemia and Epel, 2000).

The Tamar estuary was the site of half the world's arsenic (As) production approximately a century ago and still contains elevated levels as a result of As-rich sediments together with inputs from mine spoils and adit drainage (Bryan and Langston, 1992). Arsenic and its compounds occur naturally, they are ubiquitous in the environment and exhibit both metallic and non-metallic properties (Chan and Huff, 1997). The most common oxidation states of arsenic are the trivalent (+3) and pentavalent (+5) forms. However no attempts were made during the present study to identify the different forms of As and other metals. Trace metals in natural waters exist in several different forms (either as different oxidation states or forms complexed or bound to inorganic and organic matter). The distribution between these forms is often referred to as the metal's "speciation", in addition it has been shown that a metal's toxicity is related to its speciation with some forms of the metal being more bioavailable and toxic.

Arsenic compounds are found in agricultural products (pesticides), livestock products (feed additives, cattle and sheep dips), electronics (solar cells, digital watches) and industrial products (glassware, antifouling paints, dyes, soaps, ceramics etc.) (Chan and Huff, 1997). Oceanic As concentrations are usually between 1 and 2 $\mu g\ l^{-1}$ and similar concentrations may be found in estuaries but these may increase to around 100 $\mu g\ l^{-1}$ or

higher as a result of mine wastes and inputs from industrial outfalls (Langston, 1985). The Environment Quality Standard (EQS) for arsenic is 25 μg/l (Council of the European Communities, 1976). During the present study concentrations of 81.4 - 103.4 μg l^{-1} were detected in water samples from the Tamar, suggesting that elevated inputs of As have been discharged into the area. The Blue Circle Industries, a cement and lime manufacturer located on the River Plym (see map figure 8.2), released <6kg of arsenic into controlled water during 1999. However this discharge was routinely monitored by the Environment Agency and was within the set limits for the company.

The average abundance of As in soils from non-mineralised areas of the UK is 18 μg g^{-1}, while much higher concentrations occur in upland mining areas, particularly near to As-bearing ores (arsenopyrites) in S.W. England (Colbourn *et al.*, 1975). Bryan and Langston (1992) reported concentrations that ranged from 5 μg g^{-1} in the Axe estuary to >1000 μg g^{-1} in estuaries such as Restronguet Creek, Cornwall and a value of 93μg g^{-1} for the Tamar estuary. Steinert *et al.* (1998) produced an Effects Range-Median (ERM) value for arsenic of 70 μg g^{-1}. It was compiled as a guide for comparison of *in situ* biomarker data (comet assay) and a measure of sediment quality. During the present study the concentration of available As in sediment ranged from 5.7-50.3 μg g^{-1} which is below the ERM value reported by Steinert *et al.* (1998). Whereas the total concentration of As in the sediment was between 77.2-205.7 μg g^{-1} which greatly exceeds the ERM value. It is believed that the high As contents in the Tamar sediment are the by-products (arsenic trioxide and arsenic trihydride) of numerous small smelting operations that are evident in the upper Tamar estuary in the Calstock area (Lindsey and Bell, 1997).

The long term consequences of exposure to inorganic forms of arsenic are important because these compounds are recognised as carcinogens especially affecting the lungs, and in some countries, drinking water contaminated through natural sources have been linked to skin cancer (Léonard, 1991). Gonsebatt *et al.* (1997) observed evidence that arsenic was a clastogenic/aneugenic carcinogen as it increased the frequency of chromatid

and isochromatid deletions in lymphocytes and of micronuclei in oral and urinary epithelial cells of humans. During the present study the induction of micronuclei in deployed mussels also showed a strong relationship with the concentration of available arsenic present in sediment from the Tamar (R^2=37.6%). Oysters (*Crassostrea virginica*) living on chromated copper arsenate (CCA)-treated wood in a residential canal contained 2-3 times more arsenic than reference oysters, in addition their gill cells contained twice as many micronuclei as did gill cells from the reference oysters (Weis *et al.*, 1995). In humans, sodium arsenite has been shown to inhibit the cell cycle progression of lymphocytes and to induce chromatid-type aberrations and sister-chromatid exchanges (SCEs) (Jha *et al.*, 1992). In addition it also potentiated the ionising (X-rays) and non-ionising (UV) radiation-induced genetic damage in human cells (Jha *et al.*, 1992). The studies suggested that the sodium arsenite interfered with DNA repair process, presumably by inhibiting the ligase activity. Warner *et al.* (1994) also showed that chronic ingestion of high levels of inorganic arsenic in drinking water was associated with an increase in the frequency of micronucleated bladder cells in humans. Elevated frequencies of micronuclei (Mn) formation have also been found in oral mucosa cells, urothelial cells and peripheral blood lymphocytes from individuals exposed to arsenic through drinking water in West Bengal, India (Basu *et al.*, 2002). Arsenic and cadmium were also shown to induce dose dependent DNA damage in human white blood cells as detected by DNA migration in the comet assay (Hartmann and Speit, 1994).

The acute toxic value (LC_{50}) of arsenic for freshwater fish have ranged from 13.3ppm (mg/L) for rainbow trout to 41.8ppm (mg/L) for bluegill and the green sunfish was relatively resistant with a toxic dose of 150ppm (mg/L) (Léonard, 1991). Acute toxicity values for salt water invertebrates exposed to arsenic have been reported to range from 0.5ppm (copepoda) to 24.7ppm (juvenile white shrimp) (Léonard, 1991). In light of the studies mentioned above the concentration of arsenic in water samples from the Tamar (during the present study) would not be toxic (ranged from 0 – 0.10 ppm).

Typically levels of Sn in the open water may range from 0.003 to 0.008 μg l^{-1} and this can generally increase by a factor of 10^0 - 10^2 in inshore and estuarine waters (Hodge *et al.*, 1979). However localised high levels of dissolved inorganic Sn can occur near industrial discharges. For example, up to 48 μg l^{-1} Sn have been recorded in Poole Harbour (Langston *et al.*, 1987). During the current studies the levels of Sn in the water from the Tamar estuary ranged between 17.8 - 49.1μg l^{-1} (44.5 - 122.75 μg l^{-1} of TBT, if Sn was in the form of TBT). These values are significantly higher than the typical levels (Hodge *et al.*, 1979) found in the open water and are in agreement with previous studies that report localised high levels of Sn due to industrial discharges (Langston *et al.*, 1987). The Environment Quality Standard (EQS) for tin is 10 μg/l (Council of the European Communities, 1976). During the present study concentrations of 17.8 – 49.1 μg l^{-1} were detected in water samples from the Tamar, suggesting that elevated inputs of Sn have been discharged into the area. An environmental quality standard (EQS) of 20 ng l^{-1} TBT was set up in 1985 by the UK for the protection of marine biota. However since this did not offer a sufficient margin of safety to protect the most sensitive species, it was reduced to 2 ng l^{-1} TBT (0.8 ng l^{-1} as Sn) in 1989 (Bryan and Langston, 1992). It has been reported that the survival of sensitive embryo-larvae stages of marine invertebrates can be significantly reduced at concentrations of 3.11 μg l^{-1} or higher of TBT (Jha *et al.*, 2000b). Therefore, if the Sn recorded during the present study was in the form of TBT (44.5 - 122.75 μg l^{-1}), serious implication to the surrounding wildlife would be expected at the concentrations recorded. Inorganic Sn, as cassiterite (SnO^2), is usually the predominant form in sediments of estuaries associated with metal mining in SW England. Concentrations of total Sn exceeding 1000 μg g^{-1} have been reported in metal-rich sediments from Restronguet Creek and the Hayle and Helford estuaries in Cornwall, although outside mineralised regions the Sn concentrations are usually two to three orders of magnitude lower. Concentrations of Sn in sediments from the Tamar ranged from 10.9 - 94.8 μg g^{-1} for available Sn during the present study compared with between 77.2 - 205.7μg g^{-1} as reported by Bryan and

Langston, (1992). As a general rule the bioavailability and toxicity of organotin compounds is dependent on their lipophilic qualities (Laughlin *et al.*, 1989). Sn (II) has been shown to be genotoxic in the SOS chromotest at cytotoxic doses (Lantzsch and Gebel, 1997). Furthermore organic and inorganic tin has been shown to increase the frequency of chromosomal aberrations, sister chromatid exchanges and to reduce cell proliferation in human peripheral blood lymphocytes (Ganguly *et al.*, 1992). Organotin compounds have also been shown to be genotoxic. Tributyltin has been reported to be capable of inducing cytogenetic damage (sister chromatid exchanges and chromosomal aberrations) to early life stages of the marine mussel *Mytilus edulis* and the polychaete worm *Platynereis dumerilii* (Jha *et al.*, 2000b, 2000c; Hagger *et al.*, 2002). Furthermore, tributyltin has been shown to induce programmed cell death or apoptosis in human lymphoblastoid cell lines as well as in the tissues of the marine sponge, *Geodia cydonium* (Batel *et al.*, 1993). As previously mentioned in Jha *et al.* (2000b) several mechanisms may account for the genotoxicity of TBT. Under *in vivo* conditions TBT has been shown to induce nitric oxide production (Kergosien and Rice, 1998) which in turn has been shown to induce DNA damage (Burney *et al.*, 1999).

Heavy metal (cadmium, chromium, copper, mercury, nickel, and zinc) genotoxicity was tested using the Ames test, the *E. coli* WP2 test, the Mutatox $^{(TM)}$ test detecting mutagenicity, and the SOS assay with *E. coli*-detecting enzyme induction (Codina *et al.*, 1995). All the metals tested (cadmium, chromium, copper, mercury, nickel, and zinc) were detected as being genotoxic by the Mutatox $^{(TM)}$ and the SOS tests. However the Ames test and the *E. coli* WP2 assay only detected chromium as a mutagenic metal. Vargas *et al.* (2001) also found that the *Salmonella*/Ames test was not as sensitive when compared to other assays (microscreen phage-induction assay) which were able to detect more mutagenic activity when they investigated the effects of heavy metals in the Sinos River, Brazil. Furthermore the results of the genotoxicity tests correlated with concentrations of heavy metals in the Sinos River water samples and there was a strong relationship between

sites heavily contaminated by anthropogenic sources of metals and the most positive genotoxic results. No arsenic-induced mutagenicity or antimutagenicity has been observed using the Ames assay (Abdullaev *et al.*, 2001).

Mytilus galloprovincialis transplanted to an area polluted with heavy metals (Scarlino, Italy) were shown to exhibit reduced lysosomal membrane stability in their digestive cells compared to those from a clean site (La Spezia, Italy) (Regoli, 1992). Haemocyte lysosomal destabilisation has also been shown to be adversely effected by exposure to Cu at concentrations between 2.5 - 20 $\mu g\ l^{-1}$ in oysters after only 18h and the effect observed was both concentration and time dependent (Ringwood *et al.*, 1998). During the present study the water samples contained copper concentrations that ranged from 0.35 - 1.11 $\mu g\ l^{-1}$. No effects of copper were noted in the neutral red retention assay during the present study. However, in comparison to the study by Ringwood *et al.* (1998) the Tamar deployed mussels were exposed to concentration of copper approximately half that of the lowest concentration 2.5 - 20 $\mu g\ l^{-1}$, that was shown to cause an adverse effect as analysed by NRR assay. Although the mussels during the present study were exposed for 3 months compared with only 18h. The effect on lysosomal stability might be excepted to increase over a longer period of time however the organism may have become tolerant or adapted to the concentrations of copper or other toxicants present. Jacobson *et al.* (1993) also showed that copper affected neutral red retention in juvenile freshwater mussels (*Villosa iris).* Neutral red was found to be effective in assessing post exposure mortality and sublethal response (valve closure) after acute exposure to copper for 24h. An EC_{50} of 29 $\mu g\ l^{-1}$ for *Villosa iris* and an LC_{50} of 83 $\mu g\ l^{-1}$ was determined using the neutral red stain. A higher concentration of copper (100 $\mu g\ l^{-1}$) was found to cause lysosomal membrane dysfunction in the mussel *Perna ciridis* (Bivalvia: Mytilidae) (Nicholson, 1999). However no correlation was found between the concentration of metals and neutral red retention time or condition of neutral red along a pollution gradient in Tolo Harbour, Hong Kong, (Cheung *et al.*, 1998).

Although there was no chromium present in the water samples from the Tamar, it was present in sediments at various sites. A porcelain plant, several textile companies and hide tanning operations in the Plymouth area contribute to the input of Cr to the estuary (Lindsey and Bell, 1997). Merk *et al.* (2000) were able to show that chromate induced DNA-protein crosslinks using the comet assay and that chromate did lead to a small but significant induction of DNA damage. Steinert, *et al.* (1998) have carried out field studies on the effects of heavy metals using the comet assay in deployed and resident *M.edulis*. Sediment chemistry and bioaccumulation data indicated that Hg, Cu and Zn were the most notable contaminants in San Diego Bay, USA. In addition the comet assay was found to respond rapidly to station contaminants. Radiation has also been shown to produce DNA damage as detected using the comet assay although few field studies have proved this link. The comet assay was used to assess DNA damage in Catfish (*Ictalurus punctatus*) from cooling ponds used by the Chernobyl Nuclear Power Plant (CNPP). A greater number of DNA strand breaks were produced in fish that were exposed to radiocesium than fish from control ponds (Sugg *et al.*, 1996). Although, one disadvantage of single strand break measurements is that they are non-specific, repairable and may potentially occur via mechanisms not related to direct genotoxicity (e.g. by activating endonucleases) and also via endogenous reactions (e.g. the action of nitric oxide and reactive oxygen species, (ROS)) (Livingstone *et al.*, 2000).

Micronuclei can be induced by both radiation and heavy metals. Ilyinskikh *et al.* (1998) reported an increase in the frequency of micronucleation in pike (*Esox lucius*) from the Tom River, Russia, which regularly received discharges of radioactive liquid wastes between 1957 - 1993. Furthermore there was a strong correlation between radiocesium concentrations and micronucleated erythrocytes frequencies in the pikes. In comparison, catfish (*Ictalurus punctatus*) from radiocesium containing cooling ponds used by the Chernobyl Nuclear Power Plant (CNPP) did not show elevated levels of micronuclei when compared to fish from controlled ponds (Sugg *et al.*, 1996). Although a study of genetic

damage in largemouth bass from the Savannah river suggested that genetic damage was greater in liver and gill tissues and that blood exhibited the least amount of damage from sites with high radiocesium levels. This reflects a limitation to the use of erythrocytes as they do not divide when in circulation. Bank Voles living in zones of high radiation at Chernobyl, Ukraine, did not contain higher frequencies of micronuclei than voles in control areas (Rodgers and Baker, 2000). The induction of micronuclei and other nuclear abnormalities were detected in the Eastern minnow *Phoxinus phoxinus* and the mollie *Poecilia latipinna* after exposure (injection) to two dose of 1.7mg/kg of cadmium which was within the range found in sediments from the Tamar (0.25-2.65 $\mu g\ g^{-1}$) (Ayllon and Garcia-Vazquez, 2000). Although, Bolognesi *et al.* (1999) found that cadmium (1.84 µg/l for 5 days) did not significantly increase the frequency of micronuclei or single strand breaks in the marine mussel *Mytilus galloprovincialis*. Cadmium compounds are weak mutagens and clastogens, but they do interfere with DNA repair processes and enhance the genotoxicity of direct acting mutagens (Beyersmann and Hechtenberg, 1997). Copper produced a dose related increase in micronuclei frequency in a range of 10-40 $\mu g\ l^{-1}$ and single strand breaks were observed for mussels treated with 40 $\mu g\ l^{-1}$.(Bolognesi *et al.*, 1999). During the present study the concentration of copper in the water samples from the Tamar was an order of magnitude less than those reported by Bolognesi *et al.* (1999) which caused DNA damage in mussels. No correlation was found between the biological parameters examined and copper concentration. For the heavy metals, arsenic and tin available in sediment were most responsible for the induction of micronuclei in haemocytes of the mussel *M.edulis* during the present study. Although the mussel is a filter feeder it will filter particulate matter and thus the biologically available metals in the sediment would be filtered by the organism. Furthermore, as the Tamar estuary is tidal and due to the positioning of the deployed mussels (at low tide) a large amount of the sediment may be mixed on a daily basis which would make the sediments available to the mussel. In addition, the Tamar estuary is often dredged which may increase the uptake of sediments

by filter feeders. During October 2000 and March 2001, dredging operations were taking place by the Ministry of Defence (MoD) to deepen the Tamar channel in order to construct a new Royal Navy docking facility for larger vessels (in particular the Vanguard submarines). The project entitled "Remote Ammunition Facility Tamar (RAFT)" excavated 200,000 cubic metres of silt at Bull Point (Sites 1 and 2) (Management Scheme, 2001) and this may have had a significant influence on the results of the present study. However, according to the result of the present study salinity had the biggest influence on the induction of micronuclei. It has been suggested that hyperosmotic stress (changes in salinity present as NaCl) caused chromosomal aberrations and DNA double-strand breaks in mammalian kidney cells (Kultz and Chakravarty, 2001a; 2001b). Chromosomal aberrations have also been induced under hypotonic culture conditions in V79 Chinese hamster cells (Nowak, 1989) and in human lymphocytes (Kalweit *et al.*, 1990). Modulation of cell cycle checkpoints and the preservation of genomic integrity are proposed as important aspects of cellular osmoprotection and as essential for cellular osmotic stress resistance as the capacity for cell volume regulation and maintaining inorganic ion homeostasis and protein stability/activity. Therefore if DNA repair mechanisms assist in regulating osmotic stress it is also possible that osmotic stress may alter the efficiency and accuracy of DNA replication or repair consequently leading to an increase in chromosomal aberrations. Galloway *et al.* (1987) reported that hyperosmotic medium may affect chromatin structure and / or enzyme activities leading to alterations in DNA repair mechanisms. Furthermore, it is important to mention that salinity is an important factor in natural environments as it determines the bioavailability of heavy metals in intertidal sediments (Du Laing *et al.*, 2002).

During the present study there appeared to be no radiation-induced effects and in particular the concentrations of tritium in the environment were extremely low (background levels). However the behaviour of radionuclides, in particular tritium, in the environment is of particular concern at present (Wallis, 2000) and will continue to be of

concern due to the impending increase in tritium discharges by DML (Environment Agency, 2001). The tissue fluids of the freshwater clam (*Anodonta nuttallina*) were found to equilibrate quickly with the tritium levels in the water of an artificial pool (Harrison and Koranda, 1971, cited in Bruner, 1973). The viscera of the clam had the highest specific activity (0.53 μCi/g), with the gill next at 0.43 μCi/g and the mantle, muscle and calcareous tissue approximately 0.3 μCi/g. Furthermore it was found that the specific activity (ratio of tritium atoms to hydrogen atoms) increased with length of residence in the tritiated pool (Harrison and Koranda 1971, cited in Bruner, 1973). This suggests that organisms that are indigenous to areas of tritium release will have a specific activity in their tissues almost equal to that in the water. Harrison *et al.* (1973) showed that the tissue-free-water tritium of clams increased to about 95% of the level of the pool-water tritium and then followed a decrease in pool-water tritium (40 to 27 nCi/ml) during the experiment indicating the rapid exchange between the tissue-water and pool-water. This suggests that even though mussels/shellfish may experience a similar tritium level in the tissue-water as that of the surrounding seawater, if the levels of tritium were to decrease the mussels/shellfish would also contain a reduced amount of tritium. Thus, if the concentration of tritium increases, so to will the levels of tritium in the organisms. Furthermore, as well as exposure to tritium in aquatic habitats, tritium may also be incorporated into organisms as food. Two species of snails (*Lymnaea reflexia* and *Helisoma trivolis*) were found to incorporate higher levels of tritium into their bound tissue water after being fed on algae grown in tritiated water, in comparison to snails that were fed on normal food, although both groups of snails were grown in tritiated water (Bruner, 1973). Also snails raised from eggs hatching in these tanks developed higher specific tritium activities than did their parents. In comparison, it has been postulated that exposure to low doses of radiation may be beneficial to organisms as the results of numerous studies indicate that cells can become refractory to the detrimental effects of ionising radiation when previously exposed to a low "adapting dose" (Wojcik and Shadley, 2000). It has been suggested that the induced radioresistance be due

to the induction of DNA repair systems that efficiently protect the adapted cells from the effects of a subsequent, high "challenging dose". In summary, organisms in the Tamar Estuary might increase their body burden of tritium when discharges of tritium are increased by the dockyard. This will be due to increased levels of tritium in the water that they inhabit and also an increase in tritium concentrations in their food. However since the organisms are already being exposed to very low doses of tritium it is possible that they may be protected from subsequent higher challenging doses.

In conclusion the statistical approach adopted to evaluate the effects of different physical and chemical factors on the biological response did not show a significant correlation with the presence of low levels (background) radiation following deployment of adult mussels for 3 months. However, apart from salinity, it did appear that the heavy metals (e.g. arsenic and tin) were responsible for an increase in the induction of micronuclei.

The main aim for all ecotoxicologists is to understand how organisms respond to exposure to environmental toxicants and whilst laboratory studies are very informative they often lack the complexity of natural environments (Sugg *et al.*, 1996). Only with information from natural settings can we refine our predictive powers for assessment of ecological and human health risks.

Chapter 9

Effects of tritium on the developmental and reproductive success of *Platynereis dumerilii*

Hypothesis

Tritium has a detrimental effect on the development and thus the reproductive output of the marine polychaete *Platynereis dumerilii*.

9.1 Introduction

In both mammals and aquatic species there are three main potential effects of exposure to genotoxic substances (Anderson and Harrison, 1990b). Reduced fertility may occur if genetic damage induces cell death in dividing gametes. Reproductive success may be impaired if dominant- and recessive-lethal mutations are induced, causing embryo mortality or abnormality and thirdly exposure to genotoxic contaminants may cause cancer (Anderson and Harrison, 1990b). However Kurelec (1993) suggested that in natural species, the manifestations of mutational events were actually of a much greater biological and ecological importance than the induction of tumors. These manifestations include impairments in enzyme function, altered protein turnover, impairments in general metabolism, production of initiators of cytotoxic injuries, inhibition of growth, degenerative processes and atrophy in tissue and organs, decreased scope for growth in organisms, faster ageing, decreased fitness and well-being, impairments in immunoresponse and reproduction, increased frequency of diseases and neoplasia, impairments in adaptation, survival, and succession, and finally, extinction of species. Collectively these pathophysiological changes have been called the genotoxic disease syndrome (Kurelec, 1993).

Changes in the fitness of an organism or changes that affect its overall reproductive capability such as those manifested as the genotoxic disease syndrome which include, premature death, ability to mate, fecundity, viability of offspring, etc., can have the greatest influence on effects at the higher levels of biological organisation (Evenden and

Depledge, 1997). Anderson and Wild (1994) also believe that altered fertility, development, and embryonic survival are the most environmentally significant alterations because they can reduce reproductive success and thus alter a population's size or structure. Reproductive success has been extensively studied in the natural environment. Methods for assessing reproductive success have included, hatching success of black crowned night heron eggs after organochlorine exposure (Rattner, 2001) and the effects of PCB contamination on plumage colour of tree swallows which lead to earlier breeding and hence a larger clutch size (McCarty and Secord, 2000). The failure of nests were used to monitor reproductive success in tree swallows after exposure to pulp and paper mill effluent discharges (Harris and Elliot, 2000). DDE, a polychlorinated biphenyl, has been shown to be a risk factor in the hatching success and thus the reproductive success of double crested cormorants (Custer *et al.*, 1999). Successful egg hatchability and fry survival have been used in fish as well as in birds, (e.g. lake trout in the Great lakes, USA) (Mac and Edsall, 1991). In addition the development of the ovary has been used as an indicator of reproductive success in benthic fish (the English sole) after exposure to fluorescent aromatic compounds (Johnson *et al.*, 1993).

In light of the examples mentioned above it is clear that exposure to contaminants may produce physiological and biological alterations at various levels of biological organisation. In addition these alterations may alter the ability of an organism to survive, thrive, and reproduce, which in turn may lead to permanent alterations or an adaptation response (Dawkins, 1982). An adaptation may be expressed at any level of organisation from sub-cellular through the ecosystem. An adaptation may encompass morphology, physiology, development and behaviour changes. Evolutionary adaptation is a change in the genetic make-up of populations that have been passed on to successive generations. In general terms, an evolutionary mutation has occurred in an organism that has made that organism better adapted to their environment which in turn means that their selected genes are passed on to their offspring. Thus producing more viable and better-adapted young and

hence so increasing their proportion in the population (Withers, 1971). A change in the genetic constitution may be advantageous for certain populations that are living in stressful conditions (Wurgler and Kramers 1992). However as well as enhancing a population, mutations may also put a population at risk of extinction (Higgins and Lynch, 2001, Lande, 1998a). Usually anthropogenic factors are the primary causes of species decline, endangerment and extinction, however in small populations stochastic factors such as inbreeding depression, loss of genetic variability and fixation of new deleterious mutations may be more influential (Lande, 1998b). Mutations can cause inbreeding depression by maintaining potentially adaptive genetic variation in quantitative characters (Lande, 1995).

From previous studies, as discussed in chapter 4, there was a significant increase in abnormality of *P.dumerilii* embryo-larvae after exposure to tritium, however there was no statistical increase in mortality after the 72h sampling period. Altered fertility, development, and embryonic survival are environmentally significant because they can reduce reproductive success and thus alter the population size and structure (Anderson and Wild, 1994). Furthermore, cytogenetic and cytotoxic assays (in chapter 4) produced strong differences between tritium exposed embryo-larvae compared to the controls. These effects of radiation at different levels of biological organisation have been suggested to be an important indicator to link genotoxic responses to effects on reproductive success and potentially alterations to populations (Anderson and Harrison, 1990a).

In light of all the information mentioned above and in previous chapters it is clear that organisms must be able to adapt to challenges to its fitness that may arise from environmental changes and stresses. Therefore the present study will attempt to predict the ecological impacts of genotoxic responses in the polychaete worm *P.dumerilii* by analysing the developmental and reproductive success (fertility and fecundity) of worms exposed as embryos to tritium.

9.2 Methods

Figure 9.1. describes the experimental protocol for assessing the developmental and reproductive effect of tritium on 16h post-fertilised *P.dumerilii* embryo-larvae.

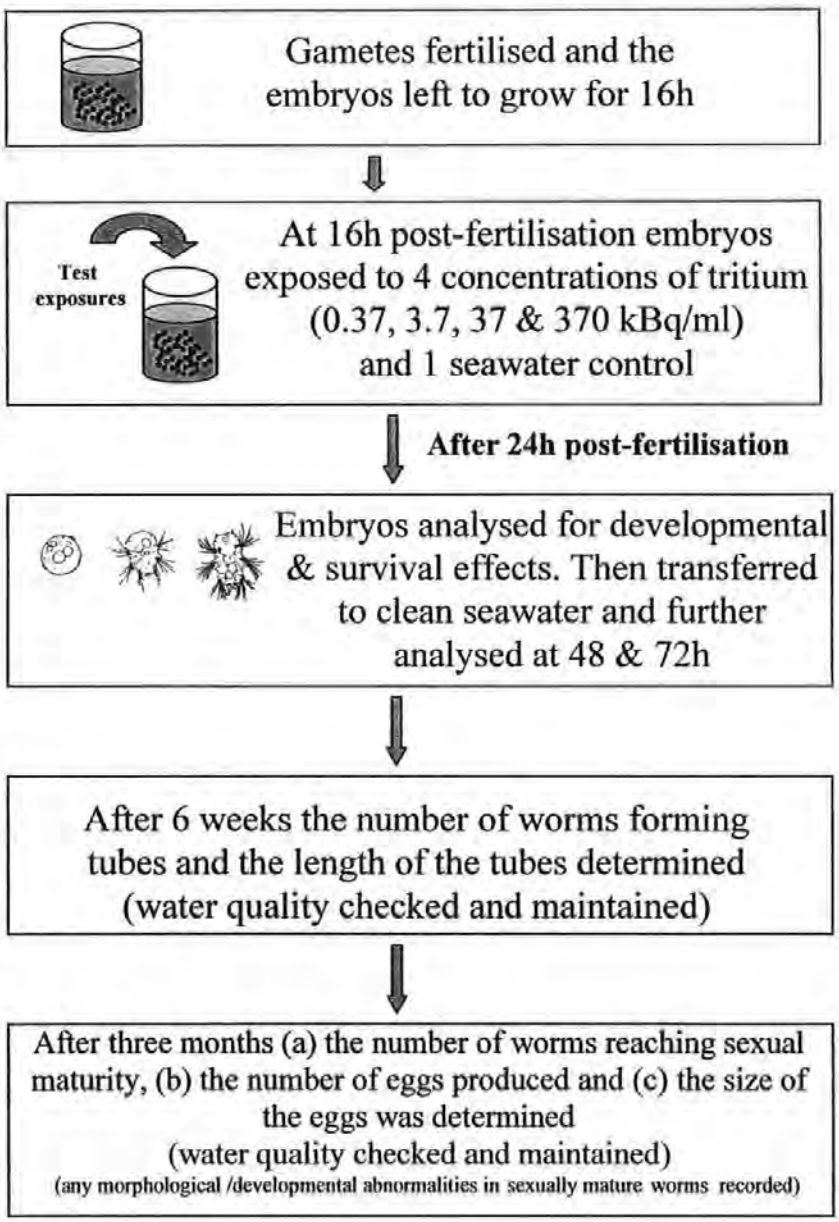


Figure 9.1. Experimental protocol to study the reproductive success of *P.dumerilii* following exposure to tritium.

In brief embryo-larvae were exposed to tritium 16h post-fertilisation as previously carried out in chapter 4. The embryo-larvae were then transferred to clean seawater and allowed to develop until maturity in order to investigate the potential effects of tritium on sexual reproduction and thus the ecological significance of radioactivity.

9.2.1 Embryo collection, developmental and mortality effects

2 sexually mature males and females were allowed to spawn naturally in 50 ml of filtered seawater. Approx. 11,000 embryos were produced with an 95.7% fertilisation rate. The embryos were allowed to grow for 16h in growth incubators at 20°C ± 1 °C. After which they were divided into exposure vessels (2 embryos per ml in 500 ml) and exposed to four concentrations of tritium (0.37, 3.7, 37 & 370 kBq/ml) and 1 negative control containing just filtered seawater. When the embryos reached 24h post-fertilisation, they were divided into replicates and placed into clean seawater (without tritium) and analysed for developmental and survival effects as described in section 2.3.1.1. Further analyses of morphology, behaviour and mortality were assessed at 48 and 72h post-fertilisation. A subset of larvae were removed after 24, 48, and 72h and fixed in a 10% formalin/seawater solution for further examinations using image analysis, approximately 50 embryo-larvae were assessed per concentration for each time period. The length of the 24, 48 and 72h post-fertilised larvae was measured using image analysis (Image Pro® Plus version 4.1, Media Cybernetics® USA.) and was calculated from the head to the tail of larvae. Figure 9.2. shows an example of normal and abnormal 72h post-fertilised embryo-larvae and indicates the variation in length.

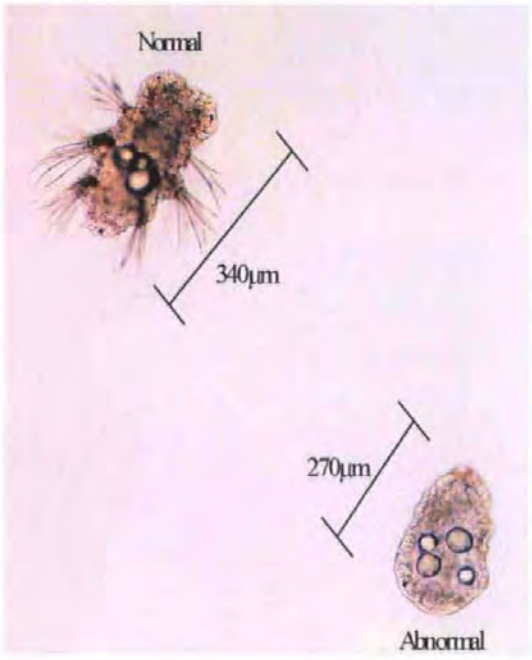


Figure 9.2. Normal and abnormal 72h post-fertilised *P.dumerilii* larvae after exposure to tritium.

9.2.2 Development of juveniles

After 72h the larvae were placed into tanks (25 x 10 cm) with 1 litre of clean seawater as shown in figure 9.3. The embryos were fed 1 ml of prepared spinach (see section 2.1.1 for details of spinach concentration) every week for four weeks. Following which it was increased to 1 ml twice a week until they were 2 months in age when it was further increased to 2 ml twice a week.

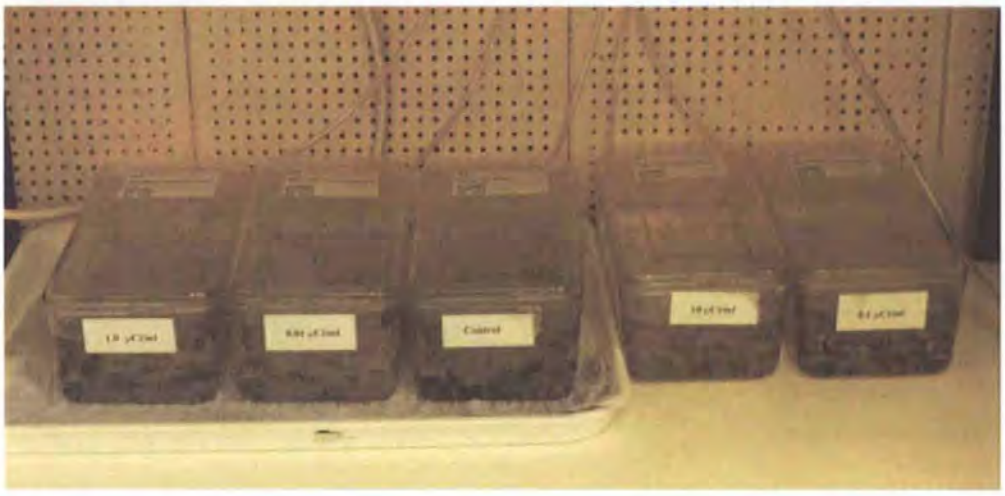


Figure 9.3. Example of tanks containing 72h+ post-fertilised larvae.

Water quality parameters (pH, temperature, salinity, and oxygen) were analysed weekly. Water was added to each tank, as and when needed, until the worms had formed tubes after which the tanks were cleaned and replaced with fresh seawater once a week, a few days after feeding. No attempt was made to analyse the worms prior to tube formation as the worms naturally vary greatly in size/segment length after 72h post-fertilisation. However the worms were checked regularly for any developmental abnormalities. Following formation of the tubes the number and length of the tubes were analysed using image analysis (approximately 6 week old juveniles) to indicate any change in the growth or survival of the worms. A digital photo of the tanks was taken and this was scanned into the image analysis package (Image Pro Plus ®). A ruler situated next to the tanks was used to calibrate the image analysis package after which a line was traced on the picture in order to calculate the length of the tubes (from which the number of tubes was determined (figure 9.4.)

Figure 9.4. Digital image of a tank of worms used to measure the length and number of tubes produced by the worms.

No attempt was made to measure the growth of the worms after 72h post-fertilisation as handling might cause stress and may lead to misleading results. Furthermore no attempts were made to re-measure the tube lengths and numbers of tubes after 6 weeks. As once the tubes are formed they may be inhabited by more than one worm or the worm may move to a larger tube which would not be a true indicator of its growth.

9.2.3 Evaluation of gamete production

The worms reached sexual maturity asynchronously between 14-19 weeks after fertilisation. The tanks were checked daily and any sexually mature worms that were swimming freely in the tanks were collected. Male worms were fixed immediately in a 10% formalin/seawater solution for further morphological examinations. Female worms were encouraged to release their eggs by physical stimulation/or the addition of a few drops of sperm (if sexually matured males were also available at the same time). The worms were fixed immediately in a 10% formalin/seawater solution for further morphological examinations using image analysis. In addition the eggs were also fixed immediately in a 10% formalin/seawater solution for examinations using image analysis.

The area of 20 eggs per female was calculated using image analysis. The outline of the egg was traced and the area within the outline determined.

9.2.4 Evaluation of morphology of sexually matured *P.dumerilii*

The length of the male and female worms was obtained using image analysis as shown in figure 9.5. Digital photos of the worms were taken which were scanned into the image analysis package, Image Pro Plus ®. A ruler situated next to the worms was used to calibrate the image after which a line was traced on the picture using the image analysis package to calculate the length of each worm. The eyes of the worms were also analysed for morphological abnormality using a Leica Quantimet Q570 image processor (Cambridge Instruments, UK). The worm were placed into a glass tube that was rotated under a Kyowa tri-nocular stereo microscope microscope (x1.0). An image of the eye was obtained and analysed using image analysis for differences in area, length, convex area and the roundness of the eye. Area, length and the convex area are all measured in µm. Roundness is an arbituary unit where a value of one represents a round object and any value greater than 1 represents irregular shapes (not round).

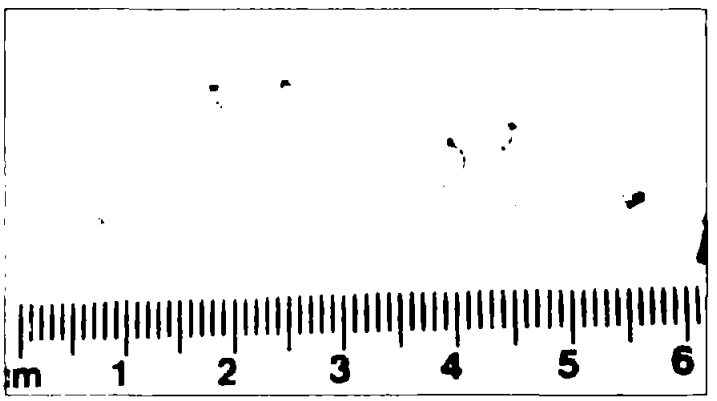


Figure 9.5. Digital photo of fixed *P.dumerilii* worms. Ruler used as a scale bar to calibrate image analysis package.

9.3 Results

9.3.1 Scintillation counts

Scintillation counts of tritium concentrations prior to exposure and post exposure of embryo-larvae were analysed as described in section 2.7.1. Readings displayed in counts per minutes were converted to kBq/ml as described in section 4.3.1. There was little variation (0.26 - 1.88%) between the pre and post-exposure concentrations of tritium for all concentrations, with less than 2% of the original concentration being removed by the end of the exposure.

Table 9.1. Nominal & definitive concentrations (kBq/ml) of tritium in seawater before and after exposure of *P.dumerilii* embryo-larvae (16h post-fertilisation).

Nominal Concentration of tritium in samples (kBq/ml)	Definitive pre-exposure concentration (kBq/ml)	Definitive post-exposure concentration (kBq/ml)	Difference in concentration (kBq/ml)	Percentage of initial concentration removed (%)
Seawater	0.0022	0.0023	+0.0001	+105
0.37	0.260	0.257	0.003	-1.15
3.7	2.66	2.61	0.05	-1.88
37	26.68	26.50	0.18	-0.67
370	258.32	257.65	0.67	-0.26

9.3.2 Dose received by embryo-larvae

The dose received by embryo-larvae (table 9.2) was calculated following the formulae by Strand *et al.* (1977) as described in section 2.7.2.

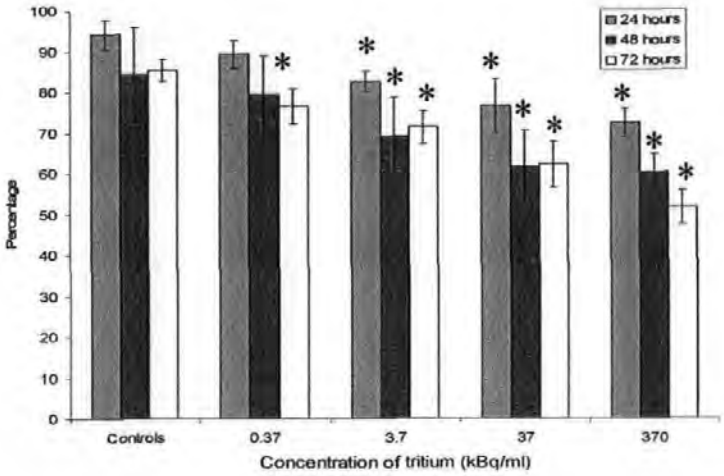
Table 9.2. Dose (mGy) received by *P.dumerilii* embryo-larvae during exposure to tritium, 16h post-fertilisation for an exposure period of 8h.

Concentration (kBq/ml)	**Dose (mGy)**
0.37	**0.01**
3.7	**0.1**
37	**0.97**
370	**9.68**

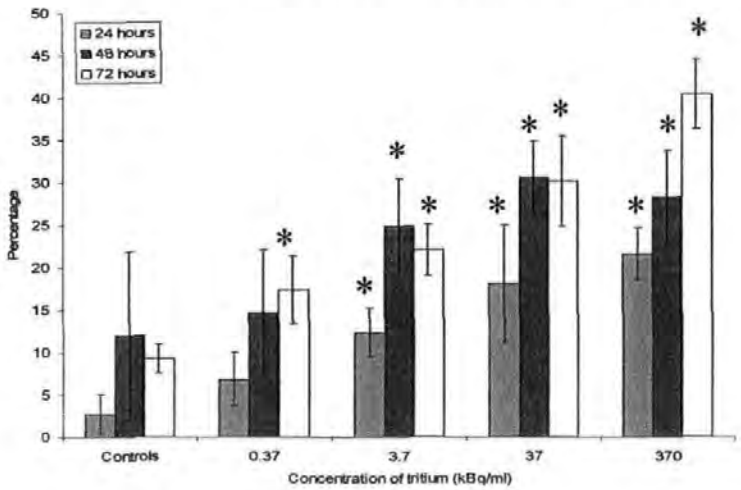
9.3.3 Developmental and survival/mortality effects of 24, 48 & 72h *P.dumerilii* embryo-larvae

Figure 9.6. represents the percentage of normal, abnormal and dead P.*dumerilii* embryo-larvae at 24, 48 and 72h post-fertilisation following exposure to tritium.

(a)



(b)



(c)

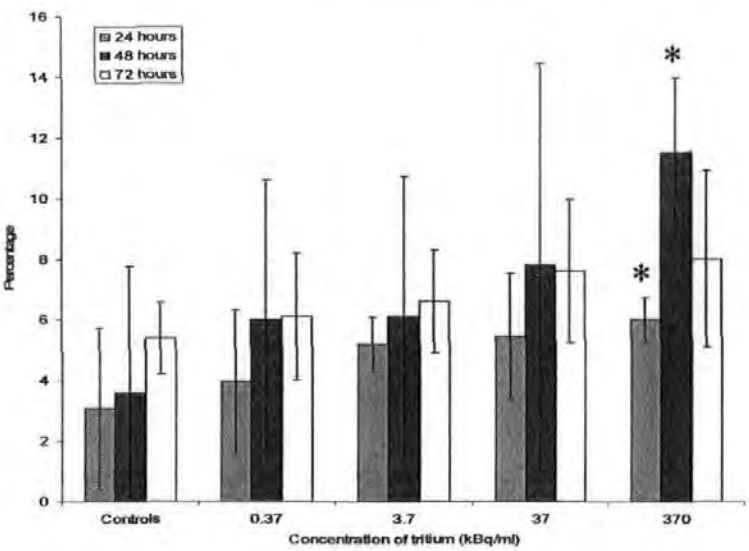


Figure 9.6. Percentage of (a) normal, (b) abnormal, and (c) dead *P.dumerilii* embryo-larvae after exposure to tritium 16h post-fertilisation. Error bars represent 2 x standard error (n = 5) (* = significantly different from control using ANOVA, $p<0.05$).

Figure 9.6.a represents the percentage of normal *P.dumerilii* embryo-larvae. There was a statistically significant difference between the percentage of normal embryo-larvae between concentrations at 24, 48 and 72h (P=0.00005, P=0.01, P=0.00005 respectively). The lowest concentration (0.37 kBq/ml) was only significantly different from the control at 72h. At 72h the decrease in the percentage of normal embryo-larvae with increasing concentrations of tritium was dependent on dose (R^2 = 58.25%).

Figure 9.6.b represents the percentage of abnormal *P.dumerilii* embryo-larvae at 24, 48 and 72h post-fertilisation. There was a statistically significant difference at 24 and 48h between the concentrations (P=0.00005 and P=0.0074 respectively) although the lowest concentration of 0.37 kBq/ml was not statistically different from the control. At 72h the number of abnormal embryo-larvae had significantly increased in the highest concentration of tritium with approximately a four-fold increase in comparison to the controls. As in the previous time periods there was a statistical difference between the samples (P=0.00005) and there was a strong dose relationship (R^2 = 59.8%).

Figure 9.6.c represents the percentage of dead *P.dumerilii* embryo-larvae. At 24 and 48h there was no statistical difference in the percentage of dead embryo-larvae between the various concentrations (P=0.2272 and P=0.3308). However there was a significant elevation in mortality in the embryo-larvae that were exposed to the highest concentration of tritium (370 kBq/ml) in comparison to the controls. By 72h there was no significant difference for the percentage of dead between any of the concentrations (P=0.4256; R^2=8.76%).

There was a statistically significant difference in the length of the 72h larvae between the concentrations (P = 0.00005). Figure 9.7. represents the mean length of 50, 72h *P.dumerilii* larvae after exposure to tritium. The length of the larvae was statistically reduced after exposure to all four concentrations of tritium although the reduction was not related to concentration (R^2=1.77%).

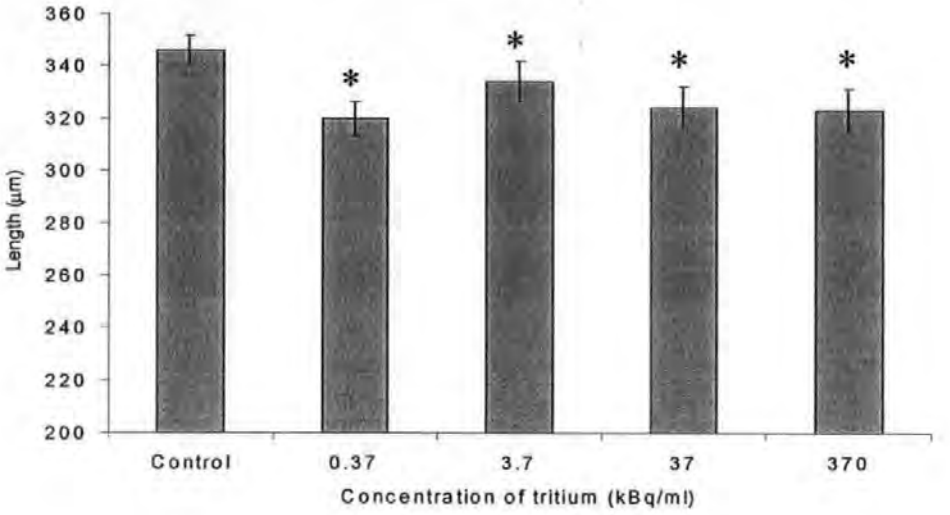


Figure 9.7. Mean length of fifty 72h old *P.dumerilii* larvae after exposure to tritium Error bars represent 2 x standard error (n = 50). (* = significantly different from controls using ANOVA, $p<0.05$).

9.3.4 Tube forming capability of juvenile *P.dumerilii*

Figure 9.8. shows the number of worms that formed tubes at 6 weeks post-fertilisation.

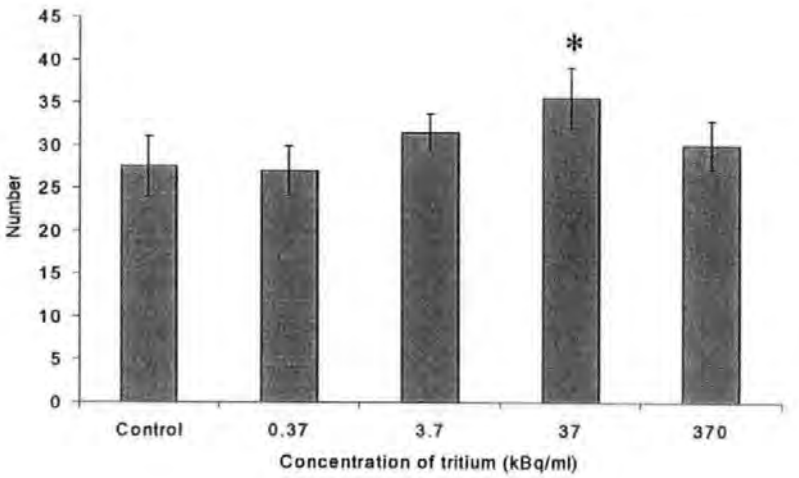


Figure 9.8. Number of 6 week old *P.dumerilii* forming tubes after exposure of embryo-larvae (16h post-fertilised) to tritium. Error bars represent 2 x standard error (n = 2 indicating replicate tanks) (* = significantly different from control using ANOVA, $p<0.05$).

There was a significant increase in the number of tubes produced by embryo-larvae exposed to 37 kBq/ml in comparison to the control and the lowest concentration of tritium (0.37 kBq/ml). However, in general there was no statistically significant difference between the mean number of worms producing tubes between the various concentrations (P=0.1616).

Figure 9.9. represents the average length of tubes formed by 6 week old *P.dumerilii* after exposure to tritium. There was no statistical difference between the length of the tubes formed between those worms exposed to tritium and the controls (P=0.0812). Although there was a significant increase in the length of tubes produced from worms exposed to 3.7 kBq/ml of tritium and the control worms.

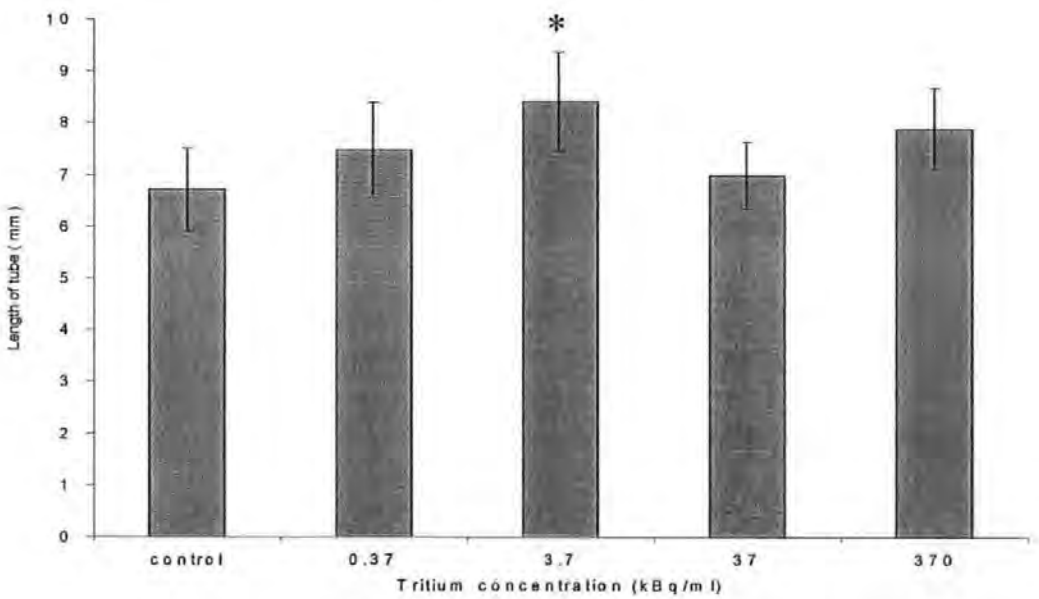


Figure 9.9. The average length of tubes formed by 6 week old *P.dumerilii* after exposure of embryo-larvae (16h post-fertilised) to tritium. Error bars represent 2 x standard error (n = 2 indicating replicate tanks). (* = significantly different from control using ANOVA, $p<0.05$).

9.3.5 Sexual maturity and morphology of *P.dumerilii*

In total 61 worms reached maturity over the five concentrations. Table 9.3. shows the number of *P.dumerilii* that reached sexual maturity after exposure to tritium as embryo-larvae.

Table 9.3. The total number of male and female *P.dumerilii* that reached sexual maturity following exposure of embryo-larvae to tritium.

	Control	**0.37 kBq/ml**	**3.7 kBq/ml**	**37 kBq/ml**	**370 kBq/ml**
Males: Tank 1	4	4	4	2	3
Males: Tank 2	2	2	4	4	5
Total Nº males	*6*	*6*	*8*	*6*	*8*
Females: Tank 1	2	6	4	2	2
Females: Tank 2	2	2	3	2	2
Total Nº females	*4*	*8*	*7*	*4*	*4*
Total Nº of worms	**10**	**14**	**15**	**10**	**12**

There was no statistically significant different (ANOVA) in the number of males or females that reached sexual maturity in comparison to the controls (P=0.8184 and P=0.4415 respectively). Furthermore there was no statistical difference in the total number of worms reaching sexual maturity between the worms exposed to the various concentrations of tritium and the controls (P=0.7205; R^2=0.28%). The date of spawning (i.e. early or delayed release of gametes) was also not affected by exposure to tritium (P=0.5618) (figure 9.10).

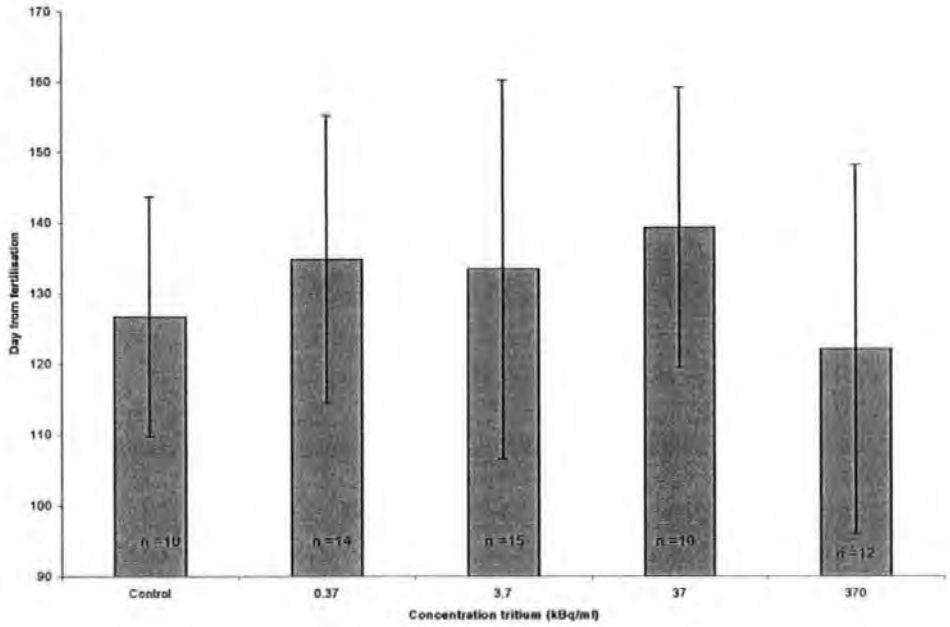


Figure 9.10. Day of spawning of sexually mature worms. Error bars represent standard deviation between individuals (* = significantly different from control using ANOVA, $p<0.05$).

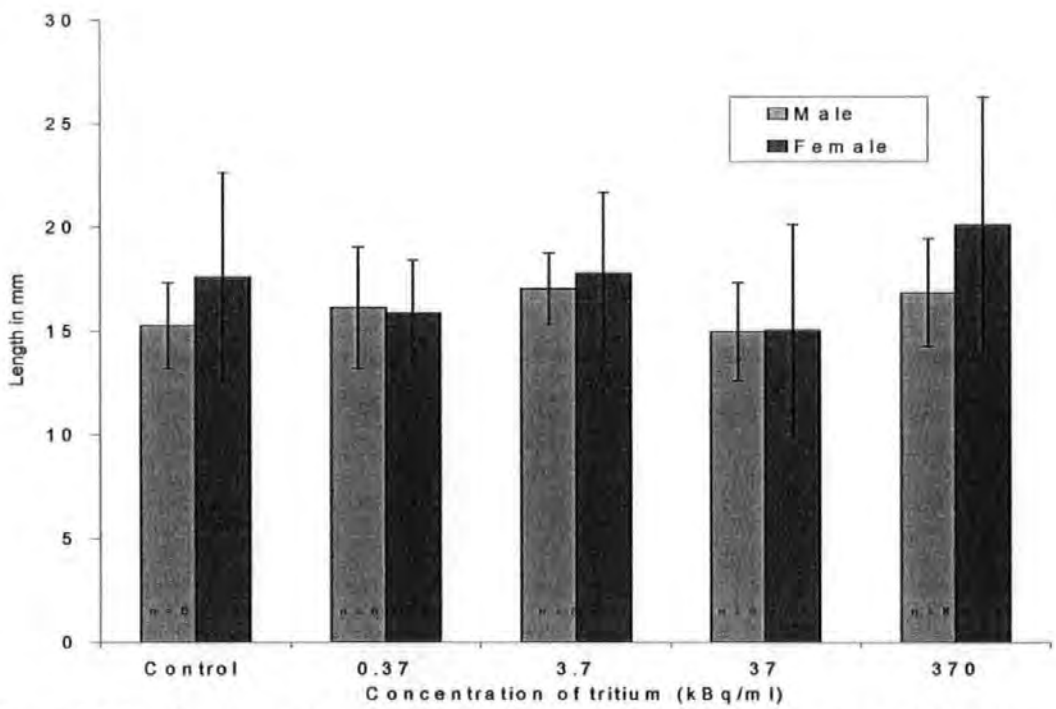


Figure 9.11. The average length of sexually mature *P.dumerilii* males and females produced after exposure of embryos to tritium. Error bars represent 2 x standard error (* = significantly different from control using ANOVA, $p<0.05$).

There was no statistically significant difference in the length of mature worms (figure 9.11.) due to exposure to the various concentrations of tritium (P=0.3082). Furthermore there was no difference in length of mature worms due to the sex of the worms (P=0.2515).

Out of the 61 worms that reached sexual maturity 6 were abnormally developed (9.83%). One worm that developed after exposure to 3.7 kBq/ml did not develop any eyes and another worm exposed to 370 kBq/ml of tritium had one eye much larger than the other. Four worms exposed to 0.37, 3.7 and 370 kBq/ml of tritium had deformed bodies, this included under developed segments and chaete, expanded head regions and protrusions from the mouth region. Figure 9.12. shows an example of a morphological abnormal adult worm after exposure to tritium.

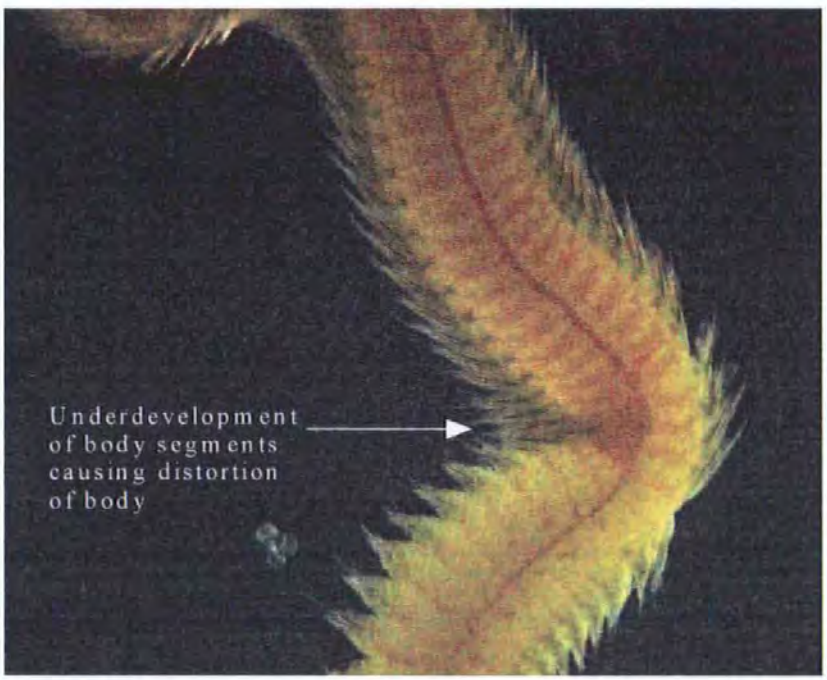


Figure 9.12. Distorted body section of a female *P.dumerilii* due to underdevelopment of segments following exposure of embryo-larvae to tritium.

Further investigation of the eyes from a selection of the worms (30 in total) concluded that there was generally no significant difference (ANOVA) in the area, length, roundness and the convex area of the eyes ($P<0.05$ for all perimeters tested) (data presented in table 9.4). Furthermore there was no dose relationship of the perimeters when examined using simple regression.

Table 9.4. Mean area, length, roundness and the convex area of the eyes of sexually mature *P.dumerilii* ± 2 x standard error.

Conc^{n} (kBq/ml)	N =	Area (μm^2)	Length (μm)	Roundness	Convex area (μm^2)
Control	5	20931 ± 7052	193.8 ± 38	2552.4 ± 852	24793.2 ± 7713
0.37	4	24494 ± 2424	199.2 ± 11	2093.5 ± 419	2698.01± 2778
3.7	8	25798 ± 6634	224.3 ± 23	2977.7 ± 1716	30158.6 ± 6486
37	7	22950 ± 3978	202.1 ± 37	2696.7 ± 1123	26611.1 ± 7649
370	6	20576 ± 11804	190.9 ± 74	3163.1 ± 1508	24511.7 ± 1361

9.3.6 Reproductive success

Figures 9.13. represent the average number of eggs produced by sexually mature female *P.dumerilii* following exposure of embryo-larvae to different concentrations of tritium.

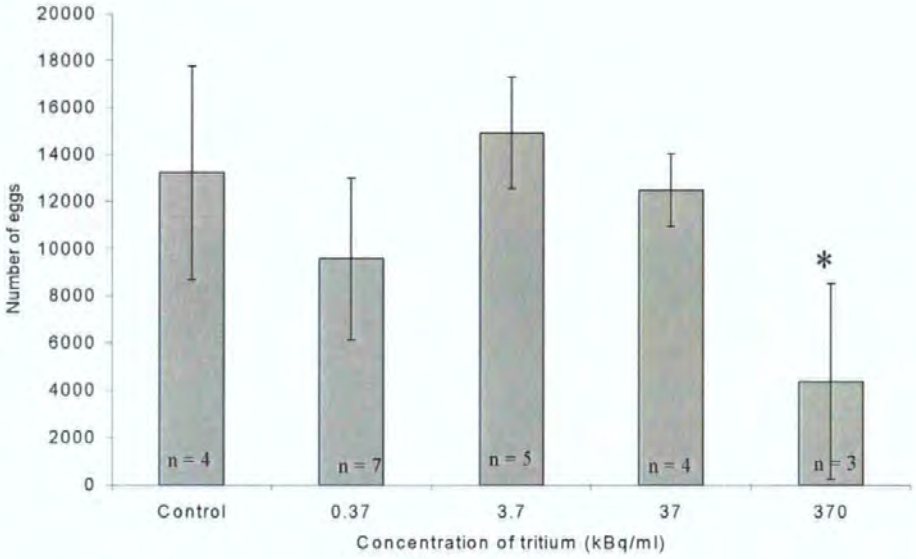


Figure 9.13. Average number of eggs produced by sexually mature female *P.dumerilii* after exposure to tritium (* = significantly different from control $p<0.05$). Error bars represents 2 x standard error of the mean of the individuals.

There was a statistically significant difference between the number of eggs produced by sexually mature females (P=0.0101). Although, only the highest concentration (370 kBq/ml) of tritium produced significantly less gametes in comparison to the controls. There was a slight dose dependent decrease in the number of eggs produced (R^2=31.16%).

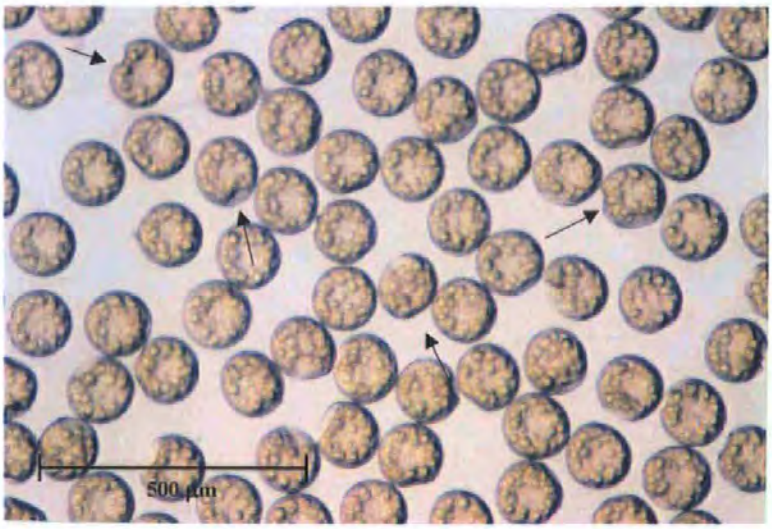


Figure 9.14. Example of eggs produced by tritium exposed *P.dumerilii* female. → represents examples of abnormal eggs.

Figure 9.14. shows examples of eggs that were abnormal in shape (irregular shapes, not round, membrane pulling away from cell wall etc.) that were produced by a female that had been exposed to tritium. Figure 9.15. shows the average area of the eggs produced from sexually mature female *P.dumerilii*. The area of the eggs were not related to tritium concentration (P=0.3698). Although the area of eggs was related to the length of the female (P=0.0203) with larger females producing larger eggs. In addition the number of eggs produced also significantly influenced the area/size of the eggs (P=0.0087) with smaller eggs being produced in larger clutch sizes.

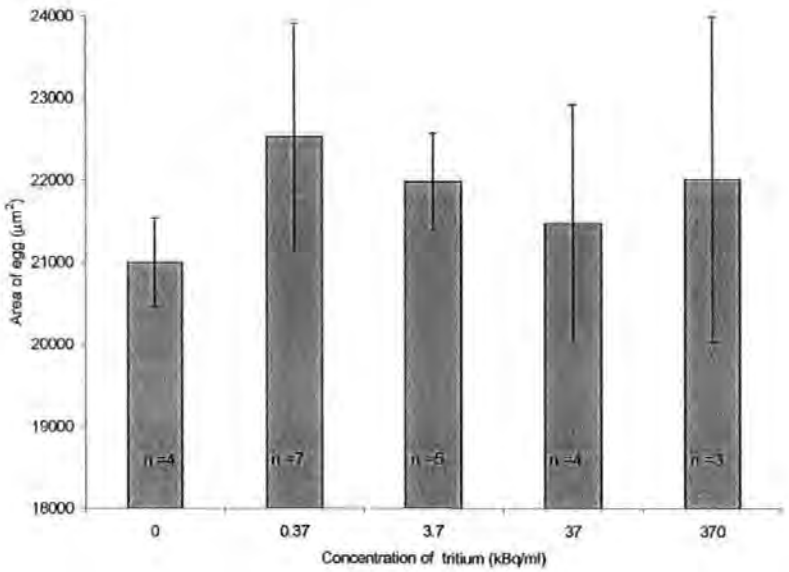


Figure 9.15. Mean area of 20 eggs produced from sexually mature female *P.dumerilii* after exposure of embryo-larvae to tritium (* = significantly different from control using ANOVA, $p<0.05$) (n = number of females).

9.4 Discussion

Approximately 450 embryo-larvae per tank were distributed after exposure to tritium and approximately 5-8% of these worms could form tubes and only 2-3% of the worms reached sexual maturity (including the controls). A study on a population of *P.dumerilii* from the Firth of Clyde, Scotland, showed that the most frequent age at maturity was 6-8 months and 57% of individuals reached sexual maturity (Grant, 1989). Therefore during the present study the worms developed slightly faster than those from

Scotland but a significantly lower percentage of worms reached maturity during the present study. The percentage of survival may be due to limitations in the size of the tanks. The mature worms from Scotland were between 15-30 mm long and the eggs produced ranged from 178-180 μm, in comparison there was little difference in the worms during the present study where mature worms ranged from 9.15-27.6 mm and the eggs ranged from 142-218μm. Of the worms that reached sexual maturity approximately 10% were morphologically abnormal after exposure to various concentrations of tritium, although the abnormalities may not have been due to exposure to ionising radiation and may be a result of injuries caused by another worm or handling (e.g. cleaning tanks). However previous experiments carried out in fish showed that external irradiation of gametes produced major eye malformations and vertebral column abnormalities in progeny of the fish (Anderson and Harrison, 1990a), which does support the findings in the current study.

Detrimental reproductive effects due to exposure to ionising radiation have been observed in fishes and invertebrates. Hoppenheit (1973) observed reduced egg-production rate in the amphipod (*Gammarus dubeni*) at 2.2 Gy, and Harrison and Anderson (1988) observed a decrease in fecundity of the polychaete *Neanthes arenaceodentata* at a dose greater than 1 but less than 5 Gy (^{137}Cs external gamma radiation). Furthermore they showed that chromosomal aberrations were induced in larvae of the polychaete, which lead to cell death in gametes and thus caused a reduction in the brood size. During the present study exposure of embryos to a dose of 9.68 mGy produced a decrease in the number of eggs produced. A reduction in brood size was observed after exposure of adult *N.arenaceodentata* to doses between 5 and 10 Gy (^{137}Cs external gamma radiation). A number of explanations may account for the differences in results between the present study and those carried out on *N.arenaceodentata.* During the present study the embryos were exposed to the beta emitting radionuclide tritium present in the water that the worms inhabited, in comparison *N.arenaceodentata* were exposed to an external source of the gamma emitting radionuclide caesium, which may not be as harmful to embryo-larval

stages as tritium. Furthermore the high dose level experienced by *N.arenaceodentata* was probably due to the length of exposure period (entire life cycle), in contrast to only 8h for *P.dumerilii* embryo-larvae from the present experiment. One possible explanation of the apparent sensitivity of *P.dumerilii* compared to *N.arenaceodentata* is the difference in reproductive strategies between the two polychaete species. A typical brood size of *N.arenaceodentata* ranges between 19-534 (Harrison and Anderson, 1988) in comparison *P.dumerilii* generally produce in excess of 10,000 eggs per female (Hutchinson *et al.*, 1995). Therefore it would appear that *P.dumerilii* produces excess number of eggs which may be more sensitive to pollutants in comparison to the *N.arenaceodentata*. However, although a reduction in brood size was observed after exposure of adult *N.arenaceodentata* to doses ranging between 5 and 10 Gy, embryonic mortality was observed at much lower doses of 0.5 Gy. Hence exposure to ionising radiation was shown to be more detrimental to embryo-larvae/juvenile stages in comparison to adults (Harrison and Anderson, 1994a). The increased abnormality and mortality of embryos was speculated to be due to the induction of lethal mutations in the germ cells during gametogenesis, as earlier studies had showed that chromosomal aberrations were induced in the larvae and juveniles of the species at about 2 Gy (Harrison and Anderson, 1994a). Based on their studies, Anderson and Harrison (1990b) proposed a model to predict the interrelationship between selected effects of ionising radiation on the marine polychaete worm *N.arenaceodentata* at subcellular, cellular, organismal and population levels. They hypothesised that DNA repair was not very active in gametes of this species and that DNA damage in gametes or selected gametogenesis stages is cumulative. Therefore they concluded that they had provided convincing evidence, for this species, that the gametes are the critical cells that determine the sensitivity of the species to ionising radiation.

The effect of tritium on embryo-larvae did not affect the survival and development of the worms in reaching maturity. A small proportion of the worms that were exposed to tritium did develop morphological abnormalities, however there was no statistical

difference between exposures. There was a significant reduction in the number of eggs produced by sexually mature females after exposure to the highest concentration of tritium (370 kBq/ml) although no difference was observed in the number of gametes produced from the worms exposed to the other concentrations of tritium. Pregnant rats maintained at constant body activities of tritiated water ranging from 1-100 μCi/ml (37-3700 kBq/ml) of body water throughout pregnancy (Cahill and Yuile, 1970) were externally and internally normal regardless of the activity levels of tritium. However statistically significant changes were observed including, microencephaly, sterility, stunting, litter size reduction and increased resorption of the fetuses (Cahill and Yuile, 1970) which supports the present study where the adults appeared normal but there was a reduction in the number of offspring. However during the present study no statistical difference occurred in the size of the eggs from sexually mature adults following exposure to tritium in comparison to the worms from the controls. Woodhead (1977) also found no effect on the survival or sex ratio of offspring of the tropical guppy *Poecilia reticulata* after exposure to an external gamma (^{137}Cs) source (dose rates 0.17, 0.40 and 1.27 rad/h), yet there was a decrease in the size of the brood.

Although during the present study sexually mature females exposed as embryo-larvae to 370 kBq/ml showed a decrease in the number of gametes produced, no attempt was made to assess the effects of exposure of embryo-larvae to ionising radiation in subsequent generations. Studies on the cytotoxicity of ionising radiation have shown that reproductive cell death may occur as many as five or six generations after exposure (Kronenberg, 1999). This phenomenon, known as delayed expression of lethal mutations, has been observed in many mammalian cell lines. Although studies that quantified radiation induced mutations at early times after exposure suggested that while ionising radiation can cause point mutations, the majority of radiation-induced mutations exhibit large-scale loss of genetic material through either deletional or recombinational mechanisms (Kronenberg, 1999). Theodorakis *et al.* (1997) showed that there was an

inverse correlation between DNA strand breaks and fecundity of mosquitofish (*Gambusia affinis*) from retention pools heavily contaminated with radionuclides. Which was evidence that genetic diversity (changes in allozyme frequency and band intensities as detected using random amplified polymorphic DNA (RAPD) techniques) is increased in a population of fish exposed to radionuclides in comparison to fish from clean reference sites (Theodorakis *et al.*, 1999). Certain band patterns were shown to be more prevalent in fish from contaminated sites and these fish experienced a higher fecundity (brood size divided by body length) and a lower degree of strand breakage measured by agarose gel electrophoresis. These studies provide strong evidence that DNA damage detected at molecular levels can be manifested at genotype levels and therefore potentially alter the population.

However several factors may modify the magnitude of the cytogenetic effects of ionising radiation at each level of biological organisation. For example, cytogenetic responses at any given dose may vary depending on the stage of the cell cycle at the time of irradiation, DNA repair capacities of each cell type, oxygen tension, dose rate and temperature (Anderson and Harrison, 1990b). Amongst the range of cellular defence mechanisms, that might be responsible for the apparent health of the worms, DNA repair is particularly important (Depledge, 1994). Cronkite *et al.* (1973) speculated that at low levels of exposure to tritium there is a longer time period for repair mechanisms to operate. Veatch and Okada (1969) showed that in the mammalian cell line, mouse leukemia ($L5178^{y}$) one tritium "absorption event" represented 0.3 rad and produced about one single strand break/cell (G_1 stage) and 0.1 double strand breaks, hence only one in ten involved cells will retain residual injury. However it is worth considering that the rate of damage may potentially increase in cells that are proliferating more rapidly such as embryo-larvae cells. Small differences in the efficiency of DNA repair mechanisms among normal individuals can account for differences in susceptibility to genotoxicity. During the present study repair mechanisms may account for the apparent health of the worms, although a

more likely explanation for the seemingly harmless effect of tritium on the reproductive output may be natural selection. Therefore it is probable that the worms most damaged by the effects of tritium did not survive to reproduce. The Darwinian fitness of an affected individual is influenced by a reduction in the natural variation found in the gene pool or the result of genetic damage (gene mutations, alterations in gene expression, or the alteration of gene frequencies) (Evenden and Depledge, 1997). Therefore the alteration in fitness of the worms is influenced by the genetic susceptibility of the individuals and this will determine its survival potential. The sensitivity of the worms to ionising radiation may also have varied. Baird *et al.* (1990) showed that the sensitivity of *Daphnia magna* clones varied by three orders of magnitude after exposure to cadmium and since *Daphnia* are clonal organisms it is the genotypes that vary in there sensitivity.

As mentioned above the effects of environmental stresses and pollutants have been shown to affect populations of species by directly reducing reproductive success. In addition a few laboratory based studies have shown without any doubt that radiation induced reproductive effects cause alterations and possibly extinction of populations. Harrison and Anderson (1994b) found that continuous exposure of parental females (P_1) to external gamma irradiation (^{137}Cs) resulted in first generation (F_1) pairs that produced significantly less live embryos. Furthermore chronic gamma (^{60}Co) irradiation (dose rate of 25 rads/hr) was shown to lead to the elimination of egg production in the aquatic snail *Physa heterostropha*, which in turn lead to the extinction of the population within one generation (Cooley and Miller, 1971).

Therefore although the original damage caused by toxicants is generally at the molecular level there are emergent effects at the level of populations that are not predictable based solely on the knowledge of the mechanism of toxicity of the contaminant. The study of evolutionary toxicology, which encompasses the population-genetic effects of environmental contaminants, should therefore be an important focus of ecotoxicology in future studies (Bickham *et al.*, 2000). From the present study it can be

concluded that the effect of tritium on embryo-larvae did not affect the survival and development of the worms in reaching maturity. There was a significant reduction in the number of eggs produced by mature females after exposure to the highest concentration of tritium (370 kBq/ml). Although there was no difference in the number of gametes produced from the worms exposed to the other concentrations of tritium and furthermore there was no statistical difference in the size of the eggs produced in comparison to the other concentrations. It is not possible to state from this work if tritium exposure will have a potential long-term effect on the population of the worm *P.dumerilii*. Although fecundity was reduced at high levels of radiation further studies are needed in order to investigate if subsequent generations will be affected.

Chapter 10:

General Discussion

10.1 Philosophy behind thesis

The introduction or release of radioactive material into the environment, either under controlled, authorised conditions, or as a result of an accident, has the potential to increase radiation exposure of native wild organisms above that of local background levels (Woodhead, 1998). The environmental effects and consequences of radiation exposure in natural ecosystems are hard to accurately predict (Evenden and Depledge, 1997). The primary goal in assessing the effects of radiation is to relate effects observed in individuals to changes at a population level (Anderson and Wild, 1994). Unlike comparative studies on humans, mammals and higher vertebrates, there are currently no tried and tested methods available for assessing genetic susceptibility for invertebrates and plants (Evenden and Depledge, 1997) and little has been done to explore the linkage between genotoxic responses and changes in reproduction and development, which are vital for the functioning of ecosystems (Anderson and Wild, 1994). This situation is somewhat surprising given the potential for animals in ecosystems to serve as sentinels for effects in humans (Anderson *et al.*, 1994). Currently the environmental protection of natural ecosystems against the effects of radiation is largely predicted upon human radiological protection regimes. It is assumed that if humans are protected then so too are other species and hence the environment as a whole is protected (Pentreath, 1998). Non-human organisms have been incorporated into regulatory and assessment frameworks but only in the recognition of their role as diverse critical pathways of radionuclide transfer to human populations and not as important members of natural ecosystems (Johnston *et al.*, 1996). Thus a multispecies ecosystem level of approach needs to be adopted which incorporates "keystone" species, for example *P.dumerilii* and *M.edulis*. In addition the effects on the whole dynamics of the ecosystem needs to be considered which would include a full range of sub-lethal effects, differential life stage sensitivity, reproductive sensitivity and interspecific interactions (Johnston *et al.*, 1996). During the current study some aspects of

this approach were adopted to assess the effects of ionising radiation on aquatic invertebrates.

A reduction in the release of radionuclides in conjunction with radioactive decay and environmental dispersion processes has resulted in a gradual decrease in concentrations of anthropogenic radionuclides. In terms of dose to humans they now account for only a small contribution above natural levels in the general environment (MacKenzie, 2000). However the problem still remains of specific locations where unusually high concentrations of contaminant radionuclides persist as a result of previous waste disposal. A variety of radionuclides are discharges into the marine environment every year. Among these radionuclides, tritium (^{3}H) is one of the most important compared to other radionuclides (^{99}Tc, ^{60}Co). For example, in the UK, ^{3}H is discharged in huge quantities by different sectors and this discharge has been relatively unchanged since 1979 (DEFRA, 2002). While studies have been carried out to evaluate the potential effects of other radionuclides there has been little information on the effects of tritium on the natural biota. This was primarily due to the fact that tritium is considered to be less harmful, as it is a soft β emitter. Recently, however, tritium has been the focus of much scientific and public concern. In May 2000 Devonport Royal Dockyard Limited (DML) submitted an application to the Environment Agency for a variation to their radioactive waste discharge authorisations granted under the Radioactive Substances Act 1993 (Environment Agency, 2001). A proportion of the radioactive waste produced by DML is discharged through a pipeline to the Hamoaze (a stretch of water which connects the River Tamar and the River Lynher with the Plymouth Sound, Plymouth UK). The variations were granted by the Environment Agency in February 2002 and the authorised limit for disposal of tritium to the Hamoaze was increased from 120 GBq to 700 GBq and cobalt-60 was reduced from 6 GBq to 0.8 GBq (Environment Agency, 2001). Currently seawater, sampled and analysed by Environment Agency contractors in close vicinity to DML's discharge point in the Hamoaze, have shown activity concentrations for tritium, as tritiated water, ranging from

<10 - 311 Bq/L, with an average of 26 Bq/L. In comparison, however, to other nuclear facilities the levels of discharge from DML are comparatively lower.

Tritium concentrations recorded in effluents from Amersham Plc. (Cardiff) ranged between 467,000-23,010,000 Bq/L (0.467-23.01 kBq/ml) and BNFL (British Nuclear Fuels) Sellafield are reported to discharge significantly more tritium than any other site in England and Wales (NCAS, 2001). They discharge tritium, in the form of tritiated water, at an approximate concentration of 184 kBq/ml. During the present study concentrations of 0.37-370 kBq/ml of tritiated water were used, this range therefore incorporated the concentration released by Amersham Plc. and BNFL Sellafield but was approximately 1000-1,000,000 greater than the levels detected in the river Tamar. Thus the levels of tritium used in the current study were environmentally realistic in comparison to the release of tritium from some sources in the UK.

The present study (chapters 4 and 5) demonstrates measurable biological effects (genotoxic, cytotoxic and developmental) in the embryo-larval stages of two ecologically relevant organisms. Although the concentrations used in the current study, which were adopted from earlier studies on barnacles and fish larvae (Abbott and Mix, 1979; Strand *et al.*, 1977), could be considered environmentally unrealistic for certain discharge points and realistic for others, the doses delivered during the present experiments were extremely low. This dose range (0.007 – 29.04 mGy) was capable of inducing damage to the genetic material in terms of chromosomal aberrations and sister chromatid exchanges. This study could be considered to be one of the few studies which support a very small number of reports (Abbott and Mix, 1979; Strand *et al.*, 1977) which showed measurable effects in aquatic larvae after exposure to very low doses of β radiation emitted by tritium. The study also throws some light on the potential effects of tritium on the natural environment. While tritiated water, in its own right, is toxic, it has also been found to be converted into organically bound forms and hence is accumulated higher up in the food chain. Although there is no reason for organisms to evolve mechanisms for hydrogen or hydrogen isotope

enrichment. In addition hydrogen is not an essential element. Thus the accumulation of tritium may have severe implications for both human and ecosystem health.

10.2 Effects of ionising radiation on embryo-larvae stages

A few studies have attempted to elucidate the potential impact of genetic damage induced by radiation or chemicals to higher levels of biological organisation. Anderson and Wild (1994) showed that there was a link between genotoxic responses and reproductive success and these mutations were associated with gamete loss, abnormal development, embryonic mortality or heritable mutations in a variety of animal models. After development of *P.dumerilii* embryo-larvae to sexual maturity there appeared to be no effect on the number or sex of the worms that reached adulthood in comparison to control worms (chapter 9). Although at the highest concentration of tritium (370 kBq/ml) there was a reduction in the number of eggs produced from sexually matured female worms. However this study had a very small sample size and was not continued beyond the parental generation into subsequent generations.

P.dumerilii and *M.edulis* embryo-larvae exposed to an environmentally relevant cocktail of radioactive waste (provided by DML) showed an increase in the percentage of abnormal embryo-larvae with increasing concentrations of radionuclides (chapter 6). The cocktail of radionuclides produced a cytotoxic effect, with the proliferative rate index being reduced in both species. The cocktail of radioactive waste was also deemed genotoxic for both the species after analysis using sister chromatid exchanges and chromosomal aberrations. In general *M.edulis* appeared to be more sensitive to ionising radiation than *P.dumerilii* embryo-larvae. In comparison, after exposure of embryo-larvae of both species to the chemical tributyltin, *P.dumerilii* was found to be more sensitive in terms of developmental and genotoxic effects. Thus from the results on experiments on the embryo-larval stages it was shown that the same concentration range of a chemical or radiation may produce differential cytotoxic and genotoxic effects in different species. In conclusion the embryo-larvae stages of the two marine invertebrates examined were

extremely sensitive to exposure by ionising radiation resulting in an increase in cytogenetic damage which in turn lead to an increase in developmental abnormality and/or mortality. Thus in order to protect the environment it is not only important that the most sensitive species are protected but also that the most sensitive life stages are identified and protected.

10.3 Effects of ionising radiation on adult life stages

After exposure of adult *M.edulis* to tritium (chapter 7) there was an increase in the levels of single strand breaks and in the induction of micronuclei in haemocyte cells although no attempts were made to assess the long-term consequences of this exposure in this species. The potential effects of environmentally realistic levels of radioactive waste were then undertaken by analysis of deployed adult *M.edulis* in the vicinity of radioactive discharges (chapter 8). The techniques validated in laboratory conditions were applied to *in situ* monitoring of adult mussels to detect the genotoxic effects of the contaminants and these effects were then correlated with concentrations of heavy metals and radionuclides. While some of the sites demonstrated an increase in the levels of genetic damage (in terms of Mn) in deployed mussels after 3 months *in situ*, the statistical approach adopted to correlate the different environmental factors with observed biological effects showed no correlation between the health of the deployed mussels and the levels of environmentally realistic radioactivity in sediment and water samples. However a significant correlation was demonstrated with concentrations of arsenic and tin and to changes in salinity. No attempt was made to measure the presence of persistent organic pollutants (POPs) in sediments and water and these may have interacted with the radionuclides (see section 10.5.3). Therefore the detrimental effects of radiation on marine organisms from the Tamar estuary can not be unequivocally discounted.

10.4 Population effects of ionising radiation

For wild organisms it is generally the population that is the object of concern in respect of hazards posed by human activities such as ionising radiation. The survival of a population requires that it be maintained though reproduction and growth within the normal range of variability resulting from natural stresses. Therefore in addition to individual morbidity and mortality caused by ionising radiations the process of gametogeneis and embryonic development that underlies reproductive capacity must also be observed (Woodhead, 1998). Effects of contaminants at both community and populations levels are much more ecologically relevant than effects at molecular levels, however they are often difficult to quantify and the techniques used are often expensive and time and labour intensive (Theodorakis *et al.*, 2000). To be of greatest value in determining the implications for ecosystems of exposure to pollution, biological markers should be chosen so that they reflect changes in the fitness of an organism or, in more simple terms, changes that affect its overall reproductive capability (premature death, ability to mate, fecundity, viability of offspring, etc.), as these can have the greatest influence on effects at the higher levels of biological organisation (Evenden and Depledge, 1997). Altered fertility, development, and embryonic survival are environmentally significant because they can reduce reproductive success and thus alter a population's size or structure (Anderson and Wild, 1994). From the current studies ionising radiation was shown to be genotoxic to sensitive embryo-larvae stages of marine invertebrates. However further studies need to be carried out to correlate the effects seen at molecular levels with the potential long-term effects observed at population and community levels of these species.

Genotoxic chemicals can damage the genetic material of humans as well as that of organisms living in the environment (Wurgler and Kramers 1992). With respect to adverse effects, alterations induced in the germline, leading to alterations in the genetic make-up of populations, are of primary concern in ecosystems, because somatic changes, even if they

lead to a loss of individuals, will not be critical in populations with a large reproductive surplus, such as *M.edulis*. This is different in human toxicology where genetic alterations in germ cells as well as in somatic cells of any individual are of concern (Wurgler and Kramers 1992). Exposure to ionising radiation has long been suspected to increase mutation load in humans. Clean-up teams (or 'liquidators') of the Chernobyl reactor were among those who received the highest doses, presumably in some combination of acute and chronic forms. Children born to liquidator families (currently either in the Ukraine or Israel) conceived after the Chernobyl accident (parental exposure to radiation) were screened for the appearance of new fragments using multi-site DNA fingerprinting. Siblings conceived before exposure served as critical internal controls, in addition to external controls (non-exposed families). An unexpectedly high (sevenfold) increase in the number of new bands in exposed individuals compared with the level seen in controls was recorded. These results indicate that low doses of radiation can induce multiple changes in human germline DNA (Weinberg *et al.*, 2001). Multiple genetic changes are required for the development of a malignant tumor cell and many environmentally induced cancers show a delayed onset of > 20 years following exposure (Carls and Schiestl, 1999). Irradiation of male mice was shown to cause a significantly higher frequency of large fur spots in the offspring, indicative of the induction of DNA deletions early in embryo development. However these deletion events occurred many cell divisions after irradiation which indicates that exposure of the germline to ionising radiation results in induction of delayed DNA deletions in offspring mice (Carls and Schiestl, 1999). Yauk *et al.* (2002) showed that induced germline mutation was due to the radiation-induced mutant alleles being present in sperm rather than unrepaired damage leading to the appearance of mutants in embryos. In addition radiation induced (x-rays and fission neutrons) germline instability persisted for at least two generations in male mice, this raises important issues and concerns of risk evaluation in humans, as well as in natural biota (Barber *et al.*, 2002).

Increased frequencies of mutations and related genetic alterations in the gene pools of individual species or populations in ecosystems have to be judged against the background of spontaneous mutations that have enabled species to survive and adapt in changing environments since the beginning of life on our planet, and which have played an important role as the substrate for evolutionary developments. There did not appear to be a radiation-induced detrimental effect on the health of the adult mussels investigated in the vicinity of radionuclide discharge although radiation-induced germline mutations might be observed in later generations. From laboratory studies it has been shown that radiations cause cytogenetic and developmental effects in embryo-larvae of *M.edulis,* therefore a problem might arise in subsequent generations and in recruiting and maintaining a population of mussels in the vicinity of the discharge.

10.5 Radiation effects on marine ecosystems

Various problems are associated with determining the potential effects of ionising radiation in marine ecosystems. For example, what species can be chosen to best detect the potential effects of ionising radiation on the whole community and at what life stages are the effects most pronounced or most relevant? At what level of biological organisation might the effects of ionising radiation be detected and what may be the potential consequences at the subsequent levels? Furthermore, can effects observed in laboratory studies be used to extrapolate the potential problem in natural ecosystems and how can the research into the biological effects of ionising radiation be used in the environmental management of natural ecosystems?

10.5.1 Bioindicator species for radiation exposure

Aquatic organisms and their habitat, play a vital role in the Earth's ecosystem (Jha, 1998), however, there are many difficulties associated with using aquatic organisms to

determine the effects of ionising radiation. One consideration that needs to be addressed is the selection of the most appropriate organism for analysis.

For a species to be selected as a useful indicator species in field conditions (sentinel species), specific criteria needs to be fulfilled. Such as, (a) is it a common or widespread organism, (b) is it ecologically important, (c) is it likely to receive a significant exposure, (d) does it represent a range of lifestyles and feeding habits, (e) is their biology understood, (f) is it experimentally amenable, (e) is it readily cultured in the laboratory and (f) is it sedentary or territorial (Taylor *et al.*, 1999)?

However separate criteria of organism selection is needed for assessing the cytogenetic effects of ionising radiation in aquatic organism. In particular, chromosomal based techniques are often limited in aquatic organisms. Most aquatic organisms have large numbers of very small chromosomes that usually hinder their use in genotoxic assay. Therefore for a species to be considered as a useful indicator of genetic damage it needs to possess a suitable karotype. Invertebrates that match this criteria include the polychaetes *Platynereis dumerilii* (Jha *et al.*, 1995b) and *Neanthes arenaceodentata* (Pesch *et al.*, 1981), and molluscs *Mytilus edulis* and *Mytilus galloprovincialis* (Dixon and Flavell, 1986; Jha *et al.*, 2000b). In addition, the induction of cancer has been relatively well characterised in bivalve molluscs compared to other invertebrates (Dixon *et al.*, 2002) and bivalve molluscs may represent a suitable sentinel species for neoplastic effects in humans due to the recent discovery that a herbicide caused an increase in the incidence of gonadal tumours in both bivalves and man (Van Beneden, 1994).

In terms of ideal bioindicator species, the effects of radiation have been extensively assessed in molluscs species (Nelson, 1971; Longwell and Stiles, 1972; Bonotto *et al.*, 1983) and polychaete worms (Anderson *et al.*, 1990a; 1990b; Knowles and Greenwood, 1994; 1997). In addition *Mytilus* has been used as a bioindicator species to study the long-term variations in radionuclide concentrations (Charmasson *et al.*, 1999).

The present study reconfirms the suitability of *P.dumerilii* and *M.edulis*. While embryo-larvae of both species were suitable for chromosome studies, the haemocytes from adult mussels also provided valuable biological samples to evaluate cytogenetic (Mn) and DNA strand breaks. Attempts to obtain blood or coelomcytes from adult worms were carried out but were not successful due to rapid healing of ruptured areas and coagulation of cells. Despite the numerous number of aquatic species only a few are suitable for ecotoxicological studies (Dixon and Wilson, 2000). Once the appropriate species is selected, the next stage in assessing the significance of ionising radiation is to select the relevant parameters.

10.5.2 Biomarkers of radiation exposure and effect

Biomarkers are defined as the "biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples, or at the level of whole organisms, to provide evidence of exposure and/or effects from one or more contaminants (Depledge, 1994). It is generally considered that physiological responses are capable of quantifying an organism's condition, its performance and the efficiency with which it functions under conditions of environmental stress and pollution (Widdows, 1985). There is general agreement among ecotoxicologists that measuring a suite of biomarkers across levels of biological organisation is often necessary to assess ecological integrity (Clements, 2000). The effects of contaminants on aquatic organisms may be manifested at all levels of biological organisation and these indicators should include biochemical, molecular, individual, population, community and ecosystem responses in order to provide different types of information necessary for ecological risk assessment. Increasing levels of biological organisation results in a decrease of the understanding of the causal mechanism but also results in an increase in the levels of ecological significance (Adams, 2001). A selected suite of measurements along this continuum of levels of organisation is recommended in the design of bioassessment studies in aquatic ecosystems (figure 10.1).

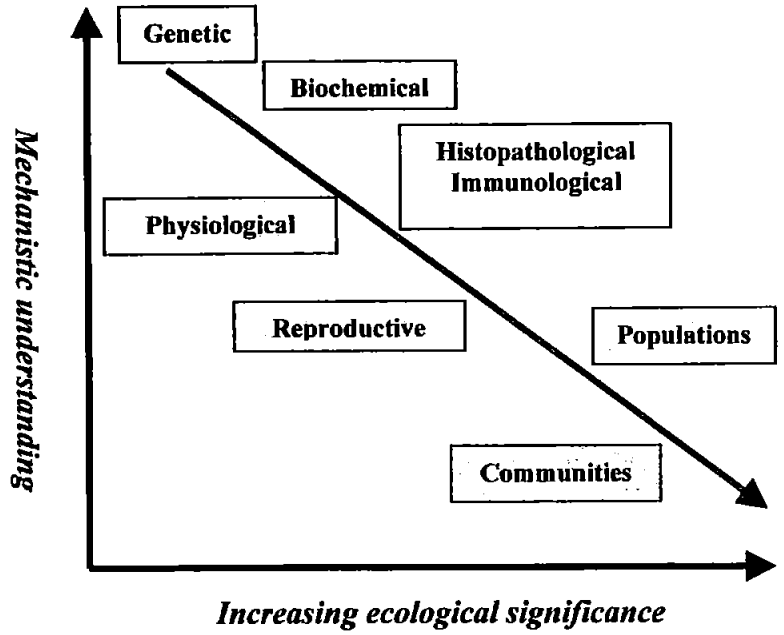


Figure 10.1. Establishment of causal relationships between different levels of biological organisation (adapted from www.esd.ornl.gov website).

The use of indicators of exposure and effects at each of these levels of organisation has particular advantages and drawbacks (Theodorakis *et al.*, 2000). Molecular effects of contaminant exposure are generally manifested within days, hours or even minutes after the onset of exposure and furthermore molecular effects are relatively easy to measure using standard laboratory assays. The effects of contaminants at lower levels of organisation occur more rapidly and may provide early warnings of toxicological effects on populations (Clements, 2000). During the current studies single strand DNA breaks induced after 1h, as detected using the comet assay, were found to correlate with the induction of micronuclei after 48h in haemocytes of *M.edulis* following exposure to ethylmethane sulphonate (EMS), a reference genotoxic agent. This highlights the variation in time that different degrees of DNA damage may be detected at two different levels of biological organisation. In addition the frequency of chromosomal aberrations were shown to correlate with an increase in abnormality and mortality of *P.dumerilii* and *M.edulis* embryo-larvae following exposure to ionising radiation. This again shows that DNA

damage is related to changes at higher levels of biological organisation. Although it is more difficult to determine the ultimate consequences of the manifestation of molecular effects at population or community levels (Theodorakis *et al.*, 2000). A further advantage of molecular biomarkers is that they can be specific indicators of contaminant exposure for a particular class of chemical e.g. trace metals (Geffard *et al.*, 2002; Ivankovic *et al.*, 2002), genotoxicants (Nacci *et al.*, 1996; Perry and Evans, 1975; Schmid, 1975), oxygen free radicals (Benzie and Strain, 1996) etc. What is clear about the field of biomarker research is that it has developed as a response to the need for more sensitive indicators of sublethal ecological effects (Bickham *et al.*, 2000).

DNA damage has been associated with perturbations in fecundity, longevity and growth of affected organisms (Jha *et al.*, 2000a; Steinert *et al.*, 1998; Theodorakis *et al.*, 1999) and may therefore have repercussions on population and higher levels of organisation (Theodorakis *et al.*, 2000). During the current study it was demonstrated that exposure of *P.dumerilii* embryo to tritium (370kBq/ml) for 8h resulted in a reduction in the number of eggs when the female worms reached sexual maturity. However, DNA is the most important target for the action of ionising radiation (UNSCEAR, 1988). The potential exposure to genotoxic substances is one of the most serious concerns of modern day society. Carcinogens and mutagens have the capacity to affect both the structural integrity of DNA and the fidelity of its biological expression (Shugart and Theodorakis, 1994). Ideally for cytogenetic assays there needs to be a rapid turnover of cells and therefore, for most chromosomal based techniques as during the present study the embryo-larvae stage of the species is used. Attempts were made during the current experiments to obtain metaphase spreads from gill cells of adult *M.edulis*, however the number of spreads obtained were very low due to the low cell turnover rate. To overcome the problem of unsuitable karotype and low cell turnover in adult organisms, other techniques may be used that study DNA damage at molecular level or at cellular levels, (i.e. DNA strand breaks, micronuclei induction etc.), thus avoiding the analysis of chromosomes. Because the

techniques and methods that are unique to genetic ecotoxicology are extremely sensitive and specific it is anticipated that their implementation into studies concerned with the mechanism of action of genotoxicants will provide a stronger scientific basis for the assessment of risk exposure (Shugart and Theodorakis, 1994). However it is imperative that the assays are properly validated against a range of concentrations of reference genotoxic agents to ensure sensitivity, reproducibility and reliability of the biomarkers in a specific bioindicator specie (Dixon *et al.*, 2002).

As mentioned above the most important and reliable parameter in terms of quantifiable damage and significance is the analysis of chromosomal aberrations however they are not generally applicable to field studies and thus are limited to laboratory work. This leads to the inherent problem of assessing the effects of ionising radiation on marine organisms in relevant environmental situations.

10.5.3 The effects of radiation on a complex environment

During the present study tritium was shown to induce both single strand DNA breaks (comet assay) and micronuclei in laboratory based experiments. In field based experiments, *M.edulis* transplanted in an area of radioactive waste discharge was also found to contain elevated levels of single strand DNA breaks and micronuclei, although there was no statistical evidence to suggest that this was a response to exposure to ionising radiation. Instead the DNA damage correlated to changes in salinity and concentrations of the heavy metals arsenic and tin. Complex interactions of physical, chemical and biological factors may have acted to disperse, dilute or concentrate the radioactive substances in the estuary, for example studies with rainbow trout (*Oncorhynchus mykiss*) have suggested that heavy metals reduced the accumulation of radionuclides (Ausseil *et al.*, 2002). Furthermore as well as the interaction of radionuclides with other radionuclides and chemicals the effects of radiation in terms of bioaccumulation may also be influenced by environmental factors such as salinity and temperature. Experiments on the blue crab

found that radiation did interact with salinity and temperature resulting in an alteration of ionic regulation (Engel, 1973). Temperature dependent bioaccumulation of radiation has been reported in macroalgae species and the brittle star *Ophiothrix fragilis* (Hutchins *et al.*, 1996) and changes in salinity have been shown to influence bioaccumulation in isopods, macroalgae, green mussels and fish species (Ke *et al.*, 2000; Topcuoglu, 2001). Therefore there is a possibility that if *M.edulis* were transplanted into the same location during the summer months there may be an increase in the uptake of the radionuclides due to the increase in temperature. This aspect needs further elucidation in the natural environment.

In addition to the physical, chemical and biological factors that may disperse or affect bioaccumulation of radioactive substances in estuarine and marine environment, these same factors may also influence the toxicity of the radiation. Recent toxicology studies, particularly in the discipline of aquatic toxicology, have reported that polycyclic aromatic hydrocarbons (PAHs) may become toxic or substantially more toxic upon co-exposure to UV irradiation (300-400 mm) (Arfsten *et al.*, 1996). For example, enhanced toxicity of *Daphnia magna* was reported after exposure of the organic PAH pyrene and retene (7-isopropyl-1-methylphenanthrene) to UV irradiation (Nikkila *et al.*, 1999; Huovinen *et al.*, 2001). UV was shown to enhance the phototoxicity of PAH contaminated sediments by five to eight-fold after toxicity testing using amphipods compared to PAH contaminated sediments not exposed to UV irradiation (Boese *et al.*, 2000). It is also possible that the genetic damage observed in *M.edulis* during the *in situ* transplant study may have been a result of the interaction of UV irradiation and PAHs. As mentioned in chapter 6, combined alpha, beta and gamma irradiation produced higher mutation rates in barley pollen compared to similar doses from an external gamma source (^{60}Co) in isolation (Bubryak *et al.*, 1992). Furthermore several studies have suggested that different radiations may interact synergistically or additively to produce genetic damage in humans (Holmberg and Jonasson, 1974; Holmberg and Strausmanis, 1983). Again this aspect needs further elucidation in the natural environment. For example, it is not clear how the β-radiation of

different qualities, emitted by ^{3}H and ^{14}C and accumulated in mussels, interacts to produce biological effects.

Variation in individuals, communities and populations may be attribute to the underlying genetic makeup of the organisms or it might be due to abiotic factors that depend on the geography of the ecosystem and the physiochemical interaction of the contaminant (Evenden and Depledge, 1997). Thus, in a complex environment where contaminants occur, in all probability it is almost impossible to state that ionising radiation alone is causing detrimental effects (in field studies), as it could be other substances or a combination of factors.

Laboratory studies under controlled conditions have traditionally been used in environmental management to assess the potential toxicity of contaminants. Results from laboratory based studies are then used to ensure that concentrations of contaminants do not exceed safe or acceptable levels in the environment (Evenden and Depledge, 1997). Although this approach has often been criticised as it does not consider the variation in the susceptibility for the toxic effects of contaminants that can be exhibited by a large number of animal and plant species found in the natural environment. The United States Environmental Protection Agency (USEPA) use a "Triad" approach to evaluate contaminated sediment, this is a weight-of-evidence approach that incorporates measures of sediment chemistry, sediment toxicity, benthic community composition (Chapman *et al.*, 1987). The use of chemical and biological monitoring improves the overall understanding of contaminant effects in complex environments (Jha *et al.*, 2000a). While performing the transplant studies, it would have been useful if this "triad approach" could have been adopted. This would have provided more detailed information pertaining to potential toxic effects of contaminants.

10.6 Effects of ionising radiation on the food chain

The use of wildlife species as sentinels of environmental problems is the conceptual basis for linking ecological effects and human health however it is difficult to directly link the two (Bickham *et al.*, 2000). There is a possible direct link between wildlife species and humans after exposure to ionising radiation through the food chain, via accumulation of radionuclides and hence enhancement of toxicity. Beta particle emitting radionuclides, in particular tritium, are of increasing concern if they are ingested. Due to their short wavelength they may cause damage to tissue and organs if they are internally present in the body. Numerous discussions have occurred regarding the importance of environmental tritium and the possibility of uptake via the food chain (Moghissi *et al.*, 1973). Figure 10.2 represent a basic interpretation of the possible routes of uptake by tritium in the environment.

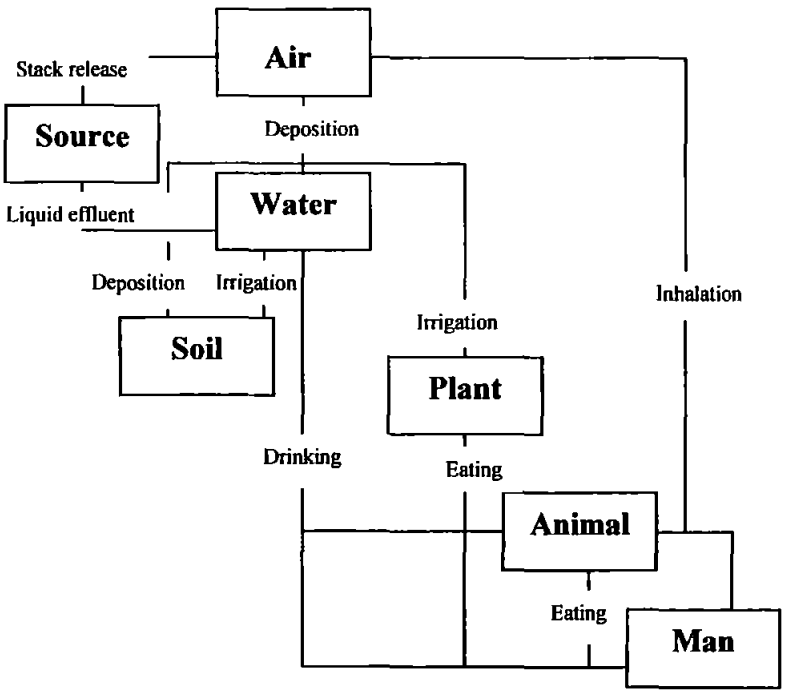


Figure 10.2. Simplified model for the possible transport of tritium in the environment (adapted from Moghissi *et al.*, 1973).

During the present study no attempts were made to link the effects of tritium on the food chain, however both *P.dumerilii* and *M.edulis* are of ecological importance. *M.edulis* is of both commercial and ecological importance as they may be consumed by higher predators and directly by humans. Hence any radionuclides present in the tissues of mussels may be directly passed on to humans. Whilst *P.dumerilii* is not of direct nutritional value to humans, due to their numerical abundance, digestibility and high energy content, polychaetes, in general, play a fundamental role as prey items for a wide range of commercially important fish species and, in mudflats and similar coastal environments, for wading birds (Hutchinson *et al.*, 1995). Thus from the present study, ionising radiation was shown to have a detrimental effect on *P.dumerilii* and *M.edulis*, in terms of cytogenetic and developmental. However, the radionuclides might also bioaccumulate up the food chain, causing addition damage. Alarmingly high levels of Technetium 99 (Tc-99) were detected in seaweed samples from the West Cumbrian coast in 1997 due to inputs from BNFL Sellafield (Environment Agency, 1998). Via the food chain Tc-99 is now found in duck eggs, and the use of locally harvested seaweed as a garden fertiliser has led to the discovery of Tc-99 in locally grown spinach. Irish Sea lobsters have shown a similar alarming rise from 210 Bq/Kg in 1993 to 52,000 Bq/Kg in 1997 – over 40 times the EU Food Intervention Level set as a safety level for foodstuffs contaminated following a nuclear accident (Environment Agency, 1998).

Although tritiated water is not supposed to bioaccumulate, some studies have reported an increase in accumulation. Brine shrimp (*Artamia salina*) and Japanese killifish (*Oryzias latipes*) that were grown in tritiated water and fed on a diet of tritiated exposed diatoms (*Chaetoceros gracilis)* were shown to accumulate tritiated water by 40 and 110% respectively compared to shrimp and fish that were grown solely in tritiated water (Komatsu *et al.*, 1981). Questions have also arisen as to the possible cause of elevated levels of tritium reported in fish and shellfish from the River Severn, in Cardiff (Edwards, 1998; Pearce, 1999; Walland, 1998). Fish and shellfish from the Cardiff area showed

tritium concentrations of 748 and 605 times the seawater concentration respectively. However no other reports of significant bioaccumulation of tritium have occurred following analysis of effluent discharges from other nuclear licensed sites (NCAS, 2001). Possible reasons for the increases in total tritium concentrations in fish and shellfish from Cardiff, are that there was an increase in discharges of organically bound tritium (OBT) or that there was a change in the form of OBT discharged which was preferentially taken up by the fish and shellfish. Other possibilities are that the OBT was recycled in the Severn Estuary perhaps due to movements of sediments or it may have been due to natural variability in fish (NCAS, 2001). At DML the process giving rise to tritium discharged is one in which water in the primary cooling circuit of the nuclear reactor in a nuclear submarine is activated by capturing neutrons originating from the nuclear reaction in the reactor core (Environment Agency, 2001). Therefore the tritium present is in the form of tritiated water. The only means by which OBT could be formed is if organic contaminants (e.g. oil) are present in the primary circuit or in effluents when the circuit is drained. However oil and other organic contaminants are not normally present in the water due to the very tight quality control of the primary circuit. Therefore it is highly unlikely that tritium produced from DML would be converted to and occur as OBT in significant amounts in the effluents (Environment Agency, 2001). However more information needs to be provided as to the chemical fate (speciation, partitioning, transport and degradation) of tritium in the marine environment.

In particular, the transfer of tritium between the atmosphere and the terrestrial and aquatic environments involves more complex processes than that of most other radionuclides, primarily because of the fundamental role played by hydrogen in biological systems (Higgins *et al.*, 1996). The absorption and transfer to the blood of ingested tritiated water is assumed to be rapid and complete with 100% absorption occurring (Harrison and Stather, 1998). In addition distribution throughout the body is taken to be uniform although it is believed to have a biological half life in the body of 10 days in adults with excretion

occurring via urine, sweat, faeces and exhalation through the lungs (Harrison and Stather, 1998). Tritium in organic forms is likely to be present in a wide variety of chemical compounds including proteins, carbohydrates, fats and nucleic acids. It is assumed that organically bound tritium is completely absorbed after ingestion and that 50% of the absorbed tritium will become organically bound in the body and is retained with a biological half life of 40 days (Harrison and Stather, 1998). However, Harrison and Stather (1998) also state that there is little evidence that the distribution of tritium within the cells after intakes of tritiated water and organically bound tritium will lead to preferential delivery of dose to DNA and increased relative biological effectiveness (RBE). During the present study tritiated water was found to induce DNA damage in embryo-larvae cells of *P.dumerilii* and *M.edulis* and in haemocyte cells of adult *M.edulis*. However it is not clear whether the tritium taken up by the cells and producing biological effects were tritiated water or if they were converted into organic tritium.

Other radionuclides have also been reported to accumulate and magnify in the food chain. For example, cesium-137 was found to be biomagnified by a factor of 3-4 in tissue of caribou after consumption of caesium exposed lichens in Arctic regions of Canada (Thomas and Gates, 1999). The cesium present in the lichens was a result of uranium mining impacts. The caribou are a staple source of food for many native people in Northern Canada and the lichen-caribou-human food chain is likely to expose humans to elevated levels of radiation (Thomas and Gates, 1999). Dietary studies and whole-body measurements were made in different groups of people in Norway after the Chernobyl accident to estimate the intake of radiocesium and the radiation dose received (Strand *et al.*, 1992). Freshwater fish, milk, and reindeer meat were the major sources for radiocesium intake. Dietary advice, together with agricultural decontamination measures, resulted in a considerable reduction in the exposure level of the population. A majority (40-80%) of the specially selected groups (farmers-hunters and Sami reindeer herdsman) changed their diet significantly after the accident. Without dietary changes, specifically a reduction in the

consumption of freshwater fish and reindeer meat, the Sami group would have had a 400-700% higher radiocesium intake, and the farmers-hunters' intake would have been up to 50% higher than what they actually had experienced (Strand *et al.*, 1992).

10.7 Ionising radiation in the 20th century and beyond

10.7.1 Environmental contamination by ionising radiation

Before 1942, human exposure to ionising radiation was limited to natural radioactivity and medical diagnosis (Ahier and Tracy, 1995). Since, then the uses of nuclear energy have become more diverse and widespread, encompassing medical diagnosis and treatment, nuclear power, and consumer and industrial applications (Ahier and Tracy, 1995). The discharges of radionuclides have been documented worldwide and a few examples are given below.

In Canada, the greatest contribution to total radiation exposure to residents of the Great Lakes Basin is natural background radiation, with the largest input from anthropogenic sources arising from fallout from atmospheric nuclear weapons tests conducted (Ahier and Tracy, 1995). The Great Lakes basin is an area of radiological interest due to both actual and potential exposures that may be received by its 36 million residents that are supported by the basin. The radionuclides of greatest concern in the basin, from a health perspective and in terms of the potential for normal and accidental release from the nuclear fuel cycle facilities are ^{3}H, ^{14}C, ^{90}Sr, ^{131}I, ^{137}Cs and ^{226}Ra. However water samples collected from the Great Lakes indicate a general decrease in levels at a rate of 2 to 5% per year (Ahier and Tracy, 1995). A further concern for most Canadians is the transfer of radionuclides in the food chain. As previously mentioned the most critical food chain in the world for concentrating airborne radionuclides is the lichen-caribou-human food chain, as lichens are the main winter forage for caribou, which in turn are a main dietary staple for many northern Canadians (Thomas and Gates, 1999). Thus

airborne radionuclides, particularly ^{137}CS, ^{210}Pb and ^{210}Po are transferred efficiently through this simple food chain to people elevating their radiological dose.

In the UK, Sellafield in Cumbria releases the highest levels of diverse radioactivity to the sea compared to other licensed nuclear facilities in the UK. (Watson *et al.*, 1999). The activity concentration of the radionuclides ^{137}Cs, ^{238}Pu, $^{239,240}Pu$ and ^{241}Am have been measured in root mat and vegetation samples collected from tide washed pastures in 17 estuaries spanning the eastern seaboard of the Irish Sea (Sanchez *et al.*, 1998). Spatial distributions of the radionuclides correlated with the transport of radionuclides discharges to the Irish Sea from the Sellafield Nuclear Reprocessing Plant with highest activities occurring closest to Sellafield. Radionuclides (^{137}Cs and ^{40}K) in harbour porpoises, *Phocoena phocoena*, showed that there were elevated concentrations of ^{137}Cs in porpoises originating from the Irish Sea compared to porpoises from the Celtic Sea, the Atlantic Ocean and the North Sea (Berrow *et al.*, 1998). Watson *et al.* (1999) carried out analysis of radionuclides (^{134}Cs, ^{137}Cs, ^{238}Pu, $^{239,240}Pu$) in liver and muscle tissue from dead seals and porpoise found stranded around the UK coast. The activity concentration of radiocesium in the tissues of both seals and porpoises was found to decrease with distance from Sellafield. The marine mammals concentrated radiocesium from their environment by a factor of 300 relative to the concentration in seawater and the importance of using marine mammal tissue as an indicator of radionuclide contamination was highlighted (Watson *et al.*, 1999). However the overall average dose received from radiocesium was less than 10% of the dose from the naturally occurring radioisotope of potassium ^{40}K (Berrow *et al.*, 1998; Watson *et al.*, 1999).

The largest accidental release of radionuclides occurred on the 26th of April 1986, when the Chernobyl Nuclear Power Plant exploded and caught fire (Jagoe, *et al.*, 1997). At least 2-4 x 10^{18} Bq of radioactivity was released. There have been a number of studies in radionuclides in the Chernobyl area since the accident. Samples of fish and sediments collected from waters within 10K of the Chernobyl Nuclear Power Plant have been

analysed for radiocesium (Jagoe *et al.*, 1998). Radiocesium concentrations in the muscle of fish were highest in ponds from sites downwind to Chernobyl and were well correlated with the concentration of radiocesium in sediments. Chesser *et al.* (2000) found that dose rates of radionuclides near the Chernobyl reactor, in some areas exceed those reported to impede reproductive success in mammals.

After ionising radiation and radioactivity were discovered at the end of the 19th century, their social perception has alternated between enthusiastic acceptance and rejection. This stemmed from the recognition that radiation was useful for medical applications and for technical and scientific aims, secondly that they had beneficial effects at low levels and finally that at high levels they were harmful (Jaworowski, 2002). The fact that ionising radiation was hazardous to man was first published in 1896 and the first fatal victim of ionising radiation was a German engineer, F. Clausen in 1900 (Jaworowski, 2002). However it was not until the 1920s that the concept of radiation protection was first considered with the introduced of a "tolerance dose" to humans. Originally the annual dose was set at 700 mSv although today the value is only 1 mSv (Jaworoski, 2002). This indicates that our knowledge of the effects of radiation has improved and as a consequence so too has the need for legislation on the use of radiation for the protection of human health and the environment. In order to reduce the emission of greenhouse gases more dependence on nuclear power is anticipated (Royal Commission on Environmental Pollution, 2000). In addition, advancement in military capabilities will also require increased use and discharges of radionuclides in the environment (e.g. depleted uranium, tritium etc.).

10.7.2 Legislation for radiological protection of humans

From the information presented above it is clear that the use and disposal of ionising radiation needs to be carefully regulated to reduce the risk of radiation exposure to humans and the environment. The United Kingdom Atomic Energy Authority (UKAEA) was

created in 1954, from the recognition that new legislation was needed to provide adequate protection of the environment against pollution that might result from the expanding nuclear development programme (DEFRA, 1999). The Atomic Energy Authority Act (1954) removed responsibility for the control of radioactive discharges from local authorities and provided a system of central control, whereby no such discharges could be made from UKAEA sites unless authorised by the relevant Government Ministers. The Radioactive Substances Act 1960 was the first comprehensive legislation applying to all radioactive discharges in the UK (RSA, 1960). Today, radioactive wastes, including discharges, are closely regulated under the Radioactive Substances Act 1993, by the relevant competent authorities (RSA, 1993). These authorities are the Environment Agency in England and Wales, the Scottish Environment Protection Agency in Scotland, and the Environment and Heritage Service in Northern Ireland (DEFRA, 1999). It has been the UK's policy since 1986 that members of the public should not be exposed to a dose of more than 1 mSv/y from all man-made radiation sources, other than from medical exposure. This was the dose limit recommended by the International Commission on Radiological Protection (ICRP) in 1990 (ICRP 60) and is also the dose limit set in the 1996 Euratom Basic Safety Standards Directive (DEFRA, 1999).

The International Commission on Radiological Protection (ICRP) is an independent Registered Charity that was founded in 1928 to advance the science of radiological protection for the public's benefit. In order to achieve this it provides recommendations and guidance on all aspects of protection against ionising radiation (Lindell *et al.*, 2002). In the UK the government relies on the Environment Agency to regulate and control the discharge of radiation into the environment (DEFRA, 1999). For the Agency to make a comprehensive decision they need to consider complex arguments about radiation protection, epidemiology and radiobiology and to achieve a well rounded, conclusive decision they often rely on advice given by the National Radiological Protection Board (NRPB).

The NRPB was created by the Radiological Protection Act 1970 and has a statutory responsibility for advising UK government departments, and those with responsibility for using ionising and non-ionising radiation, on the risks to human beings imposed by the use of such radiations (Bodmer, 2000). This applies in all areas: medical, public health, occupational and environmental and is generally achieved by means of research (Dunster, 2000). In 1995 the Director of NRPB set up the Advisory Group on Ionising Radiation (AGIR). The main responsibility of the AGIR was to review work on the biological and medical effects of ionising radiation relevant to human health in the occupational, public health, medical and environmental fields and to advise on research priorities (NRPB website, 2002). In order to achieve the general aim of the AGIR they have initially focused their efforts on four priority areas of work (i) genetic heterogeneity of response to radiation (ii) guidance on the promotion of further optimisation of medical exposures (iii) epidemiology of secondary cancer and (iv) reassessment of the risks of radiation-induced cancers in the UK (NRPB website, 2002).

There has been a long history of nuclear development in the UK. As a consequence of this the UK has been able to build an internationally recognised base of expertise in radiation protection (DEFRA, 1999). However due to the extensive use of radiation in the UK there is now a historic legacy of radioactive waste and of contaminated plants and equipment which needs to be dealt with in a way that protects both human health and the environment (DEFRA, 1999).

10.7.3 Legislation for radiological protection of the environment

A pivotal point for international co-operation to combat marine pollution in the North-East Atlantic occurred with the grounding of the Torrey Canyon in 1967 which released 117,000 tonnes of oil into the environment with disastrous consequences (Petrow, 1968). This incidence stimulated the signature, in 1969, of the Agreement for Cooperation in Dealing with Pollution of the North Sea by Oil (the "Bonn Agreement") (OSPAR,

1993). The members of the Bonn Agreement were Belgium, Denmark, European Community, France, Germany, the Netherlands, Norway, Sweden, the United Kingdom of Great Britain and Northern Ireland.

On the 16th of July 1971 a Dutch ship, the "Stella Maris" sailed from the port of Rotterdam to dump chlorinated waste in the North Sea, but because of the combined weight of public opinion and of the Governments of several countries it was obliged to return to port on the 25th of July without carrying out its mission (OSPAR, 1993). Within 8 months of this event, in February 1972, the agreement and signature of the Convention for the Prevention of Marine Pollution by Dumping from Ships and Aircraft (the "Oslo Convention") was carried out and was entered into force in 1974 (Tromp and Wieriks, 1994). The task appointed by the Oslo Commission (Belgium, Denmark, Finland, France, the Federal Republic of Germany, Iceland, Ireland, the Netherlands, Norway, Portugal, Spain, Sweden and the United Kingdom of Great Britain and Northern Ireland) under the Oslo convention was to regulate and control the dumping at sea of industrial wastes, sewage sludges and dredged material and the incineration at sea of liquid industrial wastes (OSPAR, 1993).

It was also felt necessary at this time to draw up a similar document, dealing not with the prevention of marine pollution by dumping, but instead with the prevention of marine pollution by discharges of dangerous substances from land-based sources, watercourses or pipelines (OSPAR, 1993). Negotiations on this topic resulted in the completion of the Convention for the Prevention of Marine Pollution from Land-Based Sources (the "Paris Convention") which was signed in June 1974. The Commission appointed under the "Paris convention" regulated and controlled inputs of substances and energy to the sea via the atmosphere and from land-based sources: rivers, pipelines, direct discharges and also offshore platforms (Perex, 1989). It also embarked on a series of measures to protect parts of the maritime area which were affected by nutrients and which have been linked to the occurrence of abnormal algal blooms. The Convention entered into

force on the 6th of May 1978. It has been signed by Luxembourg, and has been ratified by: Belgium, Denmark, the European Economic Community, France, the Federal Republic of Germany, Iceland, Ireland, the Netherlands, Norway, Portugal, Spain, Sweden and the United Kingdom of Great Britain and Northern Ireland (OSPAR, 1993).

A meeting of the Oslo and Paris Commissions at Ministerial level held in Paris on 21-22 September 1992 gave rise to the adoption of a new Convention for the Protection of the Marine Environment of the North-East Atlantic (the "OSPAR Convention") (Tromp and Wieriks, 1994).

Contained within the OSPAR Convention, as adopted in 1992, were a series of annexes that dealt with the following specific areas:

- Annex I: Prevention and elimination of pollution from land-based sources;
- Annex II: Prevention and elimination of pollution by dumping or incineration;
- Annex III: Prevention and elimination of pollution from offshore sources; and
- Annex IV: Assessment of the quality of the marine environment.

The contracting parties (Belgium, Denmark, European Union, Finland, France, Germany, Iceland, Ireland, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom) to the OSPAR convention have adopted the following objective and strategy for the purposes of directing the future work of the Commission with regard to radioactive substances:

> *"To prevent pollution of the maritime area from ionising radiation through progressive and substantial reductions of discharges, emissions and losses of radioactive substances, with the ultimate aim of concentrations in the environment near background values for naturally occurring radioactive substances and close to zero for artificial radioactive substances"* (OSPAR, 1993).

Since the introduction of the general legislation to regulate the use of radioactive substances (the Radiological Substances Act 1960) (RSA, 1960) the UK has consistently

applied the radiological protection principles and regulatory arrangements in order to reduce the levels of radioactive discharges and the doses of ionising radiation to both humans and the wider environment (DEFRA, 1999). In addition to the OSPAR strategy the UK is also developing its own national strategy for reducing radioactive discharges (DEFRA, 1999). The UK strategy will implement the OSPAR strategy for radioactive substances that was agreed at the 1998 meeting of the OSPAR Commission in Sintra, Portugal. The OSPAR strategy requires that, by the year 2020, discharges and losses of radioactive substances will be reduced to levels where the additional concentrations in the marine environment above historic levels, resulting from such discharges, emissions and losses, are close to zero (DEFRA, 2002).

The OSPAR Strategy introduced the factor of environmental concentrations into a mechanism for controlling radioactive discharges that had previously been driven primarily by considerations of human radiation protection. The challenge in implementing the OSPAR Strategy with regard to Radioactive Substances is to strike an appropriate balance between measures to protect human health (including the health of workers at nuclear installations) and measures to protect the environment, whilst taking account of other legitimate uses of the sea (DEFRA, 2002). At present, an internationally accepted method for assessing the environmental impact of ionising radiation does not exist. Up to now the approach taken has relied on recommendations from the ICRP first made in 1977, and modified in 1990 (Copplestone *et al.*, 2001). The ICRP states that the standard of environmental control needed to protect humans will ensure that other species will not be at risk (ICRP, 1991) as it considers man to be the most radiosensitive organism (DEFRA, 1999). However as previously mentioned this statement can be challenged due to the lack of cited evidence from the ICRP to support it. Furthermore, it fails to protect environments where no humans inhabit (e.g. oceans) and biota in certain habitats might be exposed to more harmful doses below the recommended human exposure limits (Copplestone *et al.*, 2001). At present, for ionising radiation, the research carried out into aquatic organisms

after exposure to ionising radiation is not generally used in respect to environment management strategies. The ICRP 60 states that the standard of environmental control needed to protect man to the degree currently though desirable will ensure that other species are not put at risk (Pentreath, 1998). Although they later added that individual members of non-human species might be harmed but not to the extent of endangering whole species or creating imbalance between species.

After consideration of the factors mentioned above the results of research into the effects of ionising radiation on marine organisms can be used to assess the risk assessment of the ionising radiation and the consequential recommendations to protect mankind and his environment. However, environmental management is undoubtedly a difficult occupation (Evenden and Depledge, 1997). The field of risk assessment has evolved to the point where ecological indicators are employed as well as traditional environmental chemistry and human health assessments (Bickham *et al.*, 2000). Many consider it impossible for researchers to accurately predict the consequences of exposures to natural ecosystems to particular levels of contamination (Evenden and Depledge, 1997). Although with the ever expanding use of nuclear fuel, our knowledge, via research into the detrimental effects of radiation, will also expand and with this increase in understanding we will be able to ensure that the environment in which man inhabits will also be protected as well as mankind itself.

Appendix 1

Average Generation Time (AGT) for embryo-larvae cells of *P.dumerilii*

Based on previous work carried out by Dr Awadhesh Jha at Astra Zeneca Ltd., in Brixham, Devon, and Ivett and Tive (1982) the average generation time of *P.dumerilii* embryo-larvae cells was calculated as followed.

Gametes and subsequently embryo-larval stages of *P.dumerilii* (cultured in the laboratory) were obtained as discussed in chapter 2. 2 male and 2 female worms were allowed to spawn. Gametes were fertilised and approximately 17,200 embryos were produced with a 97.2 ± 1.3% fertilisation rate. Following fertilisation, the developing embryos were allowed to grow in an incubator at 20 ± 1° for 24h. A minimum amount of 5-bromodeoxyuridine (BrdU: 1x 10^{-5} M) dissolved in seawater was added to the embryo-larvae at 9, 7 and 4.5hrs prior to the end of the 24h. Following the 24h period the embryo-larvae were processed for analysis of proliferative rate as described in section 2.3.3. The generation time was calculated by dividing the number of hours in BrdU by the proliferative rate index (PRI) e.g. 9 / 2.6 = 3.46.

Sample	Hrs in BrdU	PRI	Generation time
1	9	2.6	3.46
2	7	2.36	2.97
3	4.5	1.74	2.59

The average generation time (AGT) was calculated by taking an average of the generation times (3.46 + 2.97 + 2.59 = 9.02) (9.02 / 3 = 3.00h). Thus the average generation time for 24h *P.dumerilii* cells was therefore calculated to be approximately 3h.

Appendix 2

Do Not
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Appendix
2, 3, or 4

Environmental and Molecular Mutagenesis 35:343–350 (2000)

Tributyltin Induces Cytogenetic Damage in the Early Life Stages of the Marine Mussel, *Mytilus edulis*

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Using an integrated approach, the genotoxic potential of bis(tri-n-butyltin), an antifouling agent known to disrupt endocrine system in marine invertebrates, has been evaluated in the embryo-larval stages of the edible mussel, *Mytilus edulis*. While evaluating the genotoxic potential, the toxicity of tributyltin was also taken into account by determining the maximum tolerated dose. The study suggested that tributyltin is capable of inducing cytogenetic damage (sister chromatid exchanges and chromosomal aberrations) in this target species. The study emphasises the need for further investigations of the potential genotoxic effects of this and other endocrine disrupters on ecologically relevant aquatic invertebrates, which contribute to the maintenance of ecosystems and that could potentially be harmful to human health via the food chain. Environ. Mol. Mutagen. 35:343–350, 2000. © 2000 Wiley-Liss, Inc.

Key words: organotin compounds; endocrine disrupting agents; genotoxicity; cytotoxicity; developmental toxicity; marine mussel

INTRODUCTION

Tributyltin compounds (viz. bis(tri-*n*-butyltin)oxide (TBTO) and tributyltin fluoride) have been actively used in antifouling paints. In addition to their use as biocides, these organometallic compounds have also been utilized as catalysts for the production of polyurethane foams. However, these exclusively anthropogenic compounds are also considered to be one of the most toxic agents entering the marine environment. The effects of the leachate from antifouling paints on marine organisms first became apparent in the late 1970s when it was suspected to be the cause of declining oyster production in France and subsequently in different parts of the world [Alzieu, 1991]. This led to the implementation of legislation in Europe and North America to restrict the use of tributyltin (TBT)-based paints on small boats (< 25 m in length), and in aquaculture in order to reduce concentrations around marinas and in estuaries. However, TBT from larger vessels, which were excluded from the ban, is also known to have an impact on marine populations and therefore remains an environmental problem of concern in different parts of the world [Morgan et al., 1998].

TBT compounds are known to produce a variety of pathologic conditions in marine organisms. In terms of sensitivity, however, none rivals that of the "imposex" response in gastropod mollusks, wherein male sex organs, notably a penis and sperm duct (vas deferens), are superimposed onto the female of gonochoristic gastropods. This masculinization phenomenon is considered to the best example of disruption of the endocrine functions among invertebrates unequivocally linked to a specific environmental pollutant. This dramatic effect on the reproductive organs is reported to be caused by an increased level of testosterone titers that masculinize TBT-exposed females, although the mechanisms by which increased levels of testosterone are produced has not been fully elucidated [Matthiessen and Gibbs, 1998]. In this context, it is well known that some hormones, especially estrogens, act as cellular proliferators and therefore could act as cancer promoters or epigenetic carcinogens [Tsutsui and Barrett, 1997]. Growth-stimulating effects may, in addition, promote cancer by increasing the proliferation rate of cells that have acquired a mutation either spontaneously or after exposure to xenobiotic agents. However, new evidence indicates that hormonal metabolites may directly induce damage in the genetic material, both under in vivo and in vitro conditions and therefore can initiate the multistage process of carcinogenesis [Tsutsui and Barrett, 1997; Service, 1998; Roy and Liehr, 1999]. In view of these studies, it is perhaps not surprising that the presence of environmental contaminants that inadvertently influence the hormonal metabolism has caused concern with

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respect to increasing evidence of some cancers, damage to reproductive systems, and developmental problems reported in both humans and wild life.

While exposure of wild species to potentially genotoxic contaminants could be a threat to human health via the food chain, such damage, if induced in the genetic material of natural biota, might have significant implications for their short- and long-term survival and consequently the ecosystem [Wurgler and Kramers, 1992; Anderson et al., 1994]. While several field and laboratory studies have demonstrated the abnormal development (including those of sex organs) and adverse effects on the genetic and population structure of marine populations after exposure to TBT [Alzieu, 1991; Matthiessen and Gibbs, 1998; Tanguy et al., 1999], in light of the work overviewed above, there has been no serious attempt to evaluate potential genotoxic effects of agents (causing hormonal imbalance) in wild species. In this study, therefore, we aimed to evaluate the potential genotoxic effects, if any, of TBT using the embryo-larval stages of an ecologically relevant invertebrate species, the edible mussel, *Mytilus edulis*. This species has been widely used for ecotoxicologic and genotoxicologic studies in the past [Harrison and Jones, 1982; Dixon and Prosser, 1986; Jha et al., 2000). Using an integrated approach by linking different levels of biologic organization and taking into account the toxicity as a major confounding factor for genotoxicity testing, we have evaluated the induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (CAbs) in the embryo-larval stages of this target organism. The cytotoxicity was evaluated in terms of the proliferation rate of the embryo-larval cells using sister chromatid differential (SCD) staining whereas growth, development, and survival of the individual embryo-larvae determined developmental toxicity.

MATERIALS AND METHODS

Collection and spawning of animals

Adult specimens of mussel (*Mytilus edulis)* (collected from Whitsand Bay, Cornwall, UK, a site considered to be uncontaminated), were maintained in the aquarium to obtain gametes and subsequently embryo-larval stages, as discussed elsewhere in details (Jha et al., 2000). After fertilization, the developing embryos were allowed to grow (in duplicate) in the incubator (15 ± 1°C) for 12 hr. These 12-hr-old embryos were then exposed to different concentrations of TBT for a period of either 6 or 8 hr. This exposure period corresponded to approximately 1.5 and 2 cell cycles, respectively (Jha et al., 2000). At the end of these exposure periods, subsamples of the growing embryo-larvae were collected for chromosome preparations and the rest of the samples were allowed to grow for 3 days to evaluate the development and survival of the individual embryo-larvae. Initially, TBTO (obtained from Lancaster Synthesis Ltd., UK) was dissolved in acetone to provide a stock solution of 100 mg l^{-1} (97.3 mg l^{-1} as TBT), which was later diluted for use. A concurrent solvent control (0.01% (v/v) acetone) was also used in the experiments. A minimum amount of 5-bromodeoxyuridine (BrdU; 1×10^{-5} M) dissolved in seawater was used to elucidate SCD staining and analyses of SCEs. The growing embryo-larvae were also concurrently exposed to a single concentration of a reference mutagen, methyl methane sulfonate (MMS; 1.0×10^{-3} M) that served as positive control for the cytogenetic end points [Jha et al., 1996, 2000). The experimental protocol, including the evaluation of developmental, cytotoxic, and genotoxic effects, and linking different levels of biologic organization, is summarized in Figure 1.

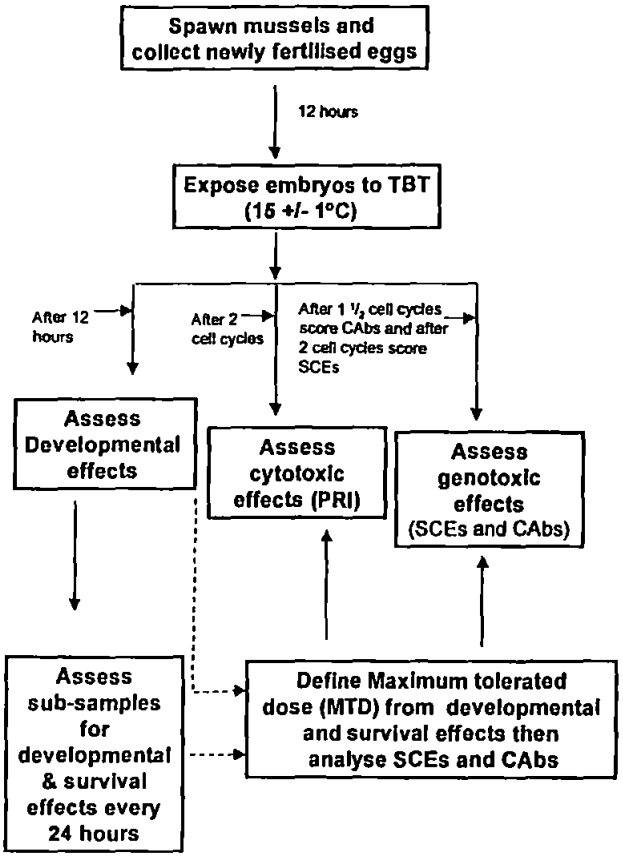

Fig. 1. Diagrammatic representation of the protocol followed to study the genotoxic effects of tributyltin (TBT).

Determination of maximum tolerated dose

Toxicity of chemicals is known to be one of the major confounding factors in interpreting results of testing for genotoxicity [Mendelshon et al., 1992]. In mammalian in vivo genotoxicity assays, for a test material to be described with confidence as negative, it is necessary to evaluate it at a sufficiently high dose level. In general, mammalian regulatory guidelines recommend the use of a maximum tolerated dose (MTD). This dose level is expected to produce some indication of cytotoxicity either on the target cells for the assay (e.g., bone marrow micronucleus assay or rat liver unscheduled DNA synthesis assay) or at the whole animal level in terms of survival [Mackay, 1995]. We adopted this well-accepted approach to determine the MTD in this study. As described in Figure 1, 12-hr-old embryos (postfertilization) were exposed to different concentrations of TBT dissolved in seawater for 8 hr to evaluate the developmental and survival effects. The range of TBT concentrations used in the experiments was adopted from earlier work reported on mussels and oyster embryos [Dixon and Prosser, 1982; Alzieu, 1991]. Test concentrations were, however, modified and selected from the semilogarithmic series (i.e., 0.31, 0.54, 1.75, 3.11, 5.44, 10.51 μg l^{-1}; w/v, etc.) routinely used for aquatic toxicity testing. At the end of the exposure period, the embryo-larvae were transferred into clean seawater and allowed to grow at 15°C. Samples of

these growing embryos were analyzed for gross developmental and survival effects immediately and at 24-hr intervals for 3 days as per the criteria described by the ASTM [1989]. The abnormal embryos lacked all or some of the developmental features present in the majority of the embryos growing in the seawater controls. The percentage of abnormal embryos was calculated for each treatment as described elsewhere in detail [Hutchinson et al., 1998].

Cytogenetic analyses and determination of preexposure and postexposure levels of TBT

As mentioned earlier, at the end of 6- and 8-hr exposure periods (18 and 20 hr postfertilization time), subsamples of the growing larvae were collected and processed for metaphase spread preparation as described in detail elsewhere [Harrison and Jones, 1982; Jha et al., 1996]. In order to determine the proliferative rate index (PRI) and evaluate the induction of SCEs, the slides were processed for SCD staining as per the method described elsewhere in detail [Jha et al., 1996]. For the analyses of CAbs, prepared slides were stained with 5% (v/v) Giemsa stain in Gurr buffer (pH 6.8). The stained (either SCD or Giemsa) slides were mounted in DPX mountant and analyzed under a microscope at a final magnification of ×1000. For SCD-stained slides, metaphases were classified as first (M1), second (M2), or third or subsequent (M3+) division cells. For the evaluation of cytotoxicity, the PRI was calculated using the formula: PRI = [(1 × M1) + (2 × M2) + (3 × M3 =)]/number of cells analyzed. This formula was originally proposed by Schneider and Lewis [1981] to evaluate the effect of an aging environment on the induction of SCEs. Different authors including Lamberti et al. [1983], later adopted this formula to evaluate the cytotoxicity of chemicals in genotoxicity experiments. At least 100 complete metaphases were analysed for the evaluation of cytotoxicity (PRI) and CAbs, while SCEs analyses were conducted on at least 30 complete metaphases per treatment. CAbs were scored as per the criteria recommended by the United Kingdom Environmental Mutagen Society [Scott et al., 1990], and expressed as the percent aberrant cells and total aberrations (chromosome plus chromatid type excluding gaps) per treatment, while SCEs were expressed as the SCEs frequency per cell.

In addition to the standard water quality parameters (i.e., dissolved oxygen, pH, salinity, and temperature), which were found to be within acceptable ranges, the levels of TBT were determined in preexposure and postexposure seawater samples using high-performance liquid chromatography coupled to inductively coupled plasma-mass spectrometry (HPLC-ICP-MS), as described in detail elsewhere [Hill, 1997].

RESULTS

The frequency of dead and abnormal embryos increased with increasing TBT concentration and observation period. At highest concentration, i.e.. 5.44 μg l^{-1}, after 3 days observation (15 ± 1°C), 100% of the larvae were either dead or abnormal (distinctly underdeveloped). During this observation period (3 days), 64% ± 4.20% of the embryo-larvae were normal or had reached the typical D-shaped stage in the seawater controls (Fig. 2). At higher concentrations, many of the growing embryo-larvae appeared as a shrunken mass of necrotic tissue within the outer membrane. These features allowed the ready identification of normal vs. abnormal individuals in each sample and allowed the percentage of normal individuals to be evaluated as an index of developmental toxicity for each treatment [Hutchinson et al., 1998]. Based on developmental abnormalities and survival, as in regulatory mammalian in vivo genetic toxicology assays [Mackay, 1995], and adopted for embryo-larvae of other marine invertebrate [Hutchinson et al., 1998], the MTD for the embryo-larvae of *M. edulis* after exposure to TBT was determined to be 5.44 μg l^{-1}.

The protocols adopted to prepare and stain metaphase spreads provided plenty of well spread metaphases to analyze PRI, SCEs, and CAbs (Figs. 3 and 4). As discussed above, the evaluation of developmental toxicity of TBT was complemented with cytotoxicity by analyzing PRI from the SCEs experiments. At the end of the exposure period for SCEs experiments (8 hr; 2 cell cycles) the metaphase spreads prepared from the collected subsample of the growing embryo-larvae showed a concentration-dependent reduction for PRI values. The PRI values for controls (seawater and solvent) ranged between 2.02 and 2.23, while for the two highest concentrations (i.e., 3.11 and 5.44 μg l^{-1}), the values ranged between 1.3 and 1.1, indicating a 1.6-fold decrease (Fig. 5). The mean baseline frequencies for the induction of SCEs in seawater and solvent controls were found to be extremely low (0.53 ± 0.96 and 0.63 ± 0.98, respectively). The phenomenon of low base or spontaneous level SCEs has been recognized in all earlier studies using early life stages (< 24 hr old) of marine invertebrates [Harrison and Jones, 1982; Brunetti et al., 1986; Dixon and Prosser, 1986; Jha et al., 1996; 2000). The low levels of SCEs observed in the present study were therefore in agreement with the earlier studies. The range of SCEs in the control treatments ranged between 0 and 4. In contrast, all the treatments showed a sharp increase for the induction of SCEs. At the top two concentrations (i.e., 3.11 and 5.44 μg l^{-1}), the mean values of SCEs per cell were 4.80 ± 0.75 and 4.30 ± 2.06 and at these concentrations the cells showed up to 7 SCEs per cell. Single concentration of MMS, used as a positive control, showed a mean value of 10.0 ± 8.05. The statistical analyses (ANOVA) of the data suggested a clear concentration-dependent increase for the induction of SCEs ($P < 0.0001$).

The chromosome complement of *M. edulis* (2n = 28) facilitated the analyses of both chromosome- and chromatid-type aberrations (Fig. 4). A clear dose-response relationship for both aberrant metaphases and total aberrations was found (Fig. 6). While the seawater control showed no aberrations (or aberrant metaphases), the solvent (acetone) control showed 1% aberrations and aberrant metaphases. With increasing concentrations, however, the frequency of aberrant metaphases containing more than one aberration increased. Overall, after TBT treatment, 70.37% aberrations were chromosome- and chromatid-type breaks (deletions), while 29.63% were chromosome- and chromatid-type exchanges. A single concentration of MMS used in the study showed 34% aberrant cells. A number of MMS-treated cells were found to be highly damaged, and from these, the exact frequency of total aberrations could not be scored. Consequently, the frequency of total aberrations for MMS treatment is shown to be lower than aberrant metaphases (Fig.

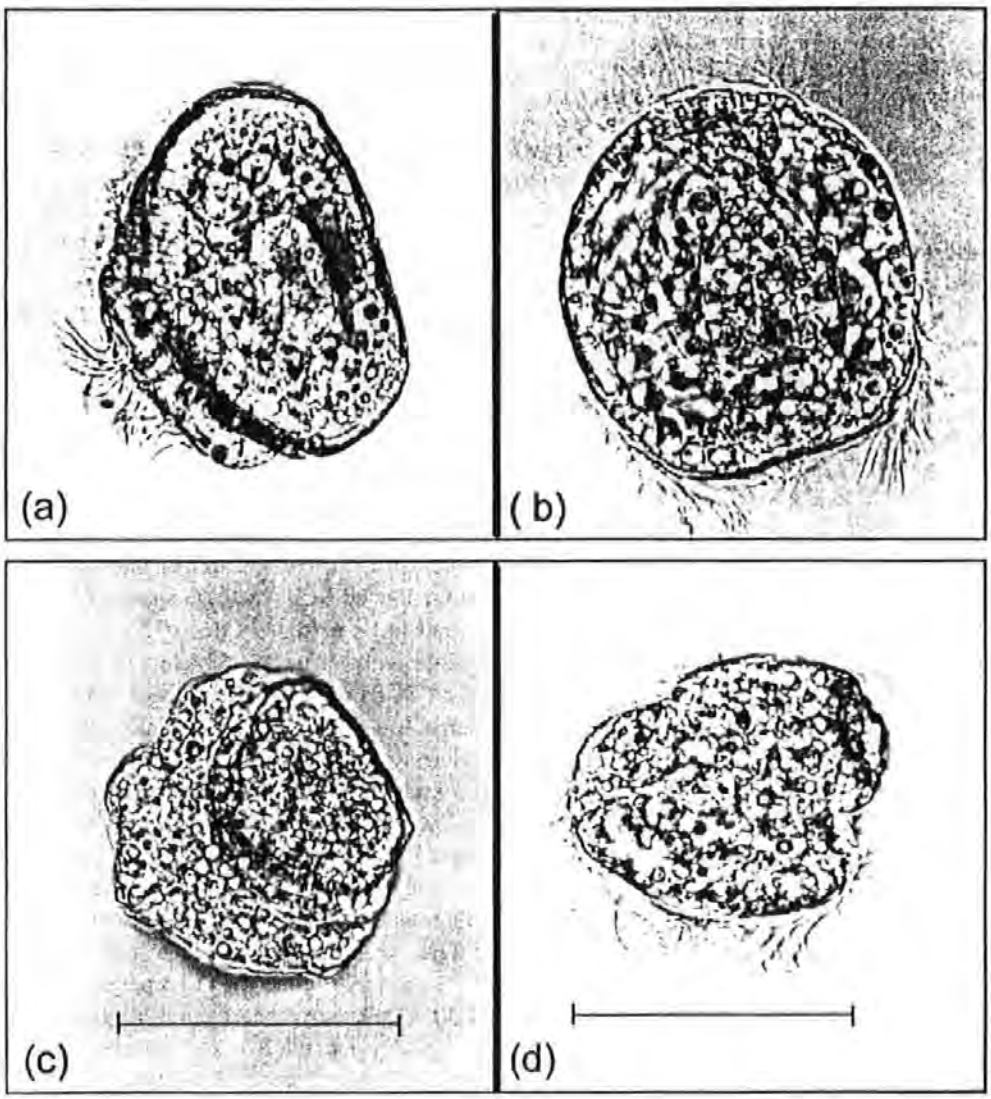

Fig. 2. Determination of developmental toxicity in the embryo-larval stages of *Mytilus edulis* (**a**). Typical D-shaped, normal embryo-larvae and (**b**), normal trochophore larvae (**c**), abnormal D-shell and (**d**) abnormal trochophore larvae (scale bar represents 80 μm).

5). The statistical analyses (ANOVA) of the results showed ı concentration dependent-increase for the induction of total ıberrations and aberrant metaphases ($P < 0.001$).

Analysis of seawater samples using ICP-MS suggested a lecrease for the concentrations of TBT in postexposure amples (data not shown). The overall recovery of the ırocedure was found to be 90% ± 6%. No TBT was letected in the seawater and solvent controls.

ıISCUSSION

Using a well-validated in vivo genotoxicity assay based n the embryo-larval stage of an ecologically relevant target rganism (*M. edulis*), we have evaluated the potential geno-ıxic hazard of an important environmental contaminant. he developmental toxicity results, in general, are in agreement with earlier studies carried out on mussel embryo-larvae [Dixon and Prosser, 1986]. Although originally proposed for application to highly differentiated and synchronously dividing mammalian cells under in vitro conditions, the PRI was found to provide a useful means to evaluate the cytotoxicity, as in earlier studies using embryo-larvae of marine invertebrates [Jha et al., 1996, 2000). In contrast to mammalian cells growing under in vitro conditions, however, the PRI for the embryo-larvae probably represents an average figure, based on a mixed population of differentiating cells with differing mitotic rates over the exposure period described.

While a large amount of information pertaining to the toxic effects of TBT can be found in the literature, there has been a lack of studies involving potential genotoxic effects of TBT in aquatic organisms. In the late 1980s, a multi-

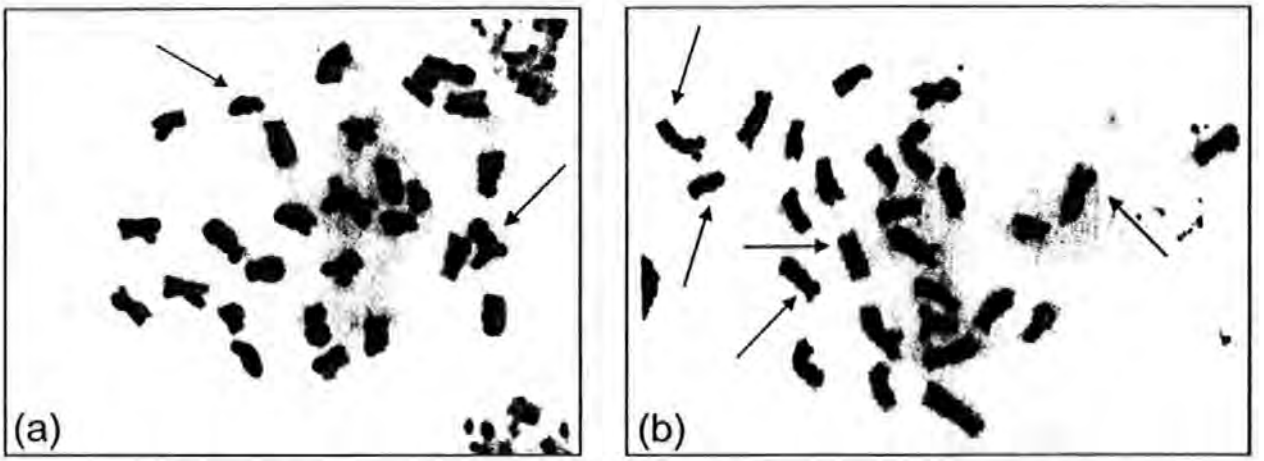

Fig. 3. a,b: Metaphase spreads prepared from the embryo-larvae of *Mytilus edulis* showing sister chromatid differential (SCD) staining and sister chromatid exchanges (SCEs). Arrows indicate SCEs.

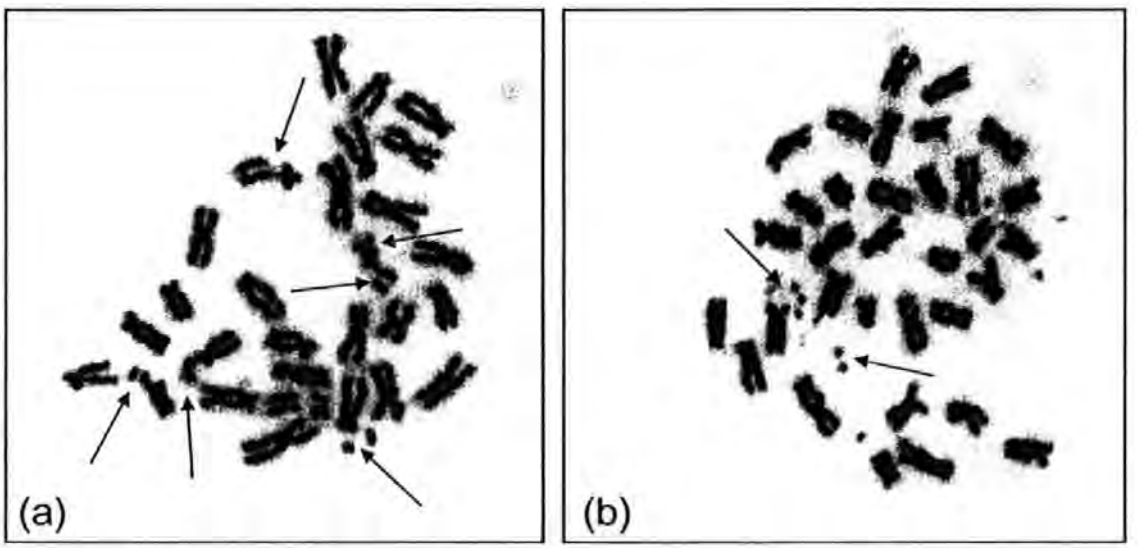

Fig. 4. a,b: Methyl methane sulfonate (MMS)- and tributyltin (TBT)-induced chromosomal aberrations in the embryo-larvae of *Mytilus edulis*. Arrows indicate chromatid-type breaks.

center study evaluated the potential genotoxic and embryotoxic effects of TBTO as a potential molluskicide, on human health [Davis et al., 1987]. In general, the study had negative results in short-term bacterial and yeast test systems using various genetic end points. However, at cytotoxic concentrations, it was found to be mutagenic in one bacterial strain, clastogenic in Chinese hamster ovary (CHO) cells in vitro, and produced micronuclei in mouse bone marrow cells in vivo. In view of the adverse biologic effects and its known capacity to interfere with morphogenetic differentiation processes, the study recommended that careful evaluation must be made before TBT is released in large quantities into the aquatic environment as a molluskicide [Davis et al., 1987]. Furthermore, TBT has been shown to induce programmed cell death or apoptosis in human lymphoblastoid cell lines as well as in the tissues of marine sponge, *Geodia cydonium* [Batel et al., 1993]. The positive genotoxic and embryotoxic effects observed in the multicenter collaborative study [Davis et al., 1987] largely support the present study carried out in the embryo-larval stage of a target marine invertebrate. However, in a similar cytogenetic study, with embryo-larvae of mussel, Dixon and Prosser [1986] could not find any genotoxic effects. Several differences between the two studies may account for this discrepancy. For example, in our study, after determination of MTD, we selected the concentration on a semilogarithmic scale, whereas the concentrations in the previous study were selected on a arithmetic scale (0.05, 0.1, 0.5, and 1.0 $\mu g\ l^{-1}$) without taking into account the MTD, the highest concentration for genotoxic evaluation being 1. 0 $\mu g\ l^{-1}$. Remembering that induction of cytogenetic damage is a cell cycle-dependent phenomenon, we exposed the growing

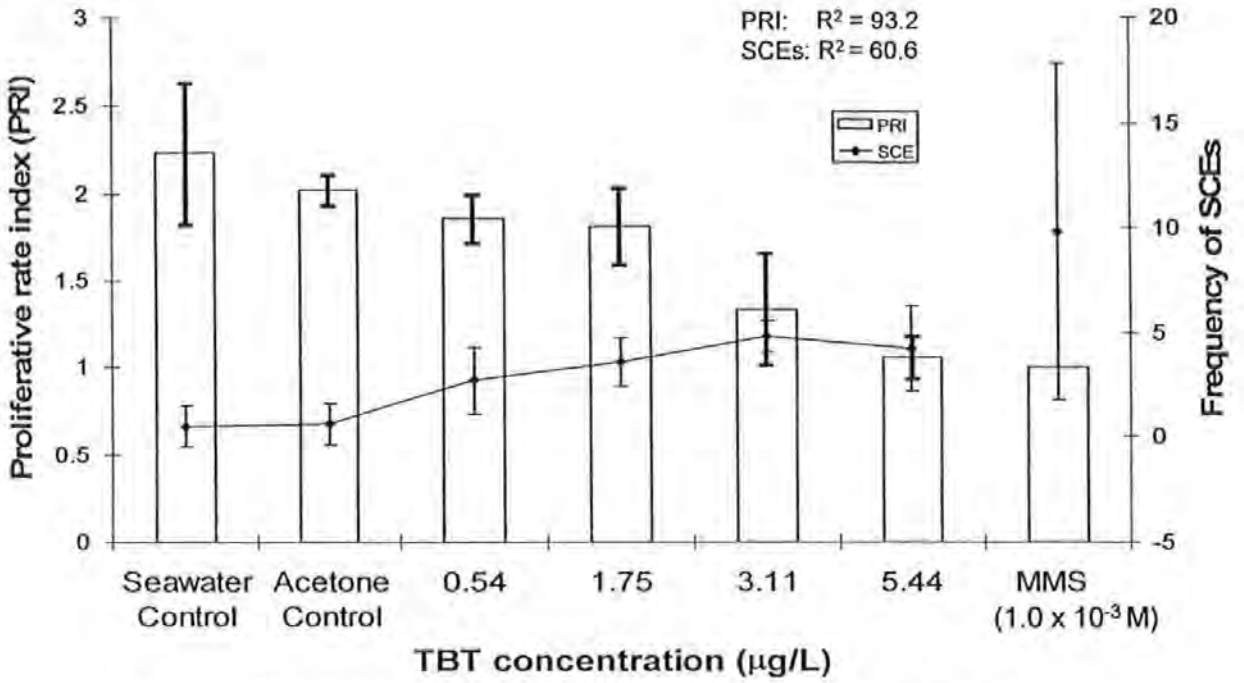

Fig. 5. The effects of tributyltin (TBT) and methyl methane sulfonate (MMS) on proliferation rate index (PRI) and induction of sister chromatid exchanges (SCEs) in the embryo-larvae of *Mytilus edulis*. Bars represent standard deviation (SD).

cells only for a specific period (1.5 (6 hr) and 2 cell cycles (8 hr) for the cytogenetic analyses. In the study by Dixon and Prosser [1986], however, cells were exposed for a period of 12 hr to analyze both the cytogenetic end points. In the earlier study, CAbs were scored and pooled from (incomplete) metaphases that had divided more than once. This would have further diluted the observed effects. However, in a subsequent study, Dixon and McFadzen [1987] reported that TBT in its own right does not induce but promotes the induction of SCEs induced by other agents (mitomycin C and crude oil). This is an important aspect and should be explored further, given the complex nature of the environment inhabited by natural biota.

Several mechanisms could account for the genotoxic potential of TBT. For example, TBT and its metabolites have been shown to bind with cellular macromolecules including thiol group of active sites of the enzymes [Lee, 1991; Matthiessen and Gibbs, 1998; Fent and Bucheli, 1994]. A large number of metallic compounds that have binding capacity with macromolecules (viz. DNA and repair enzymes) are known to induce genetic damage [Hartwig, 1998]. If TBT is capable of binding to either DNA or the repair enzymes, it will induce both S-phase–dependent and S-phase–independent cytogenetic effects in common with other metallic and organometallic compounds [Jha et al., 1992] that could also lead to programmed cell death as shown by Batel et al. [1993]. Furthermore, metals such as copper and iron, which were traditionally not considered to be genotoxic, have also been shown to induce oxidative damage to the DNA, generating double-strand breaks, base damages, and intrastrand cross-links, either independently or via cytochromes P450 enzymes [Lloyd and Phillips, 1999]. As mentioned previously, steroidal hormones, whose metabolism is influenced by TBT (because they share common metabolic pathways [Lee, 1991]), are known to induce different types of damages to the genetic material either by covalently binding to DNA or by free radical action [Tutsui and Barrett, 1997; Service, 1998; Roy and Liehr, 1999]. Additionally, cytochromes P450 are known to catalyze a diversity of reactions leading to production of several agents including nitric oxide [Mansuy, 1998]. Because TBT is known to influence several oxidative and nonoxidative reactions catalyzed by P450 [Fent and Bucheli, 1994; Matthiessen and Gibbs, 1998], it is not surprising that such byproducts could also induce genetic damage. Indeed, it has been shown that TBT can generate nitric oxide under in vivo conditions [Kergosien and Rice, 1998], which in turn is well documented for inducing damage to DNA [Burney et al., 1999].

As mentioned previously, the choice of TBT concentration used in this study was based on previous studies using embryo-larvae of mussels and oysters [Alzieu, 1991; Dixon and Prosser, 1986]. Under natural environmental conditions, the concentration of TBT in coastal areas varies tremendously, particularly where influenced by shipping activities. Although a global decline for TBT concentrations in coastal areas is now being emphasized [Evans, 1999], in several instances levels have been found to be several order

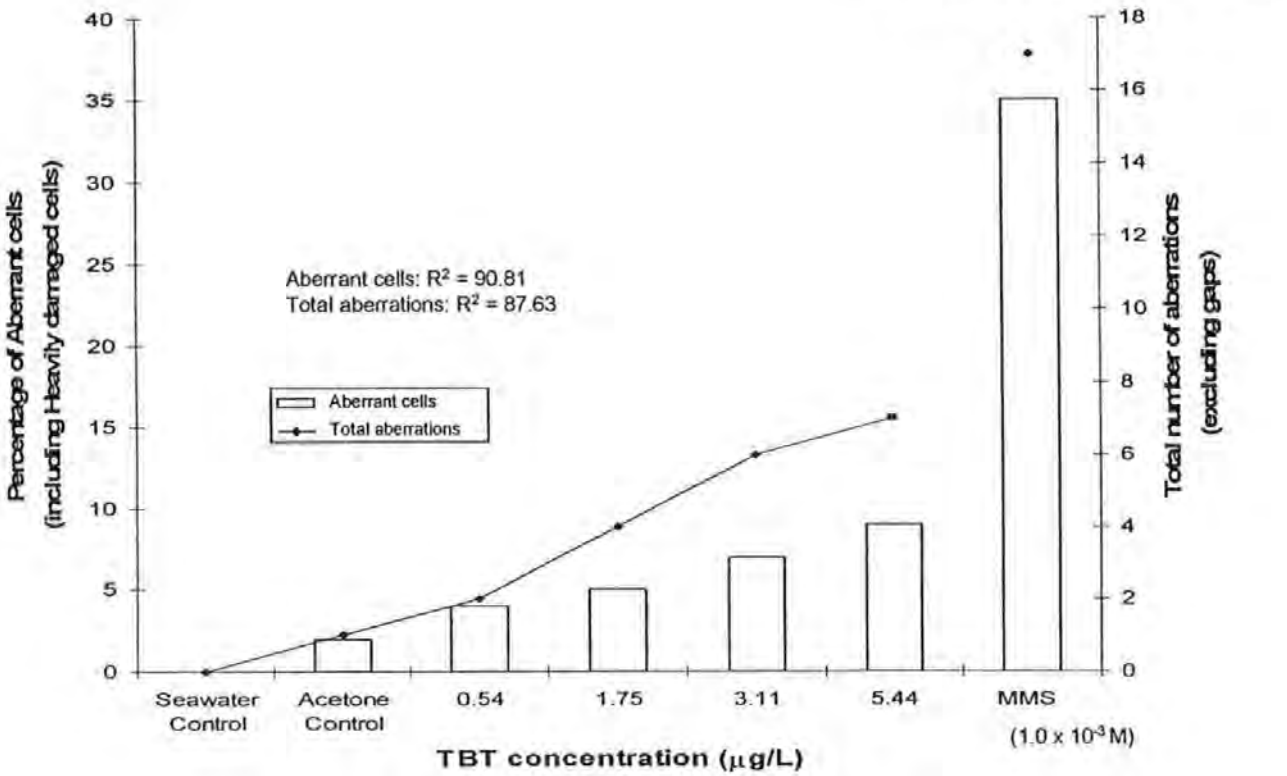

Fig. 6. The effects of tributyltin (TBT) and methyl methane sulfonate (MMS) on the induction of chromosomal aberrations (CAbs) in the embryo-larvae of *Mytilus edulis*.

of magnitude higher than those used in this work. For example, within the Severn Estuary and Bristol Channel (UK), there are large quantities of TBT associated with the sediment, both in suspension (up to 100 g l^{-1} at certain points in the tidal cycle) and as deposits (an estimated 270×10^6 tons) [Morgan et al., 1998]. In other cases, the concentrations of TBT have been found to range between 0.23–0.65 µg l^{-1} [Langston et al., 1987], a concentration range comparable to the lower end of the present study. However, while short-term exposure demonstrates detrimental effects, there is also a need to understand low-level long-term effects. This is particularly important given the fact that TBT is well known to accumulate in biota and that the accumulative pattern can be influenced by food chain magnification [Takahashi et al., 1998, 1999]. In addition, it should be also remembered that under natural environmental conditions, potential genotoxins probably occur as complex mixtures and the risk of such mixtures cannot be adequately anticipated on the basis of the effect and behavior of individual components. Furthermore, in an ecological context, the study also emphasizes the need to account for the differences in susceptibility between target and nontarget species or taxonomic groups [Alzieu, 1991; Taylor and Harrison, 1999] in order to elucidate the ecotoxicological assumptions about cross-taxa predictivity valid for this and other relevant environmental contaminants.

While there are a few clear examples of endocrine disruptive effects resulting from environmental chemical exposure, in most cases a causal link between the observed abnormalities and chemical exposure has not been established [Taylor and Harrison, 1999]. In this study, which adopted an integrated approach, we suggest that TBT, a known endocrine disrupter could lead to the induction of genetic damage and might therefore play a role in the observed effects at individual and subsequently at population levels. In the emerging field of "genetic-ecotoxicology" or "eco-genotoxicology" [Wurgler and Kramers, 1992; Anderson et al., 1994], there are not many well-validated tools for linking genetic damage to end points of direct importance to the ecological viability of marine populations. This hazard assessment study gives a practical example of such an integrated approach and goes toward the broader objectives of this stimulating and challenging field of research.

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Appendix 3

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MARINE ENVIRONMENTAL RESEARCH

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Genotoxic, cytotoxic and developmental effects of tributyltin oxide (TBTO): an integrated approach to the evaluation of the relative sensitivities of two marine species

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Abstract

Adopting an integrated approach the potential genotoxic, cytotoxic and developmental effects of tributyltin oxide (TBTO), a known endocrine-disrupting agent for neogastropods, have been evaluated in two ecologically relevant invertebrates: *Mytilus edulis* (blue mussel) and *Platynereis dumerilli* (rag worm). Following determination of the maximum tolerated dose (MTD) in terms of developmental and survival effects, the embryo-larval stages of these organisms were exposed to a range of concentrations of TBTO, and analysed for cytotoxic (proliferation rate index) and genotoxic (sister chromatid exchanges and chromosomal aberrations) effects. The study suggested that: (1) TBTO is both toxic and genotoxic to embryo-larval stages of both species; (2) at comparable concentrations, for developmental and genotoxic effects, *P. dumerilii* (non-target species) is more sensitive compared to *M. edulis* (target species); and (3) genotoxic effects are more closely tied with the development and survival of the organisms. The study emphasises the need of the evaluation of genotoxic potential of other endocrine-disrupting agents in different taxonomic groups.

Keywords: Tributyltin; Endocrine-disrupting agents; Genotoxicity; *Mytilus*; *Platynereis*

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1. Introduction

Tributyltin compounds (TBTs) are considered to be one of the most toxic anthropogenic substances to enter the marine environment. The most dramatic effect of TBT has been the irreversible sexual abnormality in female neogastropod snails. This phenomenon, known as "imposex", is a masculinisation process involving the development of male sex organs in females due to hormonal imbalance. This hormonal imbalance leading to imposex formation prevents normal breeding activity, ultimately causing population disappearance. In this context, elevated levels of estrogens are known to produce adverse effects, such as embryotoxicity, teratogenicity and carcinogenicity (IARC, 1979). In particular, the link between female hormones and cancer is hard to miss. Initially, it was supposed that steroid hormones, especially estrogens, act as growth factors in promoting cancers. However, new evidence suggests that such hormones are metabolised into a variety of compounds that may trigger the initiation process (Service, 1998). Further studies have suggested that the genotoxicity of steroid hormones must be an important contributor to the induction of adverse effects because receptor-mediated events alone cannot explain their carcinogenic and non-carcinogenic properties. Therefore, in recent years, there has been a strong resurgence of research into the genotoxicity of hormones and their metabolites (Roy & Liehr, 1999).

If endocrine-disrupting agents (or their metabolites) are capable of inducing genetic damage, it could potentially affect both the short- and long-term survival of the exposed natural biota (Anderson et al., 1994). A number of important issues associated with exposure of natural biota to endocrine-disrupting agents remains to be elucidated. These include differences in susceptibility among taxonomic groups, in particular among the invertebrates and the exact mechanisms of their action at molecular and cellular levels (Taylor & Harrison, 1999). In the light of aforementioned information, the objective of the study has been to evaluate genotoxic (if any), cytotoxic and developmental effects of tributyltin oxide (TBTO) in the embryo-larval stages of two ecologically relevant marine invertebrates, viz. marine mussels: *Mytilus edulis* (target species) and rag worm: *Platynereis dumerilii* (non-target species). The study has therefore also aimed to account for the potential differences in susceptibility between target and non-target species or taxonomic groups to elucidate the ecotoxicological assumptions about cross-taxa predictivity valid for this relevant environmental contaminants, the need for which is being emphasised (Taylor & Harrison, 1999). In this context, it will be worth mentioning that in a recent workshop to formulate the research strategy for investigating the ecological significance of endocrine disruption, *P. dumerilii* has been recommended as a sentinel annelid species, which plays an important role in the marine ecosystem (Taylor, Holmes, Duarte-Davidson, Humfrey & Harrison, 1999).

In this study we have also inherently linked different levels of biological organisation (i.e. chromosomal, cellular and individual levels) to elucidate the mechanisms of action and effects of TBTO. In addition, while evaluating genotoxic potential, we have taken into account the toxicity of the contaminants (TBTO) as one of the major confounding factors in interpreting the results for genotoxicity. For a test

material to be described with confidence as negative, in regulatory mammalian genotoxicological assays, it is necessary to perform the evaluation at sufficiently high dose levels. In general, regulatory guidelines recommend the use of a maximum tolerated dose (MTD) in terms of survival of the animals or a dose level producing some indication of cytotoxicity at the target organ for the assay (Mackay, 1995). Although there is some discrepancy when defining an MTD in mammalian regulatory guidelines, in aquatic genotoxicology it is relatively easier to evaluate the toxic effects by observing the behaviour, development and morphology of the developing embryo-larvae obtained from the broadcast spawners, which we have adopted in this study.

2. Materials and methods

Adult specimens of mussel (*M. edulis*) were collected from a clean site and acclimatised in the aquarium (at 15°C). Gametes and subsequently embryo-larval stages were obtained as per the procedure described in detail elsewhere (Harrison & Jones, 1982; Jha, Cheung, Foulkes, Hill & Depledge, 2000). *P. dumerilii*, a cosmopolitan species is being maintained (at 20°C) in our laboratory and reproduces throughout the year *via* photoperiod manipulation (Jha, Hutchinson, Mackay, Elliot, Pascoe & Dixon, 1995; Jha, Hutchinson, Mackay, Elliot & Dixon, 1996). In both species fertilisation is external and embryo-larval stages, which are considered to be critical in terms of environmental injury, are easy to handle under laboratory conditions. These developing stages provide a large number of metaphases following exposure to hypotonic solutions and colchicine to evaluate cytogenetic damage (Jha et al., 1995, 1996, 2000).

Following 'range-finding' experiments, studies were carried out to determine the MTD for the embryo-larval stages of these two species in terms of survival and the developmental effects as described in detail elsewhere (ASTM, 1989; Hutchinson, Jha, Mackay, Elliot & Dixon, 1998). Following determination of the MTD, 12-h-old (post-fertilisation) embryo-larvae of both the species were exposed to different concentrations of TBTO in the presence of thymidine analogue, 5-bromodeoxyuridine (BrdU). The exposure period was either for a duration of 1.5 cell cycles for the analyses of chromosomal aberrations or for two cell cycles to analyse sister chromatid exchanges (SCEs) (one cell cycle for mussels and worms = 4 and 6 h, respectively, at the experimental temperature). At the end of the exposure periods, sub-samples of the embryo-larvae were collected, exposed to different grades of hypotonic solutions, colchicine and processed for chromosome preparations. The metaphase spreads were further processed for sister chromatid differential staining and finally for cytogenetic analyses as described elsewhere (Jha et al., 1995, 1996). Sub-samples of embryo-larvae were allowed to grow until 72 h (mussels at 15°C and worms at 20°C) to evaluate the mortality and developmental abnormalities. The presence of minimum concentration of BrdU in the seawater facilitated the sister chromatid differential staining which allowed the evaluation of cellular proliferation rate or proliferative rate index (PRI) of the growing embryo-larvae and SCEs. In

addition to growing the embryo-larvae at different concentrations of the TBTO on a semi-logarithmic scale, a single concentration (1×10^{-3} M) of methyl methane sulphonate (MMS) was also used to serve as a reference genotoxin (positive control) for comparison. Pre- and post-exposure concentrations of TBTO in the seawater were also determined by high-performance liquid chromatography-inductively coupled plasma-mass spectrometry.

3. Results and discussion

While determining the MTD in terms of developmental toxicity and mortality, the results suggested that the toxicity of TBTO increases as a function of concentration and time (Fig. 1 a, b). The study also revealed that compared to *Mytilus*, *Platynereis* is more sensitive to the exposure of TBTO. While >90% of *Platynereis* embryo-larvae were found to be either dead or abnormal at 3.2 μg/l TBTO, the same degree of toxicity for *Mytilus* could be found at 5.6 μg/l. The spontaneous level of abnormality (seawater controls) was found to be higher in *Mytilus* compared to *Platynereis*. Moving down from individual to cellular level in the hierarchy of biological organisation, the PRI, which collectively represents frequencies of cells divided once, twice, thrice or more than thrice in the presence of BrdU, complemented the developmental toxicity and mortality data. In contrast to the developmental toxicity results, for cytotoxicity, *Mytilus* appeared to be more sensitive than *Platynereis*, although the basal PRI value (spontaneous level) was also lower in the *Mytilus* (Fig. 2). For the induction of SCEs, a cytogenetic biomarker for exposure, TBTO showed a concentration-dependent increase for both the species, *Platynereis* being more sensitive than *Mytilus* (Fig. 3). For the induction of chromosomal aberrations, a cytogenetic biomarker for effects, a concentration-dependent increase was also observed (Fig. 4). *Platynereis* again appeared to be more sensitive than *Mytilus*, both in terms of total aberrations (excluding gaps) and aberrant metaphases. At certain concentrations, some of the metaphases analysed from *Platynereis* were heavily damaged. It was difficult to score the number of total aberrations from these heavily damaged cells and therefore the frequency of total aberrations decreased at these dose points for *Platynereis* as shown in Fig. 4a. However, the total number of aberrant metaphases clearly demonstrated that *Platynereis* is more sensitive than *Mytilus*. The results of genotoxicity endpoints therefore unequivocally indicated that TBTO is capable of inducing damage to the genetic material of the embryo-larval stages of marine invertebrates, and *Platynereis*, a non-target species, is more sensitive compared to *Mytilus*, a target species. Chemical analyses of pre- and post-exposure seawater samples showed a loss of TBTO as a function of time and concentration.

The results therefore suggest that the genotoxic effects are more closely tied with the development and survival of the organisms and the same concentration-range of a chemical may produce differential cytotoxic and genotoxic effects in different species. Apart from the genetic make-up, reproductive pattern and programmed developmental commitment of the growing embryo-larvae, several factors

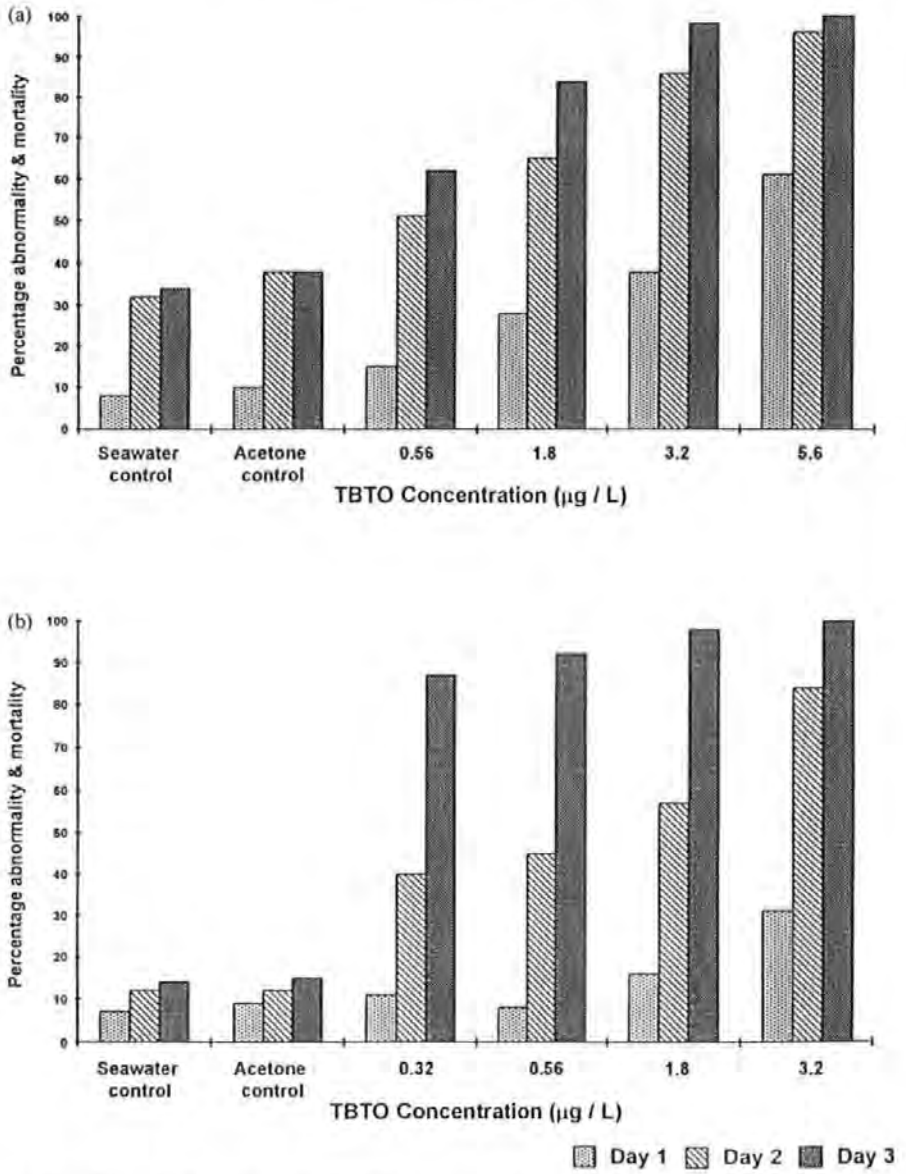

Fig 1. The effects of tributyltin oxide (TBTO) on the development and survival of embryo-larvae of (a) *Mytilus edulis* and (b) *Platynereis dumerilii*.

could account for differential sensitivity for developmental toxicity, cytotoxicity and genotoxicity for the two organisms representing two different phylogenetic groups. It is difficult to pin-point any particular factor to account for this differential sensitivity.

Several mechanisms can be used to explain the possible mode of action of TBTO as genotoxic agent. For example, TBTO has been shown to bind with cellular

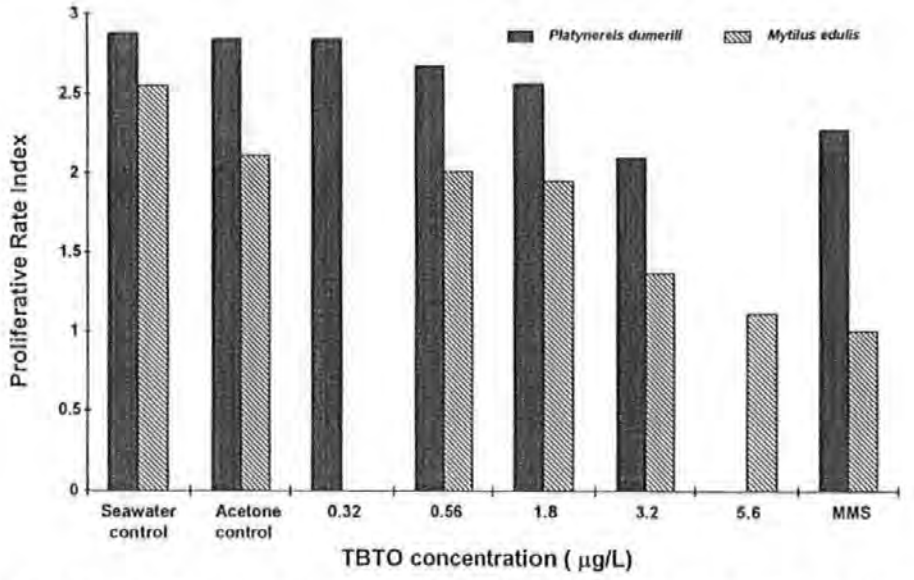

Fig. 2. The effects of tributyltin oxide (TBTO) on the proliferative rate index in the embryo-larvae of *Platynereis dumerilii* and *Mytilus edulis*.

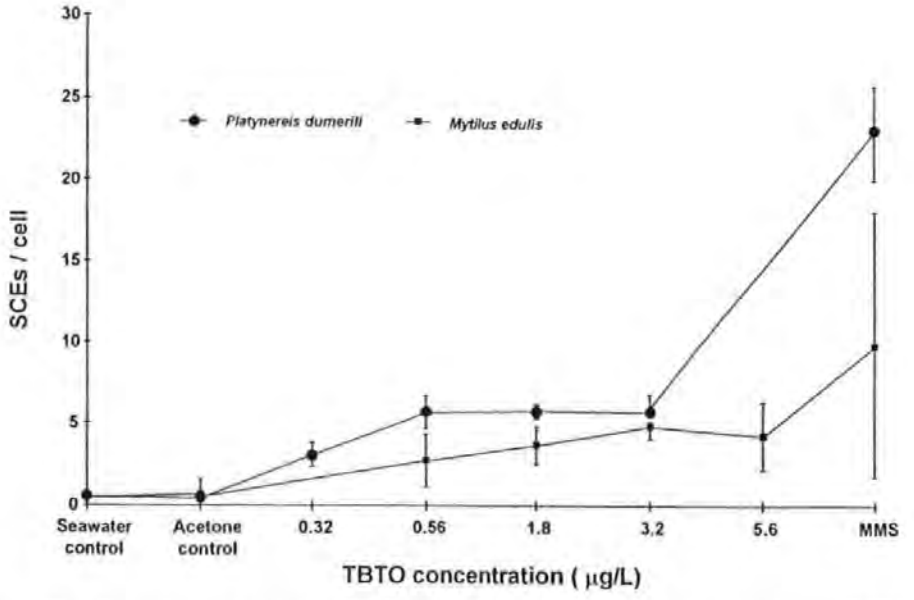

Fig. 3. The effects of tributyltin oxide (TBTO) on the induction of sister chromatid exchanges (SCEs) in the embryo-larvae of *Platynereis dumerilii* and *Mytilus edulis*.

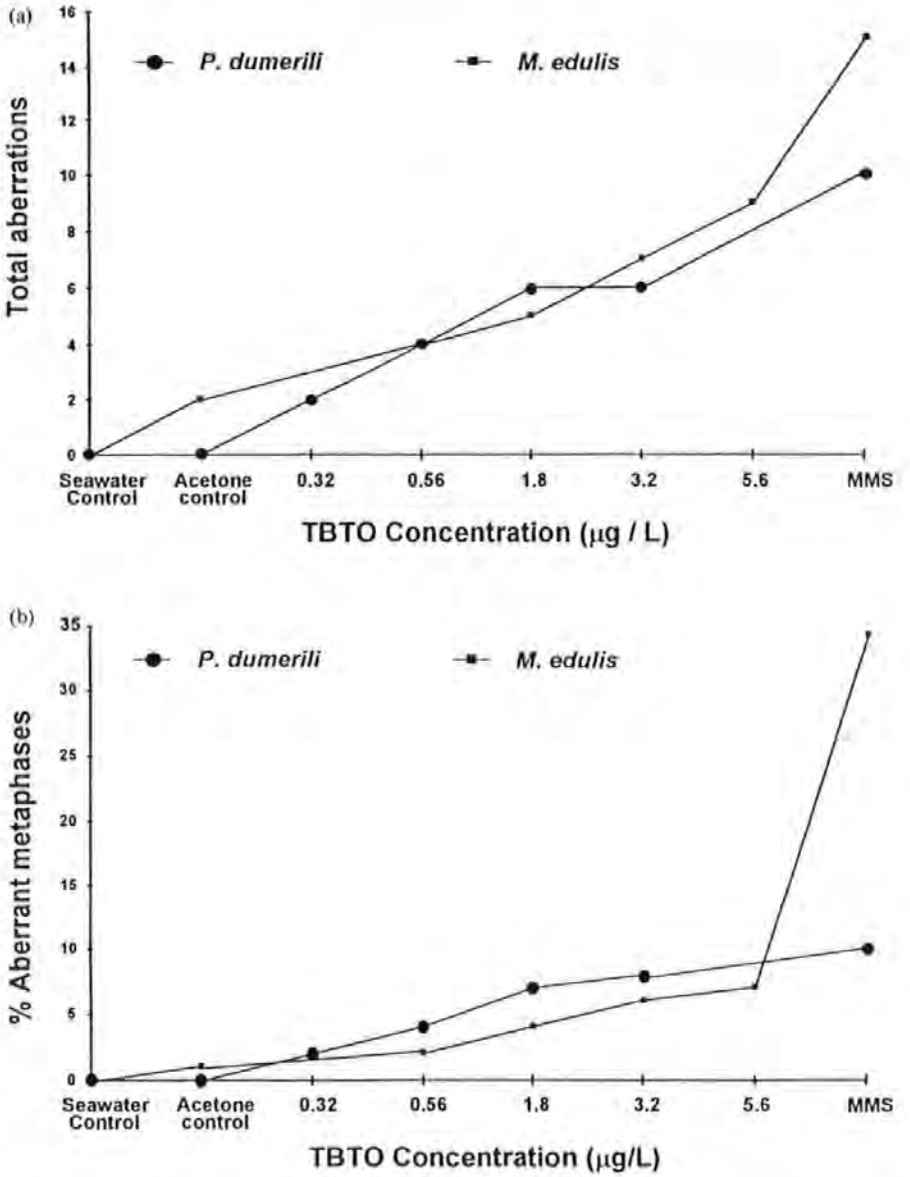

Fig. 4. The effects of tributyltin oxide (TBTO) on the induction of chromosomal aberrations in the embryo-larvae of *Platynereis dumerilii* and *Mytilus edulis*: (a) total aberrations and (b) aberrant metaphases.

macromolecules (Lee, 1991). A large number of metallic compounds, which have the binding capacity with cellular macromolecules, such as DNA and repair enzymes, have been shown to induce genetic damage (Hartwig, 1998; Jha, Noditi, Nilsson & Natarajan, 1992). If TBTO is capable of binding to either DNA or the repair enzymes, it will induce both S-phase dependent and independent cytogenetic effects

(Jha et al., 1992). Metals such as copper, iron and nickel have also been shown to induce oxidative damage to the DNA, generating double-strand breaks, base damages and intrastrand cross links, either independently or via cytochrome P450 enzymes (Lloyd & Phillips, 1999). As mentioned earlier, steriodal hormones, whose metabolism is influenced by TBTO as they share common metabolic pathways (Lee, 1991; Matthiessen & Gibbs, 1998) are also known to induce DNA or chromosomal damage and gene mutations (Roy & Liehr, 1999; Service, 1998). Finally, our conclusion is that TBTO, a known endocrine-disrupting agent induces damage to the genetic material and this damage could have adverse effects on the exposed population and eventually to the ecosystem.

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Appendix 4

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Aquatic Toxicology 57 (2002) 243–255

AQUATIC TOXICOLOGY

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Genotoxic, cytotoxic and ontogenetic effects of tri-*n*-butyltin on the marine worm, *Platynereis dumerilii* (Polychaeta: Nereidae)

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Abstract

The genotoxic, cytotoxic and ontogenetic (embryo-larval) or developmental effects of tri-*n*-butyltin (TBT), were investigated in *Platynereis dumerilii*. Following the determination of maximum tolerated dose with regard to ontogenetic effects and mortality, early life stages of *P. dumerilii* were exposed to a range of TBT concentrations. Subsequently, the embryo-larvae were analysed for evidence of genotoxicity and cytotoxicity. Genotoxicity was assessed using cytogenetic endpoints that included the frequency of sister chromatid exchanges and chromosomal aberrations from metaphase spreads. Cytotoxicity was evaluated by determining the proliferative rate index of the growing embryo-larval cells using 5-bromodeoxyuridine labelling of the chromosomes or fluorescence plus Giemsa staining technique. TBT-exposed embryo-larvae of *P. dumerilii* exhibited sensitivity similar to that of other invertebrates, indicating that *P. dumerilii* is a suitable ecotoxicity test species. The results also suggested dose-dependent effects for genotoxic and cytotoxic end points in relation to TBT exposure. The present study highlights the need to elucidate the relative importance of direct genotoxic and indirect effects through production of genotoxic hormonal derivatives.

Keywords: Tributyltin; Endocrine disrupters; Developmental toxicity; Cytotoxicity; Genotoxicity; *Platynereis dumerilii*

1. Introduction

Tributyltin compounds have been widely used as the active ingredients in marine antifouling paints. They are considered to be one of the most toxic agents entering the marine environment (Goldberg, 1986). Documented effects include impairment of growth, development, reproduction, and survival of many marine species (Heard et al., 1986). Despite a ban on tri-*n*-butyltin (TBT)-based anti-fouling paints for smaller vessels in various parts of the World, organotin compounds

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ntinue to affect wildlife around ports (Morgan al., 1998; Takahashi et al., 1998, 1999). Of rticular concern has been the induction of 'im-sex' in neogastropods. This involves the super-position of male characteristics on exposed males. Normal egg laying is prevented and ulti-ately results in a population decline (Bryan et , 1986). The biochemical mechanisms by which BT compounds exert endocrine disrupting ef-cts are not fully elucidated. However, it has en suggested that organotin compounds act as mpetitive inhibitors of cytochrome P450-medi-ed aromatase (Matthiessen and Gibbs, 1998), ading to hormonal imbalance (Lee 1991; atthiessen and Gibbs, 1998). In this context, rmonal imbalance has been shown to results in nbryotoxicity, teratogenicity and carcinogenicity ARC, 1979). In an ecological context, genetic mage that leads to heritable mutations may ve enormous significance for the long-term sur-val of exposed populations (Wurgler and ramers, 1992; Anderson et al., 1994a). Our re-nt study has suggested that TBT can induce togenetic damage in the common mussel, *ytilus edulis* (Jha et al., 2000b). In the light of s and the information already presented, the esent study was performed to further assess the togenetic (embryo-larval) or developmental ef-cts and the genotoxic potential of TBT, in the n-target polychaete worm, *Platynereis dumerilii*. With a cosmopolitan distribution, *P. dumerilii* a member of an ecologically important group of arine invertebrates, several of which have been dely used for monitoring the bioaccumulation d ecotoxicological effects of marine contami-nts (Hartmann-Schroder, 1971; Pocklington d Wells, 1992). This species is amenable to boratory culture and, via photoperiod manipu-ion, a continuous supply of different life stages n be obtained for experimental purposes (aver-e life cycle, approximately 3–4 months) auenschild and Fischer, 1969). In addition, *P. nerilii* has a stable and cytogenetically conve-nt karyotype ($2n = 28$) and constitutes a sensi-e, reproducible and reliable test system for the aluation of the genotoxic potential of contami-nts destined for marine disposal (Jha et al., 95, 1996, 1997; Hutchinson et al., 1998). As a result of the aforementioned criteria, *P. dumerilii* has been identified as a sentinel species for the indication of endocrine disruption by the United Kingdom Medical Research Council's Institute for Environment and Health (Taylor et al., 1999). Despite numerous advantageous attributes compared with several other polychaete species, only limited information exists for environmental monitoring and ecotoxicity testing for this species. Therefore, in order to assess its usefulness as a tool for laboratory-based ecotoxicity testing, it was also aimed to determine the relative toxicity of early life stages of this species against TBT. A schematic life history of *P. dumerilii* (adapted from Hauenschild and Fischer, 1969; Hutchinson et al., 1995 with some modifications) is shown in Fig. 1.

2. Materials and methods

2.1. Chemicals and dose-selection of TBT

TBT was obtained from Lancaster Synthesis (Lancaster, UK) in the form of bis(tri-*n*-butyltin) oxide (TBTO). Methanol, glacial acetic acid, acetone, Giemsa stain, DPX were obtained from BDH (Poole, Dorset, UK). Unless specified otherwise, all other chemicals used in the study were obtained from Sigma-Aldrich (Gillingham, Dorset, UK).

To determine the maximum tolerated dose (MTD) prior to evaluation of potential genotoxic effects, embryo-larvae of *P. dumerilii* were examined for developmental abnormalities and survival. Determination of MTD is considered to be necessary in regulatory genotoxicological assays, bearing in mind that the toxic potential of chemicals is considered to have a major confounding influence in interpreting the results for genotoxicity evaluation (Mackay, 1995). As mentioned earlier (Jha et al., 2000a,b), in regulatory mammalian in vivo genotoxicity assays, for a test material to be described with confidence as negative, it is necessary to evaluate it at sufficiently high dose level (Mackay, 1995). In general, the regulatory guidelines recommend the use of a MTD. This dose level is expected to produce some indication

of cytotoxicity either on the target cells for the assay (e.g. bone marrow micronucleus assay or rat liver unscheduled DNA synthesis assay) or at the whole animal level in terms of survival (Mackay, 1995). Although there is some discrepancy and ambiguity in defining MTD in mammalian regulatory guidelines, in aquatic genotoxicology, especially with the use of invertebrates, it is relatively more simple to determine apparent toxic effects by evaluating various behavioural, physiological, morphological and developmental endpoints following exposure to a contaminant (Hebel et al., 1997; Jha et al., 2000a,b).

Following preparation of stock solution of TBTO in acetone (100 mg/l TBTO ≡ 97.3 mg/l TBT), 12-h old (post-fertilisation) embryo-larvae were exposed to different concentrations of TBT for 12 h. Following this exposure, the embryo-larvae were analysed for developmental abnormalities and survival at intervals of 24 h over 3 days. The selection of TBT concentration range was based on earlier developmental toxicity studies carried out with mussel and oyster embryos (Dixon and Prosser, 1986; Alzieu, 1991). Test concentrations were, however, slightly modified to adopt the semi-logarithmic scale routinely used for aquatic toxicity testing and that were used in earlier experiments using embryo-larval stages of mussels (Jha et al., 2000b). Following an initial 'range finding' experiment, it was found that a TBT concentration at and above 3.11 μg/l on the semi-logarithmic scale had 100% dead and abnormal embryo-larvae for all the stages scored (i.e. 24, 48 and 72 h). The effects on the growing embryo-larvae above this (i.e. 3.11 μg/l) concentration had the same detrimental effects in terms of mortality and abnormality. The MTD based on these detrimental effects on the embryo-larvae of *P. dumerilii* was therefore determined to be 3.11 μg/l. Subsequent experiments to evaluate the developmental and survival effects and genotoxic and cytotoxic potentials of TBT were then carried out at this concentration or lower concentrations as per the protocol described in detail by Hutchinson et al. (1998) and Jha et al. (2000a,b). The experimental protocol is summarised in Fig. 2.

2.2. Exposure scenario, analyses of developmental toxicity and survival

Cultures of *P. dumerilii* were maintained in the laboratory as described in detail elsewhere (Hauenschild and Fischer, 1969; Hutchinson et al., 1995; Jha et al., 1995). Experiments were initiated following collection of sexually mature individuals and subsequently obtaining newly fer-

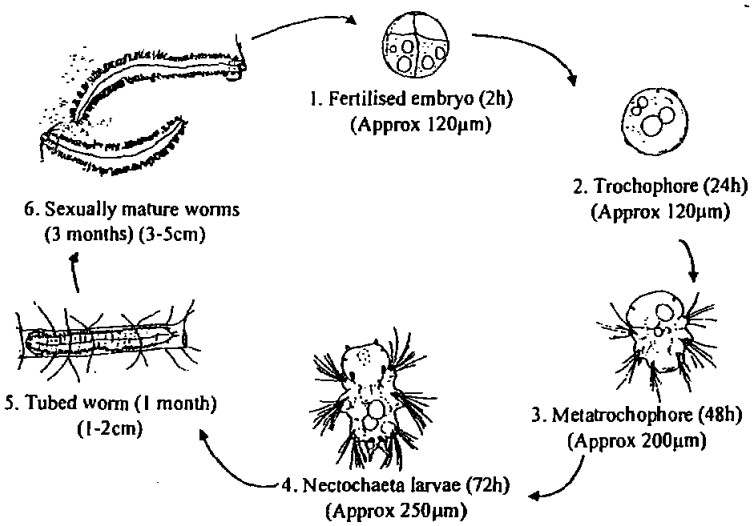
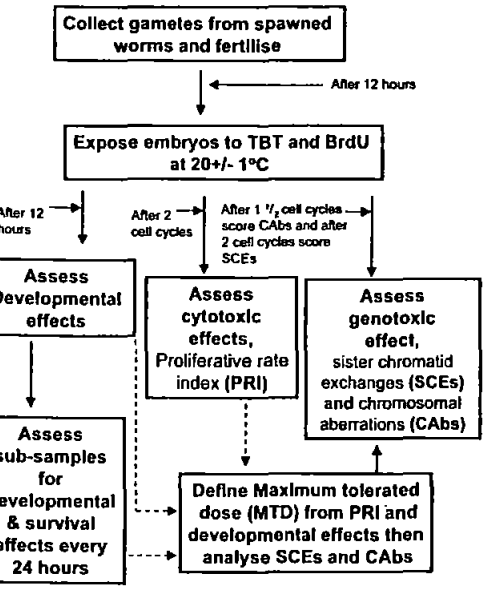


Fig. 1. Life cycle of the marine polychaete worm *P. dumerilii*.



. The protocol for analyses of developmental, genetic ytotoxic effects on *P. dumerilii*.

d eggs. Embryos aged 12 h were mixed gently a perforated plunger and distributed to test ls (glass beakers) with an approximate den- of 30 embryos/ml. They were exposed to four ent concentrations of TBT, a single concen- n of acetone (solvent control, 0.02%) and 0^{-3} M methyl methane sulphonate (MMS), erence genotoxin that served as positive con- Following addition of the chemicals to the essels, the embryos were allowed to develop $) \pm 1$ °C) in filtered (10 μm) seawater (salin- pproximately, 35 ‰). The concentration of S was selected based on earlier studies (Jha et 996, 2000a,b). In addition, a minimum con- ation (1.0×10^{-5} M) of 5-bromod- uridine was also added in all the test vessels tain sister chromatid differential staining of chromosomes following fluorescence plus sa staining technique. To analyse chromoso- aberrations (CAbs), sister chromatid ex- ges (SCEs) and proliferative rate index (PRI), amples of embryo-larvae were collected at nd of 8 and 12 h exposure periods. These ure periods corresponded to approximately d two cell cycles, respectively, for the grow- mbryo-larval cells of *P. dumerilii* (Jha et al., 1996), bearing in mind that induction of cytogenetic damage is a cell-cycle dependent phenomenon. The collected sub-samples were processed for metaphase spread preparations and subsequently for cytogenetic analyses as described in Section 2.3.

After a 12 h exposure period (24 h post-fertilisation old embryo), the growing embryo-larvae were transferred into clean seawater and checked for developmental abnormalities and survival under the microscope (100 × magnification). Developmental abnormalities and survival were also analysed at the 48 and 72 h post-fertilisation period as described by Hutchinson et al. (1995, 1998).

Following fertilisation, development and growth of the embryo-larvae is highly synchronous until the nectochaeta stage (Figs. 1 and 3). Normal larvae reached the actively swimming trochophore stage at 24-h post-fertilisation. Morphologically, the trochophores appeared spherical in shape with peripheral bands of cilia and internal fat droplets (Fig. 3a). By 48 h, normal larvae reached the metatrochophore stage of development, which is characterised by the appearance of a segmented body, a wide head region, eyespots and chaetae (Fig. 3b). At 72 h, normal larvae had developed three segments, with distinct parapodia and eyespots (nectochaeta stage) (Fig. 3c). In contrast, an abnormal trochophore (24 h), metatrochophore (48 h) and nectochaeta (72 h) larvae lacked one or more of these features (Fig. 3d–f). Dead embryos at these developmental stages comprised a mass of degenerating cells. These features collectively allowed the ready identification of normal versus abnormal and live versus dead larvae. Data obtained were used to calculate an index of developmental toxicity (Hutchinson et al., 1995, 1998).

2.3. Preparation of metaphase spreads and evaluation of cyto- and genotoxicity

At the end of 8- and 12-h exposure periods, sub-samples of embryo-larvae were treated with colchicine dissolved in seawater (0.025%, w/v) and then exposed to a series of hypotonic (seawater/0.075 M KCl) treatments. They were then fixed in

Carnoy's fixative (methanol:glacial acetic acid, 3:1) and used to prepare metaphase spreads as described by Harrison and Jones (1982) with some modifications (Jha et al., 1996). The prepared slides were either stained with Giemsa for the analyses of CAbs or processed for sister chromatid differential (SCD) staining for the analyses of SCEs and PRI. Following staining, the cells were analysed under a bright-field microscope (final magnification, 1000 ×). The SCD stained metaphases were classified into first (M1), second (M2) and third or subsequent (M3+) division cells (Fig. 6). The proliferative rate index was used to calculate the cytotoxic effects on the growing cells using the formula: PRI = [(1 × M1) + (2 × M2) + (3 × M3)]/number of cells examined (as proposed by Lamberti et al., 1983). A minimum of 100 cells was analysed per treatment for the evaluation of PRI and Cabs, and at least 30 second-division metaphases were analysed for SCEs. While the results for SCEs were expressed as SCEs frequency/cell, CAbs observations were expressed as the percent aberrant cells and total aberrations (chromosome and chromatid type excluding gaps) per treatment. Details of metaphase spread preparations, staining procedures and scoring criteria have been described in earlier studies (Jha et al., 1995, 1996).

2.4. Determination of pre- and post-exposure concentrations of tributyltin

As well as measuring the standard water quality parameters (i.e. pH, salinity and temperature), the levels of TBT were determined twice in pre- and post-exposure seawater samples using inductively coupled plasma-mass spectrometry, as described in detail elsewhere (Hill, 1997). The concentration of the standard solution used for exposure experiments was 2500 ng/ml as TBT. Prior to determination of TBT in the samples, a pre-concentration technique was employed (using a C_{18} cartridge) offering enrichment factors of the order of 100 (85–140).

3. Results

3.1. Evaluation of survival and developmental toxicity

As per the criteria described in Section 2.1, a concentration- and time-dependent increase in de

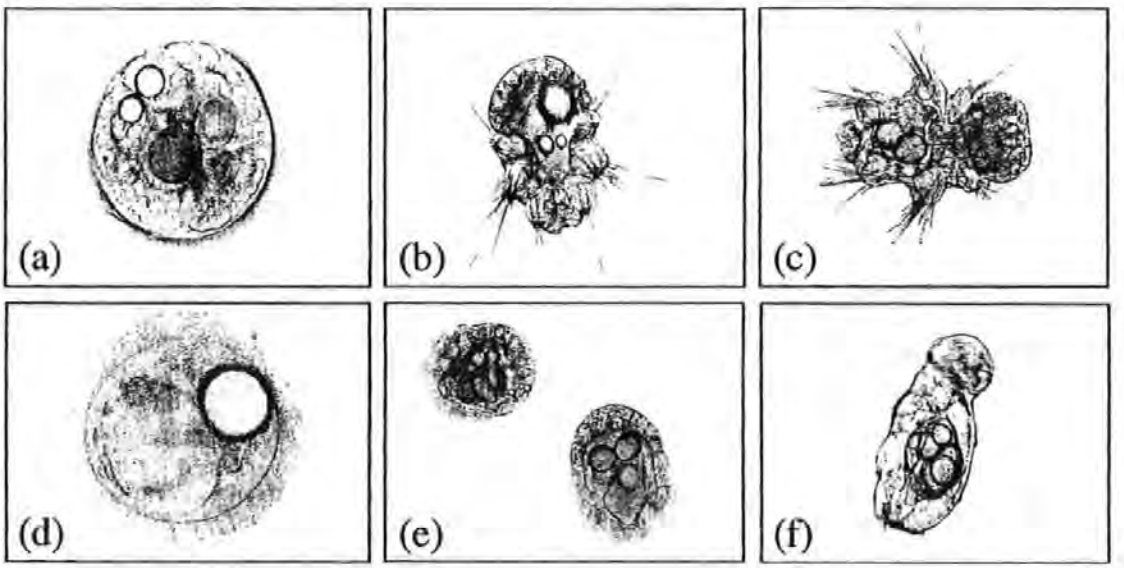
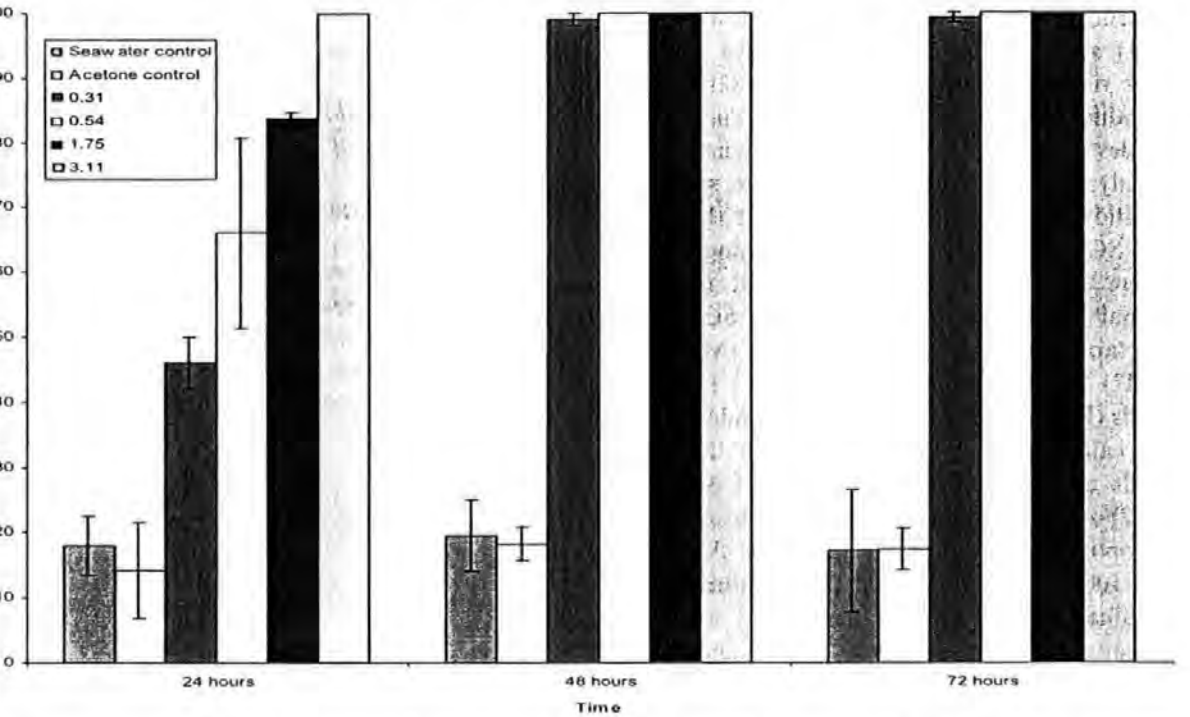


Fig. 3. The developmental stages of *P. dumerilii*; (a) normal trochophore larvae after 24 h; (b) normal metatrochophore larvae after 48 h; (c) normal Nectochaeta larvae after 72 h; (d), (e), (f) dead and abnormal embryo-larvae.



4. The percentage of abnormal and dead *P. dumerilii* over 3 days following 12 h exposure to TBT, Error bars, standard tion. TBT concentration in micrograms per litre.

pmental abnormalities and mortality in TBT osed embryo-larvae was found. Developmen- nd survival effects have been collectively rep- nted in Fig. 4. Significance of differences as a tion of concentration, investigated using anal- of variance (ANOVA), suggested no differ- between seawater and solvent controls, while ificant differences for these endpoints were rved for all but the lowest (0.031 μg/l) con- ration of TBT ($P < 0.01$). To compare sensi- y of early life stages of *P. dumerilii* with other ne invertebrates (Table 2), LC_{50} values (in s of mortality) for 24, 48 and 72 h larvae found to be 5.89, 1.34 and 0.61 μg/l, ctively.

Evaluation of cyto- and genotoxic effects

totoxic effects were assessed by calculating PRI. As represented in Fig. 5, there was a dose-related decrease for PRI values with increasing TBT concentration. The highest concentration of TBT (3.1μg/l) produced a PRI value of 2.10. This value was lower than the value obtained for MMS exposure (PRI = 2.27). The PRI results were not normally distributed, hence they were analysed using the Kruskal–Wallis non-parametric test. The ANOVA indicated a statistically significant difference ($P < 0.000\,05$) as a function of TBT concentrations. The R^2 statistic (coefficient of determination) for a linear dose-response model, which is used to judge the adequacy of a fitted regression line, showed a value of 80.62%. This indicates that the fitted model explains 80.62% of the variability in PRI. Usually, a value of R^2 of 70% or greater is considered to be a good fit. However, the PRI value for lowest concentration of TBT (0.31 μg/l) was not significantly different from the controls. The chromosomal complement of *P. dumerilii* allowed relatively easy analyses of both cytogenetic endpoints, namely SCEs and CAbs from complete

metaphases (Fig. 6). For SCEs, a dose-related increase was found (Fig. 7). The induction of SCEs was also analysed using the Kruskal–Wallis test, and was significantly different ($P < 0.000\,05$; $R^2 = 49.87\%$) for various concentrations compared with controls. For CAbs, a dose-related increase in the number of aberrations/cell was found (Fig. 8). At the two highest concentrations of TBT, some cells were highly damaged, which hindered scoring of total number of aberrations. The data for total aberrations and aberrant cells were normally distributed and therefore the results were analysed using ANOVA. Statistical treatment of the data revealed no differences between the frequency of total aberrations (chromosome and chromatid type excluding gaps) and aberrant metaphases in seawater and solvent controls, but a statistically significant difference ($P < 0.000\,05$; R^2 for total aberrations, 75.08%; R^2 for aberrant metaphases, 86.78%) was observed between the controls and TBT treatments.

3.3. Determination of pre- and post-exposure concentrations of tributyltin

Because of technical limitations (e.g. small size and number of embryo-larvae used in the study), it was not possible to measure the TBT levels in the embryo-larvae as a function of exposure time. However, the limited study of pre- and post-exposure concentration of TBT indicated uptake of the TBT by *P. dumerilii* during the trial (Table 1). The measurements in the samples were carried out twice, and the values presented in Table 1 for each treatment are an average of two measurements. The individual values are presented in parentheses in Table 1 for each treatment. At the lowest concentration, there was no apparent significant difference in the concentration (statistical tests were not possible given the limited volume of the sample available), but at the higher concentrations there would seem to be a concentration-dependent uptake.

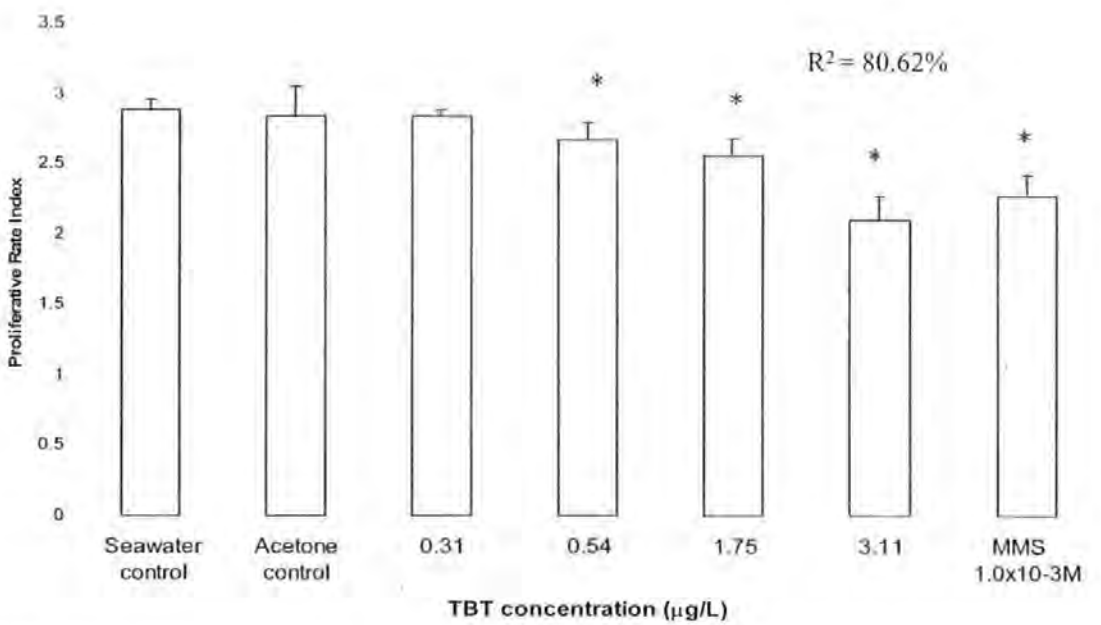
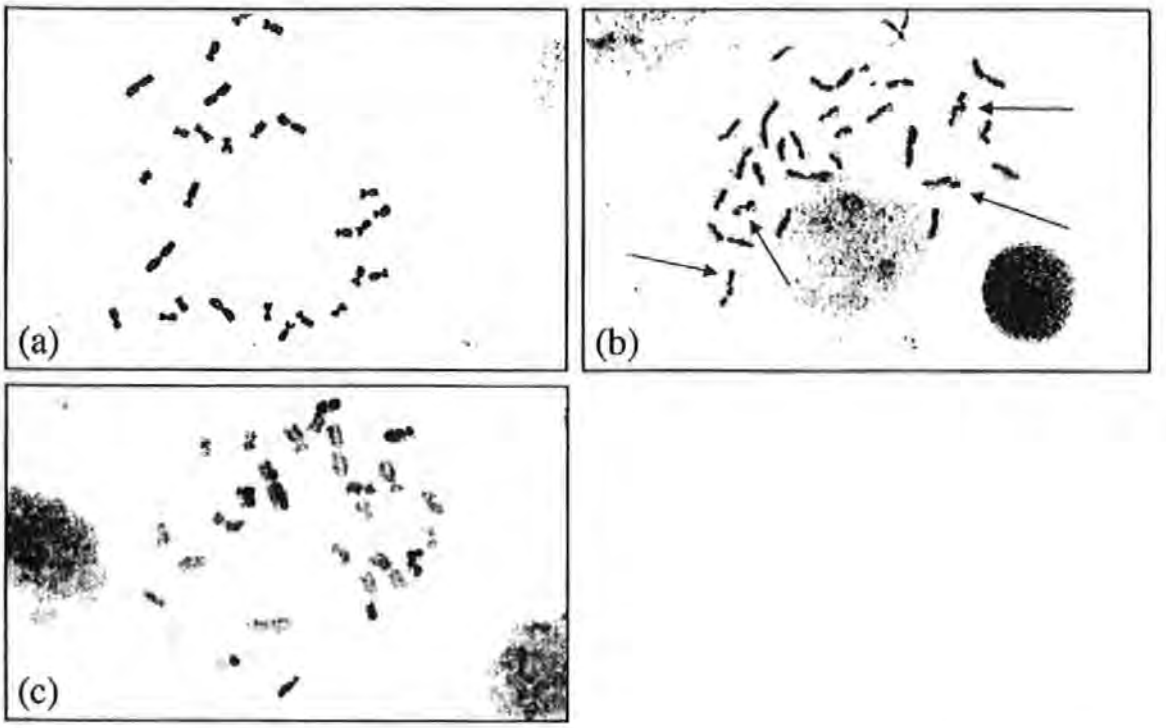


Fig. 5. The effect of TBT on the proliferative rate index (PRI) of the worm *P. dumerilii*. * Significant difference ($P < 0.000\,05$). Error bars, standard deviation.



g. 6. First (a), second (b) and third (+) (c) division cells from embryo-larvae of *P. dumerilii* following sister chromatid differential CD) staining (arrows indicate SCEs).

Discussion

A previous study suggested that *P. dumerilii* is nsitive to organic reference chemicals used in e 1991 International Paris Commission (PAROM) ring test for fertilisation rate, embryo-larl development and larval survival. The study d also indicated that, for these reference materis (i.e. Bioban P-1487, 3,5-dichlorophenol and ntocil IB), early life stages of *P. dumerilii* are of nilar sensitivity to other marine invertebrate ecies (e.g. *Crassostrea gigas*, *Mytilus edulis*, *ysidopsis bahia*, *Tisbe battagliai*, etc.) (Hutchinn et al., 1995). Table 2 summarises the available formation pertaining to toxic effects of TBT in fferent marine invertebrates. This provides furer confirmation that early life stages of *P. merilii* are similar to other species in terms of ortality and developmental toxicity. The LC_{50} lues at different life stages (24, 48 and 72 h) of *dumerilii* suggested a gradual decrease in tolerance (developmental abnormality and survival) as a function of developmental age ($24 < 48 < 72$ h). It is also likely that many of the 24-h old (trochophore) larvae that apparently appeared morphologically normal under the microscope might have been inflicted with 'internal injuries' or damage, which were expressed at the later stages of the development in terms of either abnormality or mortality. At lower concentrations, despite distinct developmental abnormalities resulting from TBT exposure, the larvae continued to survive until 48 h (metatrochophore stage), after which survival decreased rapidly. This is reflected by the lowest value of LC_{50} for 72 h-old larvae.

Developmental toxicity tests have proved to be quite sensitive in several other marine invertebrates (e.g. mussel, sea urchin, etc.) (ASTM, 1989; Anderson et al., 1994b; Garman et al., 1997). Given the fact that such tests are of interest both from ecotoxicological and mechanistic perspectives, they have been recommended for wide use

in environmental management (ASTM, 1989; Anderson et al., 1994b; Garman et al., 1997). Despite playing an important role in the marine ecosystem, however, only a limited number of polychaete species has been exploited for use in developmental toxicity assays. One of the reasons for this is that, unlike *P. dumerilii*, where fertilisation is external and subsequent development takes place freely in water, in most other potentially useful test species (e.g. *Neanthes arenaceodentata*, *Ophryotrocha diadema*, etc.), fertilisation and subsequent development takes place either inside a tube or in a brood. Therefore, it is very difficult to ascertain the post-fertilisation developmental stages and age of the larvae in these species. This technical difficulty associated with the lifestyle of the organism limits their use for developmental toxicity assay. Having external fertilisation, similar sensitivity to environmental contaminants and ease of culturing under laboratory conditions, *P. dumerilii* has therefore enormous potential for use in marine ecotoxicology for both the acute and chronic ecotoxicity of chemicals and effluents.

Originally used for mammalian cells growing under in vitro conditions (Lamberti et al., 1983), the proliferative rate index proved to be a useful indicator to evaluate cytotoxicity. In contrast to highly differentiated mammalian cells growing under in vitro conditions, PRI values in the embryo-larval stages of aquatic organisms would represent an average figure for proliferating and differentiating cells of very early life stages over the exposure period described (Jha et al., 1996). The cytogenetic damage induced by TBT, which complements the developmental and cytotoxicity results in this non-target species, confirms our earlier study in a target species, *M. edulis* (Jha et al., 2000a,b).

Despite being a well-known toxic and endocrine disrupting agent, the genotoxic potential of TBT has not been adequately evaluated. In a World Health Organisation-supported collaborative study, which used a variety of in vitro and in vivo tests, the majority of the short term tests (with bacteria and yeasts) gave negative results (except in one bacterial strain at cytotoxic dose level). However, TBT was found to be clastogenic in Chinese hamster ovary cells in vitro and produced micronuclei in mouse bone marrow cells in vivo (Davis et al., 1987). The report pertaining to clastogenic effects of TBT in mammalian systems broadly supports our findings in early life stages of target (Jha et al., 2000b) and non-target marine invertebrates (present study). Therefore, it ap-

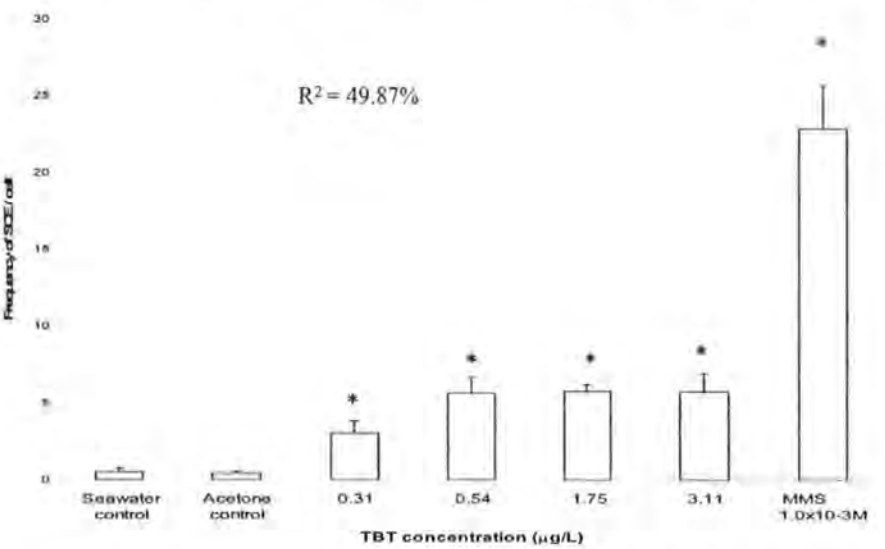
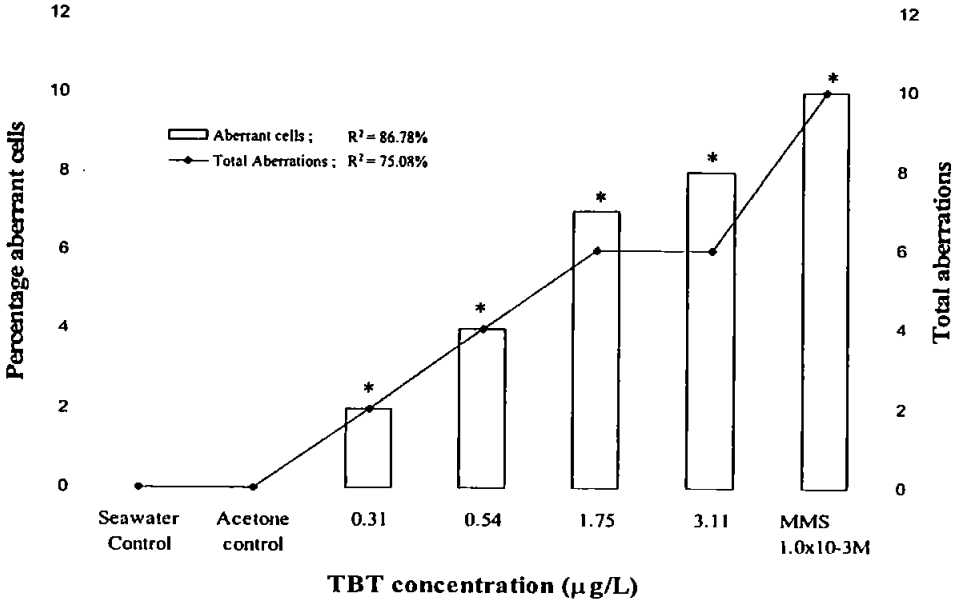


Fig. 7. The effect of TBT on the induction of sister chromatid exchanges (SCEs) in metaphase spreads of *P. dumerilii* *, Significant difference ($P < 0.000\,05$). Error bars, standard deviation.



The effect of TBT on the frequency of aberrant cells and total aberrations (excluding gap). * Significant difference 000 05).

that TBT has a significant genotoxic potenicross phylogenetically disparate group of isms and cell types. In view of its genotoxic tial, the results have wider implications for short- and long-term survival of natural (Wurgler and Kramers, 1992; Anderson et 994a), and also for human health, since in residues have even been found in the of human and other terrestrial mammals hashi et al., 1998, 1999). While evaluating evelopmental and genotoxic effects of TBT embryo-larvae of marine mussels, *M. edulis*, and Prosser (1986) did not, however, find a ve response either for the induction of SCEs CAbs. Several factors, such as dose selecexposure period, scoring criteria, etc., could nt for this discrepancy (Jha et al., 2000b). ver, in a subsequent study, when mussel (*M. edulis*) were exposed either to a refernutagen (mitomycin C) or a water-accomed fraction of crude oil in the presence of Dixon and McFadzen (1987) found that night act as an enhancer of SCEs induction. ears therefore that due consideration should en to the ability of TBT not only to induce genetic damage in its own right, but also to enhance the potential genotoxicity of other contaminants available in the complex environment.

Several mechanisms could explain the action of TBT as a genotoxic agent as described elsewhere in detail. Briefly, TBT has been shown to bind cellular macromolecules (Lee, 1991; Matthiessen and Gibbs, 1998). A large number of metallic compounds that have the binding capacity with cellular macromolecules, such as DNA and repair

Table 1
Nominal and average definitive concentrations (µg l^{-1}) of TBT in seawater samples before and after exposure of *P. dumerilii* embryo-larvae. Numbers in parentheses indicate values from repeated sampling

Nominal concentration	Definitive pre-exposure concentration	Definitive post-exposure concentration
0.31	0.27 (0.25, 0.29)	0.31 (0.27, 0.33)
0.54	0.41 (0.43, 0.39)	0.38 (0.37, 0.38)
1.75	1.55 (1.58, 1.52)	1.20 (1.17, 1.24)
3.11	2.80 (2.76, 2.83)	2.30 (2.26, 2.35)

Table 2
LC_{50} values and toxic effects on embryo-larvae/juvenile stages of different marine invertebrates following exposure to TBT

Species	Nominal test concentrations (as TBT)	Developmental stage	Exposure conditions	Reference
Platynereis dumerilii	1.34 µg/l	48 h	LC_{50}	Present study
Crassostrea virginica	1.16 µg/l	48 h	LC_{50}	Roberts (1987)
Mercenaria mercenaria	1.01 µg/l	48 h	LC_{50}	Roberts (1987)
Mytilus edulis	2.24 µg/l	48 h	LC_{50}	Thain (1983)
Arenicola cristata	2 µg/l	96 h	100% survival	Walsh et al. (1986)
	4 µg/l	96 h	100% mortality	
Neanthes arenaceodentata (body burden)	<3 µg/g	10 weeks	No effects	Moore et al. (1991)
	>6 µg/g	10 weeks	Growth and reproduction effected	
	>17 µg/g	10 weeks	Survival reduced	
Penaeus japonicus (nauplii)	1.98 µg/l	24 h	LC_{50}	Lignot et al. (1998)
	0.86 µg/l	48 h	LC_{50}	
Penaeus japonicus (juveniles)	752 µg/l	24 h	LC_{50}	Lignot et al. (1998)
	689 µg/l	48 h	LC_{50}	

enzymes, have been shown to form DNA-protein crosslinks and induce genetic damage in mammalian and aquatic systems (Jha et al., 1992; Garman et al., 1997; Hartwig, 1998). If TBT is capable of binding to either DNA or the repair enzymes, as demonstrated by earlier workers (Fent and Bucheli, 1994), it will induce both S-phase-dependent and -independent cytogenetic effects, in common with other metallic compounds (Jha et al., 1992), as observed in the present study. Metals (e.g. copper, iron, arsenic, nickel, etc.) have also been shown to induce oxidative damage to DNA, generating double-strand breaks, base damage and intrastrand crosslinks (Lloyd and Phillips, 1999), as well as DNA-protein crosslinks (Dong and Luo, 1993; Garman et al., 1997). It will therefore not be surprising if TBT is also capable of inducing such oxidative damage in the cellular DNA. As mentioned earlier, TBT is known to alter the metabolism of steroidal hormones. These hormones (or their metabolites), either directly or through the production of reactive oxygen species, are known to induce DNA or chromosomal damage (Service, 1998; Roy and Liehr, 1999). In addition, TBT has also been shown to induce programmed cell death or apoptosis in human lymphoblastoid cell lines as well as in the tissues of the marine sponge, *Geodia cydonium* (Batel et al., 1993). This further supports the assumptions that TBT might act as a genotoxic agent across different species and cell types.

In conclusion, the present study has demonstrated the genotoxic, cytotoxic and developmental effects of TBT in a marine worm, *P. dumerilii*. Genotoxic effects of TBT precipitated other effects at higher levels of organisation, indicating that TBT, a well-known endocrine disrupter for some gastropods, is capable of inducing damage in a non-target species. The findings emphasise the need for evaluation of genotoxic potential of other environmental endocrine disrupters, which has attracted wide attention in recent years. In addition, it is becoming increasingly clear that, in common with other toxicants (Hebel et al., 1997), endocrine disrupting agents may exert their effects via several mechanisms simultaneously. In this context, TBT, in addition to inducing genotoxic, cytotoxic and developmental effects, has also been shown to be immunotoxic to the marine organisms (Cima and Ballarin, 2000). The work therefore highlights the need to consider the potential

vironmental chemicals to cause toxicity by
al mechanisms simultaneously if a thorough
rstanding of their likely impacts is to be
ved.

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