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Appraisal and validation of rapid, integrated chemical and biological assays of environmental quality

FILLMANN, GILBERTO

Appraisal and validation of rapid, integrated chemical and biological assays of environmental quality

by

GILBERTO FILLMANN

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"De tudo, ficaram três coisas:

A certeza de que estamos sempre começando...

A certeza de que é preciso continuar...

A certeza de que seremos interrompidos antes de terminar...

Portanto devemos:

Fazer da interrupção um caminho novo...

Da queda um passo de dança...

Do medo, uma escada...

Do sonho, uma ponte...

Da procura... Um encontro..."

Fernando Pessoa

Appraisal and validation of rapid, integrated chemical and biological assays of environmental quality

Gilberto Fillmann

Abstract

To assess the significance of pollutants released into the environment it is necessary to determine both the extent of contamination and the biological effects they give rise to. This research is based on a tiered system, which commences with conventional analytical chemistry (gas chromatography), followed by the development, evaluation and application of rapid and simple immunochemical techniques and, finally, the integration of chemical and biological markers to assess pollution.

GC-ECD/FID/MS have been used to investigate the status of chemical contamination of the Black Sea by organochlorine residues, hydrocarbons and faecal sterols. Useful information is provided and problems with e.g. HCHs and sewage contamination are highlighted. Contamination by DDTs, PCBs, "total" hydrocarbons and PAHs is also reported. Next, these techniques are used to develop rapid screening methods.

Four distinct applications of immunochemical techniques are presented. Initially, the BTEX RaPID Assay® ELISA is evaluated to detect semi-volatile hydrocarbons in contaminated groundwater. Although overestimating concentrations when compared to GC-FID/PID, results are well correlated. Secondly, the effectiveness of the BTEX and c-PAH RaPID Assay® to detect hydrocarbons in sediments is tested. Once again, good agreement with GC-FID/MS confirms the ELISA to be a useful screening protocol to focus more expensive high-resolution analytical techniques. The adaptability and applicability of an ELISA (PCB RaPID Assay®) method in measuring "total" PCB levels in mussel tissue is demonstrated. An underestimation of concentrations, despite of covariability between ELISA and cGC-ECD, is discussed. Next, ELISA (RaPID Assay®) and fluorometry were successfully applied to quantify PAH metabolites in crab urine as a measure of exposure. HPLC analyses indicated that conjugate PAH metabolites were dominant in urine of crabs exposed to pyrene. Differences could also be identified between crabs taken from clean and contaminated sites.

Finally, an integration of chemical and biological techniques is used to investigate contamination and effects in mussels within a pollution gradient. Results indicate a correlation between micronucleus formation, heart rate and PCB and PAH level.

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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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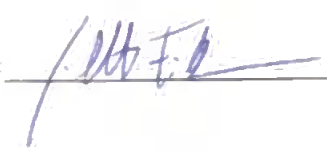
Signature 

Appraisal and validation of rapid, integrated chemical and biological assays of environmental quality

by

Gilberto Fillmann

Chapter 1

1 Strategies to assess the extent and impact of contamination

1.1 Why monitor environmental contaminants?

1.1.1 Environmental Science

Environmental science is devoted to the study of the quality of the environment and to the technology of its conservation. Therefore, it relates to the chemical, physical, and biological changes in the environment produced through contamination or modification. It also relates to the ecosystem functioning, within all environmental compartments and their interactions, and how they can become affected by man's agricultural, industrial, and social activities. It includes the application of science and technology to control and improve environmental quality (Connell and Miller, 1984).

The deterioration of environmental quality, which began when man first collected into villages and utilised fire, has existed as a serious problem under the ever-increasing impacts of an exponentially increasing population and industrialisation. Environmental contamination of air, water, soil/sediment and food threatens plant and animal communities of the ecosystem, including man.

If we are to preserve the earth for future generations, we need to address deteriorating standards of urban public health, and environmental science and technology must quickly come to play a dominant role in designing our social and industrial structure for tomorrow. Scientifically rigorous criteria of environmental quality must be developed. Based in part on these criteria, realistic standards must be established and our technological progress must be tailored to meet them. Man will continue to require increasing amounts of fuel, transportation, industrial chemicals, fertilizers, pesticides, and countless other products; and he/she will continue to produce waste products of all descriptions. What is needed is an approach focused on the development of order and equilibrium in the presently disparate segments of the human environment (Connell and Miller, 1984). Most of the skills and tools that are needed already exist, and ironically the same technology that has created environmental problems is also capable of solving them.

Our apparently limitless habitat has long been taken for granted with its supposedly vast capability to absorb wastes. In recent years it has become apparent that our environment does have limitations to the waste it can sustain or dilute to insignificance. Atmospheric wastes aggregate around our urban centres while domestic, agricultural and industrial wastes contaminate our waters, sediments/soils and biota. The response to this situation has been a massive research effort and many waste control measures have been introduced in recent years. Nevertheless, many social-economic and political factors need to be addressed, as well as scientific and engineering matters, to arrive at an effective solution to pollution problems.

Pollution occurs when substances resulting from human activities are added to the environment, causing a detrimental alteration to its physical, chemical, biological, or aesthetic characteristics. It is important to note, however, that the occurrence of pollution requires a subjective judgement as to whether a detrimental effect has resulted or not. In fact, there can be conflicting opinions on this. Environmental management agencies are now concerned with the protection of water quality for the conservation of natural ecosystems. This requires a detailed understanding of the long-term effects and this is limited at the present time.

Conventional methods for monitoring environmental pollution are problematic because they are mainly based on chemical observations and analysis. This approach is expensive and is applicable to only a small proportion of the toxic chemicals in the environment. It provides little biologically meaningful information, and overlooks the complexity of the systems under surveillance (Butterworth, 1995). Modern approaches are more comprehensive, including chemical and geochemical analysis, biodegradation and toxicity testing, biochemical, physiological and behavioural biomarkers, biomonitoring procedures and ecological survey procedures for determining the bioavailability and effects of contaminants. Again, there may be serious limitations to these approaches in terms of costs and capabilities (Wells *et al.*, 2001).

Recently, there has been increasing interest in the effects of pollutants at different levels of the ecological organisation (from a biochemical/molecular level up to ecosystems) (Fig 1.1). This has led to the development of the relatively new field of *ecotoxicology*, which is a combination of three disciplines: toxicology, ecology and environmental chemistry (Gerhardt, 2000). Consequently, an integrated environmental

monitoring regime should logically focus on the combination of chemical, toxicological and ecological field approaches.

1.2 Assessment of Aquatic Pollution

1.2.1 RAMP Programme

A diverse array of procedures exists for detecting the impacts of pollutants in marine and estuarine environments (Depledge and Hopkin, 1995; Linthurst *et al.*, 1995). These include ecological surveys to identify changes in the abundance and diversity of species comprising communities, chemical and biomonitoring procedures for assessing the concentrations and bioavailability of anthropogenic contaminants, and biochemical, physiological and behavioural biomarkers which signal exposure to, and in some cases, adverse effects of pollution (Fig. 1.1) (Depledge, 2000).

When these procedures are combined in well-designed survey programmes, they can provide an insight into which pollutants are responsible for, and the extent of, environmental degradation. However, they also have a number of practical drawbacks. They are expensive to perform, they must be carried out by highly-trained personnel and, in the case of analytical environmental chemistry and biomarker analyses, they usually require the use of technologically-advanced equipment.

In developing countries, the availability of highly trained personnel and sophisticated analytical facilities is extremely limited. In contrast, in developed countries, competing diverse demands for limited public funds severely constrain the resources available for use in environmental protection and legislation, and may hinder efforts directed towards detecting pollution threats. These restrictions point to the need to develop more pragmatic environmental assessment procedures which can provide the basis for prioritising among study sites, so that resources can be expended efficiently and effectively.

To address this problem, a project entitled Rapid Assessment of Marine Pollution (RAMP) has recently been adopted as a pilot project of the Health of the Ocean (HOTO) Panel of the Global Ocean Observing System (GOOS) within the Global Investigation of Pollution in the Marine Environment programme (GIPME/UNEP) (IOC, 1996; Knap, 2000). A RAMP pilot project has been taking place in Brazil for over 3 years (Depledge, 2000; Wells *et al.*, 2001). The development of the HOTO Panel

of GOOS specifically addresses the means and ways of developing integrated mechanisms for observing and forecasting the effects of anthropogenic activities on the marine environment. One of its tasks is to provide sensitive rapid assessment of contamination caused by discharges of sewage and chemical pollutants.

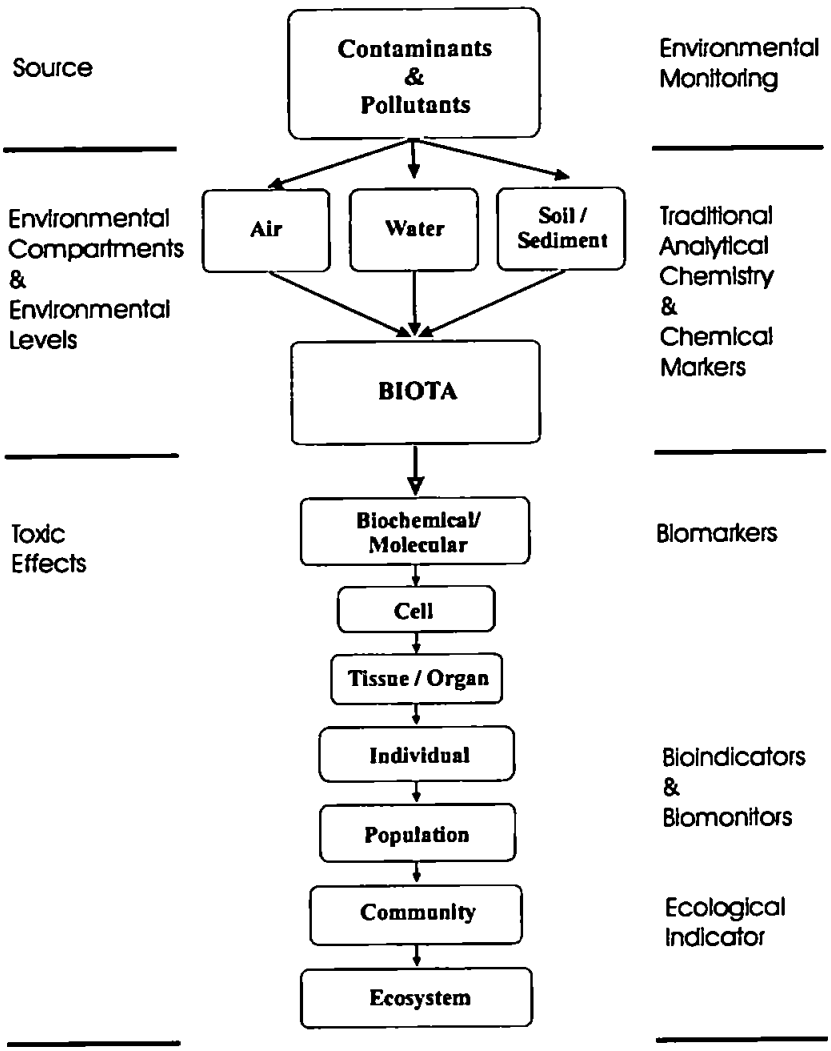


Figure 1.1. Diagrammatic illustration of the impact of pollutants on components of the ecosystem and levels of ecological organisation.

The scientific rationale underlying RAMP is based on the premise that to assess the significance of pollutants released into the environment, it is necessary to determine both the concentrations of chemicals accumulating in biota and the biological effects they give rise to. It is intended to test and provide simple and easy to perform, robust and inexpensive techniques to measure chemical and biological markers needed to assess environmental impacts, thereby providing a basis for prioritising the need for

action among sites. Specific measurements include the use of immunoassay techniques to detect and quantify levels of contaminants and simple measures of biological effects (biomarkers) (Fig. 1.1). This approach is currently being evaluated by comparing it with comprehensive analytical chemical analysis and standard toxicity test procedures to confirm the reliability of new procedures. In the context of the HOTO Module, the validation of the combined chemical and biological markers represents an important step. These techniques potentially provide cost-effective screening alternatives to the more complex procedures currently used. The limited resources available can, therefore, be better expended improving environmental management. This is important since environmental managers, although faced with the task of evaluating the extent of contamination and the degree of ecological damage in coastal regions, are usually handicapped by the lack of resources for conducting field work and to perform state-of-the-art chemical and biological assays, especially in developing countries.

1.2.2 Analytical Chemistry

Chemical analyses lies at the heart of almost all environmental investigations whether they are devoted to monitoring the distribution of a xenobiotic, evaluating its persistence and toxicity, or determining its partitioning between environmental matrices (Neilson, 1994). Monitoring environmental contaminants traditionally involves analytical chemistry. In order to assess the potential harm that environmental pollutants pose, we need to know the identity of the chemicals concerned. Environmental concentrations of those chemicals are important in determining their toxic potential. It is well established that we need to know exactly which and how much of a chemical is present before we can assess its likely deleterious effects upon organisms with which it might come into contact. In addition, chemical monitoring helps to determine how the use of such chemicals causes their concentrations to rise and when we should attempt to restrict (or even ban) their use for fear of harm to the environment resulting from their increased concentrations (Shaw and Chadwick, 1999).

In order to analyse environmental samples, the single or group of contaminants (analyte) must first be extracted from the sample (matrix) so that it can be concentrated and subjected to an appropriate analytical method. Having extracted the analyte from the environmental sample, the extract needs to be purified to remove as many interfering molecules as possible. There are numerous techniques to quantify pollutants and they are generally based on chromatography. This is by far the most commonly

applied analytical method to identify and quantify environmental organic contaminants. Compared to the very simple separation of inks and plant extracts using absorbent paper that first gave the name to this technique, nowadays chromatographic techniques can be sophisticated and are commonly used in environmental analysis.

Capillary gas chromatographs (cGC) equipped with either a flame ionisation detector (FID) or an electron capture detector (ECD) and high performance liquid chromatographs (HPLC) equipped with a light absorption detector (spectrophotometer), a diode array detector (DAD) or a fluorescence detector are common quantification techniques. Most of these techniques rely heavily on the specific retention time of each compound for identification. This is by no means a definitive identification of a peak in a chromatogram of an environmental extract. Spectroscopic techniques can give information about the molecule itself, which helps to identify the unknown substance. The most useful spectroscopic techniques are those which can be interfaced with the chromatographic procedures mentioned above. This is important because it allows the resolving capacity of the chromatography to be linked with spectroscopic identification. Mass spectrometry (MS), which can be interfaced with both GC and HPLC, is by far the most commonly applied procedure for the identification of unknown environmental contaminants.

The MS detector works by bombarding a vaporised molecule within a vacuum with either electrons (most commonly) or ions which results in fragmentation of the molecule. The molecular fragments are charged and their mass to charge ratio (m/z) determines their mass separation as they move through four rods with an electric field between them. As the ions enter the field, they interact with it in such a way that only ions of a specific mass/charge ratio will pass through to the detector. The above describes a quadrupole mass spectrometer. The detection is performed almost exclusively using an electron multiplier. The molecular fragmentation pattern is characteristic of each molecule, which enables a positive identification.

These analytical procedures only correspond to a fraction of those used by the environmental chemist. They represent the non-biological or pure analytical chemical techniques. Biology, however, still holds a key to analytical specificity and it is for this reason that immunoassays are also important in environmental monitoring.

1.2.3 Immunoassay

As mentioned above, it is important to know the extent to which contaminants accumulate in organisms since this provides some insight into likely causes of biological damage. Immunoassay-based methods for the analysis of environmental contaminants are relatively new on the analytical chemistry scene (Stanker and Beier, 1996; Dankwardt *et al.*, 1998). These methods are based on the use of a specific antibody as a detector for the analyte (or group of analytes) of interest. Immunoassays are rapid, sensitive, and selective, and are generally cost effective for large sample loads. They are adaptable to field use and have been applied to diverse chemical structures (i.e. triazines (Gascon *et al.*, 1997), polycyclic aromatic hydrocarbon (Knopp *et al.*, 2000), polychlorinated biphenyls (Franek *et al.*, 2001), dioxins (Shan *et al.*, 2001), selected organophosphorous (Oubina *et al.*, 1997) and organochlorine pesticides (Ragab *et al.*, 1997)). They have also been applied to various matrices (i.e. water (Barceló *et al.*, 1998), groundwater (Knopp *et al.*, 2000), soil/sediment (Waters *et al.*, 1997b), food (Roda *et al.*, 1999; Jaborek-Hugo *et al.*, 2001), and biota (Zajicek *et al.*, 2000)). These characteristics make immunochemical analysis a valuable tool to fulfil the aims of projects like RAMP (Wells *et al.*, 2001) and for use by environmental analytical chemists (Aga, 1997). The principles of immunoassay technique have been previously described (Kemeny, 1991) (Aga, 1997) (Sherry, 1997).

In contrast to conventional analytical methods, however, the immunoassay has not yet been extensively characterized. At the moment, several agencies in the USA (EPA, USDA, AEIC, AOAC) and Europe (German Immunoassay Study Group, UK ESCA) are involved in the evaluation of immunoassays and producing guidelines into their use (Meulenberg *et al.*, 1995). As for any analytical method, quality control and assessment of the stability of materials and equipment are required. In addition, immunoassay evaluations involve defining the working range, sensitivity, precision, accuracy, linearity, specificity, and matrix effects. Another import step in the evaluation of an immunoassay comprises validation by comparing results with established conventional methods. Furthermore, in order to recognize the immunoassay as a reliable analytical method, there is a need for interlaboratory tests (Meulenberg *et al.*, 1995). Most of the immunoassays developed, however, are retained in their particular laboratory or institute. Consequently, comparative experiments and interlaboratory validation are comparatively rare. More independent evaluations of commercially available kits

should, however, enhance the acceptance of this technology (Waters *et al.*, 1997a; Kramer, 1998). In this respect, commercial immunoassay kits offer many advantages (Meulenberg *et al.*, 1995).

A number of different immunoassay formats have been used. Enzyme-linked immunosorbent assay (ELISA) is the most common version of environmental immunoassay (Stanker and Beier, 1996; Dankwardt *et al.*, 1998). ELISA is an immunoassay method that uses enzyme-labeled antigens or antibodies (enzyme conjugates) to detect and quantify target compounds (antigens, otherwise known as the analytes of interest) in field samples. The antibody is capable of reacting specifically with the antigen to form an antigen/antibody complex (commonly referred to as the "lock and key" approach). The analyte portion of the enzyme conjugate can bind with the antibody as can the analyte present in the samples. ELISA tests are considered to be "competitive" assays because the sample-derived analyte competes with the enzyme conjugate analyte (which is kept at a constant concentration) to bind to the finite number of antibody sites. The greater the concentration of sample-derived analyte relative to the enzyme conjugate analyte, the larger the proportion of antibody sites that are occupied by the sample-derived analyte. The enzyme portion of the enzyme conjugate serves as a catalyst to change a colourless compound to a measurable coloured product that can be detected instrumentally. The amount of colour produced is inversely proportional to the amount of sample-derived analyte. More colour equals less sample-derived analyte. Less colour equals more sample-derived analyte, because all the antibody sites are bound to sample-derived analyte and there is less enzyme conjugate present to catalyse the colour reaction.

Immunoassays can rely on a single antibody (monoclonal) or mixtures of antibodies (polyclonal) to specifically bind with the antigen(s). Antibodies are a class of proteins known as immunoglobulins, which are produced in animals in response to a foreign substance (antigen). The small molecular weight antigen may not cause an immune response (a non-immunogenic molecule) in the animal. As a result, for the purpose of stimulating an immune response, it must be coupled to a "carrier" molecule (an immunogenic substance, e.g. bovine serum albumin), which will present the small molecule (hapten) to the immune system as a foreign substance.

An increasing number of immunoassays for residue analyses can be found on the market. Different companies (mainly in the United States and Germany) have developed

several environmental immunoassay test kits for contaminants. The Strategic Diagnostics Incorporation (SDI, Neward, DE, USA) is one of the biggest companies in the development, manufacture and distribution of environmental immunoassay-based test kits for industrial, chemical and biological contaminants. Recently (1996/1997), SDI has merged with other USA immunoassay manufacture companies (Millipore EnviroGard®, Ohmicron Environmental Diagnostics, EnSys Ris[c®]). Its EnSys™, EnviroGard®, RaPID Assay® (former Ohmicron) and DTech® branded test kits are distributed in United Kingdom (and Europe) through SDI Europe (Alton, Hampshire, UK), formerly known as EnSys™ Europe.

Those commercially available test kits are based on competitive, heterogeneous ELISA, but they vary in format, application, technology, analysis time and sample throughput (Table 1.1). EnSys™ has the antibody immobilised on the bottom of plastic tubes. DTech kits uses antibody immobilised to latex particles and RaPID Assay® onto magnetic particles. Envirogard® has the antibody coated either to tubes or 96-microwell plates. Some kits are designed to run a small number of samples, under field conditions, getting qualitative or semi-quantitative results (EnSys™, EnviroGard® (coated tubes), and DTech®). Others laboratory-based systems are designed to run many more samples in one batch to obtain semi-quantitative/quantitative results (RaPID Assay® and plate EnviroGard®) (Table 1.1).

Table 1.1. Guide of commercially available immunoassay kits manufactured by SDI (Data extracted from SDI Product Information).

	IMMUNOASSAY KIT			
	RaPID Assay®	EnviroGard®	EnSys™	DTech®
Technology	magnetic particles	coated tube/ plate	coated tube	latex particle
Data format	semi-quantitative & quantitative	semi-quantitative & quantitative	semi-quantitative	qualitative & semi-quantitative
Sample throughput	1 – 50 samples/run	1 – 14 samples/run 96 samples/run	1 - 10 samples/run	1 - 4 samples/run
Analysis time*	60 minutes/run	30 minutes/run 60 minutes/run	30 minutes/run	60 minutes/run
Application	laboratory	field/ laboratory	field	field

* -based on water sample analysis.

Inherent to the use of antibodies is a certain degree of cross-reactivity, i.e., the binding of structurally related compounds to the antibody. The degree to which a particular antibody selectively binds the analyte of choice determines its applicability. A low degree of cross-reactivity makes it suitable for single-compound assays. For very specific measurements, a monoclonal antibody is best suited. In contrast, a group-specific assay requires an antibody having a high degree of cross-reactivity. Generally, an antiserum consisting of several types of antibodies (polyclonal) shows a broader spectrum of cross-reactivity than a monoclonal antibody. Polyclonal antibodies might also be employed when analytes of interest are rapidly metabolised, providing that the structural part of the analyte being studied is not altered too much during metabolisation or degradation (Meulenberg *et al.*, 1995). For the purpose of projects like RAMP, the ideal immunoassay kit should have a broad spectrum of reactivity, being suitable to assess contamination by a wide range of chemicals (e.g. aliphatic and aromatic hydrocarbons or PCBs). Sensitivity, however, is a critical parameter.

Examples of popular commercially available immunoassays for hydrocarbon analysis are listed on Table 1.2. Suitable for both sediment/soil and water matrices, sensitivities differ for each format. RaPID Assay® is the most sensitive, but has restrictions regarding field application. The PAH RaPID Assay® can provide a 20-fold greater response than the EnviroGard® test (Waters *et al.*, 1997a). The EnviroGard® plate format is, however, quite sensitive but requires a plate reader to be used. Use of DTech® has being discouraged by SDI staff primarily because of reproducibility problems (SDI, pers. comm.). A magnetic particle ELISA (RaPID Assay®) has been shown to have better precision and sensitivity compared to formats where the antibody is passively adsorbed to polystyrene tubes (Aga and Thurman, 1993; Lawruk *et al.*, 1996).

The RaPID Assay®'s enhanced reproducibility is achieved due to better mixture/homogenisation between antibody, enzyme conjugate and sample. Mixing is less thorough when the antibody is coated to the tube/well walls. The small magnetic beads also have the benefit of a larger surface area to volume ratio when compared to the microtiter plate (which uses a plane surface for the immunochemical reaction). Hence, the shorter diffusion distances provided by the magnetic beads allow a rapid interaction between the antibody and the analyte or hapten-conjugate in each reaction mixture. This alleviates the well-to-well variability encountered with microtiter plate-

based ELISAs. As a result, the magnetic particle-based ELISA provides slightly better precision and shorter analysis time than the microtiter plate-based ELISA. However, the assay by the magnetic particle-based ELISA involves more steps than the microtiter plate-based ELISA because of repeated incubations and washing steps (Aga and Thurman, 1993).

Table 1.2. Application matrices and detection limits of SDI commercially available hydrocarbon immunoassay kits (data extracted from SDI Product Information)

		IMMUNOASSAY KIT			
Analyte	Application	RaPID Assay®	EnviroGard®	EnSys™	DTech®
BTEX/TPH*	Soil/Sediment	0.2 $\mu g\ g^{-1}$	2 $\mu g\ g^{-1}$	10 $\mu g\ g^{-1}$	2.5 $\mu g\ g^{-1}$
	Water	20 $\mu g\ L^{-1}$	100 $\mu g\ L^{-1}$	N.A.	600 $\mu g\ L^{-1}$
PAH	Soil/Sediment	0.07 $\mu g\ g^{-1}$	1.0 $\mu g\ g^{-1}$	1.0 $\mu g\ g^{-1}$	0.6 $\mu g\ g^{-1}$
	Water	0.7 $\mu g\ L^{-1}$	2.0 $\mu g\ L^{-1}$	15 $\mu g\ L^{-1}$	8.0 $\mu g\ L^{-1}$
Carcinogenic PAH	Soil/Sediment	0.004 $\mu g\ g^{-1}$	N.A.†	N.A.†	N.A.†
	Water	0.04 $\mu g\ L^{-1}$	N.A.†	N.A.†	N.A.†

* BTEX – benzene, toluene, ethyl benzene and o-, m-, p-xylene; TPH – "total" petroleum hydrocarbons.
† N.A. – not available

The higher number of "runs per kit" makes the RaPID Assay® more cost effective. The better sensitivity and quantitative performance of this format is achieved because of the prescribed protocol, which uses a wider range of standard solutions, large solution volumes, and controlled conditions achieved under laboratory conditions. The detection limit (or sensitivity) of an assay is often critical for the evaluation of the applicability of any particular assay. For instance, the European maximum for pesticides in drinking water is 0.1 $\mu g\ L^{-1}$ (EC Directive for Drinking Water, 1990) for individual compounds. Monitoring thus requires a detection limit of at least 0.05 $\mu g\ L^{-1}$ in order to yield a suitable method (Meulenberg *et al.*, 1995). The detection limits of the RaPID Assay® ELISA kits are in the low $\mu g\ L^{-1}$ (BTEX) and $ng\ L^{-1}$ (PAH and carcinogenic PAH) range for water (Table 1.2.), which seems to be adequate.

The choice of determinands amenable to detection by the rapid chemical analysis procedures is broad and is increasing. Thus, the most relevant contaminants can be selected for different study regions. PAHs, PCBs, dioxins, organochlorine and organophosphorous pesticides, selected herbicides and fungicides are commonly monitored environmental contaminants/pollutants and immunoassay kits are available

for many of these determinands. Water and sediment samples can provide information regarding the distribution and environmental concentrations of contaminants and most kits are designed for these matrices. In addition, immunoassays can also be adapted for tissue extracts, haemolymph/blood and excreta (e.g. urine) samples. This allows concentrations of chemicals to be determined in organisms, which has advantages (see Chapter 4). These values can then be related to biological effects.

1.2.4 Biomarkers

Biomarkers are defined as "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 proposed that biomarkers can be used to chart changes in fitness of organisms (Depledge, 1994). This greatly enhances the ecological significance of biomarker measurements. It is assumed that a healthy individual exposed to increasing chemical toxicity undergoes a progressive deterioration in health that is, eventually, fatal (Fig. 1.2). Early departures from good health are often not apparent as explicit diseases, but are associated with the initiation of biochemical and physiological compensatory responses (Fig. 1.2). When these compensatory responses are activated, the survival potential of the organism may already have begun to decline because the ability of the organism to mount new compensatory responses to natural and anthropogenic stressors is compromised. If an organism is exposed to a level of toxicity that causes changes which cannot be reversed, then pathological process will result in the development of the overt disease and finally, death.

The present perception of the state of knowledge of effects along the ecological spectrum of organisation is summarised in Fig. 1.3. The ability to understand and assign causal relationships is best at the lower levels of organisation and becomes increasingly poor as the level of organisation increases. The lower level effects are generally more sensitive (i.e. manifested at lower toxicant concentrations) and respond more rapidly to an exposure than effects at higher levels of organisation. The associated advantage is that biochemical or physiological indicators can be used proactively (i.e. before irreversible or major ecological harm). By the time an effect is seen at higher levels, the degradation has already occurred. It is suggested that effects at lower levels tend to be more reversible than effects at higher levels of organisation (Chapman, 1991). All these qualities seem to indicate that effects at lower levels of organisation are superior to

higher level effects when they are considered as tools for environmental management. However, the probability of falsely assigning an adverse ecotoxicological effect is increased when higher level effects are neglected in favour of lower level responses. Because our ability to relate lower level responses to ecosystem degradation is severely limited, it follows that the ecological relevance of a lower level response is much more ambiguous than that of a higher level effect (Newman, 1995). On the other hand, the comparatively modest cost of acquiring knowledge at lower levels of organisation, combined with the higher ability to extract this knowledge using less descriptive approaches (compared to higher levels), make them a valuable tool for investigating potentially contaminated environments. Their limitations, however, must be kept in mind.

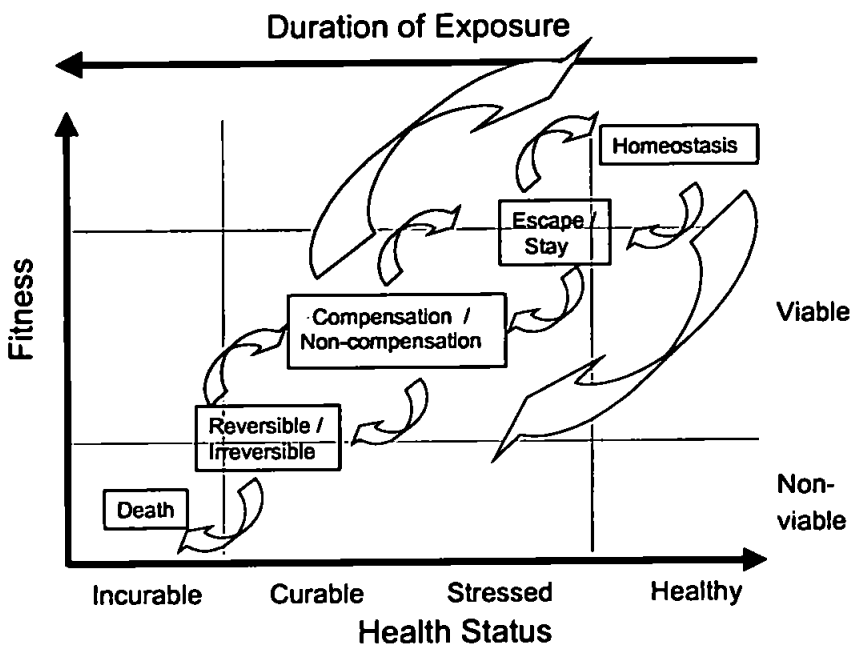


Figure 1.2. Conceptual plot of fitness versus health status as related to pollutant exposure (from Moore *et al.*, 2000).

Single biomarkers have been successfully applied to investigate pollution under many different circumstances (Lowe *et al.*, 1995; Depledge and Lundebye, 1996; Moore *et al.*, 1999). Surprisingly, it is only recently that suites of biomarkers have been included in routine environmental management procedures (e.g. The Biological Effects of Environmental Pollution in Marine Coastal Ecosystems - Beep Project), despite the fact that there is an extensive literature confirming the value of this approach (Nacci *et al.*, 1996; Burgeot *et al.*, 1996; Legras *et al.*, 2000; Sole, 2000).

It is stressed that for the purpose of this research biomarkers have been selected primarily with regard to their ease of use, low cost and relevance to known environmental problems. In some cases, more robust (and often complex) biomarker methods are available and might provide more accurate information as to the nature and extent of pollution at a particular site. However, they are more expensive, more time consuming, require more highly trained personnel, and higher quality analytical facilities than are generally available. They are thus impractical to include in a rapid assessment programme.

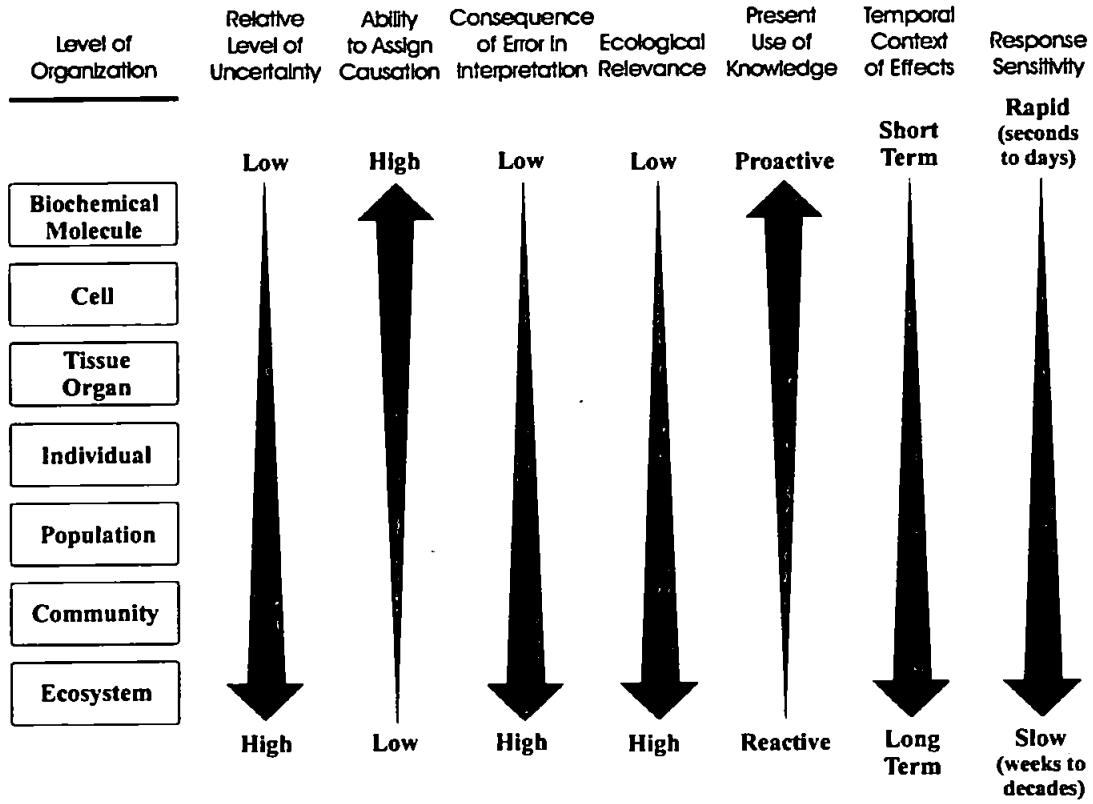


Figure 1.3. Features of ecotoxicological effects based on level of ecological organisation (from Newman, 1995).

1.3 Aim and Objectives

The approach adopted for this research involves the development, evaluation, application, combination and integration of analytical methods and chemical and biological markers in order to assess aquatic pollution.

The main objectives of this research were:

Conventional Analytical Chemistry

- To carry out and evaluate a range of contamination assessments based on conventional analytical chemistry analyses;

Chemical Markers (Immunoassay)

- To provide an independent evaluation of the performance and suitability of commercially available immunoassay kits;
- To evaluate and validate commercial ELISAs for screening of contamination in water and sediments;
- To adapt and evaluate the effectiveness of ELISA techniques in measuring contaminant levels in the biological tissues/fluids;
- To develop new applications of ELISA techniques in accordance with "Rapid Assessment of Marine Pollution" (RAMP);

Biomarkers

- To use a combination of biological markers and chemical assays to provide assessments of the relationship between anthropogenic contaminant levels, toxic damage and adverse health effects in selected invertebrates from contaminated sites.

1.4 Outline of the Thesis

The outline of this thesis is summarised in Fig. 1.4. The research is based on a sequence of events starting with the application of conventional analytical chemistry (gas chromatography), followed by development, evaluation and application of different immunochemical techniques and, finally, an environmental application of an approach which integrates chemical and biological biomarkers to assess pollution (Fig. 1.4).

Chapter 2 presents a detailed description of the conventional analytical chemistry, immunochemical and biomarkers methodology used in this research. Results elucidating the present status of chemical contamination in the Black Sea are presented in Chapter 3. Conventional chemical analyses (gas chromatography) are used to investigate concentrations of organochlorine residues, hydrocarbons and faecal sterols in surface sediments from several areas along its coast.

Chapter 4 is divided into four sections covering distinct applications of immunochemical techniques. Initially, an evaluation and validation of an ELISA kit to

detect semi-volatile hydrocarbons (BTEX) in contaminated groundwater is presented. Secondly, the effectiveness of two different immunoassay kits to detect hydrocarbons (aliphatics and aromatics) in sediments is tested and is compared with results obtained using chromatography. The adaptability and applicability of an ELISA method in measuring PCB levels in mussel tissue is also demonstrated. Finally, the effectiveness of ELISA for measuring PAH metabolite levels in the urine of aquatic crabs (*Carcinus maenas*) exposed to PAHs is determined. The latter two sections are especially interesting since they represent highly novel applications for ELISA.

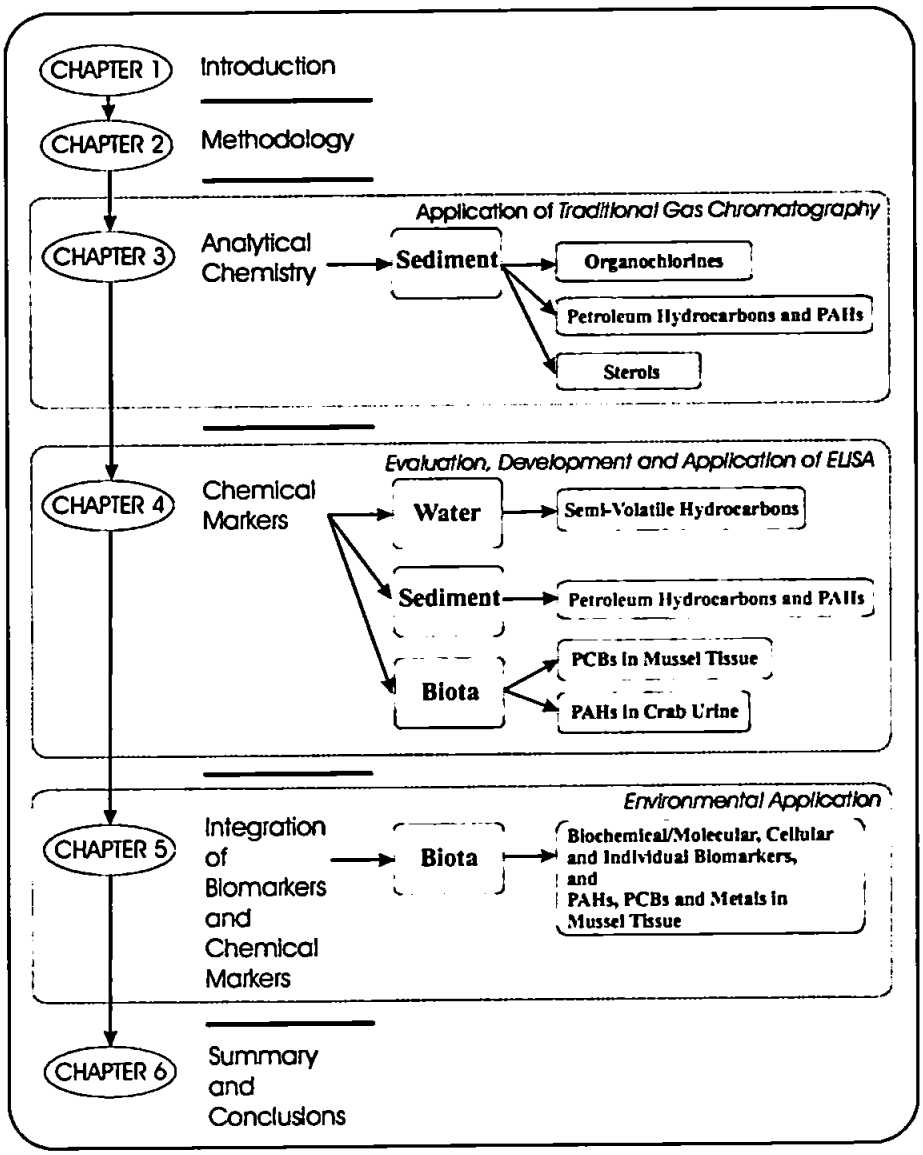


Figure 1.4. Outline of the thesis.

A rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays is presented in Chapter 5. A combination of biomarker techniques,

covering different levels of ecological organisation (biochemical/molecular, cellular and individual), and immuno and analytical chemistry are used to provide an assessment of the concentrations and adverse effects in mussels from contaminated sites.

Chapter 6 provides a summary and conclusions from this research. It includes discussions regarding information provided by conventional analytical chemistry, and compares this approach with an integrated analytical and biological approach. Recommendations for future work are also presented.

Chapter 2

2 Material and Methods

This chapter focuses on the individual description of the methodology (apparatus, instrumentation, supplies, reagents and analytical procedures) of conventional analytical chemistry (Chapter 3), immunoassay (Chapter 4) and biomarkers (Chapter 5) used in this research.

2.1 Conventional analytical chemistry (Chapter 3)

2.1.1 A comprehensive study of contamination in the Black Sea

2.1.1.1 Materials

Certified solutions of PCB congeners (CLB-1) were purchased from NRCC (Halifax, Nova Scotia, Canada). Other PCB congeners were acquired from QMx Laboratories Ltd (Safron Walden, UK). Authentic individual organochlorine pesticide standard solutions (~ 100 mg L^{-1} in hexane) and standard mixtures (CLP-216/CLP-226B; ~ 5-50 mg L^{-1} in hexane) were acquired from Ultra Scientific (North Kingstown, RI, USA). A standard reference solution of 24 aromatic hydrocarbons (NIST-SRM 1491) was purchased from Promochem (Herts, UK). Aliphatic hydrocarbons (n-C_{14}-C_{35}, phytane and pristane) were purchased from Promochem (Herts, UK). Authentic sterols standard were purchased from Sigma-Aldrich Ltd. (Gillingham, UK). Internal standards (PCB29, εHCH, 9,10-dihydroanthracene, $C_{18:1}$, and 5α-androstan-3β-o)l were obtained from QMx Laboratories Ltd (Safron Walden, UK). Silica (70-230 mesh), alumina (70-230 mesh), Florisil® (60-100 mesh) and anhydrous sodium sulphate were purchased from BDH Merck LTD (Lutterworth, UK). Solvents of glass distilled grade were bought from Rathburns Chemicals Ltd (Walkerburn, UK). Solvents were batch tested for PCB and PAH contamination.

2.1.1.2 Sample Collection

Surface sediment (0-2 cm) samples were collected from the locations shown in Fig. 2.1 using a stainless-steel grab. Individual sediment samples were well mixed and stored frozen (-20°C) in pre-cleaned glass jars until analysis. Samples from Ukraine

were collected in September 1995 during a cruise on the research vessel "V. Parshin". The Hydrometeorological Institute in Sochi collected samples from the Russian Federation in December 1995. Samples from Turkey were collected by the Institute of Marine Sciences of the Middle East Technical University (Erdemli, Turkey) in September 1995. In 1993, sediment samples were collected by the Romanian Institute of Marine Research in Constantza (EMIR). These Romanian samples were analysed only for organochlorine contaminants. Site descriptions are given in Table 2.1.

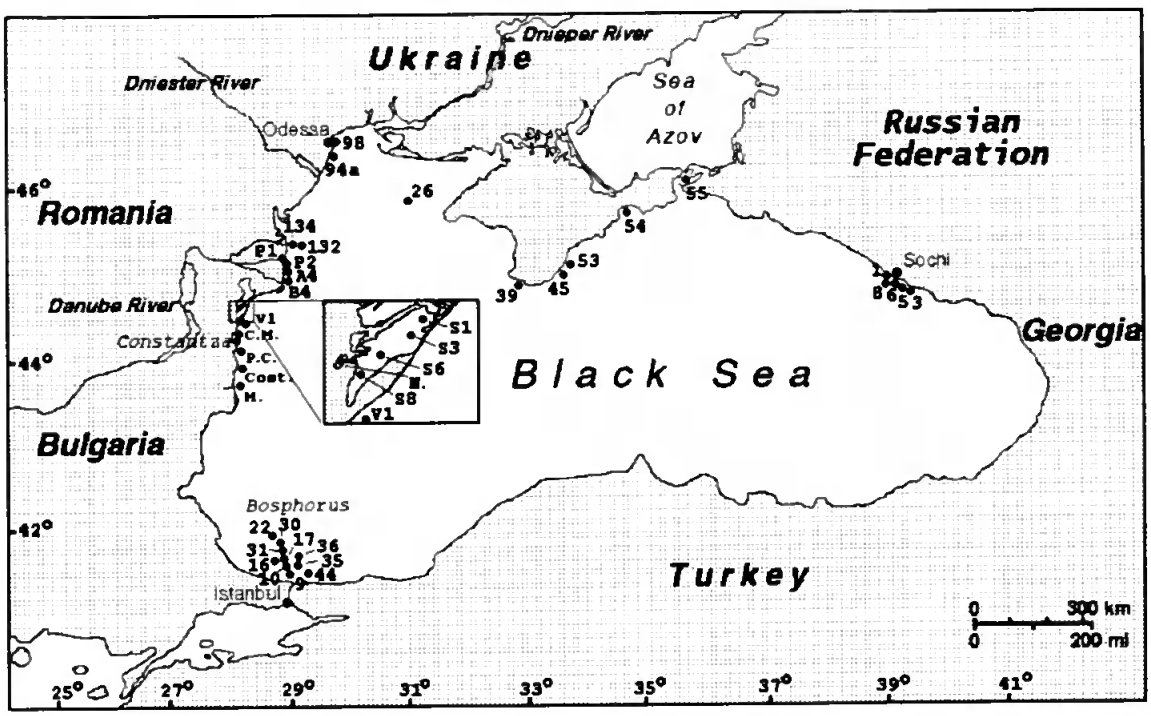


Figure 2.1. Location of sampling sites

2.1.1.3 Sediment Extraction

Sediments were freeze-dried, dry/wet weight ratios determined and then sieved (250 μm stainless steel). Each sediment sample (10 - 20 g) was spiked with internal standards: 2,4,5-trichlorobiphenyl (for PCBs), εHCH (for organochlorine pesticides), $C_{18:1}$ (for the aliphatic hydrocarbon fraction), 9,10-dihydroanthracene (for the aromatic hydrocarbon fraction) and 5α-androstan-3β-ol (for the sterols). These standards were used to quantify the overall recovery of the procedures. Samples were Soxhlet extracted for 8 hours into hexane (250 mL) followed by re-extraction into dichloromethane (250 mL) for 8 hours. The dichloromethane and the hexane were then combined and concentrated down to a few millilitres using rotary evaporation followed by gentle nitrogen "blow down". Sulphur was removed by shaking the extracts with mercury. The

extracts were then separated into aliquots: 1/3 for hydrocarbons and sterols and 2/3 for chlorinated hydrocarbons. Extractable Organic Matter (EOM) was determined by evaporating a small measured volume of the extract on the pan of an electrobalance.

The clean-up and fractionation of hydrocarbons and sterols was performed by passing the extract through a silica/alumina column (silica and alumina were activated at 200°C for 4 hours and then partially deactivated with 5% water). The chromatography column was prepared by slurry packing 10 mL of silica, followed by 10 mL of alumina and finally 1 g of sodium sulphate. Elution was performed using 20 mL of hexane to yield the first fraction (which contains the aliphatic hydrocarbons), then 30 mL of hexane/dichloromethane (90:10) followed by 20 mL of hexane/dichloromethane (50:50) (which combined contain the polycyclic aromatic hydrocarbons (PAHs)). Sterols were then eluted with 40 mL of dichloromethane/methanol (90:10).

2.1.1.4 Hydrocarbons

The hydrocarbon fractions were analysed by UV-fluorescence spectrophotometry (UNEP/IOC/IAEA, 1992) and by gas chromatography (GC) using a Hewlett Packard HP5890 series II with a flame ionisation detector (FID). A SE54 fused silica capillary column was used (HP-Ultra 2 crosslinked 5% Ph Me Silicone, 25 m length, 0.32 mm i.d., 0.17 μm film thickness). The temperature was programmed from 60°C to 290°C at 3°C min^{-1} and was then maintained at 290°C for 25 min. Helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}. Confirmation of peak identity was obtained for selected extracts using GC with mass spectrometric detection (GC-MS) (Hewlett-Packard 5889B MS "Engine").

2.1.1.5 Faecal Sterols

Fractions containing the sterols were derivatised just prior to GC analyses. A solution of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was used as the silylation reagent (Readman *et al.*, 1986b). 100 μL was added to the concentrated sample and the mixture maintained warm (40-50°C) for 60 min to react. The sample was then evaporated to dryness and toluene added as a solvent for the GC analysis. Gas chromatography (GC) was performed with a Hewlett Packard HP5890 series II equipped with a flame ionization detector and split/splitless injector. A DB5 fused silica capillary column was used (30 m x 0.25 mm i.d.; film thickness 0.25 μm). Helium was the carrier gas (1.2 mL

min^{-1}). The oven temperature was programmed from 60°C (0.5 min hold) to 290°C at 6°C min^{-1}. Injector and detector temperatures were, respectively, 270°C and 320°C. Stanones and sterols were quantified by comparison with external standards and recoveries were corrected using the internal standard (5α-androstan-3β-ol). Confirmation of peak identity was obtained for selected extracts using GC with mass spectrometric detection (GC-MS) (Hewlett-Packard 5889B MS "Engine").

2.1.1.6 Organochlorines

Clean-up and fractionation of chlorinated compounds was performed by passing the extracts through a Florisil® (17 g) column, which had been activated at 130°C for 12 hours and partially deactivated with 0.5% water. From this column, three fractions were collected: a first fraction with 70 mL of hexane; a second fraction with 50 mL of hexane/dichloromethane (70:30), and a third fraction with 40 mL of dichloromethane. Each fraction was concentrated and injected into a GC (Hewlett Packard HP5880) equipped with an electron capture detector and split/splitless injector. The capillary column used was an SE54 fused silica (HP-Ultra 2 crosslinked 5% Ph Me Silicone, 25 m length, 0.2 mm i.d., 0.33 μm film thickness). The oven temperature was programmed from an initial temperature of 70°C (2 min hold) to 260°C at a rate of 3°C min^{-1} and was then maintained at 260°C for 20 min. Injector and detector temperatures were maintained at 250°C and 300°C, respectively. Helium was used as the carrier (1.5 mL min^{-1}) and nitrogen as the make-up (60 mL min^{-1}) gas. Concentrations of individual organochlorines were quantified relative to the peak area of the respective external standards following calibration with authentic standards. Confirmation of peak identity was obtained for selected extracts using GC with mass spectrometry (GC-MS) (Hewlett-Packard 5889B MS "Engine").

2.1.1.7 Quality Control

Appropriate blanks and reference material IAEA-357 (sediment) were analysed simultaneously with each batch of samples. This sediment has certified concentrations of chlorinated compounds and hydrocarbons. Although concentrations of sterols are not certified, this sediment has been used as an "in-house" reference material for internal quality control.

The sediment analyses were carried out by the IAEA laboratory during 1996 and 1997.

Table 2.1. Description of the Black Sea stations.

Country	Sample code *	Position	Depth (m)	Comments
Ukraine	26	45°41.00'N - 32°00.00'E	33	Central North-Western area (background)
	39	44°28.00'N - 33°35.00'E	76	Sevastopol Region - Balaklava town
	45	44°31.00'N - 34°17.08'E	57	Yalta near Crimea waste treatment plant.
	53	44°35.00'N - 34°25.00'E	78	Yalta town
	54	45°05.00'N - 35°30.00'E	18	Feodosiya town
	55	45°16.00'N - 36°28.00'E	6	Kerch near Geleznorudnyi complex
	94a	46°18.05'N - 30°42.01'E	17	Odessa near port Illichevsk.
	98	46°30.00'N - 30°46.05'E	11	Centre of Odessa bay
	132	45°20.00'N - 29°50.00'E	12	Danube delta
	134	45°20.03'N - 29°45.08'E	3	Bistrayaarm of Danube delta
Russian Federation	1	43°34.82'N - 39°43.24'E	8	Sochi port
	3	43°24.61'N - 39°55.35'E	40	Canyon Adler
	5	43°29.00'N - 39°50.58'E	25	Chosta
	6	43°31.17'N - 39°48.00'E	28	River Matchesta
	8	43°33.64'N - 39°41.72'E	33	River Sochi
Turkey	9	41°21.29'N - 29°04.00'E	80	Bosphorus
	10	41°23.00'N - 29°03.11'E	84	Bosphorus
	16	41°26.20'N - 28°57.25'E	85	Bosphorus
	17	41°27.18'N - 29°01.00'E	88	Bosphorus
	22	41°36.00'N - 28°55.18'E	131	Bosphorus
	30	41°33.18'N - 29°01.18'E	107	Bosphorus
	31	41°31.00'N - 29°01.18'E	94	Bosphorus
	35	41°25.18'N - 29°13.18'E	95	Bosphorus
	36	41°29.00'N - 29°13.18'E	113	Bosphorus
	44	41°20.00'N - 29°28.18'E	86	Bosphorus
Romania	P1	45°08.94'N - 29°45.61'E	-	Sulina arm of Danube delta
	P2	45°09.80'N - 29°43.35'E	-	Sulina arm of Danube delta
	A4	45°05.00'N - 29°41.00'E	-	Between Sulina and Sfintu Gheorghe arms
	B4	45°00.00'N - 29°41.00'E	-	Between Sulina and Sfintu Gheorghe arms
	V1	44°27.00'N - 28°49.00'E	-	Vadu
	S1	44°39.00'N - 28°59.00'E	-	Sinoe lagoon
	S3	44°37.00'N - 28°57.00'E	-	Sinoe lagoon
	S6	44°33.00'N - 28°59.00'E	-	Sinoe lagoon
	S8	44°31.00'N - 28°51.00'E	-	Sinoe lagoon
	C.M.	44°15.00'N - 28°40.00'E	-	Cazino Mamaia
	Consta.	44°12.50'N - 28°43.00'E	-	Constantza
	P.C.	44°07.00'N - 28°40.00'E	-	Port Constantza
	Cost.	43°57.00'N - 28°40.00'E	-	Costineti
	M.	43°48.00'N - 28°37.00'E	-	Mangalia
	N.	44°31.00'N - 28°47.00'E	-	Lake Nuntasi

* Sample code numbers are those recorded as part of the Black Sea Environmental Programme and whilst they are not consecutive, original numbers have been retained to avoid confusion and afford identification of banked samples by scientists in the region.

2.2 Evaluation, development and application of immunoassay techniques (Chapter 4)

2.2.1 Semi-volatile hydrocarbons (BTEX) in groundwater

2.2.1.1 Chemicals

A reference standard mixture (benzene, toluene, ethylbenzene, m+p xylene and o-xylene) was obtained from Restek Corporation (gasoline range organic at 1000 mg L^{-1}, purity greater than 99%). Stock solutions of 100 mg BTEX L^{-1} prepared in methanol were further diluted with methanol to obtain the calibration solutions for GC-PID/FID in the range of 1 to 50 μg L^{-1} in water. Dichloromethane and *n*-hexane used for sample processing and analyses were ultra resi-analysis grade (J.T. Baker) and the methanol was HPLC grade (Vetec).

2.2.1.2 Sample Collection

Groundwater samples (depth 1.99-3.25 m) were pumped from 10 monitoring sites in a Petrol station from Tijuca, an urban area in Rio de Janeiro (Brazil). Prior to the sampling, corrosion of the gasoline storage tanks had led to continued fuel leakage contaminating the soil and groundwater. Soil prospections in the area had indicated strong heterogeneousity and differences in permeability. Filtering procedures had been applied to water pumped from site 10. Following the sampling, water aliquots were separated in 45 mL screw cap glass vials (PTFE-faced silicone septum) containing 50 μL of HCl (Merck, P.A.) to adjust the pH to ≤ 2 as recommended in EPA Method 502.2 (USEPA, 1997). Samples were collected by Pontifícia Universidade Católica do Rio de Janeiro (PUC-RJ, Rio de Janeiro, Brazil). The samples were immediately stored in the dark at ≤ 4°C before further processing within a week of sampling.

2.2.1.3 Sample Processing and Analytical Chemistry

Samples were sub-divided in aliquots for application of the immunoassay and for analytical chemical analysis.

Quantitative analysis of 5 mL aliquots of groundwater extracts was performed using a Hewlett Packard 5890 gas chromatograph fitted with a LDVI injector using a purge and trap system for sample introduction and photoionization/flame ionization detectors (PID/FID). The quantification analysis was performed with the photoionization

detector. A J&W DB-624 capillary column of 75 m x 0.53 mm i.d. with 3.0 μm film thickness (cyanopropyl, phenyl methyl polysiloxane) was used. Ultra pure helium carrier was maintained at a constant flow rate of 10 mL min^{-1}. The oven temperature was programmed for a initial hold at 30°C (1 min), then from 30 to 100°C at 5°C min^{-1} and from 100 to 220°C at 8°C min^{-1}. The transfer line operated at 100°C. The purge time was 11 minutes for a flow rate of 22 mL min^{-1}. Desorption temperature was 180°C and desorption time was 4 minutes.

2.2.1.4 Quality Control

Throughout the study, a standard solution of 10 μg BTEX L^{-1} was analysed daily to check the instrument conditions and the chromatographic behaviour of the standard mixture. Analytical performance was assessed by blanks and standard solutions. Detection limits derived from replicate procedural blanks were in the order of 0.6 μg L^{-1} for all the analytes. The calibration curves were characterised by a correlation coefficient of 0.99 (r^2).

2.2.1.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Water aliquots were left decanting over 4 hours before taking 1 mL of the supernatant for the immunoassay testing. Since samples were preserved with HCl, they were neutralised (NaOH solution) prior to assay.

A commercial BTEX RaPID Assay® (SDI Europe, Alton, UK) was used to measure contamination in groundwater. RaPID Assays® are tube-based immunoassays where the polyclonal antibodies are immobilised onto paramagnetic particles. These kits are based on the competitive heterogeneous ELISA. The RaPID Assay® was used according to the manufacturer's instructions. Briefly, samples were analysed in triplicate together with concurrent standard calibrations and blanks. Appropriate amounts of samples or standards, antibody-coated microbeads (anti-analyte antibodies immobilised onto paramagnetic particles) and enzyme conjugate (analyte-horseradish peroxidase) were mixed and incubated to allow competition for binding to the antibody (20 to 30 min). After washing twice with kit buffer (using a magnetic rack to retain the antibodies), substrate (hydrogen peroxide) and chromogen (3,3',5,5'-tetramethylbenzidine) were added and incubated (30 min). Stop solution (2 M sulphuric acid) was added and 150 μL of final solution was transferred to a microplate. The colour produced was measured at 450 nm using an Optimax microplate reader (Molecular Devices, Menlo Park, CA).

Sample absorbance was compared to a linear regression equation using a log of the concentration versus logit B/Bo (Ln [B/Bo/(100-B/Bo)]) standard curve to calculate the final concentration (where B/Bo is the absorbance observed for a sample or standard divided by the absorbance at the zero standard).

The BTEX RaPID Assay® was evaluated in a quantitative mode as described in the manufacture's instructions. Analytical results were calculated from a standard curve of 0, 0.54, 2.1 and 18 mg BTEX L^{-1} (r^2 = -0.993; slope = -0.486; interception = -0.087). The BTEX RaPID Assay® was developed using equal proportions of 6 compounds (benzene, toluene, ethylbenzene and m-, o- and p-xylene) as the antigen. Hence the different compositions within the environmental extracts renders the "semi-quantitative" measure and is expressed as BTEX "equivalents".

The method detection limit (MDL), as estimated at 90% B/Bo for the BTEX calibration dilutions, was 0.06 mg L^{-1}. The 50% B/Bo (concentration required to inhibit one-half of the colour produced by the negative control) was 4.86 mg L^{-1}.

ELISA kits were stored at 4°C, but temperature and time are important parameters that must be controlled for ELISA to work properly. In all cases, solutions of immunoassay and samples were allowed to equilibrate to room temperature before using, and reaction times were consistent throughout the experiment.

2.2.2 Petroleum hydrocarbons and PAHs in sediment

2.2.2.1 Materials

A certified standard reference solution of 24 aromatic hydrocarbons (NIST-SRM 1491) and authentic standards of aliphatic hydrocarbon (*n*-C_{12}-C_{35}, phytane and pristane) were purchased from Promochem (Herts, UK). Internal standards ($C_{18:1}$ and 9,10-dihydroanthracene) were acquired from QMx Laboratories Ltd (Safron Walden, UK). Solvents of glass distilled grade were obtained from Rathburns Chemicals Ltd (Walkerburn, UK). Solvents were batch tested for PCB and PAH contamination. Silica (70-230 mesh), alumina (70-230 mesh) and anhydrous sodium sulphate were purchased from BDH Merck LTD (Lutterworth, UK).

2.2.2.2 *Sample Preparation*

Fortified sediments, standard reference sediments and environmental sediment samples were analysed in order to assess the performance of the methods. Soxhlet extracted dry sediments (10 g) were fortified with either PAH (SRM 1491) or *n*-C_{12}-C_{35} standard mixture solutions prepared in hexane to yield sediment concentrations from 41 to 1650 ng g^{-1} or 14 to 112 µg g^{-1}, respectively. Spiked sediments were left stabilising overnight prior to analysis.

Five different standard reference sediments were selected including the HS-4B and HS-5 (National Research Council of Canada), the IAEA 383 (International Atomic Energy Agency - Marine Environment Laboratory, Monaco), and the QPH 16MS and QPH 17MS (Quasimeme Laboratory Performance - Studies Round 12, UK).

The environmental samples used included 16 sediments collected from the Patos Lagoon estuary (southern Brazil). Samples were collected in December 1999 and then keep frozen until analysis.

2.2.2.3 *Analytical Chemistry.*

Aliphatic and aromatic hydrocarbons were analysed using a sample preparation method modified from UNEP (1992). Oven dried (50°C) sediment samples (~25 grams) were spiked with internal standards: $C_{18:1}$ for the aliphatic hydrocarbon fraction, and 9,10-dihydroanthracene for the aromatic hydrocarbon fraction. Samples were Soxhlet extracted for 16 hours into 100 mL of dichloromethane/hexane (50:50). The extracts were then concentrated down to few mL using rotary evaporation followed by pure nitrogen "blow down". Sulphur was removed by shaking the solution with activated copper. Clean-up and fractionation was performed by using a silica (3g)/alumina (1.5g) column. Silica and alumina were both activated at 200°C for 4 hours and partially deactivated with 5% water prior to use. Elution was performed using 10 mL of hexane to yield the first fraction (containing the aliphatic hydrocarbons), followed by 10 mL of hexane/dichloromethane (70:30) to yield the second fraction (containing the aromatic hydrocarbons (PAHs)).

The hydrocarbon fractions were analysed by gas chromatography using a Hewlett Packard HP5890 series II with a flame ionisation detector (FID) (Palo Alto, CA). A SE54 fused silica capillary column was used (HP-Ultra 2 crosslinked 5% Ph Me Silicone, 25 m length, 0.32 mm i.d., 0.17 µm film thickness). The oven temperature was

programmed from 40°C to 60°C at the rate of 40°C min^{-1}; from 60°C the temperature was increased at 5°C min^{-1} to 290°C where it was held for 10 min. Injector and detector temperatures were maintained at 280°C and 325°C, respectively. Helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}. Confirmation of peak identity was obtained for selected extracts using GC with mass spectrometric detection (GC-MS) (V.G. Masslab - Fisons TRIO 1000).

Concentrations of individual aliphatic (*n*-C12 to *n*-C35, pristine and phytane) and aromatic hydrocarbons (24 compounds) were quantified relative to the peak area of the respective external standards following calibration with authentic compounds and against the correspondent internal standard. The measure of total aliphatic hydrocarbons includes all resolved peaks and the area covered by the unresolved complex mixture (UCM). UCM is quantified against the internal standards and assumes a response factor of 1. "Total" PAH quantified using GC-FID is the sum of naphthalene; 1-methyl naphthalene; 2-methyl naphthalene; biphenyl; 2,6-dimethyl naphthalene; acenaphthylene; acenaphthene; 2,3,5-trimethyl naphthalene; fluorene; phenanthrene; anthracene; 1-methyl phenanthrene; fluoranthene; pyrene; benz(a)anthracene; chrysene; benzo(b)fluoranthene; benzo(k)fluoranthene; benzo(e)pyrene; benzo(a)pyrene; perylene; indeno(1,2,3-cd)pyrene; dibenz(a,h)anthracene; benzo(g,h,i)perylene.

Recoveries ranged from 68 ± 6% to 106 ± 10%, and averaged 92% (n = 3) for aliphatic hydrocarbons and 72 ± 5% to 108 ± 7%, and averaged 95% (n = 4) for PAHs. Detection limits (blank + 3RSD) (UNEP/IOC/IAEA, 1992) ranged from 0.6 to 2.1 ng g^{-1} (dry wt) for *n*-alkanes and 0.8 to 13.2 ng g^{-1} (dry wt) for individual PAHs. Appropriate blanks were analysed and, in addition, reference material IAEA-357 was analysed simultaneously. Results for all hydrocarbons quantified in the reference material were within 93 ± 15% (n = 3) of the certified values.

2.2.2.4 Sediment Extraction

A simple extraction was performed prior to analysis using the SDI extraction kit for PAHs (SDI Europe, Alton, UK) (Hottenstein *et al.*, 1995; Lawruk *et al.*, 1996). Ten grams (10 g) of sediment and 20 mL of 100% methanol were added to an extraction jar (with 3 SS bearing per jar) and capped. The extraction jar was shaken vigorously for 5 min and allowed to settle for 15 min. Approximately 1 mL of the extract supernatant

was filtered using a filtration plunger with a fibre glass filter. Then, filtered extracts were immediately analysed using RaPID Assay® ELISAs.

2.2.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Two commercially available ELISAs were tested: BTEX RaPID Assay® and carcinogenic PAH (c-PAH) RaPID Assay® (SDI Europe, Alton, UK).

Sediment extracts were diluted 1:10 for BTEX RaPID Assay® and 1:50-500 for c-PAH RaPID Assay® with 50% v/v methanol/buffered aqueous solution (containing stabilisers and preservatives) (SDI diluent). This dilution is necessary to minimise matrix and solvent effect. Extracts were then analysed in triplicate together with 4 calibration standards for each RaPID Assay®. The BTEX and c-PAH RaPID Assay® were used according to the manufacturer's recommendation, as described in Section 2.2.1.5.

The RaPID Assays® were evaluated in a quantitative mode as described in the manufacture's instructions. Analytical results were calculated from a standard curve of 0, 0.54, 2.1 and 18 mg BTEX L^{-1} (r^2 = -0.999; slope = -0.532; interception = -0.342) for the BTEX RaPID Assays® or a standard curve of 0, 0.1, 1 and 5 ng benzo(a)pyrene mL^{-1} (r^2 = -0.997; slope = -0.580; interception = -0.550) for the c-PAH RaPID Assay®. The BTEX RaPID Assay® and c-PAH RaPID Assay® were developed using equal proportions of 6 compounds (benzene, toluene, ethylbenzene and m-, o- and p-xylene) and benzo(a)pyrene as the antigen, respectively. Hence the different composition within the environmental extracts render the "quantitative" measure a comparative on BTEX "equivalents" value or benzo(a)pyrene "equivalents" value, respectively. Sample concentrations were calculated by multiplying results by the appropriate dilution factor.

2.2.3 PCBs in mussel tissue

2.2.3.1 Materials

Certified solutions of PCB congeners (CLB-1) were purchased from the NRCC (Halifax, Nova Scotia, Canada). Other authentic standard PCB congeners were purchased from QMx Laboratories Ltd (Safron Walden, UK). Fifty-five congeners were selected for quantification based on their resolution under the selected GC conditions. Numbering of the PCB congeners followed the IUPAC system. Aroclor® solutions (~

100 mg L^{-1} in hexane) of selected PCB mixtures (Aroclor® 1242, 1248, 1254 and 1260) were purchased from Ultra Scientific (North Kingstown, RI, USA). Working solutions of individual Aroclors® were prepared in hexane for GC calibration, and in methanol for ELISA calibration and cross-reactivity determinations. Internal standard (PCB 29) was purchased from QMx Laboratories Ltd (Safron Walden, UK). Standard Reference Material® (NIST-SRM 2977 - freeze-dried mussel tissue) was obtained from the National Institute of Standards & Technology (NIST, Gaithersburg, USA). Solvents of glass distilled grade were obtained from Rathburns Chemicals Ltd (Walkerburn, UK). Solvents were batch tested for PCB contamination.

2.2.3.2 Environmental Mussel Samples

Mussel samples were taken from four sites in New Bedford Harbour (Massachusetts, USA) (*Geukensia demissa*, L.) and from one site in Whitsand Bay (Cornwall, UK) (*Mytilus edulis*, L.). Locations were selected to afford diverse levels of contamination and thus provide a robust test of the ELISA procedure. Further details about sampling sites at New Bedford Harbour (Sites 1 to 4) are given in Section 2.3.1.2 and Fig. 2.2, and elsewhere (Galloway *et al.*, *Submitted*).

2.2.3.3 Extraction of Mussel Tissue

PCBs were analysed using a sample preparation method modified from Kannan *et al.* (1995) and Nakata *et al.* (1995). Briefly, freeze-dried mussel tissue samples (~1 g), fortified samples, standard reference material and procedural blanks were spiked with an internal standard (PCB 29). Samples were Soxhlet extracted into 200 mL of hexane/dichloromethane (1:1) for 16 hours. The extracts were then concentrated down to a few mL using rotary evaporation followed by pure nitrogen "blow down". The extracts were transferred to glass columns (26 cm x 15 mm i.d.) packed with 20 g of Florisil® and then dried using a gentle flow of nitrogen. PCBs were eluted with a mixture of 120 mL acetonitrile and 30 mL hexane-washed water. The eluants were collected in a separatory funnel containing 100 mL of hexane and 600 mL of hexane-washed water. After shaking and phase separation, the hexane layer was concentrated to exactly 4 mL. The sample extracts were split (volumetrically) for analysis of PCBs by immunoassay (25%) and chromatography (75%). Extracts for ELISA were solvent exchanged into methanol (as described by Zajicek *et al.*, 1996). Samples for chromatography were treated with concentrated sulphuric acid and then cleaned-up and

fractionated using 12g of Florisil® (activated at 130°C for 12 hours). Elution was performed using 105 ml of hexane to yield the first fraction (containing the PCBs), followed by 150 ml of hexane/dichloromethane (80:20) (the 2nd fraction containing organochlorine pesticides).

2.2.3.4 Quantification using GC-ECD

Analyses of extracts were carried out using a gas chromatograph (Hewlett Packard 5890 series II) with a ^{63}Ni electron capture detector (ECD), fitted with a 60m x 0.25mm i.d. (0.25 μm thickness) HP-5MS silica fused capillary column (Hewlett-Packard, USA). The oven temperature was programmed from an initial temperature of 40°C to 160°C (held for 5 min) at the rate of 20°C min^{-1}; the temperature was then programmed to 260°C at the rate of 2°C min^{-1}; from 260°C the temperature was increased at 10°C min^{-1} to 290°C where it was held for 10 min. One μL sample volumes were automatically injected into a cold "on-column" injector. The detector temperature was maintained at 300°C. Hydrogen ("High purity" grade) was the carrier gas at a flow rate of 1.5 mL min^{-1} at 40°C. Nitrogen ("ECD grade") was used as make-up gas at a flow rate of 60 mL min^{-1}. Both H_2 and N_2 gases were further purified by moisture, hydrocarbon, and oxygen filters before use. Data were acquired and processed using Hewlett-Packard ChemStation® software. Recoveries of PCBs (examined in triplicate by spiking 4.0 μg of PCB standard (55 congeners) into Soxhlet extracted mussel tissue) ranged from 60 ± 9% to 104 ± 12%, and averaged 91%. Detection limits ranged from 0.1 to 2.0 ng g^{-1} (dry wt). Appropriate blanks were analysed and, in addition, reference material NIST-SRM 2977 was analysed simultaneously. Results for all congeners quantified in the reference material were within 94 ± 12% (n = 3) of the mean certified values.

Quantification of the individual 55 congeners was through external calibration using CLB-1. For the calculation of "total" PCBs (sum of 128 congeners), congeners for which authentic standards were not available were identified from RRTs (relative retention times) provided in the literature (Mullin *et al.*, 1984; Frame *et al.*, 1996; Erickson, 1997) and response factors (RFs) (according to Erickson, 1997), were used in quantification. Congeners with similar retention times but different chlorine substitutions were investigated by GC/MS (Hewlett-Packard Model 5890 II Plus GC

and a 5972 mass selective detector (MSD) (Palo Alto, CA)). "Total" PCBs, quantified using GC-ECD, were used for comparison with the immunoassay results.

2.2.3.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Individual sample extracts in methanol (20-200 μL aliquots) were diluted (1:1000-2000) using 50% v/v methanol/buffer solution (containing stabilisers and preservatives) as required by the method. They were then analysed in triplicate for "total" PCBs together with 4 calibration standards of Aroclor® 1254 (0, 0.25, 1.0 and 5.0 ng mL^{-1}) (r^2 = -1.000; slope = -0.663; interception = 0.569).

Commercial PCB RaPID Assays® (SDI Europe, Alton, UK) were assayed as described in Section 2.2.1.5. Sample concentrations, expressed as "total PCBs" (Aroclor® 1254 "equivalents"), were calculated by multiplying results by the appropriate dilution factor.

2.2.3.6 Statistical Analyses

A standard Student's t-test was used to examine differences between both techniques across sampling sites. Principal component analysis (PCA) and similarity analysis were performed with Primer® for Windows® (Version 5; Primer-E Ltd, Plymouth, UK).

2.2.4 PAH/PAH metabolites in crab urine

2.2.4.1 Chemicals

Pyrene (98%), 1-hydroxypyrene (98%), phenanthrene (98%) and 9-hydroxyphenanthrene (Tech.) were obtained from Sigma-Aldrich Co. Ltd. (Gillingham, UK). Pyrene-d_{10} and phenanthrene-d_{10} were acquired from Promochem (Welwyn Garden, UK). Ethanol (99% v/v) and L-ascorbic acid (99.7%) was purchased from BDH (Poole, UK). Ethyl acetate of glass distilled grade was obtained from Rathburns Chemicals Ltd (Walkerburn, UK). C_{18} cartridges were purchased from Jones Chromatography (Hengoed, UK). Acetonitrile (>99.8%) was purchased from J. T. Baker (Deventer, The Netherlands). Tris hydrochloride (buffer, pH = 9.0), acetic acid (>99.8%) and ammonium acetate (>97%) were purchased from Fluka Chemica (Buchs, Switzerland). Solvents were batch tested for PAH contamination.

2.2.4.2 *Collection of experimental crabs and laboratory conditions*

Green, male, intermoult crabs (carapace width 50-72mm) of the species *Carcinus maenas* were collected on incoming tides from Jenkins Quay on the Avon estuary at Bantham (South Devon, UK) on three separate occasions between April and July, 2000. On return to the laboratory, they were kept in holding tanks containing aerated seawater (34 PSU, 15±1°C), under a 12hr light/12hr dark regime for a period of one week in order to depurate and acclimatise. During this period, crabs were fed twice a week with irradiated cockle and their water was changed within 12hrs of feeding.

To test the techniques on environmentally exposed crabs, samples were collected from Bantham (South Devon, UK) and Sutton Harbour (Plymouth, UK). Bantham is a clean control site while Sutton Harbour is an oil-contaminated site. On return to the laboratory, urine was immediately sampled (see details below) and stored in liquid nitrogen.

2.2.4.3 *Dose-response exposure experiment*

The exposure experiment consisted of a static toxicity test (Rand, 1995). Parent PAH (either pyrene or phenanthrene) was added to the pre-filtered seawater (34 PSU) to produce six nominal concentrations (200, 100, 50, 25, 10 and 0 (control) ng mL^{-1}). Phenanthrene and pyrene were administered to the water in an acetone "carrier" (at a ratio of 1:1, w:v PAH/acetone) to increase their solubility. The experiment was kept under 12hr light/12hr dark regime at 15±1°C. Crabs were not fed during the exposure period. After the experiment, they were transferred to clean seawater to depurate (for 3 weeks) before being returned to the environment. The experiment was run in triplicate for each parent PAH.

2.2.4.4 *GC/MS analyses of water samples*

Concentrations of parent PAHs in the tanks were monitored using GC/MS analyses (Hewlett-Packard Model 5890 II Plus GC and a 5972 mass selective detector (MSD) (Palo Alto, CA)). Measured concentrations confirmed that the nominal (spiked) values were within ± 12%. Briefly, internal standard (pyrene-d_{10} and phenanthrene-d_{10}) spiked water samples (100-500 mL) were concentrated using C_{18} cartridges (IST, Hengoed, UK), which were subsequently eluted (3 times) using 3 ml of ethyl acetate. The eluent

was then concentrated down to 1 ml before analyses by GC/MS. Recoveries were 95 ± 3% (n = 18) for pyrene and 93 ± 5% (n = 18) for phenanthrene.

2.2.4.5 Urine sampling

Urine samples were taken from each crab after 48hrs of exposure using the technique described by Bamber and Naylor (1997). Briefly, crabs were removed from their aquaria and placed in a bucket containing clean seawater. After being drained, the third maxillipeds were moved aside and kept apart. Under a dissecting microscope (x10), the operculum of each antennal gland bladder was lifted and urine (20-400 µL per crab) was collected. Samples were stored at -80°C in siliconised microcentrifuge tubes until analysis. Crabs were returned to their respective aquaria immediately after sampling.

2.2.4.6 Fluorescence analyses

Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer following an adapted method of Ariese *et al.* (1993) and Aas *et al.* (2000). Standards (pyrene, 1-OH-pyrene, phenanthrene, and 9-OH-phenanthrene), blanks and urine samples were diluted (20- to 100-fold) with 50% v/v ethanol/Milli-Q water. Fixed excitation wavelength fluorescence (FF) and synchronous excitation/emission fluorescence spectrometry (SFS) measurements were carried out with excitation and emission slit widths of 2.5 nm (pyrene) and 5.0 nm (phenanthrene). The assigned wavelength pairs were $\lambda_{ex/em}$ = 345/382 (FF) for pyrene and $\lambda_{ex/em}$ = 252/357 (FF) for phenanthrene. A $\Delta\lambda$ of 42nm (pyrene) and 54nm (phenanthrene) were selected for the SFS analyses. Samples were quantified against 1-OH-pyrene and 9-OH-phenanthrene standards (200, 100, 75, 50, 25, 10, 5 ng mL^{-1}). Results are reported in terms of 1-OH-pyrene or 9-OH-phenanthrene "equivalents". Further details are provided in Watson *et al.* (*In Preparation*).

2.2.4.7 Immunoassay procedure

Urine samples (50 µL aliquots) were diluted with 50% v/v methanol/buffered solution (SDI diluent) (20- to 40-fold). Analyses were undertaken in triplicate with concurrent standard calibrations and blanks. The PAH RaPID Assay® (SDI Europe, Alton, UK) was used according to the manufacturer's recommendations, as described in Section 2.2.1.5.

The PAH RaPID Assays® were calibrated using 5 standards of 1-OH-pyrene (0, 10, 50, 200, 2000 ng mL^{-1}) or 9-OH-phenanthrene (0, 10, 50, 200, 2000 ng mL^{-1}), for the samples of crabs exposed to pyrene or phenanthrene, respectively. Standards of pyrene (1.0, 10, 50, 250 ng mL^{-1}) or phenanthrene (2.0, 10, 50 ng mL^{-1}), respectively, were analysed at the same time. Sample concentrations were then corrected for dilution factors. Results are reported in terms of 1-OH-pyrene "equivalents" or 9-OH-phenanthrene "equivalents", respectively.

2.2.4.8 HPLC/Fluorescence

High-performance liquid chromatography was performed using an ion pair elution system under acidic conditions on a reversed phase C_{18} analytical column (Vydac 201TP54 column (250 x 4.6 mm), Hesperia, CA, USA). The column temperature was maintained at 30°C in a column oven. A gradient elution of acetonitrile (CH_3CN) and aqueous buffer (10 mM ammonium acetate, adjusted to ~pH5 with acetic acid / L buffer) was used at a flow rate of 0.5 mL min^{-1} (t = 0 min, 5% CH_3CN; t = 40 min, 90% CH_3CN; isocratic at 90% CH_3CN for 10 min). The instrument used consisted of two Spectroflow 400 pumps (Applied Biosystems), a Spark-Holland PROMIS II autosampler (20µL injection loop), a GT-103 in-line degasser (Separations, The Netherlands) and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). Fluorescence detection was carried out at $\lambda_{ex/em}$ = 346/384 nm for pyrene metabolites (slit widths $\lambda_{ex/em}$ = 18/40 nm) (from Stroomberg *et al.*, 1999).

Samples were diluted 30-fold with ethanol (modified with 5 mg mL^{-1} ascorbic acid) and stored at -20°C. Further dilutions were made when necessary. Peaks were identified according to their retention times, based on the work by Stroomberg *et al.* (1999), and confirmed for the system used in this study (Howsam, personal communication). Quantification of 1-OH-pyrene was performed using a dilution series of 1-OH-pyrene external standard, while the conjugates were quantified using their relative fluorescence efficiencies compared to 1-OH-pyrene; pyrene-1-glucoside = 2.0 ± 0.31, pyrene-1-sulfate = 1.23 ± 0.09, pyrene-1-'conjugate' = 1.75 ± 0.18 (Stroomberg *et al.*, *Submitted*). All conjugates are expressed as 1-hydroxy-pyrene "equivalents". The identity of the pyrene-1-'conjugate' is still unknown, but it is not a glucuronide conjugate nor (derived from) a glutathione conjugate (Stroomberg *et al.*, *Submitted*).

2.3 Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassay (Chapter 5)

2.3.1 New Bedford Harbour Case Study

2.3.1.1 Materials

Certified solutions of PCB congeners (CLB-1) were purchased from NRCC (Halifax, Nova Scotia, Canada). Other PCB congeners were from QMx Laboratories Ltd (Safron Walden, UK). Aroclor® 1254 (~ 100 mg L^{-1} in hexane) was from Ultra Scientific (North Kingstown, RI, USA). Working solutions of Aroclor® were prepared in hexane for GC calibration and in methanol for ELISA calibration. A standard reference solution of 24 aromatic hydrocarbons (SRM 1491) was purchased from Promochem (Herts, UK). Internal standards (PCB 29 and 9,10-dihydroanthracene) were from QMx Laboratories Ltd (Safron Walden, UK). Solvents of glass distilled grade were from Rathburns Chemicals Ltd (Walkerburn, UK). Solvents were batch tested for PCB and PAH contamination.

2.3.1.2 Study site

The New Bedford Harbour site drains the Acushnet River into Buzzard's Bay. The Acushnet River borders the city of New Bedford to the east and the towns of Acushnet and Fairhaven to the west. The site is divided by two sea walls into an inner (upper and lower) and outer harbour area (Fig. 2.2). The primary objective of remedial dredging activities instituted during the last decade in New Bedford Harbour has been to reduce the public health hazard posed by exposure to contaminated sediments or consumption of contaminated seafood. The area is heavily populated and industrialised, and received large quantities of Aroclor® 1242 and 1254 and, possibly, also Aroclor® 1016 during the 1970's (Pruell *et al.*, 1990; Lake *et al.*, 1995). Prior to remedial action, sediment concentrations of PCBs as high as 100,000 μg g^{-1} were measurable in the upper harbour area (USEPA, 1996). The water flow in the estuary is influenced by the tidal movement of Buzzard's Bay and the concentration of contaminants in estuarine sediments generally show a gradual decline from both shores towards the centre of the river channel (NUS Corporation, 1984). Congener specific PCB measurement by gas chromatography, conducted after remedial dredging action by the USEPA (1996), revealed a decreasing gradient of total PCB concentrations from the upper to outer

harbour (Table 2.2). The distribution of toxic metals in sediments throughout the harbour area also showed marked gradients in contaminants loading from the inner harbour out into Buzzard's Bay (Shine *et al.*, 1995; USEPA, 1996) (Table 2.2). The highest concentrations of metals, in particular cadmium, chromium and copper, occurred in the inner harbour. More recent sediments from the outer harbour were characterised by their lead and zinc content consistent with a non-point source superimposed on a point source in the inner harbour (Shine *et al.*, 1995). The choice of study sites for the present study was made to encompass the decreasing trend of contamination from inner to outer harbour areas and is indicated in Fig. 2.2 and Table 2.2. The control site (West Island - site 1) was located in Fairhaven, MA, approximately 15km east of the harbour and has been used as a reference station for Superfund monitoring programmes. It is adjacent to marshland and surrounded by woodland. The Buzzard's Bay site (site 2) was located outside the harbour breakwater. Sites 3 and 4 were in the lower and upper inner harbour respectively. Large indigenous populations of ribbed mussels were present at all sites.

2.3.1.3 Sample collection and preparation

Specimens of *Geukensia demissa* of shell length 50-80 mm were used for all experiments. A total of 60 mussels were sampled from each of the sites, collected by hand from the sediment below the high water mark. Water temperature was 12°C and salinity between 32-35. Sites 1 and 2 were sampled at low tide on 7th April 2000. Sites 3 and 4 were sampled at low tide on 10th April 2000. All mussels were returned to the laboratory and kept in fully aerated water at 15°C for 24 hours prior to analysis. Haemolymph (0.5 – 1.0 mL) was withdrawn by needle aspiration from the posterior adductor muscle into an equal volume of physiological saline (0.02M HEPES, pH 7.4, 0.4M $NaCl_2$, 0.1M Mg SO_4, 0.01M KCl and 0.01M $CaCl_2$) (Smith *et al.*, 1990). The whole tissue was excised, weighed and frozen immediately at –80°C prior to further analysis. The mussels were collected and biomarkers performed by Dr Tamara S. Galloway, Mr Ross C. Sanger and Miss Karen L. Smith (University of Plymouth).

2.3.1.4 Neutral red retention time

The neutral red retention assay reflects the efflux of the lysosomal contents into the cytosol following damage to the membrane and, possibly, impairment of the H^+ ion pump. Neutral red retention time was measured in haemolymph using the method of

Lowe *et al.* (1995). Briefly, haemolymph (40 µL) was dispensed onto a poly-L lysine coated microscope slide (26 x 76 mm) and incubated in a humidity chamber consisting of a shallow insulated light proof vessel containing water ice (incubation temperature 10 °C) for 15 min to allow the cells to adhere. The excess solution was then carefully tipped off and 40 µL of the neutral red working solution (see details below) added to the area containing the attached cells. A 22 x 22 mm coverslip was then applied. After 15 min incubation to allow dye to penetrate the cells in an chamber, the slide was inspected under a light microscope (x400 magnification). Following a further 15 min incubation the slide was examined again and thereafter at 30 min intervals for 180 min to determine the time at which the dye that had been taken up into individual lysosomes (turning them red) had leached out into the cytosol. Following each inspection, the slide was returned to the incubation chamber. The test was terminated when dye loss was evident in 50% of the small granular haemocytes and the time recorded.

A stock solution of neutral red was made by dissolving 20 mg of dye in 1 mL of DMSO. A working solution was then prepared by diluting 10 µL of the stock solution with 5 mL of physiological saline solution (pH 7.36)

2.3.1.5 Spontaneous cytotoxicity response: immunotoxicity

Immune function was assessed in microtitre plate format by measuring the ability of haemocytes to lyse erythrocyte target cells (Raftos and Hutchinson, 1995). Haemolymph samples were centrifuged for 10 min at 1000 x g to separate cellular and liquid fractions of the haemolymph and stored on ice prior to analysis. Cells were resuspended in Tris buffered saline with calcium (TBS-Ca, 10mM Tris, pH 7.4, 150 mM NaCl, 10mM $CaCl_2$), counted and adjusted to a density of 2 x 10^6 cells mL^{-1}. Sheep erythrocytes (Tissue Culture Services, UK), diluted by cell pack volume in TBS-Ca were incubated with haemocytes in the ratio 1:1 for 1 h at 20°C in round bottomed microtitre plates. The cells were pelleted by centrifugation and the percent lysis determined by measuring the release of haemoglobin into the supernatant by its absorbance at 405nm. Spontaneous release of the red blood cells was quantified concurrently in the absence of haemocytes. Results were expressed as percent lysis relative to the maximum lysis achieved using a solution of 0.2% Triton-X 100 in distilled water.

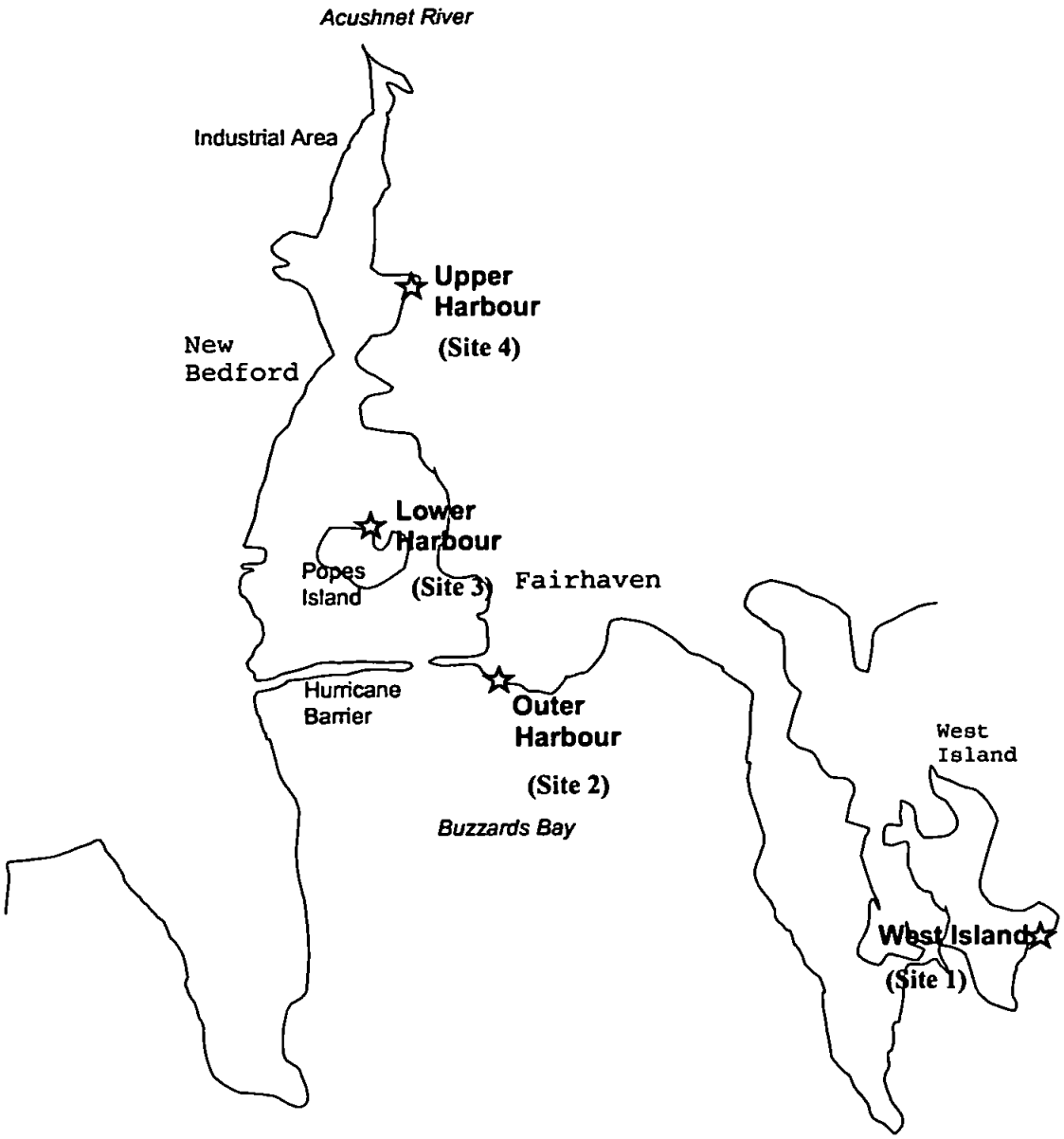


Figure 2.2. The location of study sites in the New Bedford Harbour area

2.3.1.6 Cell viability

The viability of haemocytes used for the immunotoxicity assay was determined in microtitre plate format by measuring the ability of whole (viable) cells to incorporate and retain neutral red dye (Babich and Borenfreund, 1990). Neutral red is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and

binding of neutral red. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay. Briefly, haemocytes (200 µL) diluted in physiological saline were dispensed into flat-bottomed microtitre plates and allowed to adhere for 45 min to form a monolayer. Excess liquid was discarded and replaced with a solution of 0.004% neutral red in physiological saline. The cells were incubated for 3h at 25°C, the excess solution was discarded and plates washed with a solution of 0.5% formaldehyde:1% calcium chloride to remove residual dye. Damaged or dead cells lose their ability to retain NR, which is then removed during this wash/fixation procedure. The dye is then extracted from the intact, viable cells with a solution of 1% acetic acid:50% ethanol (1:1). The plate is left to stand at room temperature for 10-15 minutes, then agitated on a microplate shaker for 30 minutes. The absorbance of solubilised dye is then determined using a spectrophotometer at 540nm. Quantitation of the extracted neutral red was expressed as absorbance per mg protein. Total protein was measured with Bio-Rad protein assay.

Table 2.2. Average metal, "total" PCB and "total" PAH concentrations (in µg g^{-1} dry weight) in the upper, lower and outer harbour sediments and at West Island compiled from the literature. PCBs were measured by GC. Metals were measured by ICP-MS of sonicated sediments. Unless indicated otherwise, results are from USEPA, 1996. The correspondence of the present study sites to these locations is shown in parenthesis.

Average concentration (µg g^{-1} dry wt)	West Island *(site 1)*	Buzzard's Bay *(site 2)*	Lower Harbour *(site 3)*	Upper Harbour *(site 4)*
Metal				
As	-	3.1	5.3	5.2
Cd	0.16[b]	0.28	12	67
Cr	19	19	190	310
Cu	19	19	450	630
Hg	0.04[b]	0.07	0.40	0.43
Ni	-	5.3	11	34
Pb	24[b]	18	130	270
Se	-	0.23	0.42	0.32
Zn	-	42	260	630
"Total" PCB	<0.1[b]	0.83	8.2	44
"Total" PAH[a]		-	20[a]	170[a]

[a] = Pruell *et al.*, 1990, [b] = Fowler, 1990, compiled from concentrations from surface layers of nearshore sediments in the North West Atlantic area.

2.3.1.7 Metallothionein analysis

Metallothionein concentration was determined by a spectroscopic method using DTNB (5,5-dithiobis-2-nitrobenzoic acid) (Viarengo *et al.*, 1997). Whole tissue previously stored at –80°C was ground to a fine powder in liquid nitrogen and 1g (wet weight) of tissue diluted into a solution of 1mM dithiothreitol containing 1 mM phenylmethylsulphonylfluoride (PMSF). The solution was ultra centrifuged at 55,000 rpm at 4°C for 70 min. The metallothionein was purified by extraction with ethanol and chloroform at 4°C, and the resulting metallothionein levels determined after addition of DTNB by recording the absorbance at 412nm in comparison to a reduced glutathione standard.

2.3.1.8 Heart rate

Following 24 h acclimation in the aquarium, the heart rate of mussels was monitored for at least 24h using the non-invasive computer aided physiological monitoring system (CAPMON) developed by Depledge and Anderson (Depledge and Andersen, 1990). In brief, a coupled infrared transmitter and detector unit, glued (Loctite 314) directly onto the shell of each mussel, beams infrared light onto the surface of the heart. As the conformation of the heart changes with each cardiac cycle, the intensity of the light reflected back to the detector fluctuates. The detected signal is fed to an analogue to digital converter and displayed on a computer screen. Prior to the start of heart rate measurement, groups of four mussels were transferred and kept in four litres of fully aerated seawater collected *in situ* for the duration of the recording. Sensors were connected to the mussels, which were then placed into tanks. After1 h acclimation, heart rate was recorded every minute and stored on the computer for later analysis.

2.3.1.9 Micronucleus detection

The presence of micronucleated haemocytes (Wrisberg and Rhemrev, 1992) was determined after adhering 100 µL of haemolymph, diluted in physiological saline as described above, to glass microscope slides coated with poly-L-lysine solution and incubation for 30 min in a humidity chamber to encourage adhesion of the cells to the glass. After incubation, the excess cell suspension was drain off. Cells were fixed with methanol (for 15 min) and allowed to air dry at room temperature (approximately 45 min). The slides were then stained with 5% (v/v) Giemsa's stain in Giemsa buffer solution for 15-25 min, rinsed with distilled water and air dried. Slides were mounted in

DPX (with 22 x 22 mm coverslip) and examined under a light microscope (x400 magnification). Micronuclei were characterised using the following criteria: well preserved cell cytoplasm, micronuclei not touching the main nucleus and of smaller size (up to 1/3 diameter) and similar or weaker staining as the main nucleus. Incomplete micronuclei in which connection to the main nucleus was still evident, binucleated cells and other aberrant nuclear structures were noted separately.

2.3.1.10 Condition index

The condition index was determined by comparing the dry weight of tissue (mg) from the mussel with its shell length.

2.3.1.11 Metal analysis

The mussel samples and shells were pulverised separately by pestle and mortar. Aliquots of 0.25g were mixed with 4 mL nitric acid, placed in a microwave digestion bomb, capped loosely and left overnight to digest at room temperature. The next day, the bombs were tightly sealed, placed in a domestic microwave oven and irradiated at 400W for 1.5 min. After cooling, digests were transferred quantitatively to acid washed 25mL volumetric flasks with indium as internal standard and analysed by ICP-MS. Semi-quantitative analysis of 60 elements was performed and the results, presented as a range to reflect the predicted accuracy to a factor of two, in $\mu g\ g^{-1}$. The fully quantitative analysis for the elements Ni, Cu, As, Cd, Hg and Pb were obtained using standard operating conditions. Results were validated against the DORM-2 dogfish muscle certified reference material.

2.3.1.12 Mussel Tissue Extracts

PCBs were analysed as described in Section 2.2.3.3 with exception of the final clean-up and fractionation step. Sample extracts for chromatography were concentrated, cleaned-up and fractionated using 12g of Florisil® (activated at 130°C for 12 hours). Elution with 105 mL of hexane yielded the first fraction containing PCBs, followed by 150 ml of hexane/dichloromethane (80:20) to yield a second fraction containing organochlorine pesticides and also polycyclic aromatic hydrocarbons (PAHs). The PCB fraction was concentrated and further treated with concentrated sulphuric acid.

2.3.1.13 Quantification of PCBs using GC-ECD

PCBs were quantified as described in Section 2.2.3.4.

2.3.1.14 PAH Analysis

The second fraction containing the PAHs was concentrated and analysed either by GC, or aliquots (50-200 µL) were transferred and solvent exchanged to methanol (avoiding dryness) for ELISA. GC analyses were carried out with a gas chromatograph (Hewlett Packard 5890 series II) equipped with a flame ionization detector using a 30m x 0.25mm i.d. (0.25 µm thickness) DB-5MS fused silica capillary column (J&W Scientific, Folsom, CA, USA). The oven temperature was programmed from an initial temperature of 40°C to 60°C at the rate of 40°C min^{-1}; from 60°C the temperature was increased at 5°C min^{-1} to 300°C where the temperature was held for 25 min. Samples (1 µL) were injected automatically as for PCBs. Filter purified helium (CP grade) was the carrier gas at a flow rate of 2.0 mL min^{-1} at 40°C. Concentrations of individual PAHs were quantified relative to the peak area of the respective external standards following calibration with authentic compounds. Confirmation of peak identify was obtained for selected extracts using GC-MS as for PCBs. Appropriate blanks and reference material NIST-SRM 2977 were analysed simultaneously. Recoveries of PAHs were examined by spiking 5.0 µg of PAH standards (24 compounds) into Soxhlet extracted mussel tissue, using 9,10-dihydroanthracene as internal standard. The results were typically > 75% ± 12%. However, losses through volatility or unacceptable chromatographic performance precluded quantification of naphthalene, acenaphthylene, benzo(a)pyrene and perylene. Instrument detection limits ranged from 0.02 to 0.1 µg g^{-1} (dry wt).

2.3.1.15 Detection of PCB and PAH by ELISA

Sample extracts in methanol were diluted by 1:1000-2000 (PCBs) or 1:100-200 (PAHs) with 50% v/v methanol/buffered aqueous solution (containing stabilisers and preservatives) (SDI diluent). Mussel extracts were then analysed in triplicate together with 4 calibration standards. The PAH RaPID Assay® and PCB RaPID Assay® ELISAs (SDI Europe, Alton, UK) were used according to the manufacturer's recommendations (as described in Section 2.2.1.5). Analytical results were calculated from a standard curve of 0, 0.25, 1.0 and 5.0 ng Aroclor® 1254 mL^{-1} (r^2 = -1.000; slope = -0.663; interception = -0.569) for the PCB RaPID Assays® or a standard curve of 0, 2.0, 10, 50 ng phenanthrene mL^{-1} (r^2 = -0.996; slope = -0.650; interception = 2.115) for the PAH

RaPID Assay®. Sample concentrations, expressed respectively as "total PCBs" (Aroclor® 1254 "equivalents") or "total" PAHs (phenanthrene "equivalents"), were calculated by multiplying results by the appropriate dilution factor.

2.3.1.16 Statistical analysis

The statistical package used was Statgraphics™ Plus Version 5 (Statistical Graphics Corporation, Rockville, MD, USA). The data for each biomarker and each chemical was analysed against the four collection sites using analysis of variance, assuming a normal distribution. Micronuclei are rare and independent events thus their distribution may be Poissonian, so the results of the micronucleus determination were additionally analysed using the chi square test. As multiple simultaneous determinations were being evaluated, multivariate statistical analysis was used to clarify correlation structures or redundancy of individual parameters. Multifactorial analysis of variance (Manova) was used to describe and detect significant differences between sites, with the level of statistical significance adjusted to account for the multiple simultaneous determinations to $p \leq 0.002$. Canonical correlation analysis was used to study the relationship between biological and environmental variables (Sparks, 2000). Principal component analysis was used to determine the similarity of patterns of individual PCBs in tissue samples with known Aroclor® mixtures (see Section 2.2.3.6). For all statistical tests, the significance level was set at 0.05.

Chapter 3

3 Conventional analytical chemistry to evaluate contamination

3.1 A comprehensive study of contamination in the Black Sea

The Black Sea is the world's largest land-locked sea. It has a surface area of 4.2×10^5 km^2 and an average depth of 1240 m. About 25 % of its area is occupied by its north-western continental shelf which is less than 200 m deep. The Black Sea is bordered by Bulgaria and Romania to the west, Ukraine to the north, the Russian Federation and Georgia to the east and Turkey to the south (Fig. 3.1). Although only these six countries surround it, the total catchment area draining into the Black Sea covers a vast area of the European Continent, being over five times the size of the sea itself and including parts of 21 countries (EEA, 1995). The north-western area is subject to the discharge of large rivers (the Danube, Dnieper and Dniester). The River Danube delivers about half of the allochthonous material to the Black Sea (Shimkus and Trimonis, 1974), hence the western Black Sea receives more terrigenous material than the eastern Black Sea. At least 171 million people live in the Black Sea basin, from which about 81 million live in the Danube basin alone (Mee, 1992).

Human activities in the catchment areas have had major effects, not only on the rivers, but also on the Black Sea itself, which has been the victim of unmanaged fisheries, unrestricted intense shipping activities, mineral exploitation and dumping of toxic wastes. The threat to the Black Sea from land-based sources of pollution is potentially greater than in any other sea on our planet (Mee, 1992). The consequences of pollution, such as reduction of tourism in the region, loss of biodiversity, changes in the hydrological balance and reduction or even collapse of fisheries have been reported (Tuncer *et al.*, 1998).

Although some studies have assessed the environmental quality of the Black Sea (Wakeham and Beier, 1991; Mee, 1992; Wakeham, 1996; Tanabe *et al.*, 1997b; Tuncer *et al.*, 1998; Maldonado *et al.*, 1999), data on chemical contamination are still scarce. Hence, comprehensive studies are needed to understand the status of contamination and pollution in the Black Sea (Mee, 1992). For elucidating the present status of its

chemical contamination, this study focuses on conventional chemical analysis (e.g. gas chromatography) of organochlorine residues (Fillmann *et al.*, *In Press a*), hydrocarbons (Readman *et al.*, *In Press*) and faecal sterols in surface sediments from several areas along the Black Sea coast.

3.2 Persistent Organochlorine Residues

Synthetic organochlorines such as DDTs, PCBs (polychlorinated biphenyls), HCHs (hexachlorocyclohexanes), CHLs (chlordanes), cyclodienes and HCB (hexachlorobenzene) are highly resistant to degradation by biological, photochemical or chemical means. They are also liable to bioaccumulate, are toxic, probably hazardous to human and/or environmental health, and most are prone to long-range transport (Tanabe *et al.*, 1994; UNEP, 1996). These compounds are also typically characterised as having low water solubility and high lipid solubility.

Organochlorines have been associated with significant environmental impact in a wide range of species and at virtually all trophic levels (Tanabe *et al.*, 1994). Many organochlorines have been implicated in a broad range of adverse human health and environmental effects, including impaired reproduction, endocrine disruption, immunosuppression and cancer (UNEP, 1996). Exposure to organochlorines has been correlated with population decline in a number of marine mammals (Tanabe *et al.*, 1994). The primary transport routes into marine and coastal environments include atmospheric deposition and surface run-off, the former being by far the greatest albeit dispersed over large areas. Because many organochlorines are relatively volatile, their remobilization and long-distance redistribution through atmospheric pathways often complicates the identification of specific sources. Nevertheless, those (the majority) used in agriculture are also washed off the land into rivers, thence to the sea, or directly into the sea via outfalls or run-off. Many organohalogens follow quite complex biogeochemical pathways (UNEP/IOC/FAO, 1992). Whilst there is substantial information concerning contamination of many industrialised countries and a number of studies have been conducted regarding organochlorine contamination in Eastern Europe and Asia (Iwata *et al.*, 1994b; Vetter *et al.*, 1995; Iwata *et al.*, 1995; Nakata *et al.*, 1997; Tanabe *et al.*, 1997b), the Black Sea remains to be studied.

Concentrations of DDTs, HCHs and PCBs in Black Sea fish and mammals are high in comparison with those reported for other regional seas (Tanabe *et al.*, 1997a; Tanabe

et al., 1997b). Due to the toxic effects of organochlorines in humans and aquatic organisms, the use and/or sale of most organochlorine pesticides has been banned or restricted in countries surrounding the Black Sea and in many other European countries. In Turkey and Romania, for example, the use of organochlorine pesticides was controlled in the late 1970s, but effective restrictions were not imposed in Turkey until the 1980's (Tanabe *et al.*, 1997b). Between 1976 and 1983, the annual use of organochlorine insecticides in Turkey was 1000-2000 tonnes (Karakaya and Ozlap, 1987). Despite this restriction, recent studies detected DDT in Turkish rivers, streams, and domestic and industrial discharges, which indicates their illegal use (Tuncer *et al.*, 1998). The use of these chemicals in other Black Sea countries is currently unclear.

The data of selected chlorinated compounds analysed in sediments from the Black Sea are presented in Tables 3.1 and 3.2 and Figs. 3.1, 3.2 and 3.3. On the basis of these data, the ranking of concentrations of the various compounds are as follows: DDTs >HCHs ≥ PCBs >HCB >cyclodienes. A similar ranking has also been observed in organisms from Black Sea (Tanabe *et al.*, 1997b).

3.2.1 PCBs

The concentration of PCBs (sum of 13 congeners) in some sediment from the Black Sea (Fig. 3.1) is low in comparison with those reported for other locations (Table 3.3). Relatively higher concentrations of 4.8 and 8.8 ng g^{-1} dry wt were, however, recorded for the Romanian stations A4 and P1 respectively. These locations are influenced by discharges from the River Danube, whose sediment has been reported to contain relatively high levels of PCBs (Equipe Cousteau, 1993). The cyclonic circulation pattern in the western basin may also aid in transporting contaminants from the Danube River southward (Aibulatov, 1987). The highest concentration of PCBs in sediment (24.3 ng g^{-1} dry wt) was found in a sample taken from Port Constantza (P.C.), on the Romanian coastline. Sediments of Odessa (stations 94a - near port Illichevsk - and 98 - centre of Odessa bay) and Sochi (station 1) were also found to contain relatively high concentrations (6.8, 5.7 and 4.7 ng g^{-1} dry wt, respectively). In the Bosphorus, the highest concentration (4.4 ng g^{-1} dry wt) was found at one of the furthest offshore stations (station 30). Substantial levels of PCBs have been previously recorded in porpoise samples from the Black Sea indicating inputs of contaminants from the surrounding countries (Tanabe *et al.*, 1997a). Some of the Eastern European countries,

Table 3.1. Concentrations of chlorinated hydrocarbons (on a dry weight basis) in Black Sea sediments (Ukrain, Russia Federation and Turkey)

		Ukraine						Russian Federation										Turkey								
Samples codes		26	39	45	53	54	55	94a	98	132	134	1	3	5	6	8	9	10	16	17	22	30	31	35	36	44
EOM	$\mu g\ g^{-1}$	25	29	16	13	37	5	200	390	90	490	290	170	54	100	290	110	76	70	160	33	180	48	42	44	85
HCB	$pg\ g^{-1}$	7	10	7	6	19	24	74	57	710	1300	260	29	21	27	79	250	50	69	130	20	120	41	29	16	120
α-HCH	$pg\ g^{-1}$	16	48	<6	<6	26	<6	390	1100	1000	460	180	190	100	110	190	100	37	100	110	22	210	85	35	32	160
β-HCH	$pg\ g^{-1}$	20	30	18	6	140	16	680	900	460	470	560	310	160	280	500	62	<6	27	130	42	100	46	82	23	220
Lindane	$pg\ g^{-1}$	16	26	12	10	23	12	180	250	550	330	73	89	26	26	37	64	44	100	72	92	790	63	32	46	98
pp' DDE	$pg\ g^{-1}$	280	100	54	34	220	74	2900	4300	1200	1500	2700	1800	600	1100	1600	1000	860	2800	1900	41	1600	1400	260	98	710
pp' DDD	$pg\ g^{-1}$	73	290	93	12	310	71	17000	33000	22000	5100	4900	1500	1300	2200	2500	2000	1500	1900	1000	63	2900	3600	220	230	3700
pp' DDT	$pg\ g^{-1}$	<10	<10	<10	<10	<10	10	11000	20000	15000	940	3700	7600	820	800	2300	1100	3000	570	450	78	960	1500	94	290	890
DDMU	$pg\ g^{-1}$	45	59	<10	15	83	10	1200	3500	620	510	510	160	160	190	260	270	220	500	260	48	230	620	62	31	410
op DDE	$pg\ g^{-1}$	<8	<8	<8	<8	<8	<8	58	170	85	42	36	130	14	8	11	22	8	48	16	<8	30	39	<8	<8	21
op DDD	$pg\ g^{-1}$	26	93	27	12	58	<10	3900	7500	4600	1600	660	270	250	300	420	330	160	350	190	13	790	640	74	37	620
op DDT	$pg\ g^{-1}$	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	360	1000	270	110	170	11	<10	<10	<10	<10	79	35	<10	<10	40
Heptachlor	$pg\ g^{-1}$	<5	<5	<5	<5	<5	<5	<5	32	<5	<5	<5	<5	<5	<5	<5	6	<5	<5	<5	<5	<5	<5	5	<5	<5
Aldrin	$pg\ g^{-1}$	<7	<7	<7	<7	<7	<7	<7	<7	<7	<7	<7	<7	<7	<7	<7	12	7	15	19	10	180	17	16	10	16
Dieldrin	$pg\ g^{-1}$	11	17	17	8	34	<10	58	36	35	15	39	15	<10	10	17	48	13	27	43	17	91	54	18	14	50
Endrin	$pg\ g^{-1}$	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	250	20	<15	18	<15
Aroclor 1254	$pg\ g^{-1}$	60	<60	<60	<60	140	180	5800	4700	850	1300	3830	930	380	600	1300	1000	300	810	600	550	4900	1130	640	430	950
Aroclor 1260	$pg\ g^{-1}$	310	<60	<60	<60	<60	<60	3600	3600	1100	4400	2450	370	230	180	540	1460	390	580	1180	<60	1210	630	170	<60	1550
PCB 44	$pg\ g^{-1}$	26	<5	<5	<5	<5	<5	120	110	<5	36	160	28	20	16	54	66	<5	52	24	71	550	80	51	55	78
PCB 49	$pg\ g^{-1}$	16	<4	<4	<4	27	11	310	280	110	200	210	42	32	27	84	52	42	100	67	91	320	75	45	41	76
PCB 52	$pg\ g^{-1}$	<6	<6	<6	<6	<6	12	340	270	93	130	270	95	53	66	120	79	87	230	150	160	710	270	120	140	260
PCB 87	$pg\ g^{-1}$	60	<9	<9	<9	9	<9	220	130	18	32	150	18	<9	14	39	36	<9	31	25	28	280	31	21	17	23
PCB 101	$pg\ g^{-1}$	<1	<1	<1	<1	33	20	650	620	66	120	380	50	12	26	99	95	43	140	81	75	510	140	65	56	120
PCB 105	$pg\ g^{-1}$	18	<5	<5	<5	25	<5	520	370	44	100	380	79	20	36	94	82	14	37	51	10	130	57	12	<5	50
PCB 118	$pg\ g^{-1}$	13	<5	<5	<5	29	21	1200	980	170	120	760	140	37	76	180	150	35	94	100	23	260	120	22	17	110
PCB 128	$pg\ g^{-1}$	<10	<5	<5	<5	<5	<5	140	110	43	130	170	41	10	16	61	53	11	28	33	<5	28	16	<5	<5	21
PCB 138	$pg\ g^{-1}$	20	<5	<5	<5	18	20	1200	930	150	370	670	130	41	71	190	340	68	170	240	39	470	260	87	52	270
PCB 149	$pg\ g^{-1}$	40	<5	<5	<5	24	12	600	540	340	340	640	94	28	48	170	220	49	140	180	38	440	180	44	24	180
PCB 153	$pg\ g^{-1}$	55	<5	<5	<5	56	19	990	890	190	430	590	110	34	59	150	310	71	180	240	26	450	220	60	28	260
PCB 180	$pg\ g^{-1}$	71	<9	<9	<9	<9	<9	330	320	110	450	200	33	15	18	60	200	40	64	180	9	120	130	22	<9	210
PCB 187	$pg\ g^{-1}$	33	<5	<5	<5	<5	<5	200	180	85	220	110	14	<5	<5	28	130	34	64	110	<5	170	54	16	<5	130

Table 3.2. Concentrations of chlorinated hydrocarbons (on a dry weight basis) in Black Sea sediments (Romania)

		Romania														
Sample code		P 1	P 2	A 4	B 4	V 1	C.M.	Consta.	P.C.	Cost.	M.	S1	S3	S6	S 8	N.
EOM	$\mu g\ g^{-1}$	250	160	490	20	8	8	3	5	10	50	60	100	160	60	50
HCB	$pg\ g^{-1}$	23000	110	5300	140	10	32	1100	1700	36	150	40	130	180	60	<10
α-HCH	$pg\ g^{-1}$	5400	410	8600	400	70	84	120	620	170	300	260	640	940	740	30
β-HCH	$pg\ g^{-1}$	550	220	2400	110	43	84	220	1600	170	140	380	690	610	1100	60
Lindane	$pg\ g^{-1}$	1800	1600	29000	2100	180	380	470	2800	1400	230	210	430	400	560	100
pp' DDE	$pg\ g^{-1}$	5300	290	8200	120	70	220	390	3700	150	360	890	4700	3100	5100	230
pp' DDD	$pg\ g^{-1}$	18200	1000	35000	400	320	1000	1700	25000	480	1800	1900	2400	2500	6800	220
pp' DDT	$pg\ g^{-1}$	7600	520	20000	200	500	1400	230	1200	330	1900	220	580	370	1250	100
DDMU	$pg\ g^{-1}$	780	53	1400	47	25	67	140	1200	38	67	280	700	360	750	20
op DDE	$pg\ g^{-1}$	170	16	330	15	9	18	29	470	9	20	31	97	80	110	10
op DDD	$pg\ g^{-1}$	3000	280	8100	99	77	250	530	6500	120	350	340	550	260	990	30
Heptachlor	$pg\ g^{-1}$	<3	<3	<3	<3	<3	<3	<3	170	<3	3	<3	<3	<3	<3	<3
Aldrin	$pg\ g^{-1}$	<10	<10	<10	<10	<10	<10	<10	180	<10	<10	<10	<10	<10	<10	<10
Dieldrin	$pg\ g^{-1}$	44	50	150	11	<7	7	8	240	8	60	24	70	70	110	10
Endrin	$pg\ g^{-1}$	<10	28	<10	<10	<10	<10	10	250	<10	<10	<10	<10	<10	10	<10
α-Endosulfan	$pg\ g^{-1}$	29	46	240	7	<7	<7	<7	270	15	<7	7	10	<7	20	20
β-Endosulfan	$pg\ g^{-1}$	<10	72	<10	<10	<10	<10	<10	100	<10	<10	<10	<10	<10	<10	<10
Endos. Sulfate	$pg\ g^{-1}$	12	44	72	10	<7	<7	<7	20	7	<7	<7	<7	<7	<7	10
Aroclor 1254	$pg\ g^{-1}$	4900	90	4300	730	240	350	440	25000	270	1040	400	1130	2650	980	160
Aroclor 1260	$pg\ g^{-1}$	27000	630	12000	<60	<60	210	620	50000	410	200	730	2530	1460	1240	<60
PCB 28	$pg\ g^{-1}$	250	4	290	6	1	<1	39	200	21	30	40	46	1300	60	40
PCB 49	$pg\ g^{-1}$	4	16	180	25	13	6	6	250	6	<4	5	48	20	20	<4
PCB 52	$pg\ g^{-1}$	220	<6	310	34	6	27	33	840	28	40	58	78	170	170	20
PCB 97	$pg\ g^{-1}$	36	6	54	10	5	4	4	130	2	<2	<2	5	10	<2	<2
PCB 101	$pg\ g^{-1}$	520	1	280	47	7	38	55	3100	32	80	73	96	270	130	20
PCB 136	$pg\ g^{-1}$	39	2	15	<1	<1	1	<1	130	<1	<1	<1	<1	<1	<1	<1
PCB 138	$pg\ g^{-1}$	2200	58	1200	150	37	53	120	6300	42	340	180	370	660	290	10
PCB 153	$pg\ g^{-1}$	2500	64	1200	87	34	45	95	6400	48	320	220	370	950	360	10
PCB 180	$pg\ g^{-1}$	3000	70	1300	110	15	30	76	6900	18	250	180	320	500	260	<5

3.2.2 DDTs

Concentrations of DDT and its related compounds in sediment from the Black Sea are shown to be generally lower than those reported for the Baltic Sea and most Asian

such as the former USSR, produced PCBs (Sovol) for use as a dielectric fluid in power capacitors and transformers (Ivanov and Sandell, 1992). A recent study showed that the composition of PCBs in Sovol was similar to that in Aroclor 1254, which contains mainly tetra- to hexa-chlorinated congeners (Iwata *et al.*, 1995). Although comparatively low concentrations of PCBs were found in this survey, significant concentrations in air, water, sediments, soil, fish and seals have been reported for other regions in the Russian Federation e.g. Lake Baikal (Iwata *et al.*, 1995; Nakata *et al.*, 1997). The usage pattern and major sources of PCBs in other countries surrounding the Black Sea remain unclear.

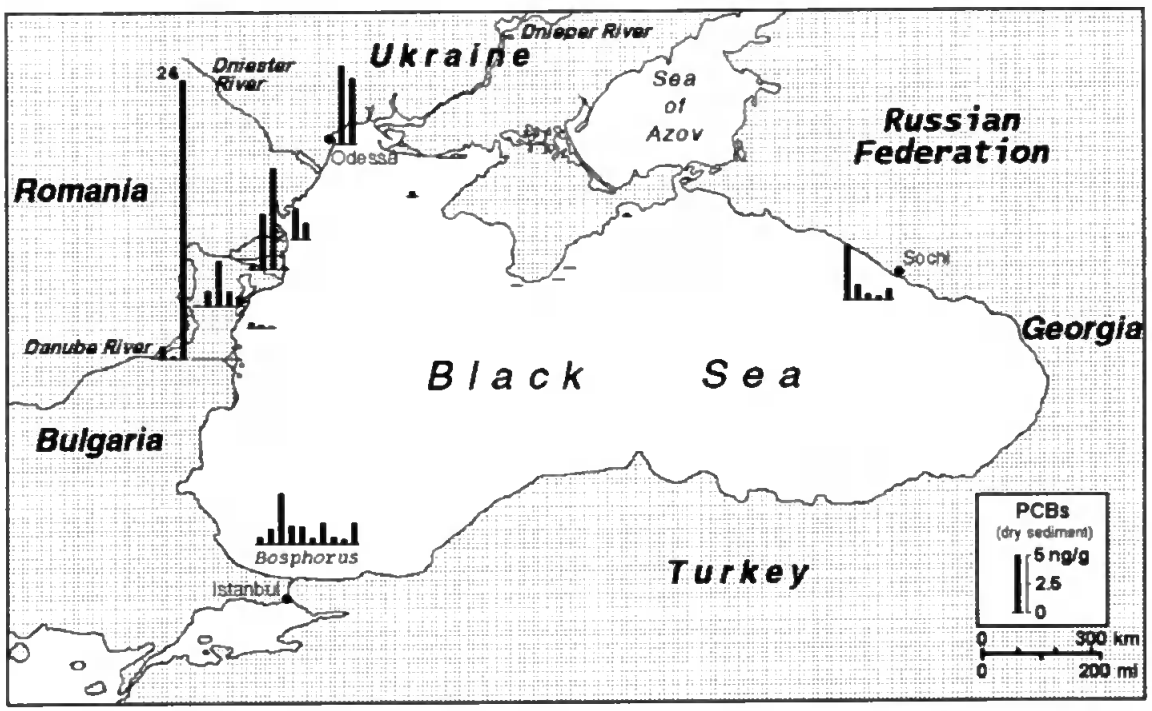


Figure 3.1. Distribution of PCBs in sediment from the Black Sea (ng g dry wt^{-1})

Among the 209 PCB congeners, some attain coplanarity and elicit highly toxic biological effects that can approach that of 2,3,7,8-TCDD (Safe, 1990). Tanabe *et al.* (1997a) have reported the presence of toxic co-planar PCBs in porpoise and fish from the Black Sea (Turkish coast) with a dominance of the highly chlorinated congeners (IUPAC No. 138, 153 and 180). Results from our sediment analyses confirm the presence of co-planar congeners, especially IUPAC No. 138 and 153 (di-*ortho*) and 118 (mono-*ortho*), which dominate (Tables 3.1 and 3.2).

sites. They are comparable to, or slightly higher than, those reported for other regions of Russian Federation (e.g. Lake Baikal), the USA and Mexico (Table 3.3). High concentrations of DDTs (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD and o,p'-DDD, excluding o,p'-DDT in the case of the Romanian samples) in the Black Sea are associated with sediments rich in lipids (EOM) in the Ukraine (43 ng g^{-1} dry wt - station 132) and Romanian stations, which are under the influence of River Danube discharges (stations P1 and A4 - 34 and 72 ng g^{-1} dry wt, respectively). The high concentrations associated with the Danube and adjacent coastal areas indicate that the river is a major source of contamination to the Black Sea. High concentrations are also reported for sediments in the vicinity of Odessa (stations 94a and 98 - 34 and 65 ng g^{-1} dry wt, respectively) and Port Constantza (37 ng g^{-1} dry wt) (Fig. 3.2).

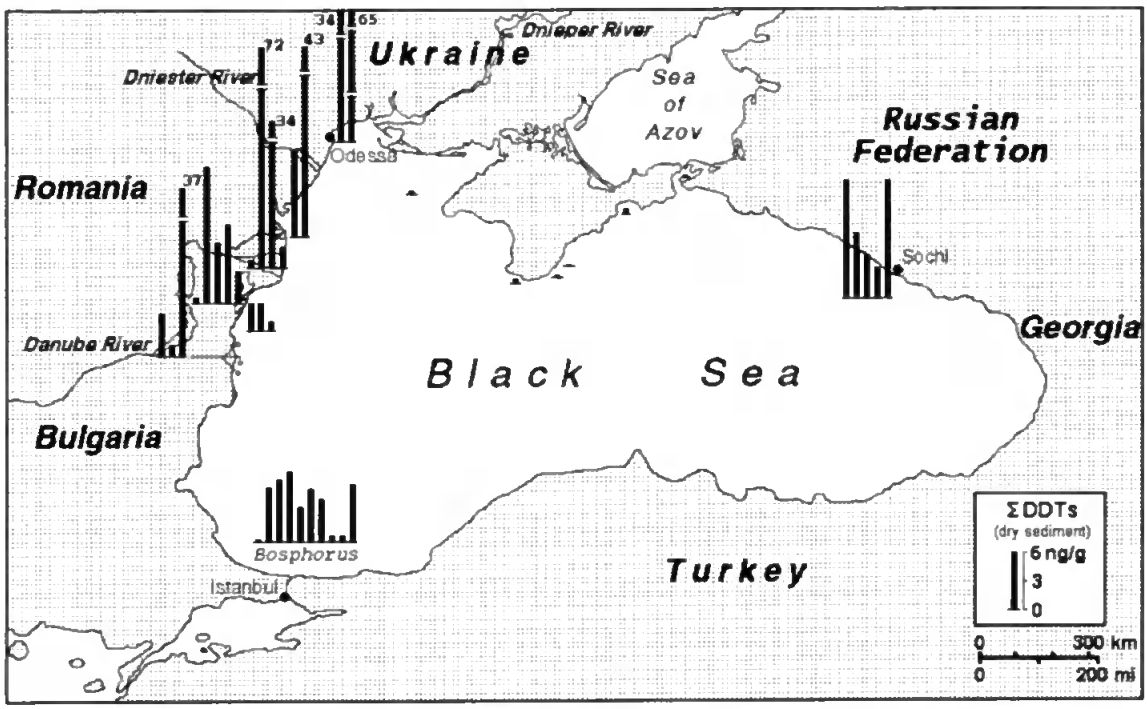


Figure 3.2. Distribution of DDTs in sediment from the Black Sea (ng g dry wt^{-1})

The DDE/DDT ratio can be used to indicate whether DDT inputs have occurred recently or in the past. Since p,p'-DDE is a metabolite of p,p'-DDT (and is not present in technical DDT), lower and higher ratios denote recent and past usage of technical DDT, respectively. A higher ratio of DDE/DDT may, however, also be influenced by long-range atmospheric transport, because of the more efficient transport of p,p'-DDE compared to p,p'-DDT (Iwata *et al.*, 1993a).

DDT compositional ratios (p,p'-DDE/p,p'-DDT) throughout the Black Sea show large variability among sites: > 7.0 along the Ukrainian north coastline; 0.2 - 1.2 at Sochi; 0.2 - 0.3 at Odessa; 0.1 - 1.6 along the Ukrainian coastline adjacent to the Danube; 0.3 - 5.0 in the vicinity of the Bosphorus and, finally, along the Romanian coastline (2.4 - 8.6 in Sinoe Lagoon; 0.4 - 0.7 in proximity to the Danube and 0.2 - 3.5 at other Romanian stations) (Fig. 3.4). Similar variations have also been observed in other studies (Ramesh *et al.*, 1989; Iwata *et al.*, 1993a; Iwata *et al.*, 1995). It is likely, however, that the low DDE/DDT values combined with the relatively high concentrations (especially in Odessa sediments and, Ukraine and Romanian sediments under influence of Danube discharges) are an indication of current DDT usage around the Black Sea. Measurements of DDT made in 1981 and 1982 indicated high water concentrations (32 to 486 $ng.L^{-1}$) in the region of Danube delta and in the Kerch Strait (8 to 20 $ng.L^{-1}$) (EEA, 1995), indicating extensive usage of this insecticide. According to Tuncer *et al.* (1998), approximately 100 tonnes of DDT are discharged annually via rivers into the Black Sea suggesting continued illegal usage of this pesticide in agriculture. This is further supported by concentrations of DDT residues reported in human adipose tissues and organisms (porpoise and fish) from the region (Tanabe *et al.*, 1997b).

3.2.3 HCHs

HCH concentrations (the sum of α, β and γ isomers) were found to be in the range of 0.02 to 40 ng g^{-1} dry wt (Table 3.3). Concentrations of lindane (γ-HCH) and the other HCH isomers are low in samples from the Ukrainian coastline, Russian Federation and Turkey (Fig. 3.3). These levels are comparable to the low to-medium range of values reported by Iwata *et al.* (1994b) for estuarine sediments from eastern and southern Asia and Oceania. However, they are much lower than values reported for India and Vietnam, which are subject to intensive sources of HCH contamination (Table 3.3). High concentrations in samples from Romanian stations (under the influence of the River Danube) indicate usage of HCH as a pesticide in the River Danube watershed. Indeed, on a global basis this study reports some of the highest concentrations recorded for HCHs in sediment (up to 40 ng g^{-1} dry wt) (Table 3.3).

With regard to the composition of the HCH isomers in sediment, a high percentage of the γ-isomer is recorded at some locations along the Romanian Coastline (56 to

81%). However, the α isomer is dominant at Sulina arm of Danube delta (P1) and, at the Sinoe Lagoon (S1-S8) and the southernmost station Mangalia (M.), none of the isomers dominate (Fig. 3.5). Elevated percentages of the γ-isomer indicate the use of lindane as a pesticide in the region. Technical HCH (which is also used as a pesticide) has been reported as a mixture of β (c. 9%), γ (c. 14%) and α (c. 70%) isomers (Ramesh *et al.*, 1989; Iwata *et al.*, 1994b; Iwata *et al.*, 1995). The percentage of the γ-isomer is between 5 and 15% in Sochi (β-isomer 53-69%), 11 and 14% in Odessa (β-isomer 40-54%), 26 and 27% along the Danube Coastline, 12 and 63% along the Ukrainian coastline (β-isomer 29-74%), and 22 and 72% in the Bosphorus (Fig. 3.5). At some stations, β-HCH appears as the dominant isomer. Of the HCH isomers, the β-isomer has the lowest water solubility and vapour pressure, and is the most stable and resistant to microbial degradation. These properties could account for higher levels of this isomer in some sediments (Ramesh *et al.*, 1991).

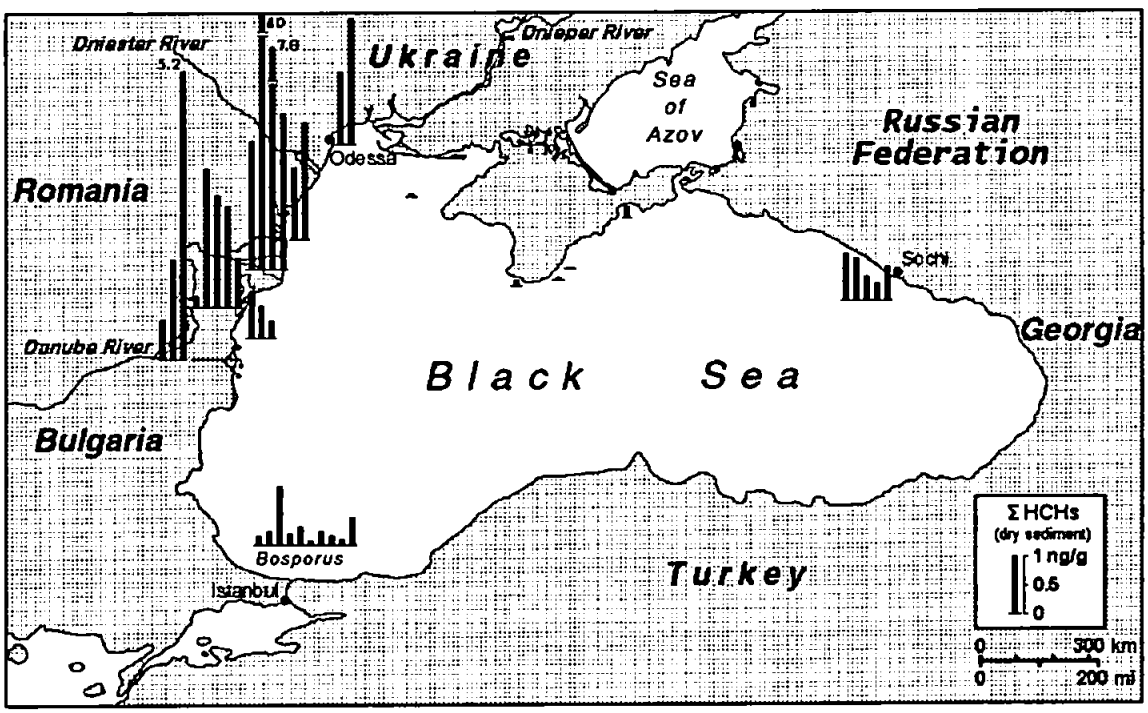


Figure 3.3. Distribution of HCHs in sediment from the Black Sea (ng g dry wt^{-1})

The ratio between the α- and γ-isomers was less than unity for sediments along the Romanian coastline (i.e. 0.1 to 0.4), with the exception for station P1 (3.0), the Sinoe Lagoon (0.3 to 2.4) and Mangalia (1.3). These low values confirm the agricultural use of lindane in the region, since the α/γ ratio is between 4 and 7 for technical HCH and nearly zero for lindane (Iwata *et al.*, 1995). On the other hand, relatively high values

(i.e. those found at Odessa (2.2 and 4.4) and Sochi (2.1 to 5.1)), and intermediate values (i.e. those found along the Ukrainian coastline (1.0 to 1.8), Ukrainian Danube coastline (1.4 to 1.8) and Bosphorus (0.7 to 1.6)) suggest that HCH contamination at some locations arises through the use of both formulations. The use of technical HCH is recorded in the former USSR and Romania (Fischer *et al.*, 1991), but information is lacking for the other countries surrounding the Black Sea (Tanabe *et al.*, 1997b).

Table 3.3. Worldwide concentrations of organochlorines in sediment (ng g^{-1} dry wt)

Area	PCB	DDTs	HCHs	References
Pearl River Delta, China	11.5-485	N.A.	N.A.	(Kang *et al.*, 2000)
North Coast Vietnan	0.5-28.1	6.2-10.4	1.2-33.7	(Nhan *et al.*, 1999)
South-Western Coast, Baltic Sea	0.1-11	<0.04-88	<0.04-1.2	(Dannenberger, 1996)
Vanuatu and Tonga, South Pacific Islands	N.A.	<0.1-1027	<0.1-0.3	(Harrison *et al.*, 1996)
Humber Estuary, UK	N.D.-84	N.A.	N.A.	(Tyler and Millward, 1996)
Irish sea, UK	0.2-42	N.A.	N.A.	(Thompson *et al.*, 1996)
San Quintin Bay, Mexico	<10	<2-15	N.D.-<1	(Galindo *et al.*, 1996)
Gulf of Bothnia, Baltic Sea	2-14	N.A.	N.A.	(VanBavel *et al.*, 1995)
Lake Baikal, Russia	0.08-6.1	0.01-2.7	0.02-0.1	(Iwata *et al.*, 1995)
Xiamen Harbour, China	0.05-7.2	4.5-311	0.1-1.1	(Hong *et al.*, 1995)
Victoria Harbour, Hong Kong	3.2-81	1.4-97	<0.1-9.4	(Hong *et al.*, 1995)
Western Coast, Australia	<10	1-22	N.A.	(Burt and Ebell, 1995)
Firth of Clyde, Scotland	0.5-500	N.A.	N.A.	(Kelly and Campbell, 1995)
Sarasota Bay, Florida, USA	N.A.	<1-69	N.A.	(Sherblom *et al.*, 1995)
Gulf of Alaska, Bering Sea, Chukchi Sea	0.1-2	0.01-0.2	0.04-0.3	(Iwata *et al.*, 1994a)
San Francisco Estuary, USA	<0.1-8.1	<0.1-9	N.A.	(Pereira *et al.*, 1994)
Cities, India	4.8-1000	8-450	0.6-38	(Iwata *et al.*, 1994b)
Cities, Thailand	11-520	4.8-170	0.5-3.1	(Iwata *et al.*, 1994b)
Cities, Vietnam	0.2-630	0.4-790	0.4-12	(Iwata *et al.*, 1994b)
Cities, Indonesia	5.9-220	3.4-42	0.04-0.1	(Iwata *et al.*, 1994b)
Cities, Papua New Guinea	3.3-54	4.7-130	0.2-0.3	(Iwata *et al.*, 1994b)
Cities, Solomon Islands	1.1-5.0	9.3-750	<0.3-2.2	(Iwata *et al.*, 1994b)
Cities, Japan	63-240	2.5-12	4.5-6.2	(Iwata *et al.*, 1994b)
Cities, Taiwan	2.3-230	0.4-11	0.3-0.8	(Iwata *et al.*, 1994b)
Cities, Australia	0.5-790	0.08-1700	0.02-17	(Iwata *et al.*, 1994b)
Danube River	0.02-85	<0.04-41	0.03-6.4	(Equipe Cousteau, 1993)
Bosphorus, Black Sea, Turkey	0.4-4.4	0.2-7.2	0.08-1.1	(This study)
Sochi, Black Sea, Russia	0.3-4.7	3.3-12	0.3-0.8	(This study)
Odessa, Black Sea, Ukraine	5.7-6.8	35-65	1.3-2.3	(This study)
Coastline, Black Sea, Ukraine	N.D.-0.4	0.06-0.6	0.02-0.2	(This study)
Danube Coastline, Black Sea, Ukraine	1.4-2.7	9.2-43	1.3-2	(This study)
Romania Coastline, Black Sea	0.1-24	0.6-72	0.2-40	(This study)

N.D.: Not detected; N.A.: Not analised.

Iwata *et al.* (1993b) have also suggested that an increase in the α/γ ratio would occur during atmospheric transport, providing further evidence that the generally low α/γ ratios recorded in the region are due to agricultural usage of HCH. Air-sea exchange represents an important process in regulating the composition and concentration of

organochlorine compounds in the marine environment. Iwata *et al.* (1993a; 1994a; 1995) clearly demonstrated that, in accordance with the volatility (Henry's law constants and vapour pressure) of these compounds, they can be either selectively lost to the atmosphere or alternatively retained in the ocean. Thus, these processes that also depend on ambient conditions need to be considered in any marine environmental study of these compounds. α-HCH has a higher Henry's law constant and vapour pressure than either the β- or γ-isomers (Suntio *et al.*, 1988), rendering atmospheric transport more important for this isomer. γ-HCH is preferentially degraded by micro-organisms (Benezet and Matsumara, 1973) and can be photochemically isomerised to the α-isomer (Malaiyandi and Shah, 1984). These factors could account for higher proportions of α-HCH in sediments distant from contamination sources.

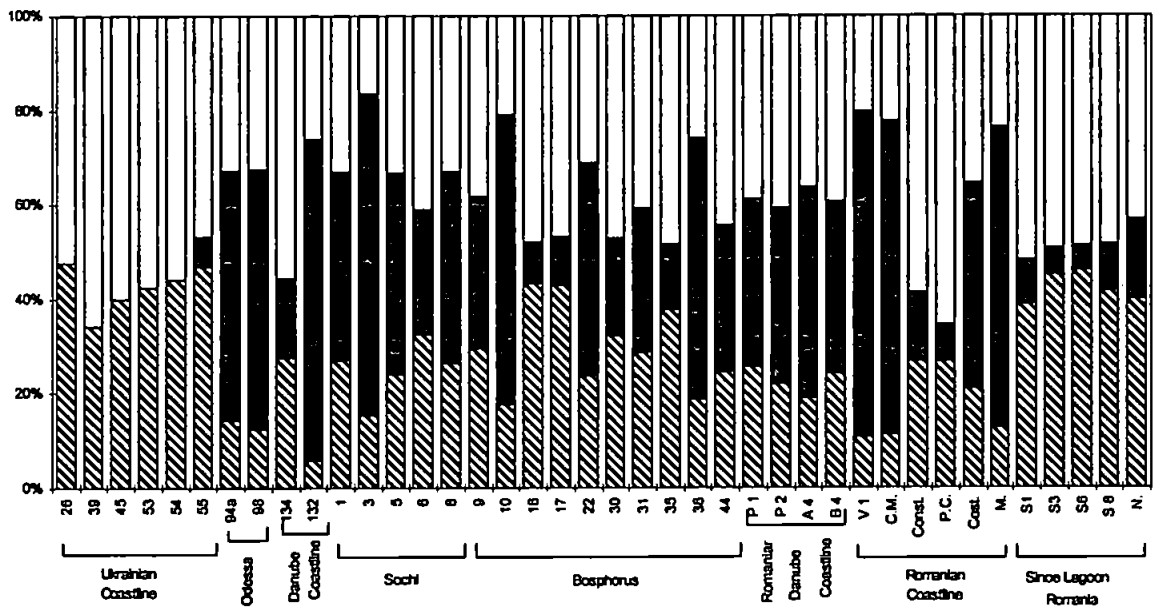


Figure 3.4. Composition of DDTs (▨ - DDE; ■ - DDT; □ - DDD) in sediment samples from the Black Sea

Although few data exist for comparative purposes, mean and maximum water concentrations of α-HCH and γ-HCH (lindane) in the Black Sea have been reported to be 5 ng L^{-1} and 23 ng L^{-1}, and 1 ng L^{-1} and 5 ng L^{-1}, respectively (Dechev, 1990; EEA, 1995). Measurements made in 1981 and 1982 revealed levels of lindane in water of 20 to 550 ng L^{-1} in the region of the Danube delta (EEA, 1995).

3.2.4 HCB and cyclodienes

HCB and certain cyclodienes were also found in the sediments from the Black Sea (Tables 3.1 and 3.2). In many samples, however, cyclodienes were not detected. The concentrations of HCB in sediments, ranging from 0.01-23 ng g^{-1} dry wt, are much lower than those recorded for other compounds. The highest values were recorded along the Romanian (5.3 and 23 ng g^{-1} dry wt) and Ukrainian (0.7 and 1.3 ng g^{-1} dry wt) coastlines adjacent to the River Danube. This chemical has been released into the environment as a by-product of industrial processes (Tanabe *et al.*, 1997b) and is also formed by combustion and during chlorination (Grimalt *et al.*, 1988). Some countries have also used it as a fungicide (Kutz *et al.*, 1991). HCB has been previously reported in porpoise and fish tissues from the Black Sea (60-610 ng g^{-1} dry wt basis) (Tanabe *et al.*, 1997b), confirming the usage of the compound in the region.

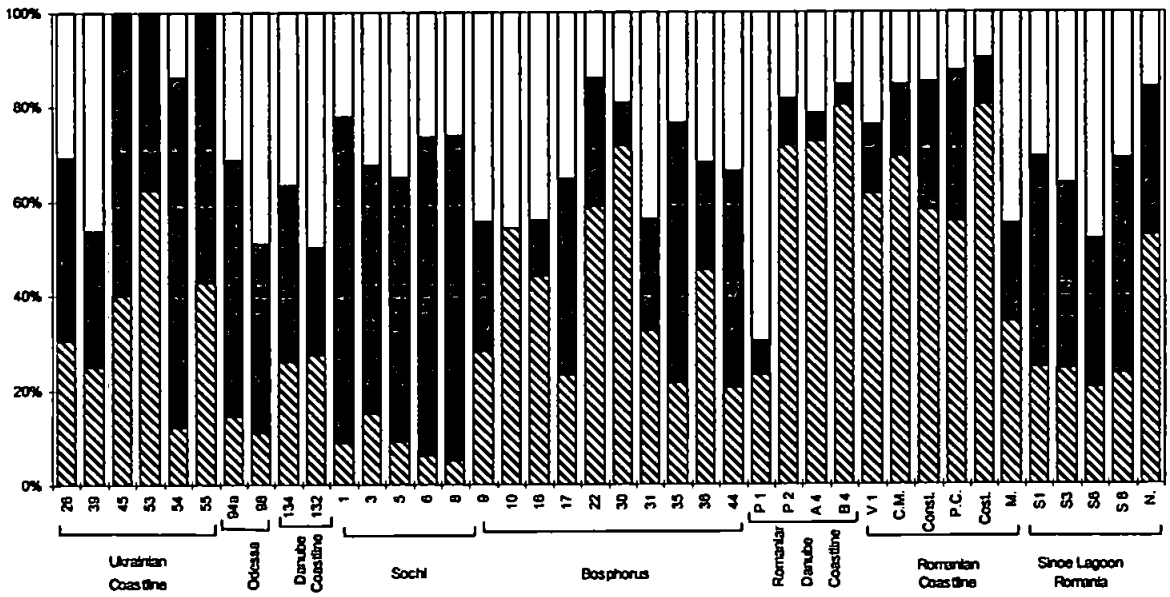


Figure 3.5. Composition of HCHs (▨ - γ-HCH (Lindane); ■ - β-HCH; □ - α-HCH) in sediment samples from the Black Sea

As previously mentioned, important characteristics of organochlorines are their hydrophobicity and lipophilicity. These factors result in organochlorines being preferentially accumulated in sediments and marine organisms, particularly those that have a high lipid (EOM) content. Thus, sediments with high concentrations of organic carbon are more likely to adsorb the lipophilic organochlorines than those with lower concentrations, such as sandy materials (Iwata *et al.*, 1994b). The most contaminated locations in this survey were those associated with lipid (EOM) rich sediments (200 to

490 μg g^{-1} dry wt) i.e. stations P1 and A4 (Romanian coast), station 134 (Ukrainian Danube coast), stations 94a and 98 (Odessa) and stations 1 and 8 (Sochi – Russian Federation). There are, however, exceptions to these findings. For example, relatively high concentrations of organochlorines were observed in sediments at Port Constantza (P.C.) on the Romanian coastline, where the concentration of lipids (EOM) in sediments is low (i.e. 5 μg g^{-1} dry wt) indicating high inputs of wastes containing organochlorines in this particular area.

3.3 Petroleum and Polycyclic Aromatic Hydrocarbons

Hydrocarbons in sediments originate from several sources which have been grouped into the following categories (UNEP/IOC/IAEA, 1992; Clark, 1997): 1) petroleum inputs; 2) hydrocarbons (especially PAH) released as the result of partial combustion of fuels; 3) PAHs originating from forest and grass fires (transported to the marine environment via aeolean (atmospheric) and fluvial (riverine) processes); 4) biosynthesis of hydrocarbons by marine or terrigenous organisms; 5) early diagenetic transformation of non-hydrocarbon natural products to hydrocarbons. Thus, the distribution of hydrocarbons in the environment can vary greatly from one area to another. Biological sources include land plants, animals, bacteria, macroalgae and microalgae. Certain hydrocarbons such as phytanes, hopenes and sterenes are produced from bacterial and chemical degradation of naturally occurring lipids. Bush fires, agricultural burn-off, vehicle exhausts and other combustion processes are also significant sources, especially of polycyclic aromatic hydrocarbons. Considerable amounts of petroleum products are discharged into the marine environment through runoff, industrial and sewage effluents, storm water drains, shipping activities, spillage, etc. Natural oil seeps can also be important in some areas. Significant changes in hydrocarbon composition can occur due to selective dissolution, evaporation, chemical and photo-oxidation and biodegradation. Short chain alkanes and simple aromatics are rapidly lost, but complex cyclic molecules such as steranes and hopanes are rarely affected and can be particularly useful in source investigations (Volkman *et al.*, 1992). Sediments (especially anoxic silts) act as repositories of hydrocarbons where the compounds can remain for years.

To estimate the severity of oil contamination, a number of indicators have been proposed: i) high concentrations (>100 μg g^{-1}) of "total" hydrocarbons; ii) C_{21}-C_{35} *n*-alkanes having no odd over even predominance; iii) complex distributions; iv) an

Table 3.4. Concentrations of aliphatic hydrocarbons (on a dry weight basis) and selected ratios for Black Sea sediments

Sampling sites	Ukraine										Russian Federation					Turkey									
	26	39	45	53	54	55	94a	98	132	134	1	3	5	6	8	9	10	16	17	22	30	31	35	36	44
EOM ($\mu g\ g^{-1}$)	25	29	16	13	37	5	200	390	90	490	290	170	54	100	290	110	76	70	160	33	180	48	42	44	85
CHR equiv. ($\mu g\ g^{-1}$)	0.9	1.8	3.2	0.9	7.4	0.6	40	230	11	320	200	55	16	54	160	18	42	8.4	34	1.9	69	5.7	4	4.6	19
ROPME equiv. ($\mu g\ g^{-1}$)	5.0	10	18	5.0	42	3.2	220	1300	66	1750	680	180	52	180	530	64	140	30	120	6.5	340	20	14	16	69
TOT- HC ($\mu g\ g^{-1}$)	2.7	5.2	6.6	4.4	5.8	2.1	110	310	49	220	170	16	7.6	15	53	69	39	38	51	16	76	35	16	12	35
RES-ALI ($\mu g\ g^{-1}$)	0.68	1.2	0.79	1.1	0.84	0.21	5.1	8.1	2.4	6.3	7.8	3.0	1.6	2.4	4.7	5.6	2.6	3.3	3.1	2.1	5.6	3.5	2.2	1.8	2.8
UCM-ALI ($\mu g\ g^{-1}$)	1.2	1.6	3.1	1.8	3.0	1.0	78	232	33	160	140	5.9	2.9	7.8	36	38	20	19	25	4.1	26	17	7	4	18
TOT-ALI ($\mu g\ g^{-1}$)	1.9	2.8	3.9	2.9	3.8	1.2	83	240	35	166	150	8.9	4.5	10	41	44	23	22	28	6.2	32	21	9.2	5.8	21
n-C_{17} ($ng\ g^{-1}$)	7.7	17	12	14	24	27	47	75	21	91	130	44	34	50	80	21	21	31	20	24	69	22	19	16	22
Pristane ($ng\ g^{-1}$)	1.2	4.5	3.9	3.2	5.5	0.4	4.8	11	23	170	160	86	76	93	100	19	21	30	14	22	92	25	25	16	24
n-C_{18} ($ng\ g^{-1}$)	3.1	8.2	5.1	4.8	6.8	2.2	9.7	23	7.5	45	110	30	25	33	48	15	16	25	13	20	64	17	14	11	18
Phytane ($ng\ g^{-1}$)	<0.3	<0.3	0.6	<0.3	2.5	0.4	37	12	35	170	160	27	16	18	60	9	5.8	10	4.9	9.9	24	9	5.7	6.1	6.1
Squalane ($ng\ g^{-1}$)	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	3.2	<0.4	<0.4	<0.4	8.8	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
$\Sigma\ n$-C_{14}-C_{34} ($\mu g\ g^{-1}$)	0.25	0.57	0.46	0.52	0.37	0.11	1.6	1.4	1.2	2.1	3.4	1.9	0.74	1.2	2.1	2.6	1.5	1.6	1.4	1.4	1.7	1.9	1.4	1.3	1.4
UCM/RES	1.8	1.3	3.9	1.6	3.6	4.8	15.3	28.6	13.8	25.4	18.0	2.0	1.8	3.3	7.7	6.8	7.7	5.8	8.1	2.0	4.6	4.9	3.2	2.2	6.4
%UCM/ aliphatics	63.2	57.1	79.5	62.1	78.9	83.3	94.0	96.7	94.3	96.4	93.3	66.3	64.4	78.0	87.8	86.4	87.0	86.4	89.3	66.1	81.3	81.0	76.1	69.0	85.7
%n-alkanes/aliphatics	13.2	20.4	11.8	17.9	9.7	9.2	1.9	0.6	3.4	1.3	2.3	21.3	16.4	12.0	5.1	5.9	6.5	7.3	5.0	22.6	5.3	9.0	15.2	22.4	6.7
C_{17}/Pr	6.4	3.8	3.1	4.4	4.4	67.5	9.8	6.8	0.9	0.5	0.8	0.5	0.4	0.5	0.8	1.1	1.0	1.0	1.4	1.1	0.8	0.9	0.8	1.0	0.9
C_{18}/Ph	>31	>82	8.5	>48	2.7	5.5	0.3	1.9	0.2	0.3	0.7	1.1	1.6	1.8	0.8	1.7	2.8	2.5	2.7	2.0	2.7	1.9	2.5	1.8	3.0
Pr/Ph	>12	>45	6.5	>32	2.2	1.0	0.1	0.9	0.7	1.0	1.0	3.2	4.8	5.2	1.7	2.1	3.6	3.0	2.9	2.2	3.8	2.8	4.4	2.6	3.9
%aliphatics/EOM	7.6	9.7	24.4	22.3	10.3	24.0	41.5	61.5	38.9	33.9	51.7	5.2	8.3	10.0	14.1	40.0	30.3	31.4	17.5	18.8	17.8	43.8	21.9	13.2	24.7
%TOT-HC/EOM	10.8	17.9	41.3	33.8	15.7	42.0	55.0	79.5	54.4	44.9	58.6	9.4	14.1	15.0	18.3	62.7	51.3	54.3	31.9	48.5	42.2	72.9	38.1	27.3	41.2
%aliphatics/TOT-HC	70.4	53.8	59.1	65.9	65.5	57.1	75.5	77.4	71.4	75.5	88.2	55.6	59.2	66.7	77.4	63.8	59.0	57.9	54.9	38.8	42.1	60.0	57.5	48.3	60.0

Abbreviations used: CHR equiv.: Chrysene equivalents; ROPME equiv.: ROPME Oil equivalents; TOT-HC: Total Hydrocarbons;RES-ALI: Resolved Aliphatics; UCM-ALI: Unresolved Aliphatics; TOT-ALI: Total Aliphatics

Table 3.5. Concentrations of polycyclic aromatic hydrocarbons (on a dry weight basis) and selected ratios recorded for Black Sea sediments

Sampling sites	Ukraine										Russian Federation					Turkey									
	26	39	45	53	54	55	94a	98	132	134	1	3	5	6	8	9	10	16	17	22	30	31	35	36	44
RES-ARO (μg g^{-1})	0.35	0.6	0.69	0.43	0.47	0.19	2.0	2.4	1.3	3.3	2.4	4.0	0.56	1.2	2.7	3.7	3.1	3.3	3.1	2.8	14	4	2.5	2.4	2.8
UCM-ARO (μg g^{-1})	0.49	1.8	2.0	1.1	1.5	0.73	28	63	13	51	16	3.5	2.5	3.4	8.9	21	13	13	20	6.9	30	10	4.5	3.6	11
TOT-ARO (μg g^{-1})	0.84	2.4	2.7	1.5	2.0	0.92	30	65	14	54	18	7.5	3.1	4.6	12	25	16	16	23	9.7	44	14	7	6	14
NAPH (ng g^{-1})*	2.2	7.5	7.6	3.9	3.0	1.5	11	25	9.5	29	0.3	6.1	5.2	9.7	11	6.4	6	4.5	4.5	1.7	7.2	4.8	1.8	1.8	5.1
C1-NAPH (ng g^{-1})*	0.7	2.5	2.4	1.6	1.4	0.8	5.2	17	6.6	32	3.2	6.2	4.7	10	11	4.5	3	3.3	2.8	1.5	4.2	3	1.7	1.5	3.5
C2-NAPH (ng g^{-1})*	1.8	6.0	4.9	2.9	2.4	1.1	9.8	31	8.2	39	3.6	7.5	6.2	13	16	5.4	4.2	4.5	3.8	1.2	4.3	4.2	1.8	1.3	4.2
ACTHY (ng g^{-1})	<0.5	<0.5	1.1	<0.5	<0.5	<0.5	<0.5	<0.5	1.5	5.2	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.8	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
ACE (ng g^{-1})	<0.5	<0.5	3.6	<0.5	<0.5	<0.5	0.9	8.5	0.6	3.0	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2.8	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FL (ng g^{-1})	0.10	2.0	9.6	0.7	1.0	0.5	5.7	23	4.5	20	9.8	3.1	2	2.3	4.5	2.5	5.6	1.8	1.7	1	2.4	2	1.0	0.8	1.5
PHE (ng g^{-1})	1.6	10	58	2.8	7.3	2.8	28	130	8.7	77	53	20	9.1	14	24	16	69	14	11	4.2	18	8.3	6.3	4.4	13
ANT (ng g^{-1})	<0.1	<0.1	5.7	<0.1	1.3	<0.1	1.0	37	<0.1	59	1.8	0.9	0.3	<0.1	1.3	4.8	22	1.9	4.1	0.1	<0.1	0.4	1.6	<0.1	4.5
C2-PHE (ng g^{-1})	1.3	1.5	4.2	0.8	2.4	1.2	4.9	37	1.2	19	19	8.2	3	5	11	3.4	12	3.7	4.1	1.3	14	1.9	3.4	1.8	5
C1-PHE (ng g^{-1})	0.8	1.3	1.9	0.6	1.5	0.4	2.8	21	1.5	19	11	5.4	3.5	6	8.5	1.8	6.3	3.2	3.4	1.6	10	1.1	1.5	1.3	3.1
Fluoranthene (ng g^{-1})	3.3	3.0	21	0.4	1.3	1.4	11	190	1.8	67	100	25	9.5	17	37	10	145	13	32	3.3	15	6.1	6.1	4.7	30
PYR (ng g^{-1})	2.3	1.5	17	0.2	11	0.9	4.0	160	1.6	93	86	23	9	16	36	6.3	130	8.3	29	3.5	30	3.9	5.2	5.4	30
C1-PYR (ng g^{-1})	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	3.4	1.9	2.4	9.7	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	4.5	0.1	1.8	<0.1	2.1	<0.1	0.4	0.4	2
CHR (ng g^{-1})	1.3	0.2	0.9	0.3	11	0.10	1.4	46	0.4	67	56	21	7.1	13	35	0.2	49	0.4	18	0.03	3.9	0.1	1.4	1.7	13
PER (ng g^{-1})	0.1	0.1	0.2	0.3	6.2	<0.1	0.3	0.6	0.4	43	3.5	4.6	6.3	9	9.1	0.1	5	0.1	37	0.1	29	0.1	1.2	30	11
BaA (ng g^{-1})	0.4	0.1	0.5	0.2	6.1	<0.1	0.8	2.7	0.3	32	29	8.2	2.1	7.3	16	0.1	49	0.1	15	0.1	1.9	0.1	0.7	0.8	11
Bb+kF (ng g^{-1})	0.3	<0.1	0.1	0.4	7.5	<0.1	1.3	8.0	0.5	57	16	18	4.8	11	22	0.1	30	0.1	20	<0.1	5.6	0.1	0.6	2.3	18
InP (ng g^{-1})	0.2	0.1	0.2	0.4	1.3	<0.1	0.5	0.3	0.3	26	0.8	3.8	2.4	6.2	6.7	<0.1	1.7	<0.1	11	<0.1	3.9	<0.1	0.1	0.8	3.5
DBA (ng g^{-1})	0.1	<0.1	0.1	0.2	0.3	<0.1	0.1	0.1	0.1	4.1	0.2	0.8	0.3	0.9	1.2	<0.1	0.3	<0.1	1.3	<0.1	0.4	<0.1	<0.1	0.2	1
BghiP (ng g^{-1})	0.1	0.1	0.2	0.3	0.8	<0.1	0.7	0.2	0.4	37	0.6	3.6	3.6	8	6.2	<0.1	1.0	<0.1	11	<0.1	3.8	<0.1	<0.1	0.6	3.2
BaP (ng g^{-1})	0.1	<0.1	0.1	0.2	2.3	<0.1	0.1	1.3	0.1	28	6.3	6.9	2.3	6.6	11	<0.1	7.8	<0.1	9.4	<0.1	1.4	<0.1	0.1	0.6	5.4
BeP (ng g^{-1})	0.1	<0.1	0.1	0.2	1.6	<0.1	0.4	2.9	0.2	34	8.1	6.2	3.5	9.6	14	<0.1	10	<0.1	9.6	<0.1	2.6	<0.1	0.3	1.0	7.4
ΣPAH (ng g^{-1})[‡]	12.1	24.5	125.8	10.2	55.8	7.2	66.9	635.0	30.5	638.3	367.9	146.6	61.2	121.6	225.9	46.4	531.0	44.0	177.6	13.8	96.1	25.7	25.2	25.1	146.6
%ARO/TOT-HC	31.1	46.2	40.9	34.1	34.5	43.8	27.3	21.0	28.6	24.5	10.6	46.9	40.8	30.7	22.6	36.2	41.0	42.1	45.1	60.6	57.9	40.0	43.8	50.0	40.0
PHE/ANT	>15	>15	10.2	>15	5.6	>15	28.0	3.5	>15	1.3	29.4	22.2	30.3	>15	18.5	3.3	3.1	7.4	2.7	105.0	>15	20.8	3.9	>15	2.9
FLTH/PYR	1.4	2.0	1.2	2.0	0.1	1.6	2.8	1.2	1.1	0.7	1.2	1.1	1.1	1.1	1.0	1.6	1.1	1.6	1.1	0.9	0.5	1.6	1.2	0.9	1.0
ΣMPHE/PHE	1.3	0.3	0.1	0.5	0.5	0.6	0.3	0.4	0.3	0.5	0.6	0.7	0.7	0.8	0.8	0.3	0.3	0.5	0.7	0.7	1.3	0.4	0.8	0.7	0.6
BaA/CHR	0.4	0.1	0.2	0.2	0.8	<0.2	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	1.2	0.0	0.4	0.0	0.0	0.0	0.2	0.2	0.6
%PER/Σpenta	14.3	>20.0	33.3	23.1	34.6	-	13.6	4.7	30.8	25.9	10.3	12.6	36.6	24.3	15.9	>20.0	9.4	>20.0	47.9	>20.0	74.3	>20.0	>50.0	88.0	25.7

Abbreviations used: RES-ARO: Resolved Aromatics; UCM-ARO: Unresolved Aromatics; TOT-ARO: Total Aromatics; NAPH: naphthalene; C1-NAPH: 1 methyl naphthalene; C2-NAPH: 2 methyl naphthalene; ACTHY: Acenaphthylene; ACE: Acenaphthene; FL: Fluorene; PHE: Phenanthrene; C2-PHE: 2 methyl phenanthrene; C1-PHE: 1 methyl phenanthrene; ANT: Anthracene; FLTH: Fluoranthene; PYR: Pyrene; C1-PYR: 1 methyl pyrene; CHR: Chrysene; BaA: Benz[a]anthracene; PER: Perylene; Bb+kF: Benzo[b+k]fluoranthene; DBA: Dibenz[a,h]anthracene; BaP: Benzo[a]pyrene; BeP: Benzo[e]pyrene; InP: Indeno[1,2,3-cd]pyrene; BghiP: Benzo[g,h,i]perylene; %ARO/TOT-HC: %aromatics/total hydrocarbons; %PER/Σpenta: %Perylene/Σpenta-isomers. * Compound subject to losses during analyses giving higher analytical variability. [‡]ΣPAH - Σ17 isomers

unresolved complex mixture (UCM), which produces a raised baseline in the gas-chromatogram of the hydrocarbon fraction; v) biomarkers (Volkman *et al.*, 1992).

Regarding hydrocarbon contamination in the Black Sea, the European Environment Agency (1995) and Mee (1992) have highlighted severe contamination (particularly by oil) in areas subject to riverine discharges, navigation routes and ports. However, Wakeham (1996) and Maldonado *et al.* (1999) report only moderate contamination. To determine the hydrocarbon contamination in the Black Sea region, surface sediments from several areas were collected and analysed. Results from the aliphatic and polycyclic aromatic hydrocarbon analyses are summarised in Tables 3.4 and 3.5, respectively.

3.3.1 "Total" aliphatic hydrocarbons

Total extractable organic matter (EOM) in sediments from the Black Sea ranged from 5 to 37 $\mu g\ g^{-1}$ dry wt along the northern Ukrainian coastline (stations 26, 39, 45, 53, 54 and 55), from 54 to 490 $\mu g\ g^{-1}$ dry wt in the vicinity of Odessa (stations 94a and 98), Danube coastline (stations 132 and 134) and Port of Sochi (stations 1, 3, 5, 6 and 8), and from 33 to 180 $\mu g\ g^{-1}$ dry wt in the vicinity of the Bosphorus (stations 9, 10, 16, 17, 22, 30, 31, 35, 36 and 44) (Table 3.4). In most of these samples, "total" hydrocarbons accounted for a major part of the EOM (from 9.4 to 80%). With the exception of only a few sample locations, "total" hydrocarbon concentration in sediment was related to the concentration of extractable organic matter (EOM) ($r^2 = 0.78$, n = 25). Thus, in general, the hydrocarbon burdens appeared to follow dispersal trends similar to "total" lipids.

In terms of "total" hydrocarbons (Table 3.4), concentrations range from 2 to 310 $\mu g\ g^{-1}$ dry wt. In "unpolluted" intertidal and estuarine sediments, concentrations generally range from sub-$\mu g\ g^{-1}$ to approximately 10 $\mu g\ g^{-1}$ (Volkman *et al.*, 1992; Bouloubassi and Saliot, 1993), although they may be two or three times higher where significant inputs of *n*-alkanes derived from plant waxes occur. Organic-rich marine sediments may contain up to 100 $\mu g\ g^{-1}$ of "total" aliphatic hydrocarbons, but concentrations higher than these are usually associated with petroleum inputs. Indeed values of up to 2900 $\mu g\ g^{-1}$ have been reported in petroleum-contaminated surface sediments from New York Bight (Farrington and Tripp, 1977). When the concentration of hydrocarbons is < 50 $\mu g\ g^{-1}$, a more detailed analysis of the hydrocarbon constituents is generally required to

assess the magnitude of anthropogenic contamination. The most contaminated Black Sea stations (concentrations > 100 μg g^{-1} dry wt) are associated with discharges from Odessa, inputs from the River Danube and the Port of Sochi (Fig. 3.6). Samples taken from the Northern Black Sea (Ukrainian) coastline show comparatively little contamination ("total" hydrocarbon concentrations < 10 μg g^{-1} dry wt) even though some areas are subject to wastewater discharges. This is partly explained by a lower lipid content when compared to other more contaminated stations. Regarding inputs through the Bosphorus (Turkey), the level of hydrocarbon contamination is relatively low (concentrations < 100 μg g^{-1} dry wt). In this region hydrocarbons are correlated with EOM (r^2 = 0.76, n=10) and spatial variations in levels probably relate to sedimentation characteristics.

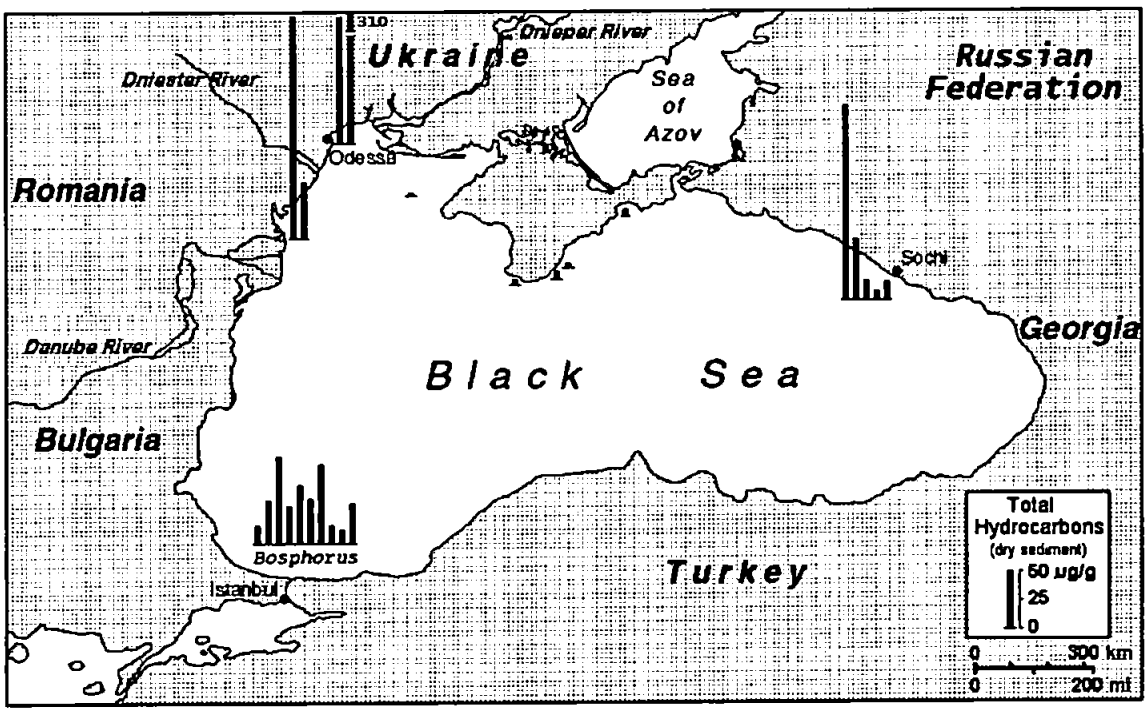


Figure 3.6. Distribution of "total" hydrocarbons in sediments from the Black Sea (μg g^{-1} dry wt)

Wakeham (1996) have reported similar levels of hydrocarbons ("total" aliphatic 10 – 153 μg g^{-1} dry wt) in sediments from the Black Sea. This author, however, worked with off-shore samples but also recorded maximum contamination associated with Danube inputs. Compared to other regions of the world (Table 3.6), levels are shown to be substantially higher than those from selected pristine environments (e.g. Antarctica and the Great Barrier Reef) and are generally comparable to levels encountered in the Mediterranean. Much higher concentrations than those reported for the Black Sea are,

however, shown for locations known to be chronically contaminated by oil (e.g. the Gulf, Hong Kong, New York Bight).

3.3.2 Unresolved complex mixture (UCM)

Gas chromatographic traces of saturated hydrocarbons can be characterised by two general features: resolved compounds and an unresolved complex mixture (UCM). The latter appeared in all samples from the Black Sea as a broad unimodal hump in the range C_{18}-C_{35}. The UCM is generally considered to be a mixture of many structurally complex isomers and homologues of branched and cyclic hydrocarbons that cannot be resolved by capillary GC columns (Bouloubassi and Saliot, 1993). However, using chemical degradation techniques it has been shown that the UCM consists primarily of linear carbon chains connected at branch points, which result in "T-shaped" molecules (Gough and Rowland, 1990). These molecules are resistant to biodegradation and thus accumulate in sediments. In general, the presence of a UCM in aliphatic hydrocarbon chromatograms is considered to be associated with degraded or weathered petroleum residues (Farrington and Tripp, 1977; Readman *et al.*, 1986a; LeDreau *et al.*, 1997). Smaller contributions, in the low $\mu g\ g^{-1}$ dry wt range (Volkman *et al.*, 1992) can, however, relate to bacterial reworking of sedimentary organic matter (Grimalt *et al.*, 1988) or weathering of ancient rocks (Volkman *et al.*, 1992).

The absolute UCM concentrations or, alternatively, its relative importance expressed as the ratio of unresolved to resolved compounds (U/R) are commonly used as a diagnostic criteria of pollutant inputs (Mazurek and Simoneit, 1984). According to this criterion, values of U/R >4 confirm the widespread presence of important petroleum-related residues (Mazurek and Simoneit, 1984; Lipiatou and Saliot, 1991). In the Black Sea, the UCM was by far the major component of the "total" sedimentary aliphatic hydrocarbons (Table 3.4). UCM concentrations varied from 1 to 232 $\mu g\ g^{-1}$ dry wt, which accounted for 57-97% of the "total" aliphatic hydrocarbons. The U/R ratio ranged from 1.3 to 28.6 (Table 3.4). The highest values were recorded in samples near Odessa, the Danube delta and Sochi, which also showed the highest UCM concentrations and hydrocarbon levels (Table 3.4) indicating high petroleum contributions to these sediments, particularly from the Danube River (Equipe Cousteau, 1993) (Table 3.6). The concentration of UCM in sediments from the Black Sea is comparable to those previously reported for other regions and show similar trends as described for the "total" hydrocarbons (Table 3.6).

3.3.3 Alkanes

n-Alkanes in the range of *n*-C_{14} to *n*-C_{35} are present in nearly all sediments. Although biogenic hydrocarbons from recent sources can dominate chromatograms in uncontaminated samples (UNEP/IOC/IAEA, 1992), Σn-C_{14} to *n*-C_{34} can provide a good indication of "fresh" oil inputs (Readman *et al.*, 1986a; Readman *et al.*, 1986b). Σn-C_{14} to *n*-C_{34} concentrations in the surficial Black Sea sediments ranged from 0.1 to 3.4 $\mu g\ g^{-1}$ dry wt (Table 3.4) constituting 0.6-22.6% of the "total" aliphatic hydrocarbons. Lowest concentrations were reported for stations on the Ukrainian coastline indicating negligible fresh petroleum inputs at these locations. Major contributions of fresh oil to the Black Sea occur at the Danube outflow (see also Equipe Cousteau, 1993) where the highest concentrations were recorded. To place these levels in perspective, concentrations are comparable to the lower values reported from around the world (Table 3.6).

Variations in *n*-alkane concentrations did not reflect those for "total" aliphatic hydrocarbons (which were quantitatively dominated by UCM). This could be explained by differential transport mechanisms, which have been reported by several authors. *n*-Alkanes (particularly the terrigenous components) have been found to be associated with coarse sediment fractions rich in plant debris. Conversely, the anthropogenic UCM has been found in association with finer particles (Bouloubassi and Saliot, 1993). In the case of sediments from Sochi, however, similar distributions of *n*-alkanes and the UCM ($r^2 = 0.82$, $n = 5$) indicate a uniform source and similarity in dispersal mechanisms of the two hydrocarbon groups.

3.3.4 Isoprenoid hydrocarbons

Pristane (C_{19}) and phytane (C_{20}) are common isoprenoids detected in coastal marine sediments. They are present in most petroleums, usually as the major constituents within a much wider range of isoprenoid alkanes. They are often considered as good indicators of petroleum contamination. The ratio of pristane to phytane varies between oils reflecting the depositional environment of the original source. Biogenic sources of the compounds are important, for example they derive from the phytol side chain of chlorophyll, either under reducing conditions (phytane) or oxidising conditions (pristane) and can also originate from lipids of zooplankton and bacteria (LeDreau *et*

al., 1997). As a rule, a high ratio of pristane to phytane or the predominance of a single isoprenoid (such as pristane) indicate a biogenic source (UNEP/IOC/IAEA, 1992).

In the Black Sea, the highest concentrations were recorded in the vicinity of the Danube and around Sochi (pristane ranging from 23-170 ng g^{-1} dry wt and phytane from 16 to 170 ng g^{-1} dry wt; Table 3.4). In these areas, and additionally at Odessa, the pristane to phytane ratios were ≤1 reflecting petroleum contamination. In sediments from the unpolluted Ukrainian coastline, concentrations of pristane ranged from 0.4 to 5.5 ng g^{-1} dry wt. High ratios of pristane to phytane were recorded in this region reflecting biogenic origins. In these samples it is noted that *n*-C_{17} dominates pristane (Table 3.4) indicating contributions derived from algae.

Other unsaturated isoprenoid alkenes, e.g. squalene (a C_{30} isoprenoid), were also recorded in some samples (Table 3.4). This compound is usually attributed to animals (UNEP/IOC/IAEA, 1992) but is also ubiquitous in microalgae (Volkman *et al.*, 1992).

3.3.5 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are primarily products of incomplete combustion processes and comprise two to six fused aromatic rings. The low molecular weight (two and three rings) PAHs have a significant acute toxicity, whereas some of the higher molecular weight PAHs are carcinogenic (Neff, 1979; Witt, 1995). Low temperature thermal alterations of organic matter, such as in the formation of fossil fuels, result in PAHs with a 2 or 3 ring structure and a large proportion of alkylated homologues. Conversely, high temperature combustion produces PAHs with a 4, 5 or 6 ring structure and minimal alkylated products. Some PAHs, however, occur naturally in minerals (e.g. coronene) (Onuska, 1989) and others (e.g. perylene) are synthesised by organisms, such as bacteria, algae and fungi. Inputs from these natural processes are generally low when compared to those from anthropogenic sources (Witt, 1995).

Anthropogenic PAHs enter the marine environment through a variety of routes including atmospheric deposition, river runoff, domestic and industrial outfalls and the direct spillage of petroleum or petroleum products. The aqueous solubility of PAHs is low (Wei and Chan, 2000) and their hydrophobic nature (log K_{ow} = 3-8) favours particulate associations. In addition, there is increasing evidence that PAH may be occluded in soot particles (e.g. Readman *et al.*, 1984) (American Chemical Society, 1997) hindering exchange and isomer specific alterations through microbial

degradation, photo-degradation and chemical oxidation. The final repository of PAH is generally sedimentary deposition.

In many environmental studies, "total" PAH concentrations are often reported as the sum of 3 to 6 ring parent compounds. This facilitates comparisons, but can underestimate the total amount of PAHs occurring in environmental samples. Moreover, this parameter does not take into account lower molecular weight petrogenic PAHs derived mainly from fossil fuels, which are characterised by a high abundance of alkylated homologues and sulphur-heterocyclics (Bouloubassi and Saliot, 1993).

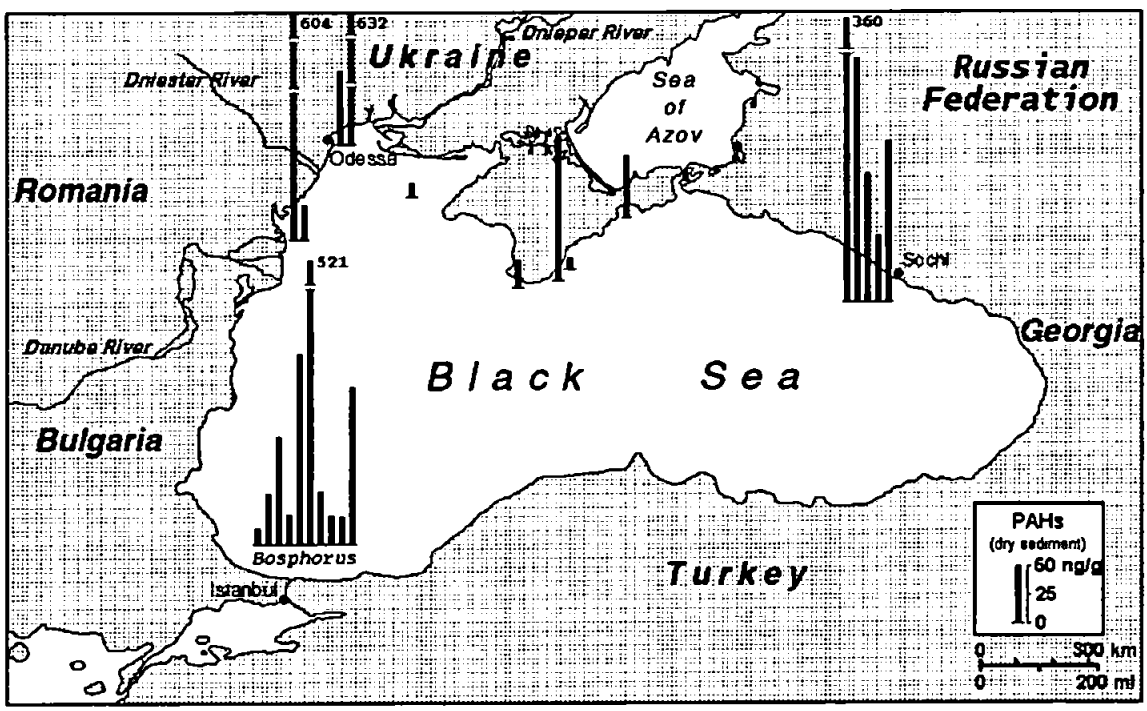


Figure 3.7. Distributions of PAHs (Σ17 isomers) in sediments from the Black Sea (ng g^{-1} dry wt).

Concentrations of "total" PAHs (the sum of 17 parental (non-alkylated) compounds) in sediments from the Black Sea are generally quite low (from 7 ng g^{-1} dry wt to 640 ng g^{-1} dry wt) (Table 3.5, Fig. 3.7). Petroleum derived PAH (containing 3 or less aromatic rings with a high proportion of alkylated homologues) and pyrogenic PAH (parental compounds with 4 or more aromatic rings) are both present. Highest concentrations were observed at sites influenced by the Danube (638 ng g^{-1} dry wt), Odessa (635 ng g^{-1} dry wt), the Port of Sochi (368 ng g^{-1} dry wt) and station 10 (Bosphorus) (531 ng g^{-1} dry wt) (Fig. 3.7). Comparable distributions have been inferred by Wakeham (1996)

working with "off-shore" sediments and Maldonado *et al.* (1999) who have also identified the importance of estuarine inputs of PAHs.

Table 3.6. Worldwide concentrations of hydrocarbons in sediments ($\mu g\ g^{-1}$ dry wt)

Area	Survey year	"total" Hydroc.	Oil "Equiv."	Σ*n*-Alkanes	Aliphatic UCM	References
Crete, Mediterranean	1994	0.5-5.7 †	-	0.1-0.9	0.3-4.8	(Gogou *et al.*, 2000)
Offshore, Gulf	1994	-	5.4-92	-	-	(Al-Lihaibi and Ghazi, 1997)
Saudi Arabia, Gulf	1991-93	11-6900	5.3-26000	0.2-28	6.4-5300	(Readman *et al.*, 1996)
Kuwait, Gulf	1992-93	40-240	72-1400	0.3-2.2	28-150	(Readman *et al.*, 1996)
Xiamen Harbour, China	1993	3.1-33 †	-	0.4-3.4	2.7-30	(Hong *et al.*, 1995)
Victoria Harbour, Hong Kong	1992	60-646 †	-	3.1-20	56-626	(Hong *et al.*, 1995)
Mississippi-Alabama, USA	1987-89	-	-	0.1-3.2	1-131	(Kennicutt *et al.*, 1995)
Alexandria, Egypt	-	-	-	7-143	54-1214	(Aboul-Kassim and Simoneit, 1995)
Western Coast, Taiwan	1990	869-10300†	-	-	-	(Jeng and Han, 1994)
Rhone River, France,	1985-86	25-170	-	2-12	18-146	(Bouloubassi and Saliot, 1993)
Kuwait, Gulf	1991	28	13	0.2	24	(Fowler *et al.*, 1993)
Saudi Arabia, Gulf	1991	19-671	5-1400	0.9-23	10-420	(Fowler *et al.*, 1993)
Bahrain. Gulf	1991	23-41	3-14	0.3-2.6	14-30	(Fowler *et al.*, 1993)
UAE, Gulf	1991	16	5-7	0.3-0.5	9-12	(Fowler *et al.*, 1993)
Oman, Gulf	1991	6-22	1-12	0.1-1.2	3-13	(Fowler *et al.*, 1993)
UK estuaries	1990	-	0.4-750	-	-	(Franklin, 1992)
Great Barrier Reef, Australia	1984	0.5-2	-	-	-	(Volkman *et al.*, 1992)
Antartica (pristine)	1988	<0.5	-	-	-	(Lenihan *et al.*, 1990)
Dee Estuary, UK	1984	-	-	1.8	10	(Readman *et al.*, 1986b)
Tamar Estuary, UK	1984	-	-	13	42	(Readman *et al.*, 1986b)
Mersey Estuary, UK	1984	-	-	11	104	(Readman *et al.*, 1986b)
New York Bight, USA	1971-75	35-2900	-	-	-	(Farrington and Tripp, 1977)
Off-shore Black Sea	1988-90	7-153 †	-	-	-	(Wakeham, 1996)
Danube River	1992	-	-	1-40	4-530	(Equipe Cousteau, 1993)
Bosphorus, Turkey	1995	12-76	6.5-340	1.3-2.6	4-38	This study
Sochi, Russia	1995	7.6-170	52-680	0.7-3.4	2.9-140	This study
Odessa, Ukraine	1995	110-310	220-1300	1.4-1.6	78-232	This study
Coastline, Ukraine	1995	2.1-6.6	3.2-42	0.1-0.6	1-3.1	This study
Danube Coastline, Ukraine	1995	49-220	66-1750	1.2-2.1	33-160	This study

† "total" aliphatic hydrocarbons

Concentrations of PAHs in these Black Sea sediments are comparable to relatively unpolluted locations in other seas (e.g. unpolluted locations in the Mediterranean Sea) and are much lower than levels reported for polluted estuaries in the UK (e.g. Mersey, Tyne, Thames) (Table 3.7). Of the areas sampled in this study, one of the most contaminated sites is situated in the River Danube delta (638 ng g^{-1} dry wt). This is explained by the fact that the river drains extensive urbanised inland areas (Equipe Cousteau, 1993). The relatively high concentrations observed for Odessa bay and the port and river of Sochi are linked to the greater industrialisation and urbanisation at these locations compared to the other sites. In contrast, PAH concentrations in most of the sediments sampled in the Northern Black Sea (Ukrainian) coastline and in the

vicinity of the Bosphorus (Turkey) are low (≤ 50 ng g^{-1} dry wt), and are typical of locations distant from extensive anthropogenic activities (Baumard *et al.*, 1998).

Table 3.7. Worldwide concentrations of polycyclic aromatic hydrocarbons (PAHs) in sediments (ng g dry wt^{-1}).

Area	Survey year	Concentration	References
Gironde estuary and Arcachon bay, France	-	3.5-853 (Σ14 PAHs)	(Soclo *et al.*, 2000)
Cotonou, Benin	-	80-1411 (Σ14 PAHs)	(Soclo *et al.*, 2000)
France, Mediterranean Sea	1996	36-6900 (Σ18 PAHs)	(Baumard *et al.*, 1998)
Spain, Mediterranean Sea	1996	1.2-8400 (Σ18 PAHs)	(Baumard *et al.*, 1998)
Majorca, Mediterranean Sea	1996	0.3-100 (Σ18 PAHs)	(Baumard *et al.*, 1998)
Kyeonggi bay, Korea	1995	9.1-1400 (Σ23 PAHs)	(Kim *et al.*, 1999)
River Thames, United Kingdom	1993-96	N.D.-6519 (Σ15 PAHs)	(Woodhead *et al.*, 1999)
River Mersey, United Kingdom	1993-94	6-6230 (Σ15 PAHs)	(Woodhead *et al.*, 1999)
River Tyne, United Kingdom	1993-96	260-43470 (Σ15 PAHs)	(Woodhead *et al.*, 1999)
Crete Sea, Eastern Mediterranean Sea	1994	14.6-158.5 (Σ28 PAHs)	(Gogou *et al.*, 2000)
North-West Coast, Mediterranean Sea	1991	86.5-48090 (Σ14 PAHs)	(Benlahcen *et al.*, 1997)
North-Western Gulf	1991-93	<20-4740 (Σ13 PAHs)	(Readman *et al.*, 1996)
San Quintin Bay, Mexico	1992	N.D.-<50 (Σ44 PAHs)	(Galindo *et al.*, 1996)
Xiamen Harbour, China	1993	70-33000 (Σ9 PAHs)	(Hong *et al.*, 1995)
Victoria Harbour, Hong Kong	1992	350-3450 (Σ9 PAHs)	(Hong *et al.*, 1995)
Baltic Sea	1993	9.5-1871 (Σ15 PAHs)	(Witt, 1995)
Sarasota Bay, Florida, USA	-	17-26771 (Σ11 PAHs)	(Sherblom *et al.*, 1995)
Western Coast, Australia	1991	1.0-3200 (Σ11 PAHs)	(Burt and Ebell, 1995)
Italy Coast, Adriatic Sea	1990	27-527 (Σ9 PAHs)	(Guzzella and DePaolis, 1994)
Rhone River, Mediterranean Sea	1985-86	1070-6330 (Σ15 PAHs)	(Bouloubassi and Saliot, 1993)
Lake Burley Griffin, Australia	1989	80-538 (Σ8 PAHs)	(Leeming and Maher, 1992)
Tabasco State Continental Shelf, Mexico	1989	454-3120 (Σ15 PAHs)	(Botello *et al.*, 1991)
Brisbane River Estuary, Australia	1986	2840-13470 (Σ17 PAHs)	(Kayal and Connell, 1989)
Dee Estuary, UK	1984	490 (Σ13 PAHs)	(Readman *et al.*, 1986b)
Tamar Estuary, UK	1984	8630 (Σ13 PAHs)	(Readman *et al.*, 1986b)
Mersey Estuary, UK	1984	5310 (Σ13 PAHs)	(Readman *et al.*, 1986b)
Boston Harbour, USA	-	487-718360 (Σ14 PAHs)	(Shiaris and Sweet, 1986)
Lake Woods, N.Y., USA	1978	12104 (Σ19 PAHs)	(Tan and Heit, 1981)
Lake Sagamore, N.Y., USA	1978	3660 (Σ19 PAHs)	(Tan and Heit, 1981)
Abyssal Black Sea	1988-90	200-1200 (Σ28 PAHs)	(Wakeham, 1996)
Danube River mouth, Black Sea	1988-90	2400 (Σ28 PAHs)	(Wakeham, 1996)
Danube River	1992	<10-3700 (Σ4 PAHs)	(Equipe Cousteau, 1993)
Bosphorus, Black Sea, Turkey	1995	13.8-531 (Σ17 PAHs)	(This study)
Sochi, Black Sea, Russia	1995	61.2-368 (Σ17 PAHs)	(This study)
Odessa, Black Sea, Ukraine	1995	66.9-635 (Σ17 PAHs)	(This study)
Coastline, Black Sea, Ukraine	1995	7.2-126 (Σ17 PAHs)	(This study)
Danube Coastline, Black Sea, Ukraine	1995	30.5-608 (Σ17 PAHs)	(This study)

The absence of correlation between "total" hydrocarbons and PAHs (r^2 = 0.04) indicates separate primary sources and/or differing transport processes for the two classes of compounds. Wakeham (1996) also report a similar lack of correlation in offshore sediment from the Black Sea. It is likely that combustion derived PAH will have aeolian components to their transport mechanisms, whereas petrogenic PAH will

be predominantly fluvial. As previously mentioned, PAH derived from the two prime sources have distinctive compositional differences. Readman *et al.* (1996) compared "typical" extracts of oil and combustion derived materials to identify the origin of PAH. On the basis of this work, it can be demonstrated that PAHs in sediments from the Black Sea originate from both pyrolytic and petrogenic sources (Fig. 3.8). A mixture of pyrolytic and petrogenic PAHs can be observed in most of the sediments, usually with a slightly pyrolytic predominance but less frequently with a petrogenic predominance (e.g. Fig. 3.8 - station 132 (Danube delta)). A substantial biogenic input of perylene was observed in some samples (e.g. Fig. 3.8 – station 30). For some stations off the Bosphorus, only a pyrolytic profile is observed (Fig. 3.8 - station 10).

A less subjective approach to investigate sources can be achieved using molecular indices based on ratios of selected PAH concentrations (Soclo, 1986; Sicre *et al.*, 1987; Garrigues *et al.*, 1995; Baumard *et al.*, 1998). Some characteristic values are given in Table 3.8. Equivalent values for the Black Sea sediments, for the following indices: phenanthrene concentration/anthracene concentration (PHE/ANT), fluoranthene concentration/pyrene concentration (FLTH/PYR), Σmethyl-phenanthrenes/phenanthrene (ΣMPHE/PHE) and benz[a]anthracene/chrysene (BaA/CHR), are presented in Table 3.5.

The degree of alkylation, as assessed by ΣMPHE/PHE, is less than 2 for all samples indicating pyrolytic origins. If, however, the assessment is tempered by using other parental PAH ratios (Table 3.5), it becomes apparent that the majority of sediment samples contain a mixture of petrogenic and pyrolytic profiles. Stations with greatest pyrolytic inputs include 45 (Ukrainian coastline), 98 (Odessa), and 9, 35 and 44 (Bosphorus). At stations 10 and 17 (Bosphorus) negligible petrogenic input is apparent. To supplement this information, values of PHE/ANT can be plotted against values of FLTH/PYR (Fig. 3.9) (Baumard *et al.*, 1998). Two standard matrices, oil and coal tar, which represent, respectively, petrogenic and pyrolytic fingerprints, are also included in Fig. 3.9. The plot confirms the dominance of the combustion derived material at most Bosphorus stations and indicates contributions of oil to sediments at Sochi. However, the diagram fails to classify e.g. station 132 into its clear petrogenic class endorsing the need to use a variety of measurements in order to provide a robust assessment.

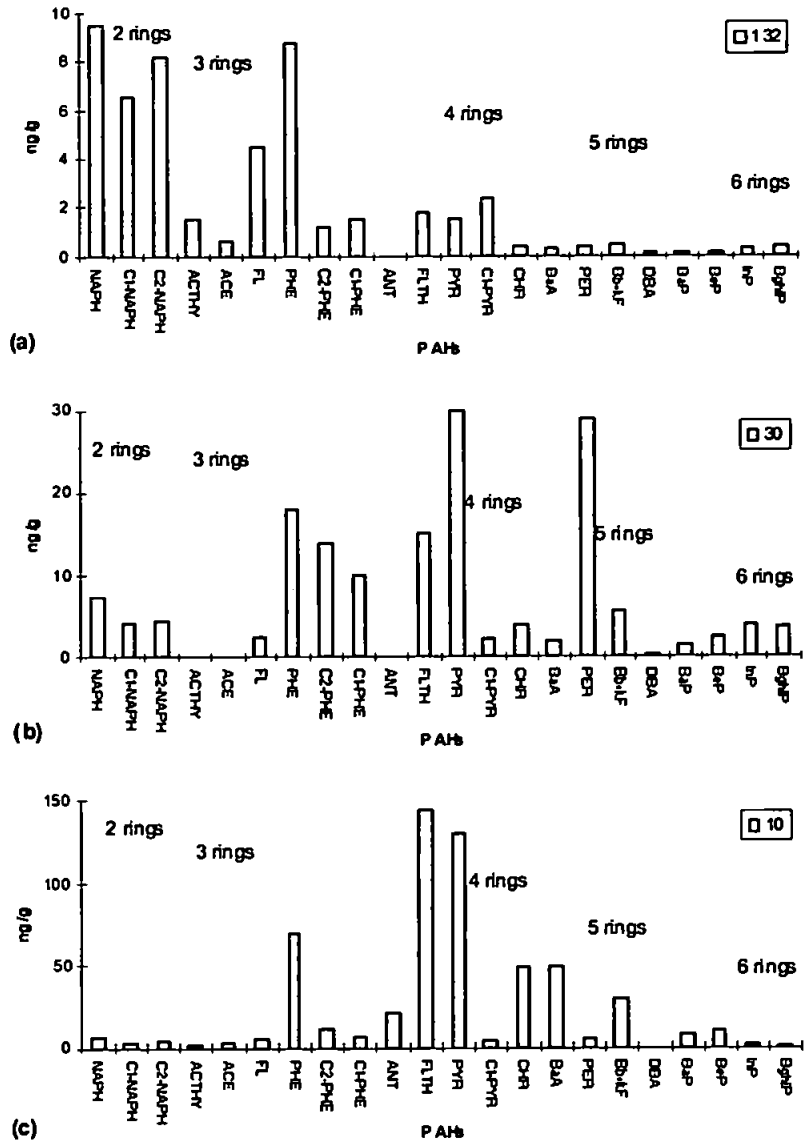


Figure 3.8. Composition of PAHs (μg g^{-1} dry wt) in sediments from the Black Sea: a) station 132 (Danube coastline); b) station 30 (Bosphorus) and c) station 10 (Bosphorus). Compositions indicate primarily a petrogenic origin at station 132, a mixture of petrogenic and pyrogenic PAH at station 30 and a dominant pyrogenic PAH input at station 10. Station 30 also includes a substantial biogenic input of perylene. * See Table 3.5 for the abbreviations

The low levels of PAH in the sediments from the Ukrainian coast might relate to an atmospheric source of the compounds with negligible fluvial inputs (Prahl and Carpenter, 1984). In addition, the cyclonic circulation pattern in the western basin would protect this area, drawing major inputs from the Danube southerly towards the Bosphorus (Aibulatov, 1987; Wakeham, 1996).

Table 3.8. Characteristic values of molecular indices for pyrolytic and petrogenic origins of polycyclic aromatic hydrocarbons (PAHs).

	PHE/ANT	FLTH/PYR	ΣMPHE/PHE	BaA/CHR	PHE/ANT / FLTH/PYR
Pyrolytic origin	<10	>1	<2	>0.9	0-10 / >1
Petrogenic origin	>15	<1	>2	≤0.4	≥10 / < 1
Reference	(Gschwend and Hites, 1981; Soclo, 1986)	(Sicre *et al.*, 1987; Baumard *et al.*, 1998)	(Prahl and Carpenter, 1983; Garrigues *et al.*, 1995)	(Gschwend and Hites, 1981)	(Baumard *et al.*, 1998)

PHE/ANT: phenanthrene concentration / anthracene concentration. FLTH/PYR: fluoranthene concentration / pyrene concentration. ΣMPHE/PHE: sum of the concentrations of the methyl-phenanthrenes against phenanthrene concentration. BaA/CHR: benz[a]anthracene concentration against chrysene concentration. (PHE/ANT) / (FLTH/PYR) (phenanthrene concentration / anthracene concentration) *vs* (fluoranthene concentration / pyrene concentration).

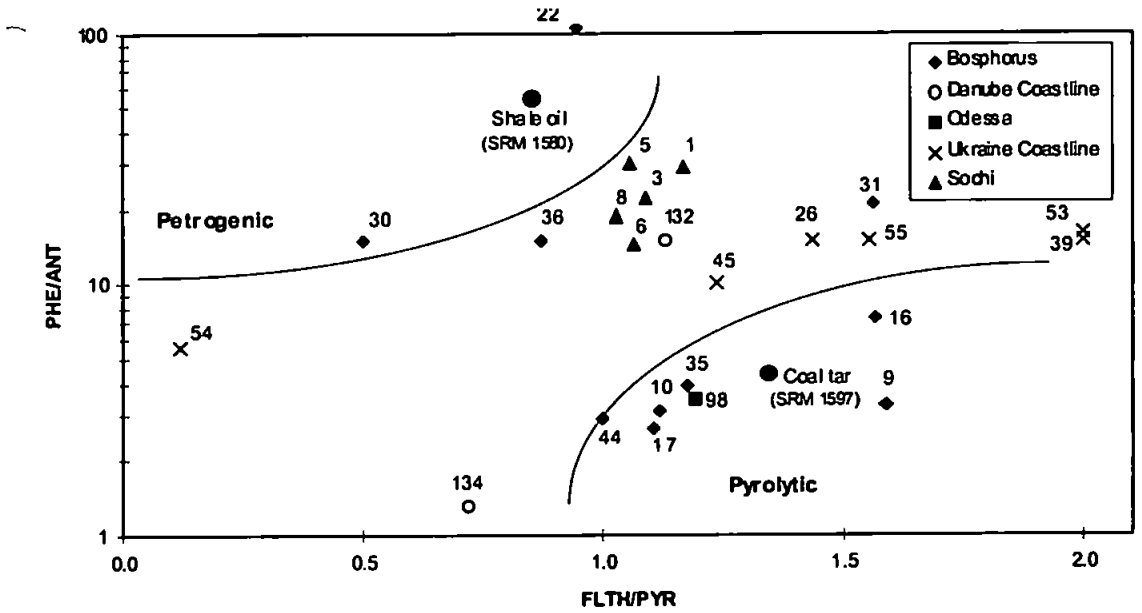


Figure 3.9. Plot of the isomeric ratios PHE/ANT (phenanthrene *vs* anthracene) *vs* FLTH/PYR (fluoranthene *vs* pyrene) for sediments from the Black Sea and for two reference matrices (●) to attempt delineation of petrogenic and pyrolytic sources (Wise *et al.*, 1988).

In addition to pyrolytic and petrogenic sources, perylene is also produced by *in situ* degradation of biogenic precursors (Venkatesan, 1988; Wakeham, 1996; Baumard *et al.*, 1998). Indeed, perylene is probably the most important diagenetic PAH encountered in sedimentary environments and, thus, a high abundance of perylene relative to the other PAHs can indicate an important natural origin of the compound. Perylene has been frequently associated with inputs from rivers and estuaries (LaFlamme and Hites, 1978;

Baumard *et al.*, 1998). These authors have suggested that concentrations of perylene which are higher than 10 % of the total penta-aromatic isomers indicate a probable diagenetic input, whereas those in which perylene is less than 10 % indicate a probable pyrolytic origin of the compound. Concentrations of perylene relative to the penta-aromatic isomers (expressed as % composition) for the Black Sea sediments are given in Table 3.5 and are shown in Fig. 3.10. A few values are < 10%, indicating a pyrolytic origin of the compound. High values are, however, observed in most of the samples from the Black Sea - the Ukrainian coastline (14-35%), Odessa (14%), the Danube coastline (26-31%), Sochi (10-37%) and Bosphorus (>20-88%), indicating a diagenetic origin for the presence of perylene at these locations. Most probably, the diagenesis of terrestrial organic matter draining into the Black Sea produces the majority of the perylene present.

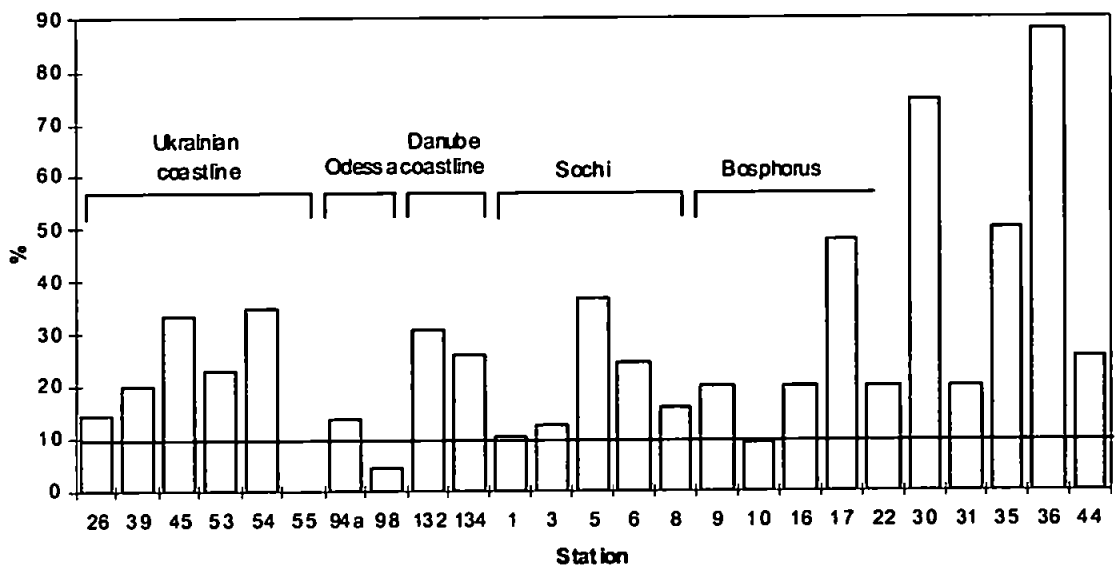


Figure 3.10. Concentrations of perylene relative to the combined concentration of all measured penta-aromatic isomers. The horizontal line at 10% delineates biogenic inputs of perylene (at > 10%) from pyrolytic sources (< 10%).

3.4 Sterol Markers to Assess Sewage Contamination

The transport and fate of urban contaminants can be traced using molecular markers of domestic wastes (Vivian, 1986; Readman *et al.*, 1986a). Among them, coprostanol (5β(H)-cholestan-3β-ol) has proven to be a successful indicator of sewage pollution in many coastal environmental studies (Goodfellow *et al.*, 1977; Readman *et al.*, 1986a; Readman *et al.*, 1986b; Grimalt *et al.*, 1990; Venkatesan and Kaplan, 1990; Leblanc *et*

al., 1992; Chalaux *et al.*, 1995). Coprostanol is produced in the digestive systems of higher animals by the microbial reduction of cholesterol and is one of the principal sterols in human and animal faeces. However, because of the multiplicity of sources of sterols in aquatic environments and the transformation/degradation processes that occur, the occurrence of coprostanol in coastal environments cannot be unambiguously attributed to urban sewage pollution. In theory, however, unpolluted sediments should not contain coprostanol, i.e. the compound should typically be below the limits of analytical detection. To enhance the reliability of pollution assessments using coprostanol (and other steroid compounds), some authors have derived (and tested) ratios between selected steroids. Grimalt *et al.* (1990) proposed the use of the 5β/(5β+5α)cholestan-3β-ol (sterols) and 5β/(5β+5α)cholestan-3-one (ketones).

With a growing population density in coastal urban centres within the Black Sea basin (at least 171 million people, about 81 mill. in the Danube basin), the need for disposal of sewage containing faecal wastes increases. Although data on sterols in the Black Sea are scarce (Gagosian *et al.*, 1979; Wakeham, 1989; King and Repeta, 1991), it is known that municipal wastes are often discharged into the aquatic environment. In order to elucidate the present status of sewage contamination, an effort has been made in this study focusing on faecal sterols in sediments from several areas along the Black Sea coast.

The concentration of coprostanol in the analysed samples ranged from 1 to 5400 ng g^{-1} dry sediment (Table 3.9 and Fig. 3.11). Differences of 1-2 orders of magnitude are observed between sediments from the Ukrainian coastline (which are shown to be uncontaminated) and contaminated areas. However, coprostanol is present in all cases. In the majority of samples, levels were comparatively low (<500 ng g^{-1}), providing evidence of minor sewage contamination. Conversely, there was evidence of contamination with sewage, grossly in the case of stations 1, 3, 8 at Sochi (up to 5400 ng g^{-1}) and station 134 on the coast adjacent to the Danube delta (2600 ng g^{-1}). For comparison, levels of coprostanol reported in dry sewage sludge include: 1,282,000 ng g^{-1} (Goodfellow *et al.*, 1977); 5,800,000 ng g^{-1} (Hatcher *et al.*, 1977); 910,000-7,800,000 ng g^{-1} (McCAlley, 1980).

Concentrations of coprostanol for the Black Sea samples are comparable to, or perhaps even lower than, those generally encountered in estuarine and coastal areas worldwide (Table 3.10). Since a previous study on the River Danube (Equipe Cousteau,

1993) identified high concentration of coprostanol in some samples, this could explain the high concentration reported at station 134.

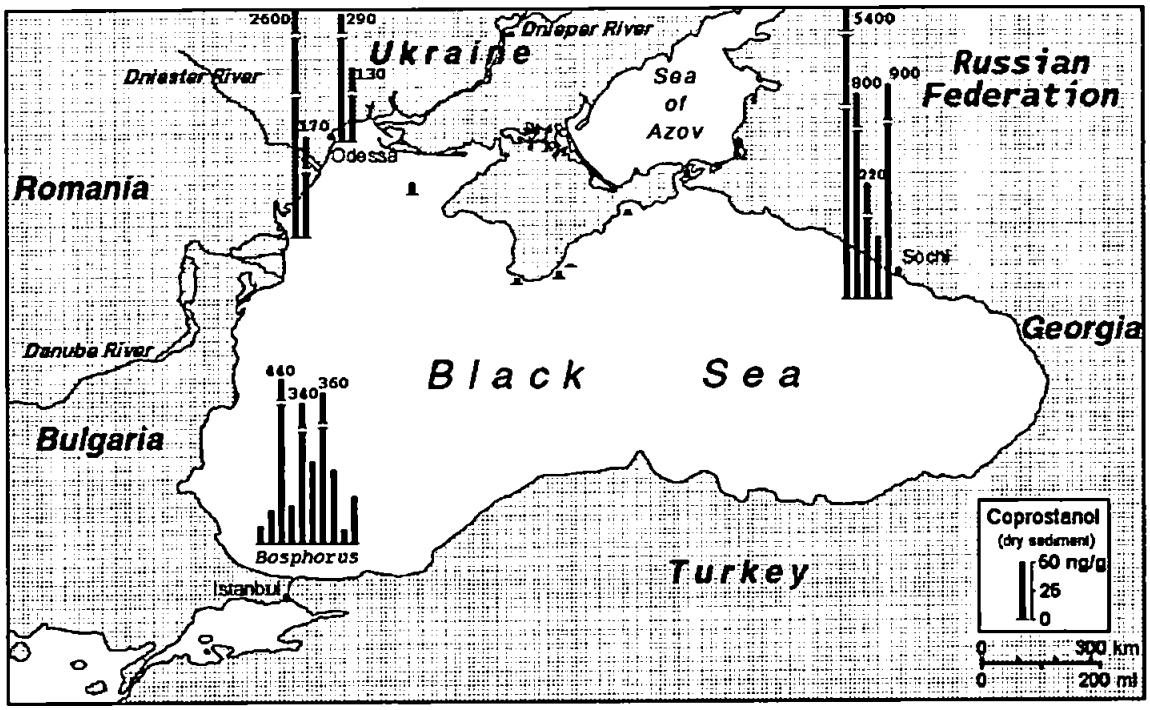


Figure 3.11. Distribution of coprostanol in sediment from the Black Sea (ng g^{-1} dry wt).

To enhance the reliability of pollution assessments using coprostanol (and other steroid compounds), some authors have derived (and tested) ratios between selected steroids. Grimalt *et al.* (1990) proposed the use of the 5β/(5β+5α)cholestan-3β-ol (sterols) and 5β/(5β+5α)cholestan-3-one (ketones). They showed that ratios higher than 0.7 (showing a clear predominance of 5β isomers) could be attributed to contaminated areas, whilst ratios lower than 0.3 indicate non-contaminated samples. The 5α stanols are thermodynamically more stable than their 5β epimers and the sedimentary reduction process of Δ^5 sterols gives rise to stanol mixtures in which the 5α isomers predominate (Robinson *et al.*, 1986; Grimalt *et al.*, 1990). Microbial assemblages from marine sediments allowed to grow in anaerobic media containing cholesterol provide nearly equal concentrations of 5α- and 5β-(H) cholestan-3β-ol. In contrast, 5β stanols are dominant in sewage sludge (Gaskell and Eglington, 1975; McCAlley *et al.*, 1981). Also, the 5β/(5β+5α)cholestan-3β-ol ratio is susceptible to change through direct inputs of 5α stanols from diverse aerobic organisms, including phytoplankton, zooplankton, and macrophyte species (Grimalt and Albaiges, 1990; Grimalt *et al.*, 1990). In this respect, intermediate ratios of 5β/(5β+5α) stanols in sedimentary records dominated by

Table 3.9. Concentrations of sterols (ng g^{-1} dry weight basis) and selected parameters in sediments from the Black Sea

		Ukraine										Russia							Turkey							
Sample code		26	39	45	53	54	55	94a	98	132	134	1	3	5	6	8	9	10	16	17	22	30	31	35	36	44
Coprostanol	ng g^{-1}	10	4.4	5.4	1.3	3.3	5.1	290	130	170	2600	5400	900	54	220	800	360	71	29	340	15	440	33	64	12	41
24-Ethyl-coprostanol	ng g^{-1}	35	17	3.0	7.3	15	40	490	110	310	1200	2100	430	71	160	440	420	88	47	330	<3.0	1400	49	87	36	56
Coprostan-3-one	ng g^{-1}	6.4	10	3.6	2.9	8.9	15	150	55	130	950	2100	280	95	160	400	140	37	8.9	240	69	300	45	48	9.9	140
Cholesterol	ng g^{-1}	56	280	13	17	360	560	570	160	390	1600	2000	760	760	580	1000	260	160	99	1000	210	16000	120	520	37	540
Cholestanol	ng g^{-1}	28	33	9.5	9.0	54	80	610	240	360	1600	1000	230	92	190	440	280	64	68	230	37	1900	100	86	43	190
Cholestanone	ng g^{-1}	7.7	11	3.9	3.0	13	5.7	120	43	48	330	320	50	40	60	150	61	23	17	65	22	120	40	21	26	65
Campesterol	ng g^{-1}	76	92	30	n.d.	29	2.0	610	57	150	370	430	26	17	45	110	170	71	94	150	130	160	170	99	92	180
Stigmasterol	ng g^{-1}	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
b-Sitosterol	ng g^{-1}	38	42	18	9.5	25	31	610	150	n.q.	950	980	690	62	170	520	340	150	130	220	94	270	190	84	50	190
Mean detection limit	ng g^{-1}	3.2	2.1	0.5	0.7	1.2	0.5	0.7	1.1	1.2	4.5	1.8	4.1	3.0	4.3	3.7	1.1	1.2	1.8	1.8	1.8	9.0	1.9	1.9	1.9	2.0
Ratios																										
(5β/(5β+5α)) cholestan-3β-ol		0.26	0.12	0.36	0.13	0.06	0.06	0.32	0.35	0.32	0.62	0.84	0.80	0.37	0.54	0.65	0.56	0.53	0.30	0.60	0.29	0.19	0.25	0.43	0.22	0.18
(5β/(5β+5α)) cholestan-3-one		0.45	0.48	0.48	0.49	0.41	0.72	0.56	0.56	0.73	0.74	0.87	0.85	0.70	0.73	0.73	0.70	0.62	0.34	0.79	0.76	0.71	0.53	0.70	0.28	0.68
cholesterol/cholesterol+5α(H)-cholestan-3β-ol		0.67	0.89	0.58	0.65	0.87	0.88	0.48	0.40	0.52	0.50	0.67	0.77	0.89	0.75	0.69	0.48	0.71	0.59	0.81	0.85	0.89	0.55	0.86	0.46	0.74

N.Q. - not quantified

phytoesterol inputs render it difficult to confirm faecal contamination, particularly in regions of high algal productivity (Grimalt *et al.*, 1990).

In contrast, the 5β/(5β+5α) stanones (ketones) ratio is not significantly influenced by algal inputs, affording a supplementary parameter for the reliable identification of sewage contamination in such areas. The sterone (ketone) composition of aquatic particulates and sediments polluted with faecal matter is essentially comprised of C_{27} and C_{29} 5β(H)-cholestan-3-ones. Hence, larger concentrations of these sterones generally correspond to a higher degree of sewage pollution. As with the stanols, 5α-cholestan-3-one predominates in anoxic depositional environments (Robinson *et al.*, 1986) because of the microbial and/or diagenetic reduction of Δ^5 sterols. In microbial reduction, 5α-cholestan-3-one is preferentially produced. In contrast, intestinal bacteria give rise to 5β(H)-cholestan-3-one due to their stereospecific mechanism of Δ^5 sterol biohydrogenation (Grimalt *et al.*, 1990).

Table 3.10. Worldwide concentration of coprostanol in sediments (ng g^{-1} dry wt).

Area	Survey year	Concentration	References
San Vicent Bay, Chile	1997	10-7,300	(Mudge and Seguel, 1999)
Venice Lagoon, South-West Italy	1992	40-4,410	(Fattore *et al.*, 1997)
Ria Formosa, Algarve, Southern Portugal	1994	100-41,800	(Mudge and Bebianno, 1997)
South-Western Coast, Taiwan	1992	<5-820	(Jeng *et al.*, 1996)
Kaoping River, Taiwan	1992	835-58,200	(Jeng *et al.*, 1996)
Tokyo Bay, Japan	1989	20-243	(Chalaux *et al.*, 1995)
Firth of Clyde, Scotland	1989	<100-176,000	(Kelly and Campbell, 1995)
Tan-Shui Estuary, Taiwan	1990	710-163,000	(Jeng and Han, 1994)
Venezia, Italy	1986	200-41,000	(Sherwin *et al.*, 1993)
Narragansett Bay, USA	1985-86	130-39,300	(Leblanc *et al.*, 1992)
Barcelona, Spain	1986-87	1,000-390,000	(Grimalt *et al.*, 1990)
Santa Monica Basin, USA	1985	500-5,100	(Venkatesan and Kaplan, 1990)
Rhone Estuary, France	1988	<1,000-24,000	(Readman *et al.*, 1989)
Narragansett Bay, USA	1984	1010-4,070	(NOAA, 1987)
Dee Estuary, UK	1984	1,400	(Readman *et al.*, 1986b)
Tamar Estuary, UK	1984	800-17,000	(Readman *et al.*, 1986a)
Mersey Estuary, UK	1984	9,000	(Readman *et al.*, 1986b)
Dry sewage sludge	-	910,000-1,282,000	(Readman *et al.*, 1989)
Danube River	1991	15-56,000	(Equipe Cousteau, 1993)
Bosphorus, Black Sea, Turkey	1995	12-440	This study
Sochi, Black Sea, Russia	1995	54-5,400	This study
Odessa, Black Sea, Ukraine	1995	130-290	This study
Coastline, Black Sea, Ukraine	1995	1.3-5.4	This study
Danube Coastline, Black Sea, Ukraine	1995	170-2,600	This study

When the C_{27} 5β(H) sterone/sterol is considered, a significant decrease with increased pollution is evident. After an initial rapid decay of coprostanol (under the influence of aerobic bacteria (Walker *et al.*, 1982; Bartlett, 1987), an equilibrium is achieved.

It is evident from the above discussions that joint quantification of sterols and evaluation of the C_{27} isomeric ratios of 5α and 5β stanols and stanones affords a useful differentiation between sewage polluted and unpolluted samples. It has been demonstrated that cross-representation of both the 5β/(5β+5α) isomeric ratio of cholestan-3β-ol and cholestan-3-one provide an even better discrimination between contaminated and uncontaminated environments (Grimalt *et al.*, 1990).

Sochi (Russia Federation), especially near to Sochi port (station 1), Canyon Adler (station 3) and the river Sochi (station 8), appear to be one of the most contaminated sites in terms of sewage. These sites display a typical sewage contamination signature, with high levels of coprostanol and high values for the 5β/(5β+5α) isomeric ratios of cholestan-3β-ol and cholestan-3-one (Fig. 3.12). Although sediments at Chosta (station 5) and river Matchesta (station 6), contained relatively low levels of coprostanol (54 and 220 ng g^{-1}, respectively), these sites exhibited high 5β/(5β+5α) cholestan-3-one ratios which suggest the presence of sewage pollution. In contrast, the 5β/(5β+5α) cholestan-3β-ol ratios are intermediate in value, probably indicating a substantial algal input (Fig. 3.12). This might arise from an increased flow of nutrients associated with the anthropogenic discharges that would enhance microbial degradation (Grimalt *et al.*, 1990).

The Ukrainian coastline was sampled throughout the north (stations 39, 45, 53, 54 and 55) and north-western (Odessa - stations 94a and 98; Danube coastline - stations 132 and 134) coastal shore of the Black Sea. Samples from the north are characterised as non-polluted sediments, showing very low coprostanol concentrations and 5β/(5β+5α) isomeric ratios (Fig. 3.12).

Odessa sediments have intermediate coprostanol concentrations and 5β/(5β+5α) isomeric ratios (Fig. 3.12), indicating minor sewage contamination and probably reduction of Δ^5 stenols to 5α(H)-stanols by anaerobic microbial transformation. The cholesterol/cholesterol+5α(H)-cholestan-3β-ol ratios of 0.48 and 0.40 (samples 94a and

98, respectively) (Table 3.9) provide evidence in support of anoxic cholesterol reduction at these sites. Cholesterol/cholesterol+5α(H)-cholestan-3β-ol (cholestanol) ratio lower than 0.5 is an indication of cholesterol reduction (Chalaux *et al.*, 1995).

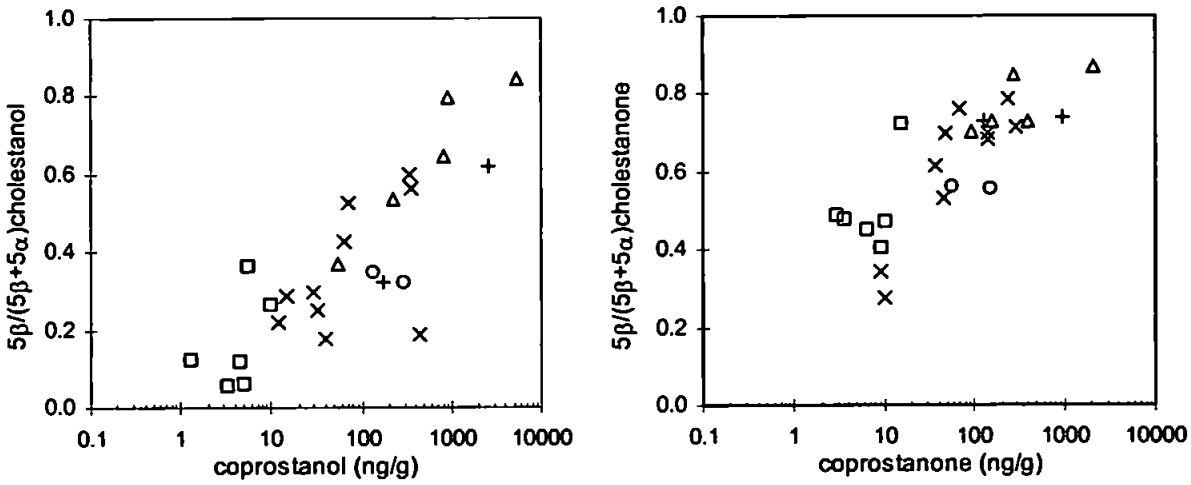


Figure 3.12. Cross-representation of the coprostanol (a) and coprostanone (b) concentrations and their corresponding C_{27} epmeric ratios for the Black Sea sediments (Δ Sochi; × Bosphorus; □ Ukrainian Coastline; O Odessa; + Danube Coastline).

The River Danube influences locations on the Danube coastline significantly. Station 134 is located in the "Bistraya" arm, near to the Danube discharge, and station 132 a little further off-shore. The coprostanol concentrations and 5β/(5β+5α) isomeric ratios are high and intermediate, respectively (Fig. 3.12). The cross-representation of both the 5β/(5β+5α) isomeric ratio of cholestan-3β-ol and cholestan-3-one (Fig. 3.13) indicate that sediments at both locations are contaminated by sewage, severely in the case of station 134. The comparatively lower cholesterol/cholesterol+5α(H)-cholestan-3β-ol ratios of 0.52 and 0.50 (samples 132 and 134, respectively) (Table 3.9) might also indicate some anaerobic microbial cholesterol reduction at these sites.

In the south-west Black Sea region, samples were taken in the vicinity of the Bosphorus (Turkey). Highest values were found near to the Bosphorus (stations 9, 10, 17 and 35), with values decreasing with increasing distance seawards. These sediments generally contained high values for both the 5β/(5β+5α) isomeric ratios of cholestan-3β-ol and cholestan-3-one, indicating sewage contamination (Figs. 3.12 and 3.13). Other locations (stations 16, 22, 31 36 and 44) are considered to be uncontaminated by sewage since their ratios of 5β/(5β+5α) cholestan-3β-ol are lower than 0.3. The level of 440 ng g^{-1} of coprostanol at station 30 could have originated from the anoxic microbial

reduction of large cholesterol concentrations (16000 ng g^{-1}) present in sediment. The low 5β/(5β+5α) cholestan-3β-ol ratio could be a reflection of high algal productivity due to nutrients availability.

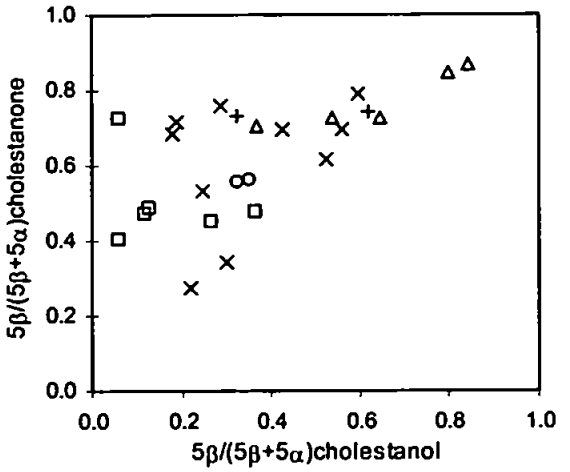


Figure 3.13. Cross-representation of the 5β/(5β+5α) isomeric ratios of cholestan-3β-ol and cholestan-3β-one for the Black Sea sediments (Δ Sochi; × Bosphorus; □ Ukrainian Coastline; O Odessa; + Danube Coastline).

The reduction of Δ^5 stenols in the anoxic sediments leading to the formation of 5α- and 5β-stanol has been reported previously in anoxic sediments and particulate matter boundaries in the Black Sea (Gagosian *et al.*, 1979; Wakeham, 1989). Reduction of Δ^5 stenols, the predominant biogenic sterols, to 5α(H)-stanols is primarily an anaerobic microbial transformation. Increased stanol/stenol ratios are used as evidence of this process, for example in sediments where stanol/stenol ratios are higher at lower redox potential (Chalaux *et al.*, 1995).

In the Black Sea, increased 5α(H)-stanol/Δ^5 stenol ratios in particulate matter at the oxic-anoxic interfaces in the water column were attributed to *in situ* microbial conversion of sterol to stanol (Wakeham, 1989). The extent of conversion varies with water-column redox potential: little stanol generation occurs under oxic conditions, whereas there is substantial conversion in anoxic waters. These results imply that anoxic waters, particularly near oxic-anoxic interfaces, are important sites of intense alteration of organic matter.

3.5 Conclusions

3.5.1 Organochlorines

The ranking of concentrations of the various organochlorine compounds in sediments from the Black Sea are as follows: DDTs >HCHs ≥ PCBs >HCB >cyclodienes.

Concentrations of PCBs in sediments from the Black Sea are relatively low in comparison with those reported for other regions of the world. The highest concentrations of PCBs are recorded for the Romanian coastline (stations influenced by discharges from the River Danube and Port Constantza), Odessa, Sochi and one of the furthest offshore stations in the Bosphorus. Among the PCBs, the toxic di-*ortho* and mono-*ortho* co-planar congeners were dominant.

Concentrations of DDT related compounds in sediments from Black Sea are shown to be generally lower than those reported for the Baltic Sea and most Asian sites. They are comparable to, or slightly higher than, those reported for other regions of the Russian Federation (e.g. Baikal Sea), the USA and Mexico. The highest concentrations of DDTs in the Black Sea are associated with lipid rich sediments in the Ukrainian and Romanian coastline, which are under the influence of the River Danube discharges. Elevated concentrations are also reported for sediments in the vicinity of Odessa and Port Constantza on the Romanian coastline. The low DDE/DDT values combined with the relatively high concentrations (especially in Odessa sediments and, in sediments under the influence of Danube discharges) indicate current usage of DDT around the Black Sea.

Concentrations of lindane (γ-HCH) and the other HCH isomers are low in samples from the Ukrainian coastline, Russian Federation and Turkey. These levels are comparable to the low to medium range of values for estuarine sediments from eastern and southern Asia and Oceania. However, they are much lower than values reported for areas that are subjected to intensive sources of HCH contamination, e.g. India and Vietnam. Elevated concentrations in samples from Romanian stations, under the influence of the River Danube, indicate substantial usage of HCH as a pesticide in the River Danube watershed. Indeed, we report some of the highest sedimentary concentrations ever recorded for ΣHCH. The composition of the HCH isomers in the sediments showed a high percentage of the γ-isomer at the highly contaminated

locations along the Romanian Coastline (56 to 81%) indicating usage of lindane in this region. Conversely, the values found at Odessa, Sochi, along the Ukrainian coastline, the Ukrainian Danube coastline and Bosphorus suggest that HCH contamination at some locations arises through atmospheric inputs or/and use of both lindane and technical formulations.

HCB and cyclodienes were also found in sediments from the Black Sea, albeit at much lower concentrations than those recorded for the other compounds. The highest values of HCB were recorded along the Romanian and Ukrainian coastlines adjacent to the River Danube.

3.5.2 Hydrocarbons

The Black Sea is only moderately contaminated by hydrocarbons. Concentrations of "total" hydrocarbons in sediments from the Black Sea are shown to be substantially higher than those from pristine environments and are generally comparable to levels encountered in the Mediterranean Sea. The most contaminated stations (concentrations > 100 $\mu g\ g^{-1}$) are shown to be associated with discharges from Odessa, Sochi and inputs from the River Danube. Samples taken from the Ukrainian coastline show comparatively little contamination ("total" hydrocarbon concentrations < 10 $\mu g\ g^{-1}$ dry wt) even though some areas are subject to wastewater discharges.

The UCM is by far the major component of the "total" sedimentary aliphatic hydrocarbons and indicates contamination by degraded/weathered petroleum. The highest unresolved/resolved hydrocarbon ratios were recorded in samples near Odessa, the Danube coastline and Sochi, co-varying with the highest UCM and hydrocarbon levels, confirming substantial petrogenic inputs to these sediments.

"Fresh" oil inputs (as indicated by $\sum n$-C_{14} to n-C_{34} concentrations) are shown to be low and comparable to relatively uncontaminated areas on a worldwide basis. A major contribution to the Black Sea is, however, shown to be associated with inputs through the River Danube. Very low concentrations were reported for some stations (generally those on the Ukrainian coastline) indicating negligible fresh petroleum inputs at these locations.

In general, the concentrations of PAHs (sum of 17 isomers) in Black Sea sediments are low by comparison with those observed in other regions. The highest concentrations

of "total" PAHs were observed at sites along the Danube coastline (638 ng g^{-1} dry wt), Odessa (635 ng g^{-1} dry wt) and Sochi (368 ng g^{-1} dry wt). A mixture of pyrolytic and petrogenic PAHs were observed in most of the sediments, usually with a slight pyrolytic predominance. This was most notable at the Bosphorus stations. Petrogenic contributions were recorded at the Danube delta and at Sochi. No correlation between "total" hydrocarbons and PAH was observed (r^2 = 0.04) indicating separate primary sources and/or differing transport processes for the two classes of compounds. High concentrations of perylene were recorded in many of the samples. The diagenetic origin of this compound was most notable in samples taken from the Bosphorus.

3.5.3 Sterols

Coprostanol is present in all sediments from the Black Sea, with variations of 1-2 orders of magnitude in concentration. Levels of coprostanol in the Black Sea are comparable (or perhaps even lower) than those generally encountered in the regions selected for comparison.

In the majority of the samples, levels were comparatively low (<500 ng g^{-1}), indicating minor sewage contamination. However, there was evidence of chronic contamination with sewage, grossly in the case of Sochi (up to 5400 ng g^{-1}) and on the coast adjacent to the Danube delta (2600 ng g^{-1}). Odessa sediments have intermediate coprostanol concentrations and 5β/(5β+5α) isomeric ratios, indicating minor sewage contamination. Very low coprostanol concentrations and 5β/(5β+5α) isomeric ratios along the Ukrainian coastline are characteristic of non-polluted sediments.

Coastal locations influenced by the River Danube exhibit high coprostanol concentrations and 5β/(5β+5α) isomeric ratios indicating sewage contamination. In addition, cross-representation of both the 5β/(5β+5α) isomeric ratio of cholestan-3β-ol and cholestan-3-one confirm sewage contamination and indicate anaerobic microbial cholesterol reduction at these sites.

In the Bosphorus, the highest values were found near shore, with values decreasing with increasing distance seawards. These sediments generally contained high values for both the 5β/(5β+5α) isomeric ratios of cholestan-3β-ol and cholestan-3-one, indicating sewage contamination.

The anoxic conditions in the Black Sea favour reduction of sterols to α-stanol by *in situ* microbial conversion. Our results indicate that anoxic waters (and particularly near oxic-anoxic interfaces) are important sites of intense alteration of organic matter including the reduction of sterols to α-stanols by in situ microbial conversion.

Chapter 4

4 Evaluation, development and application of immunoassay techniques

4.1 Introduction

This chapter incorporates data which have generated five separate manuscripts, one of which has been accepted, three are submitted and one is in the final stages of preparation. These cover different applications of immunoassay. Initially, an evaluation and validation of an ELISA kit to detect semi-volatile hydrocarbons (BTEX) in contaminated groundwater is presented. This data has been submitted to Environmental Technology (Francioni *et al.*, *Submitted*). Secondly, the performance of different hydrocarbon immunoassay kits is tested using sediment extracts and results are compared with those obtained using chromatography. A manuscript is in the final stages of preparation (Fillmann *et al.*, *In Preparation*). The third section investigates the adaptability and applicability of ELISA to measure PCB levels in mussel tissues. This study has been submitted to the Analytical Chimica Acta (Fillmann *et al.*, *Submitted a*). Finally, the effectiveness of ELISA in measuring PAH metabolite levels in body fluids of crabs (*Carcinus maenas*) is tested. The first part of this research has been accepted for publication in Marine Environmental Research (Fillmann *et al.*, *In Press b*) and complementary data including HPLC analyses of metabolites has been submitted to Environmental Science and Technology (Fillmann *et al.*, *Submitted b*).

4.2 Semi-volatile hydrocarbons (BTEX) in groundwater

4.2.1 Introduction

Aliphatic and aromatic hydrocarbons are among the most commonly detected contaminants in the aquatic environment (UNEP/IOC/IAEA, 1992). Their ubiquity together with the high concentrations frequently encountered raises environmental concern regarding ecotoxicological effects including carcinogenicity (Manahan, 1992; Harrison, 1996; Betton, 1997). Accidental contamination of soil and groundwater by petroleum products stored in underground tanks poses a threat to groundwater. Benzene, toluene, ethylbenzene and xylene (BTEX) which are found at relatively high

concentrations in most gasolines (c. 35%) (Fels, 1999) are the most commonly used indicators of dissolved phase petroleum contamination. BTEX have a relatively high aqueous solubility and, at least in the case of benzene, are considered very toxic and potentially carcinogenic (Beyer *et al.*, 1997).

Determination of BTEX in environmental matrices typically involves several steps (extraction, clean-up, and analysis with HPLC, GC or GC/MS). Analyses are, therefore, time consuming and generally expensive. Also, they do not provide a rapid response which is sometimes needed in an environmental assessment. To avoid these complex procedures, immunoassay techniques have recently been directed towards measuring environmental contaminants (Waters *et al.*, 1997b) because they are generally faster and less expensive than conventional laboratory methods (Barceló *et al.*, 1998; Kramer, 1998). Modern tests are rapid, sensitive, and selective, and are adaptable to field use. They have been applied to diverse environmental contaminants (e.g. triazines, total petroleum hydrocarbon, polycyclic aromatic hydrocarbons, organophosphorus pesticides, polychlorinated biphenyls, organochlorines) (Meulenberg *et al.*, 1995; Barceló *et al.*, 1998; Castillo *et al.*, 1998). The most common format used for environmental analyses is the ELISA (enzyme-linked immunosorbent assay) (Meulenberg *et al.*, 1995; Hage, 1999).

An important advantage of immunological methods is the possibility of the application to complex matrices with only minor sample preparation. This has generated interest in the development of rapid screening methods. A number of commercially available BTEX-immunological tests have been developed by several producers. Each kit uses different strategies, for example, the type of antibody source (poly and monoclonal), and the test format (tubes, microtiter plate, immunofiltration). Detection limits are generally in the ppm- (mg L^{-1}) or high ppb-range ($\mu g\ L^{-1}$) for single and multiple analytes. Beyer *et al.* (1997) have described most of the BT(E)X enzyme immunoassays which are commercially available or have been reported in the literature. The selected ELISA kit (BTEX RaPID Assay®), however, was not evaluated by these authors.

In contrast to conventional analytical methods, many immunoassays have not, to date, been extensively characterised. At the moment, several agencies in the USA and Europe are evaluating the use of immunoassays for regulation-linked analyses (Meulenberg *et al.*, 1995). Although immunoassay-based analytical methods are rapidly gaining acceptance, it is essential for any new analytical method to be rigorously validated

(Waters *et al.*, 1997b). Information on field-based environmental applications of immunoassays is scarce (Meulenberg *et al.*, 1995).

The goal of the present work was to investigate the performance of an immunoassay technique for the detection of benzene, toluene, ethylbenzene and xylene (BTEX) as tracers for gasoline contamination in groundwater. The case study we describe arose as a result of leakage from storage tanks in an urban petrol station in Rio de Janeiro city. Underground tank corrosion in Rio de Janeiro is aggravated due to the close proximity of the sea which is exacerbated through location of petrol stations on sandbar areas/shorelines.

4.2.2 Results and Discussion

Gasoline (or petrol) is comprised of approximately 50% aliphatic hydrocarbons and 50% aromatic (plus naphthenic) compounds (Betton, 1997). Benzene, toluene, ethylbenzene and xylenes are important constituents/markers. Whilst these BTEX compounds have relatively high vapour pressure/Henry's law constants, when trapped within the ground their release into the atmosphere is restricted and concentrations in groundwater can become elevated.

Calibration of the two analytical techniques revealed good linear regressions ($r^2 >$ 0.99) for gas chromatography and ELISA, respectively, within the calibration ranges of 0 to 50 $\mu g\ L^{-1}$ (GC) and 0 to 18 $mg\ L^{-1}$ (ELISA). Detection limits are described in Section 2.2.1.4 and 2.2.1.5 for the GC-FID/PID and ELISA techniques, respectively.

Results from analyses of the potentially contaminated samples taken in Rio de Janeiro are given in Table 4.1 and those pertaining to the two different types of analyses are plotted against each other in Fig. 4.1. It is evident that the immunoassay procedure overestimates the actual concentration of the BTEX by a factor of two or more. This probably results from interactions between the antibody and other constituents of the petrol (i.e. cross reactivity). A similar trend has been reported by Barceló *et al.* (1998) and Castillo *et al.* (1998) for PAH RaPID Assay® ELISA, although differences in that case reached one order of magnitude. Good reproducibility of the ELISA is demonstrated, ranging from 0.30 % to 11.7 % (as given by standard deviations from triplicate analyses).

In Fig. 4.1, a strong linear relationship ($r^2 = 0.997$; slope: 0.471; Y-intercept: 0.088) is demonstrated between results from the two analytical techniques. Despite this tight

regression, the ratio of [BTEX] $_{ELISA}$/[BTEX] $_{GC}$ shows more scatter at the lower concentrations. It is likely that with the ELISA, discrimination between BTEX and other hydrocarbons is less at lower concentrations when competition for binding sites increases and non-BTEX constituents can have an increasing effect producing more scatter in the results. This suggestion is upheld by the data for samples 5, 7 and 10. As can be seen in Fig. 4.2 and Table 4.1, although samples 5 and 10 have essentially the same BTEX concentration, the immunoassay gave results that are 4 times larger for sample 10. The chromatograms (Fig. 4.2) differ principally through the presence of a set of light unidentified compounds, which are eluted in the first 8 minutes of the chromatographic run. These possibly interact with the antibody producing an elevated response with ELISA. At higher concentrations (≥ 10 mg L^{-1}) the immunoassay results appear close to those given by GC-FID/PID (see, for example, sample 7). This might infer that effects owing to other hydrocarbon components are lesser.

Table 4.1. Concentration values (mg L^{-1}) of BTEX obtained by ELISA and GC-PID/FID

Sample	ELISA	GC/PID/FID						[BTEX]$_{ELISA}$/
	BTEX[a]	*BTEX*[b]	*Benzene*	*Toluene*	*Ethyl benzene*	*m+p-xylene*	*o-xylene*	[BTEX] $_{GC}$
1	<0.06	<0.005	<0.001	<0.001	<0.001	<0.001	<0.001	-
2	<0.06	0.04	<0.001	<0.001	0.02	0.02	0.01	-
3	<0.06	0.12	0.001	0.07	0.01	0.02	0.01	-
4	2.0 (0.5)[c]	1.68	0.01	0.94	0.26	0.32	0.14	1.2
5	0.52 (1.2)[c]	0.41	0.005	0.05	0.10	0.17	0.09	1.3
6	1.3 (0.11)[c]	2.10	0.90	0.23	0.58	0.22	0.17	0.6
7	78.9 (11.7)[c]	37.4	5.32	7.58	7.53	11.25	5.70	2.1
8	61.2 (2.7)[c]	29.1	2.80	7.69	5.58	8.96	4.04	2.1
9	13.7 (0.3)[c]	5.2	3.94	0.10	0.42	0.61	0.12	2.6
10	2.5 (9.7)[c]	0.44	0.13	0.06	0.09	0.11	0.06	5.7

[a] BTEX is expressed as the sum of the components by multiplying the value obtained by 6.

[b] BTEX is the sum of benzene, toluene, ethylbenzene and o-, m-, p-xylene.

[c] Coefficient of variation expressed in percentage.

The results obtained from chromatographic and immunoassay analyses agreed with respect to the distribution of the spilled fuel. Sampling stations 1, 2 and 3, in which the concentration values were below the detection limits of the immunoassay tests, were distant from the fuel spillage. In contrast, stations 7, 8 and 9 (chromatographic BTEX concentrations of 37.4, 29.1 and 5.2 mg L^{-1}, respectively) were the most affected sites.

Despite their close proximity, station 7 presented a concentration almost 85 fold higher than station 10. This is explained by a remediation treatment applied at station 10, where the groundwater had been pumped out continuously prior to our sampling campaign.

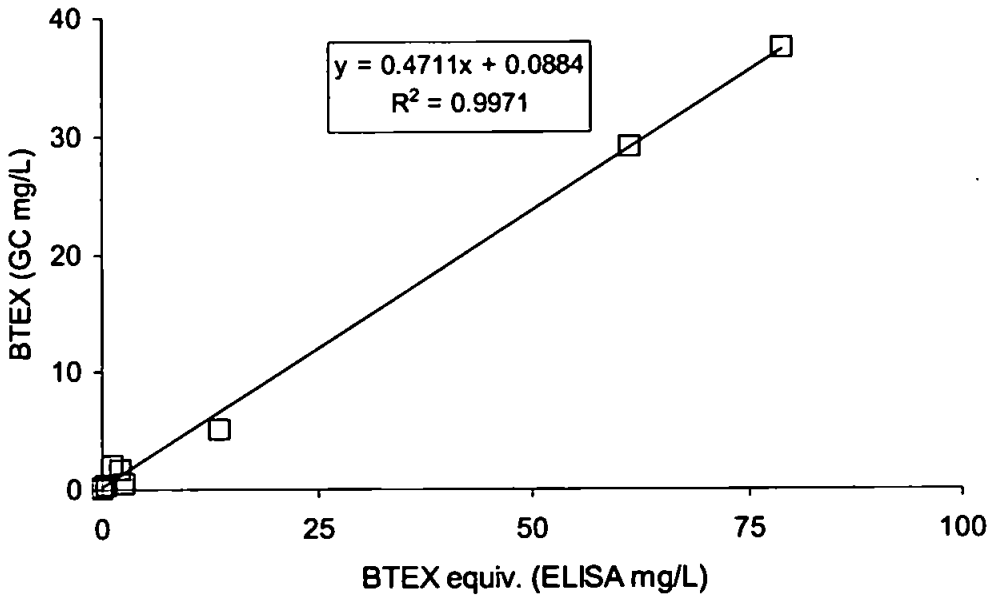


Figure 4.1. Correlation between BTEX RaPID Assay® ELISA and GC-PID/FID analyses of BTEX in contaminated groundwater

With regards to pollution levels, results indicate a critical contamination of groundwater close to the petrol station, although measurable concentrations were also found in a radial distance of approximately 100 m from the source. Benzene concentrations are up to 500 times above the maximum levels permitted for drinking water by the Brazilian legislation (0.01 mg L^{-1}) (CONAMA, 1986) and up to 1000 times above EPA standards (0.005 mg L^{-1}; US Environmental Protection Agency) (Beyer *et al.*, 1997).

The results obtained indicate that the applied immunoassay test is suitable for detecting contamination from gasoline at "hot spots" such as leakage. It is important to note, however, that the reactivity of the ELISA is lower for benzene than the other compounds and subsequently results relate primarily to other marker compounds. This is important because legislation on drinking water specifies benzene. In addition, at the lower levels the ELISA cannot reliably measure the concentrations specified by legislation. This renders compliance testing using the ELISA inappropriate. Additional

work to enhance the sensitivity of the test is required. This may be attained through pre-concentration or by altering reaction times or the amount of sample used.

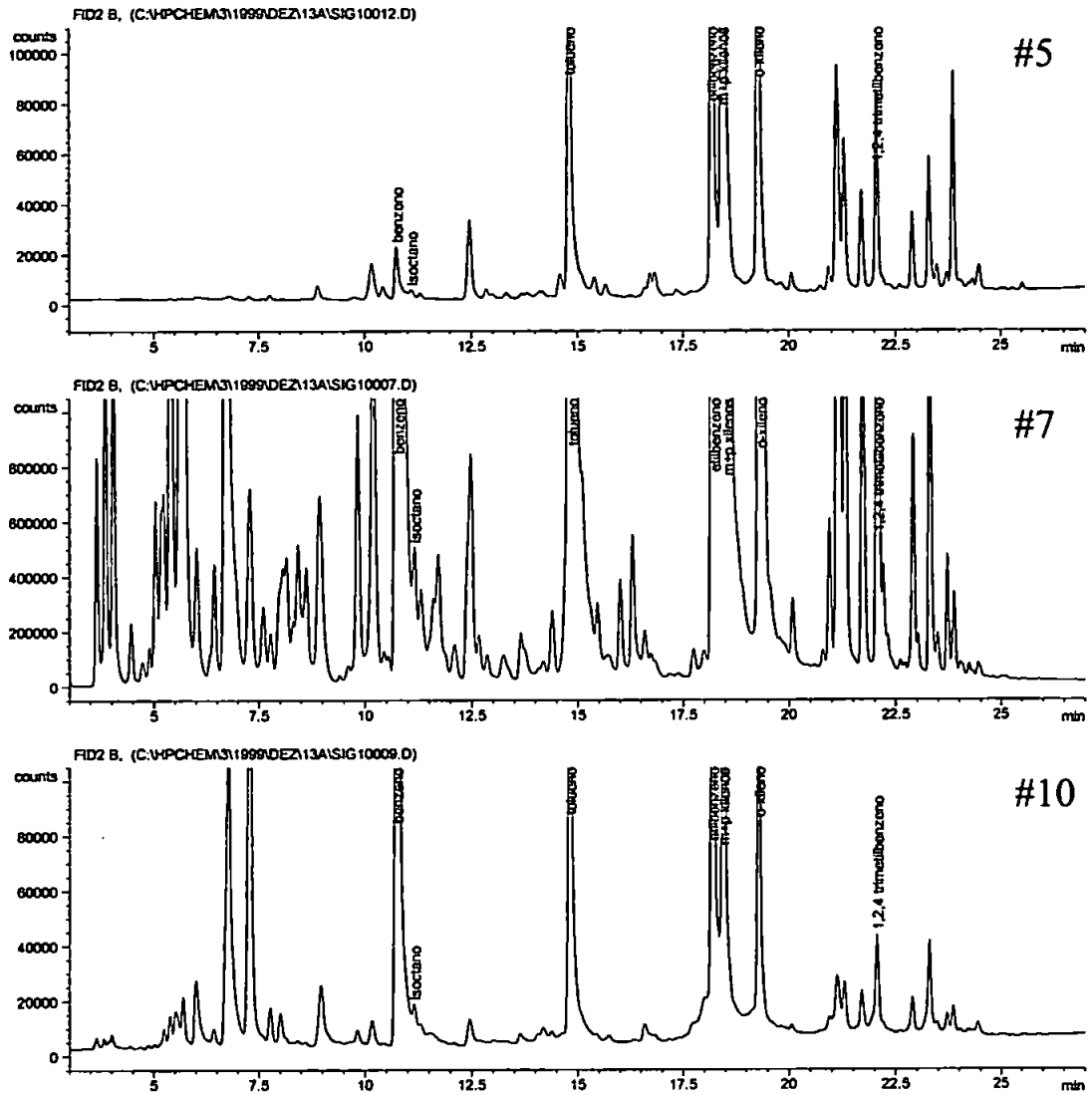


Figure 4.2. Chromatograms of GC-PID/FID in three groundwater samples:#5, #7 and #10

4.2.3 Conclusions

In general, good agreement was achieved between the conventional gas chromatography analyses and the RaPID Assay® BTEX ELISA. A linear regression of $r^2 = 0.99$ was achieved for gasoline contaminated environmental samples analysed using both techniques. The ELISA has proven very simple and rapid to use but the results obtained at lower concentrations (< 10 mg L^{-1}) revealed increased scatter. Also, the detection limits cannot (at present) achieve the concentrations specified by legislation.

This renders the ELISA technique (currently) unsuitable for compliance testing but demonstrates its worth as a rapid screening tool to investigate "hot spots" of contamination. In spite of the tendency to overestimate the actual concentration the test allows identification of problematic sites where storage tanks should be repaired or exchanged. The simple and fast application makes ELISA suitable for screening of large networks of gasoline distribution as those existing in the city of Rio de Janeiro and elsewhere.

4.3 Petroleum hydrocarbons and PAHs in sediment

4.3.1 Introduction

Aliphatic and aromatic hydrocarbons are amongst the most commonly detected contaminants in the aquatic environment, deriving from petroleum and combustion processes. Their ubiquity and frequent high concentrations creates environmental concern regarding ecotoxicological effects. Although a wealth of literature addresses these as important environmental pollutants, there is a weak link between chemical investigations and biological effect assessments using ecotoxicological methods. Often, time consuming chemical methods do not provide the response needed for rapid environmental assessments. However, immunoassay techniques have recently been directed towards measuring environmental contaminants (Meulenberg *et al.*, 1995; Waters *et al.*, 1997a). The most common format used for environmental analyses is the ELISA (enzyme-linked immunosorbent assay) (Meulenberg *et al.*, 1995; Aga and Thurman, 1997; Hage, 1999). This technique has proven to be rapid and cost effective (Barceló *et al.*, 1998; Kipp *et al.*, 1998) and can usefully complement ecotoxicological methods in environmental assessments (Galloway *et al.*, *Submitted*) (see Chapter 5).

Immunoassay-based analytical methods are rapidly gaining acceptance. However, it is necessary for any new analytical method to be rigorously validated before it can be considered as a replacement for, or adjunct to, currently used laboratory methods. The scarcity of available information on the "real" environmental applications of immunoassays was documented (Meulenberg *et al.*, 1995). Multiple and independent evaluations lend credibility to the method. Most currently available immunoassay validations have been produced by the kit manufacturers (Waters *et al.*, 1997a; Kramer, 1998). Therefore, independent evaluations of these kits are necessary to enhance the acceptance of the technology by potential users. The use of commercial immunoassays

for field-testing has been encouraged (Oubina *et al.*, 1996b). In this work, a laboratory study was conducted to evaluate the performance of two immunoassay-based analytical methods for quantification of "total" petroleum hydrocarbons and polycyclic aromatic hydrocarbons. Two commercially available ELISA kits (BTEX and carcinogenic PAH RaPID Assays®) were evaluated for quantification of hydrocarbons in estuarine sediment samples, including reference materials. The BTEX RaPID Assays® was employed to analyse aliphatic and small aromatic hydrocarbons. The c-PAH RaPID Assays® was employed to analyse the polycyclic aromatic hydrocarbons (> 3 aromatic rings). Results were validated by comparison with gas chromatographic analyses (CG-FID with GC-MS confirmation).

An estimate of "total" hydrocarbons contamination is included by screening measurements from both assays.

4.3.2 Results and Discussion

4.3.2.1 Analytical Performance

The linearity of standard calibration curves for BTEX and benzo(a)pyrene analysed by ELISA was > 0.99 for both kits. The method detection limits (MDL), as estimated at 90% B/Bo for the BTEX or c-PAH calibration dilutions, were 1.08 $\mu g\ g^{-1}$ and 5.5 $ng\ g^{-1}$, respectively. A 10% inhibition of signal was considered to be significantly different from the zero analyte concentration and was used to estimate the sensitivity of the assay giving the method detectable limit (MDL) at 90% B/Bo (Oubina *et al.*, 1996a).The coefficient of variation (%CV) for repeated analyses for a single sample was 16 ± 8% (n = 5) and 6.7 ± 4% (n = 5) for BTEX and c-PAH, respectively. These are similar to variations within conventional analytical techniques.

The 50% B/Bo (IC_{50}) (the concentration required to inhibit one-half of the colour produced by the negative control) was 63.6 $\mu g\ g^{-1}$ and 255 $ng\ g^{-1}$ for the BTEX and c-PAH RaPID Assay®, respectively.

Inherent to the use of antibodies is a certain degree of cross-reactivity, the binding of structurally related compounds to the antibody. The degree to which a particular antibody selectively binds the analyte of choice determines its applicability. A low degree of cross-reactivity makes it suitable for single-compound assays. In contrast, a group-specific assay requires an antibody having a high degree of cross-reactivity. Thus, the selection of antibody depends on the purpose of application. Generally, an antiserum consisting of

several types of antibodies (polyclonal) shows a broader spectrum of cross-reactivities than a monoclonal antibody (Meulenberg *et al.*, 1995). The ELISAs being tested are polyclonal and, thus, suitable to detect multi-compounds such as hydrocarbons.

Table 4.2. Cross-reactivities against other hydrocarbons and petroleum products in the BTEX RaPID Assay® (data provided in the RaPID Assay® SDI Product Information)

Compound	MDL* ($\mu g\ g^{-1}$)	50% B/Bo* ($\mu g\ g^{-1}$)
m-Xylene	0.6	36
p-Xylene	2.6	62
o-Xylene	4.4	94
Ethylbenzene	4.8	156
Toluene	8.8	148
Benzene	11.8	1,000
Naphthalene	0.6	11.8
Anthracene	1.2	560
Styrene	1.4	52
Hexachlorobenzene	1.6	NR
Phenanthrene	1.6	32
Acenapthene	3.4	124
n-Octane	68	NR
n-Nonane	88	NR
n-Heptane	126	NR
n-Decane	270	NR
Methylene Chloride	NR	NR
Trichloroethylene	NR	NR
Gasoline	8.6	842
Diesel	25.8	324
Kerosene	30	480
Jet-A Fuel	54	670

NR – non reactive up to 1,000 $\mu g\ g^{-1}$

* based on a 20-fold dilution of the sediment extact

In the present study, the cross-reactivity against other PAHs and other petroleum products are expressed in Tables 4.2 and 4.3 as the method detectable limit (MDL) and 50% B/Bo. However, comparisons are hindered since cross-reactivity often varies with the dose of cross-reactant due to nonparallel displacement curves (usually, but not always, with higher cross-reactivity at lower doses). In addition, the commercial assays

generally describe the cross-reactivities of an individual cross-reactant at one fixed concentration and using a clean matrix, either distilled or ground water (Oubina *et al.*, 1996a). This would affect comparisons with environmental samples. In spite of this, results indicate that the ELISAs will detect most aliphatic and small PAHs (BTEX RaPID Assay®) and >3 ring PAHs (c-PAH RaPID Assay®) (albeit with differing sensitivities) potentially affording an effective monitor to environmental hydrocarbons (aliphatics + PAHs).

Table 4.3. Cross-reactivities against other PAHs and petroleum products in the c-PAH RaPID Assay® (data provided in the RaPID Assay® SDI Product Information sheet)

Compound	MDL* ($ng\ g^{-1}$)	50% B/Bo* ($ng\ g^{-1}$)
Benzo(a)pyrene	4.0	160
Benz(a)anthracene	1.0	48
Benzo(k)fluoranthene	1.0	63
Chrysene	2.0	69
Benzo(b)fluoranthene	2.0	130
Indeno(1,2,3-c,d)pyrene	1.0	203
Dibenz(a,h)anthracene	7.0	241
Anthracene	22	2,050
Phenanthrene	135	6,720
Fluoranthene	100	6,850
Benzo(g,h,i)perylene	15	>10,000
Pyrene	100	23,300
Fluorene	1,850	34,200
Naphthalene	18,800	NR
Acenaphthylene	7,400	NR
Acenaphthalene	NR	NR
Creosote	62	838
Fuel Oil #4	1,260	30,400
Fuel Oil #5	1,000	20,700
Heating Fuel	1,000	65,300
Diesel Fuel	12,000	NR
Gasoline	10,000	NR
Kerosene	NR	NR
Jet A Fuel	NR	NR

NR – non reactive up to 50,000 $ng\ g^{-1}$

* based on a 100-fold dilution of the sediment extact

The accuracy of immunoassay-based methods, as with most analytical methods, will depend on the integrity of the standards used to calibrate them. The history of a standard is important since the concentration of the standards may change over time after the first use (Waters *et al.*, 1997a). Small discrepancies in concentrations (smaller than stated) have been reported for RaPID Assay® calibration standards (Kipp *et al.*, 1998). None of the standards supplied and used in this study appeared to contain less than the assigned concentration at first use.

The best indicator of the integrity of the standards (and reagents as a whole) is the % B/Bo. This treatment of the data effectively cancels out variations in absolute absorbance measurements resulting from inherent variability of the assay. The % B/Bo values for a particular standard concentration should remain relatively constant and can be used as an index of quality control (Waters *et al.*, 1997a). The % B/Bo values for all the BTEX standards used in the BTEX RaPID Assay® were 80.29 ± 1.42% (for the 0.54 $\mu g\ g^{-1}$ standard, n = 3), 62.78 ± 0.79% (for the 2.1 $\mu g\ g^{-1}$ standard, n = 3), 42.34 ± 1.22% (for the 18 $\mu g\ g^{-1}$ standard, n = 3). Values obtained for all the benzo(a)pyrene standards used in the c-PAH RaPID Assay® were 86.33 ± 1.56% (for the 0.1 $ng\ g^{-1}$ standard, n = 3), 65.83 ± 1.79% (for the 1.0 $ng\ g^{-1}$ standard, n = 3), 39.03 ± 2.12% (for the 5.0 $ng\ g^{-1}$ standard, n = 3).

4.3.2.2 Performance of ELISAs

Sediment Extraction. An important disadvantage of the commercially available immunoassay is the associated extraction kit. The samples are mixed with 20 ml of methanol and shaken vigorously for at least 60s (5 minutes in present research). Samples are then left to settle, filtered and diluted with buffer solution. This procedure is more or less the same for all immunoassay test kits, since methanolic solutions are compatible with the assay (Hottenstein *et al.*, 1995; Lawruk *et al.*, 1996). Knowing that even Soxhlet extraction for several hours can show incomplete extraction or have different analyte and/or matrix dependant extraction efficiencies, it is not surprising that there are differences between the results obtained with immunoassay test kit and those obtained with conventional analytical methods (Kipp *et al.*, 1998). It is suggested that lower ELISA PAH concentrations are generated because methanolic extraction cannot effectively remove all the PAH from the samples (Johnson and VanEmon, 1996; Chuang *et al.*, 1998; Johnson *et al.*, 2001). In addition, the sediment type can also

influence the efficiency of extraction, where clay type sediments have less efficient extraction of PAHs (Waters *et al.*, 1997a).

BTEX RaPID Assay® Results. A total of 34 sediment samples were analysed. Five sediment samples spiked with a mixture of *n*-C_{12}-C_{35} (14 to 112 $\mu g\ g^{-1}$) and, additionally, a sediment reference material (IAEA 383) were analysed in triplicate. Sixteen environmental sediment samples from the Patos Lagoon estuary (southern Brazil) were also analysed singly.

The concentrations of "total" petroleum hydrocarbon (expressed as BTEX "equivalents") found by the ELISA were plotted against the concentrations measured by GC/FID (Fig. 4.3). The spiked sediment extracts were differentiated from all other samples. The ELISA results for the spiked sediments gave a best-fit straight line with an r^2 value of 0.94 and slope of 0.75. Although this shows good agreement between both techniques, the slope is indicative of the higher immunoreactivity of the Σ*n*-alkane-spiked samples relative to that of BTEX standards.

Results for the unspiked samples also show a reasonable agreement between the techniques ($r^2 = 0.68$, slope = 1.21) (Fig. 4.3). In this case the TPH found by ELISA is compared with GC/FID results which include the sum of alkanes, unresolved complex mixture and ≤ 3 ring PAHs. The slope indicates a lower reactivity compared to that of BTEX standards. Since the UCM is dominant in these samples (15.5 – 98%, with an average of 73.3%), the UCM is shown to have a lower reactivity with these ELISA antibodies. The sample with the highest UCM (Esgoto, 98%) is the one showing the lowest reactivity (Fig. 4.3). Samples with less UCM (< 80%) and more ≤ 3 ring aromatics (> 7%) show increased reactivity (e.g. Mercado and Refinaria, Fig. 4.3) However, if only the alkanes and ≤ 3 ring PAHs are compared with the ELISA results, no relationship is found ($r^2 = 0.10$), indicating that it is the UCM that is cross-reacting with the antibodies. Indeed, this would concur with very recent data published by Rowland *et al.* (2001) who report on the composition and toxicity of the aromatic UCM. These authors confirm that the composition of this UCM includes the presence of alkyl benzenes and C-ring monoaromatic steroids (revealed by GC-MS mass fragmentography). These constituents would be expected to react with the BTEX RaPID Assay® antibodies. The absence of a correlation when the UCM components are removed from the GC data cannot, however, discount contributions of components (e.g.

volatiles) in the extracts which are not quantified by the selected GC analytical techniques.

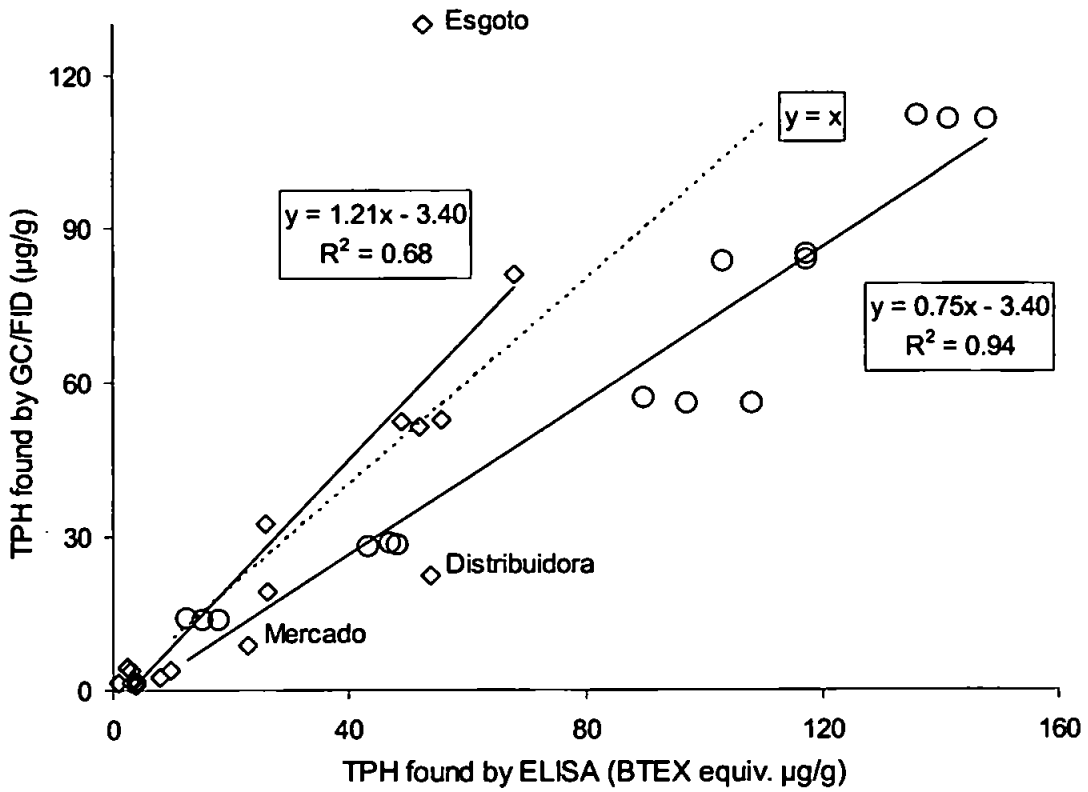


Figure 4.3. Plot of results from the BTEX RaPID Assay® ELISA and GC/FID analyses of TPH compounds in (o) Σn-C_{14}-C_{36}-fortified sediment samples and environmental sediment samples (◇) (GC results indicate the sum of alkanes, UCM and ≤ 3 ring PAHs)

The BTEX RaPID Assay® uses equivalent parts of benzene, toluene, ethylbenzene and m-, o- and p-xylene as the cross reactants set as 100%. Other hydrocarbon compounds react in weaker (or much weaker) ways compared to the main targets. Again, depending on the individual hydrocarbon composition of each sediment, the response of the total petroleum hydrocarbon ELISA will differ. Consequently this test cannot be used as a quantitative method for determining "total" petroleum hydrocarbons (TPH) in sediment. The concentrations derived from the ELISA should not, therefore, be treated as absolute and accurate measurements, but rather relative comparisons between samples.

PAH RaPID Assay® Results. A total of 25 sediment samples were analysed. Four PAH-spiked sediment samples (18, 177, 670 and 1650 ng g^{-1}), five sediment reference materials (HS-4B, HS-5, IAEA 383, QPH16MS and QPH17MS) and sixteen

environmental sediment samples (from the Patos Lagoon estuary, southern Brazil) were analysed.

The concentrations (PAH "equivalents") found by the ELISA were plotted against the concentrations measured by GC/FID (Fig. 4.4). The spiked sediment extracts were differentiated from all other samples. The ELISA results gave a best-fit straight line with an r^2 value of 0.97 and slope of 0.80. This indicates good agreement between the two techniques. The slope indicates higher immunoreactivity of the Σ PAH-spiked samples relative to that of benzo(a)pyrene calibration standard.

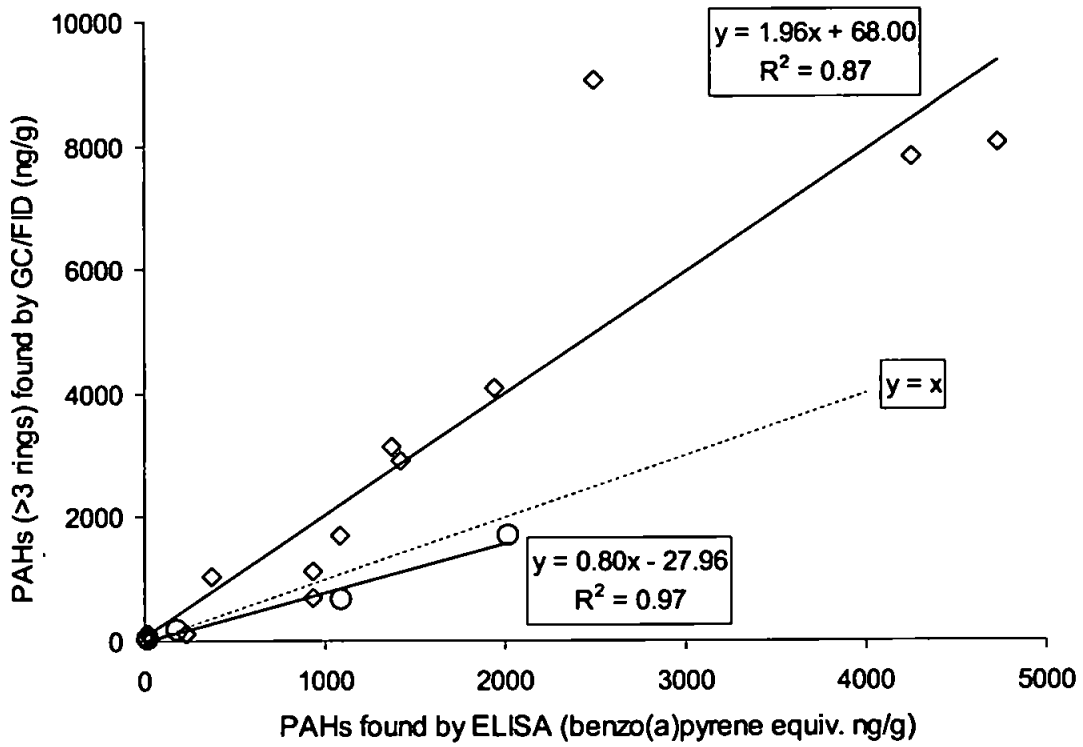


Figure 4.4. Plot of results from the c-PAH RaPID Assay® ELISA and GC/FID analyses of PAH compounds (>3 rings) in (o) Σ 24 PAHs-fortified and (◇) environmental sediment samples

The ELISA results for the other samples also showed good agreement with the GC data (r^2 = 0.87, slope = 1.96) (Fig. 4.4). In this case the c-PAHs found by ELISA are compared with GC/FID results for Σ ≥4 ring PAHs. The slope indicates a lower reactivity compared to that of benzo(a)pyrene calibrant. The sediment samples used were 0.5 – 2.0 times as reactive with the antibodies as benzo(a)pyrene (except for the samples Distribuidora (0.3 times) and Mangueira (0.2 times)). In the field samples, shifts in the relative composition of complex mixtures of PAH are apparent which alter

the ELISA response. For example, the sample from Mangueira contained phenanthrene, fluoranthene and pyrene as major constituents but no five or six ring compounds were detected. These differences in response result from the fact that the antibodies were produced against benzo(a)pyrene (a 5 ring PAH). Thus the different shaped/sized PAH will react to different degrees. The correlation between immunoassay and chromatographic PAH results, as might be expected, was better using the sum of PAHs >4 rings than the total PAH. Other chemical compounds not quantified using the selected chromatographic technique could also, in part, be responsible for the higher ELISA kit test values.

Several other ELISA kits to measure PAH are available and have been described in the literature. In Section 1.2.3 (Table 1.2), sensitivities of different kits are compared and indicate a high performance for the RaPID Assay®. Various authors have investigated the RaPID Assay® PAH-ELISA performance relative to other analytical techniques and usually report overestimation by the ELISA. Chuang *et al.* (1998), working with house dust and soil, found that the c-PAH RaPID Assay® ELISA measurements were 5.9 times higher than those from GC/MS. Waters *et al.* (1997a) showed that a 16 component PAH mixture had 3.4 - 4.5 times more reactivity to the PAH RaPID Assay® ELISA antibody than phenanthrene. Kipp *et al.* (1998) working with the PAH RaPID Assay® report ELISA results 10 times higher than those obtained with HPLC. Indeed, in most cases the overestimation of the ELISA is at least one order of magnitude (e.g. Barceló *et al.*, 1998). These results concur with those obtained using an EPA protocol involving a commercial microtiter plate ELISA for PAH (EPA SW-846 Method 4035) that also give a considerable overestimation (USEPA, 1997).

Reasons for this overestimation probably relate to differences in the reactivity of compounds in the environmental extracts. Often extracts contain many more compounds in the extract than the 16 PAHs normally quantified. These compounds most probably are isomeric or substituted analogues of the 16 quantified PAHs and, therefore, can react with the antibody leading to elevated results (Kipp *et al.*, 1998).

Although the present study reports a marginal overestimation for the "spiked" sediments, in general our results do not demonstrate the same overestimation as commonly reported. It should be noted that the kit used in our study was the carcinogenic PAH RaPID Assay® so only the work of Chuang *et al.* (1998) (who reported a 5.9 times overestimation) is strictly comparable. It is likely that discrepancies

arise owing to extraction techniques. Compounds spiked into sediments are extracted more easily than compounds actually present in environmental samples. The better reactivity within the spiked samples might, therefore, be related to better recoveries. For the environmental and reference samples, the lower reactivity (slope = 1.96, Fig. 4.4) might relate to the extraction procedures used. Dichloromethane was used for the GC analyses, whereas methanol was used for the ELISA. In addition, Soxhlet extraction was for 16 hours for the GC samples and that for the methanol was only 5 minutes (ELISA samples). Sonication would possibly improve recoveries using the methanolic extraction and provide a more effective technique to extract c-PAH from sediments (Chuang *et al.*, 1998), whilst maintaining simplicity of operation.

Analytical interferences due to the limited selectivity of antibodies (cross-reactivity) is another very important aspect to take into account when assessing ELISA performance (Oubina *et al.*, 1997). Castillo *et al.* (1998) found that many non-PAH compounds in industrial wastewater samples could be detected by the carcinogenic PAH RaPID Assay® test kit. Phthalates in particular gave high cross-reactivities. Whilst immunoassays are often used without cleaning-up the extracts, this may result in matrix interferences which affect performance. Values produced by immunoassay, therefore, only provide a qualitative/semi-quantitative estimate of contaminants in extracts. The application of limited clean-up techniques can often improve performance to an extent. To check for matrix interferences, standard additions can prove of great use owing to the fact that interferences usually have widely different reactivities than the selected determinands. When sediments from diverse sources are analyzed, matrix variations can differ and affect results. Within a pollution gradient with a single pollution source, however, the compositional variability is small and the performance of an immunoassay improves (Gascon *et al.*, 1995; Waters *et al.*, 1997a).

4.3.3 Conclusions

Results from the ELISA kits tested are shown to compare well with those obtained by GC. This confirms ELISA to be a useful screening protocol with which to focus more expensive (high resolution) analytical techniques. In addition, the selected immunoassay kits are fully portable (including the spectrophotometer), offering true field deployment. Other screening techniques such as infra red and spectrofluorimetry require laboratory based measurements. The combined use of both the BTEX and

carcinogenic PAH RaPID Assay® offers the capability to screen for a very wide range of hydrocarbon components to give a good measure of "total" hydrocarbons.

Our results indicate that the use of attenuation/correction factors can improve the ELISA performance. In addition, owing to differences in cross reactivities (especially with the PAH kit), it is essential to appreciate limitations associated with compositional differences. BTEX and c-PAH RaPID Assay® ELISA cannot strictly be used as a quantitative method for determining hydrocarbons in environmental sediments, since the antibodies used bind differently to different hydrocarbons/PAHs. The concentrations derived from the ELISA cannot, therefore, be treated as absolute and accurate measurements but rather as a rapid inexpensive and portable screening tool for environmental investigation of contamination/pollution.

4.4 PCBs in mussel tissue

4.4.1 Introduction

During the last decade, environmental immunoassays have been developed to detect selected pollutants in water and sediment/soil samples. Several commercial kits are now available for this purpose and offer rapid screening at comparatively low cost. Recently, environmental researchers have started to apply the technique to the analysis of biological media in which pollutants and their metabolites can become concentrated (Sherry *et al.*, 1989; Nam and King, 1994; Itak *et al.*, 1994; Zajicek *et al.*, 1996; Zajicek *et al.*, 2000). Biological monitoring data is often essential to provide a measure of exposure to biologically "available" contaminants. Methods to provide this data, however, are often complex.

Conventional biomonitoring methods, which use chromatographic techniques, are often time consuming, labour intensive, and expensive. Immunochemical methods, such as enzyme-linked immunosorbent assays (ELISAs), are easier and less expensive to use, can be very specific to the chemical or group of chemicals (e.g. PCBs), may offer improved limits of detection, and are easily adapted for use in the field.

The principles of enzyme-linked immunosorbent assays (ELISA) have been previously described (Kemeny, 1991; Aga, 1997; Sherry, 1997). Among several ELISA formats available, the PCB RaPID Assay® (Strategic Diagnostics Inc., Newtown, PA, USA) using magnetic particle-based immunoassay was selected for this study. This

format has been applied to the detection of contaminants in different matrices such as water (Dombrowski *et al.*, 1997; Barceló *et al.*, 1998), sediment/soil (Lawruk *et al.*, 1993a; Lawruk *et al.*, 1993b; Hottenstein *et al.*, 1995; Donnelly *et al.*, 1996), organisms (Zajicek *et al.*, 1996) and food (Nam and King, 1994; Itak *et al.*, 1994; Lawruk *et al.*, 1995). In addition, magnetic particle ELISA has shown better precision and sensitivity compared to formats where the antibody is passively adsorbed to polystyrene tubes (Aga and Thurman, 1993; Lawruk *et al.*, 1996).

Whilst primarily designed for analyses of polychlorinated biphenyls (PCBs) in water, our goal was to adapt and evaluate the effectiveness of this immunoassay method in measuring PCB levels in the biological tissues of exposed invertebrates (mussels). These evaluations included: a) adaptation of our routine sample work-up procedure to combine with ELISA; b) determination of matrix effects relating to co-extracted biogenic materials; c) assessment of cross-reactivities of the antibodies with technical PCB mixtures (Aroclors); d) assessment of reproducibility and limits of detection, and; e) determination of how well immunoassay results correlate with more definitive cGC-ECD results.

4.4.2 Results and Discussion

PCB congener patterns in contaminated environmental matrices often resemble those of the commercial/technical PCB mixtures (Aroclor® 1016, 1242, 1248, 1254 and 1260) or their combinations. As a result, immunoassay antibodies for PCB analysis have been raised and calibrated against technical Aroclors (e.g. Aroclor 1254 (Lawruk *et al.*, 1996); Aroclor 1248 (Zajicek *et al.*, 2000)). The RaPID Assay® PCB ELISA was raised and calibrated against Aroclor 1254. For these reasons, performance testing of the immunoassay procedure included other technical Aroclor mixtures.

Some organisms, however, can accumulate a modified composition of congeners depending on the extent of environmental alterations, and the bioaccumulative and metabolic capabilities of the organisms (Lake *et al.*, 1995). Results for the ELISA are, therefore, compared with GC-ECD results to better understand the ELISA response to environmental PCB compositions.

4.4.2.1 *Performance of ELISA*

Immunoassay kits for particulate analyses generally recommend methanolic extraction which results in a solution compatible with the immunosorbent assay (Hottenstein *et al.*, 1995; Lawruk *et al.*, 1996). Extractions involving polar solvents, however, are generally not as effective for complex matrices and very hydrophobic contaminants (Johnson and VanEmon, 1996; Johnson *et al.*, 2001). For this reason, Soxhlet extraction, which yields high recoveries (and is used in our routine sample preparation procedure for PCB analysis), was selected to provide extracts for this study. This does, however, coextract extraneous lipids which can introduce matrix effects with the ELISA. In addition, hydrophobic solvents (e.g. isooctane, hexane, etc.) inhibit the ELISA. For example, Zajicek *et al.* (1996) have reported significant interference by isooctane on a PCB ELISA (based on antibody-coated magnetic particles) even at trace 0.1% (v/v) concentrations. Therefore, a solvent exchange procedure (hexane to methanol) is essential when using high sensitivity ELISA such as PCB RaPID Assay®.

Matrix effects were carefully studied. A preparatory clean-up (see Section 2.2.3) was selected to remove the bulk of extraneous lipids. Ten mussel samples were diluted (to cover the entire range of the standard calibration curve) and then analysed by ELISA. Results were compared to similar curves resulting from Aroclor 1254 standards. A lack of parallelism between samples and standard curves can indicate matrix effects (Kemeny, 1991). The slope of the curves (slope = 0.663 ± 0.023, r^2 = 0.97 ± 0.04, n = 10, not shown) was unaffected by the dilutions, providing evidence that no significant matrix effects were present using the selected analytical conditions.

The method detection limit (MDL), as estimated at 90% B/Bo for the Aroclor 1254 calibration dilutions, was 0.08 ng mL^{-1}. The 50% B/Bo (concentration required to inhibit one-half of the colour produced by the negative control) was 2.9 ng mL^{-1} (Table 4.4). This sensitivity approached the estimated detection limit for the GC-ECD technique (0.1 ng mL^{-1}). The assay detection limit for mussel tissue was 0.6 μg g^{-1} (dry wt.), which is the 90% B/Bo corrected for the dilution used. Quantification using the ELISA must be within the range of the standard curve (0.6 to 40 μg g^{-1}) and appropriate dilutions must be made. The sensitivity can be improved by reducing the dilution up to a limit that guarantees no matrix effect. The coefficient of variation (%CV) within the assay was less than 11 ± 4% (n = 10), which is similar to conventional analytical variability.

Within the analytical protocol, internal standard PCB 29 (40 ng) was added to enable improved quantification by cGC-ECD (to correct for recovery). Thus the compound was also present in the extracts analysed by ELISA. Tests to investigate the potential for this to affect the immunoassay results revealed that even ten times the amount of PCB 29 added as internal standard produced no measurable ELISA response.

Although the PCB RaPID Assay® was raised and calibrated against the technical Aroclor 1254 mixture, compositions of PCBs in the environment can vary and reflect other Aroclors (or their combination) thus resulting in different ELISA responses. The ELISA was, therefore, tested against other commercial Aroclors (1242, 1248 and 1260). Results relative to Aroclor 1254 are given in Table 4.4 and demonstrate that the assay is broadly responsive to all the Aroclors tested. The ELISA response for Aroclor 1242 and 1248 is smaller indicating that these congener mixtures have lower binding efficiency than those of Aroclor 1254. Conversely, Aroclor 1260 has a higher binding efficiency than Aroclor 1254, inferring that it has a higher proportion of strongly binding congeners. These results indicate that the antibodies have increased affinity for congeners with a higher degree of chlorination. Similar relative cross-reactivities for the Aroclors have been reported by Lawruk *et al.* (1996) using a magnetic-particle PCB ELISA and Zajicek *et al.* (2000) using a EnviroGard™ tube format PCB ELISA.

Table 4.4. Specificity (cross-reactivity) of Aroclors® in the PCB RaPID Assay®

Aroclor®	% Chlorination	MDL[a] ($ng\ mL^{-1}$)	50%B/Bo[b] ($ng\ mL^{-1}$)	Cross-reactivity
1260	60	0.08	2.72	1.07
1254	54	0.08	2.90	1.00
1248	48	0.16	6.90	0.42
1242	42	0.35	14.20	0.20

[a] MDL – method detection limit (90% B/Bo)

[b] 50%B/Bo – is the concentration required to inhibiting one-half of the colour produced by the negative control.

Lawruk *et al.* (1996), using a magnetic-particle PCB ELISA, showed that PCB antibodies are most reactive to the Aroclors that largely contain 4, 5, and 6 chlorine-substituted homologues (i.e., Aroclor 1248, 1254, 1260) because Aroclor 1254 (which was used as the PCB immunogen) is comprised of 94% of these homologues. It is, however, difficult to determine which specific congeners are most reactive to the

antibodies. Carlson (1995) suggested that a PCB immunoassay had a greater specificity for congeners with 2,4,5- substitution pattern and its 2,4- and 2,5- subsets, which represent a significant portion of Aroclor mixtures. In the present study, the response of the assay was within a factor of 2.5 for Aroclors 1248 and 1260 and is in agreement with the significance of 2,4-, 2,5- and 2,4,5- substitution in these congener mixtures. In general, as the percent chlorination of Aroclors increases, so does their content of congeners chlorinated in the 2-,4-,5-,2'-,4'-,5'-positions and hence ELISA cross-reactivity (Zajicek *et al.*, 2000). Lawruk *et al.* (1996) showed, however, that a coplanar PCB (3,3',4,4',5-pentachlorobiphenyl) was the most reactive of a selection of congeners tested. Another pentachlorobiphenyl (2,3,4,4',5-) along with tetra- and hexachlorobiphenyls showed much lower reactivities.

4.4.2.2 Comparison of results from ELISA and cGC-ECD

In total, twenty-seven (27) mussel tissue samples were analysed by ELISA and GC-ECD. These included mussels from natural populations (with diverse concentrations of PCBs from 0.3 – 100 $\mu g\ g^{-1}$ dry wt; n = 20), tissues fortified with Aroclor 1254 (0.7 - 40 $\mu g\ g^{-1}$ dry wt; n = 3), and replicates of a certified standard reference material (SRM 2977, n = 4). To enable comparison of the results obtained by both techniques, the GC results were not corrected for procedural recovery since ELISA results cannot be corrected for this factor. Losses during the analytical procedure were typically less than 20% (based on recoveries of the internal standard). Since mussel extracts were processed using the same general analytical procedure for both GC and ELISA analyses, losses should be comparable. However, minor differences might be expected because the extracts for ELISA analyses went through an additional solvent exchange and dilution, whilst the GC extracts were further cleaned-up and fractionated using Florisil®.

Comparison of "total" PCB data for both techniques (Σ 128 congeners for the GC-ECD; Aroclor 1254 "equivalents" for ELISA) shows a high correlation between the immunoassay and GC results ($r^2 = 0.95$, slope = 1.28, n = 27) (Fig. 4.5). ELISA results, however, were consistently lower than those obtained by GC by a factor of 0.83 (3-29%). A reduced antibody response, due to differences in the congener composition between the mussel extracts and Aroclor 1254 (used to raise and calibrate the ELISA), provides the most likely explanation for this discrepancy.

To further investigate differences between the PCB congener compositional patterns, the cCG-ECD data were subjected to principal component analysis (PCA)) (PC1 – 29.9%; PC2 – 27.8%) (Fig. 4.6). The majority of the PCB mixtures in most mussel extracts, although differing from technical Aroclors (or their combinations), are confirmed to be most closely related to the technical Aroclor 1254. Mussel samples from Whitsand Bay, however, are shown to comprise either a mixture of Aroclors or an environmentally altered technical mixture (Fig. 4.6). The good agreement achieved between the ELISA and GC results is enhanced because the mussel extract PCB composition resembles that of Aroclor 1254. This is supported by the fact that the samples fortified with pure Aroclor 1254 exhibit ELISA/GC ratios very close to one (Fig. 4.5).

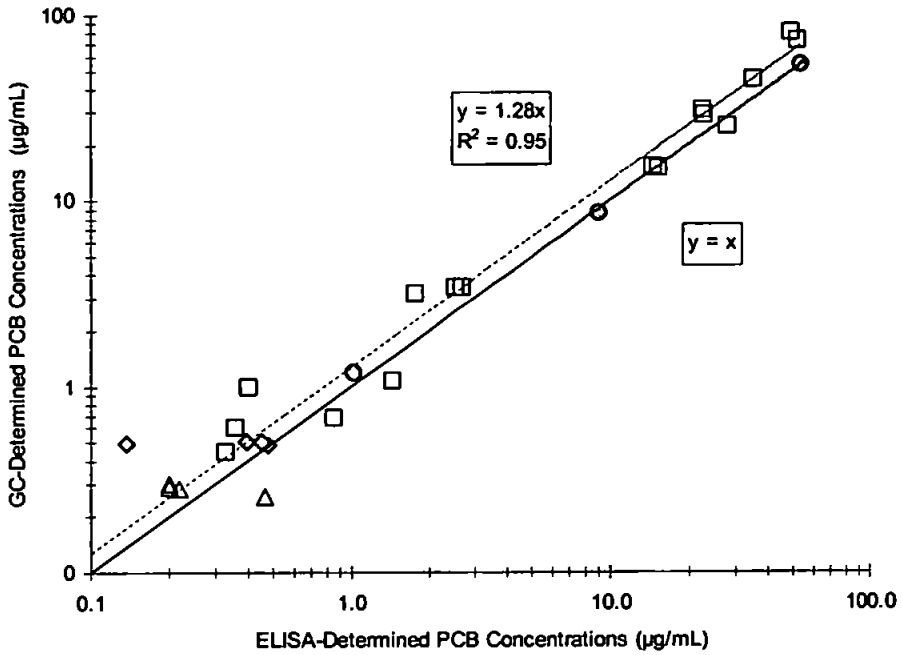


Figure 4.5. Correlation between PCB concentrations in field-contaminated samples (New Bedford Harbor (□); Whitsand Bay (◆)), Aroclor 1254-fortified mussel tissue (●) and standard reference material (Δ). GC-determined PCB concentration - Σ 128 congeners; ELISA-determined PCB concentration – Aroclor 1254 "equivalents".

Our results are consistent with levels and congener patterns previously reported for New Bedford Harbour (Pruell *et al.*, 1990; Lake *et al.*, 1995; Ho *et al.*, 1997). Whilst physical, chemical and metabolic processes can potentially discriminate between congeners, the cGC-ECD data closely matches the patterns reported for other bivalves, sediments and water in New Bedford Harbour (Pruell *et al.*, 1990; Lake *et al.*, 1995).

This is in agreement with negligible metabolism of PCBs by the mussels (Brown, 1992), although Lake *et al.* (1987; 1995) do suggest enhanced accumulation of mid range congeners. No previous data are available for mussels from Whitsand Bay, UK.

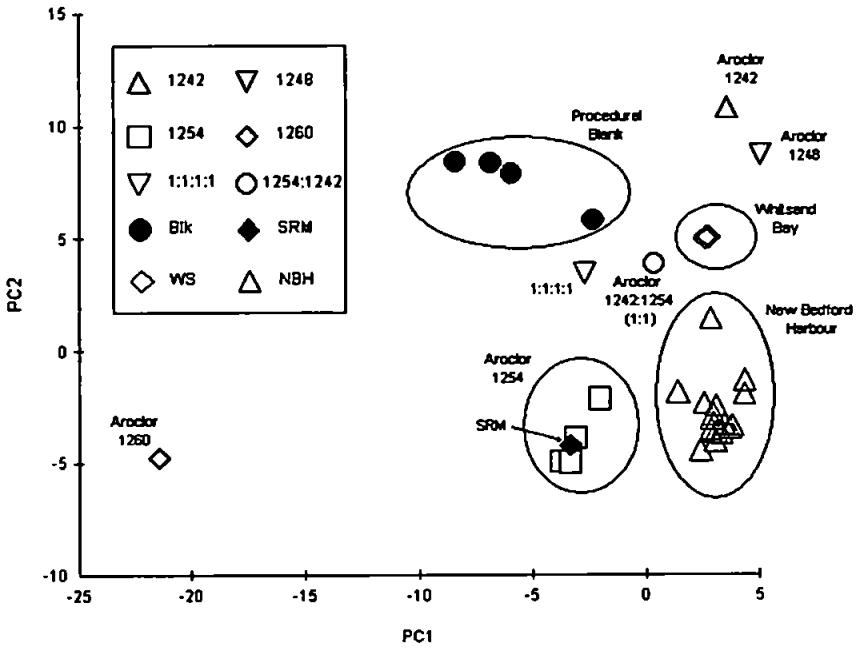


Figure 4.6. Principal component analysis (PCA) for Aroclor mixtures and environmental samples analysed by cGC-ECD. 1:1:1:1 – equal parts of Aroclor 1242, 1248, 1254 and 1260; 1254:1242 – equal parts of Aroclor 1242 and 1254; SRM – standard reference material; WS – Whitsand Bay (UK); NBH – New Bedford Harbor (USA); 1254 - Aroclor 1254 and Aroclor 1254-fortified mussel tissue.

A comparison of PCB data for the different sites and samples is presented in Fig. 4.7. Comparison of means (± 2 standard error), based on four samples for each site, shows that the techniques are comparable over a wide range of concentrations. Indeed, no significant differences were detected ($p>0.05$) with PCB concentrations up to 30 $\mu g\ g^{-1}$. It is, however, revealed that for the samples with the highest concentrations of PCBs, whilst the trend in increased concentration is shown by both techniques, results from the two methods are statistically different ($p<0.01$) (Fig. 4.7). This marginal difference probably relates to the fact that even after dilution, the measurement was made at the upper part of the calibration curve. It is notable, however, that larger differences can be observed between individual environmental samples taken from the same location (Bergen *et al.*, 2001). Thus, the lower ELISA results could potentially relate to methodological differences, especially solvent exchange and sample dilution. It was necessary to transfer the ELISA extract from hexane to methanol. This involved taking

the extract to dryness followed by redissolution of the residue in methanol. Although this can result in losses of volatiles or problems related to dissolution of hydrophobic contaminants into a comparatively polar solvent, Zajicek *et al.* (1996) have shown good and quantitative recoveries for this procedure with PCBs. It is also noteworthy that the ELISA diluent (50% v/v methanol/buffered solution) is polar and the solubility of hydrophobic chemicals is likely to be reduced. Li and Andren (1994) measured solubilities of different PCBs (PCB 3, PCB 30, and PCB 155) in mixtures of water and methanol. Concentrations of PCBs in the extracts in the present study are generally well within the solubility limits determined by Li and Andren (1994). With the most concentrated samples (Site 4, Fig. 4.7), however, limits of solvation are being approached.

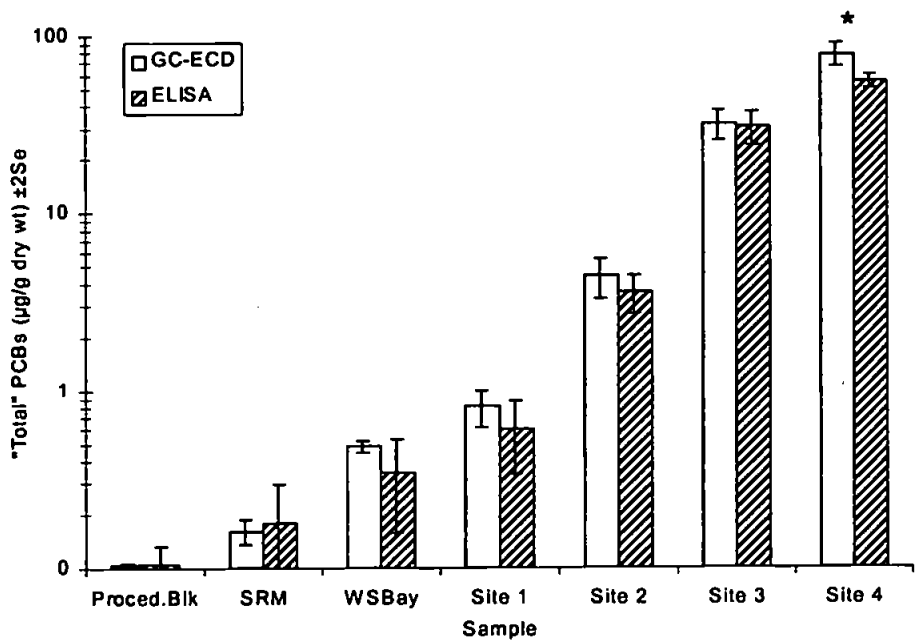


Figure 4.7. Results (µg g^{-1} dry wt) of procedural blanks, standard reference material (SRM) and environmental samples (sites 1, 2, 3 and 4 - New Bedford Harbour, USA; WSBay - Whitsand Bay, UK) analysed by ELISA and cGC-ECD. Values shown are means (± 2 standard errors, n = 4). * - *t*-test $p<0.01$. †"Total" PCB - GC (Σ 128 congeners); ELISA (Aroclor 1254 "equivalents").

Finally, a second exploratory statistical procedure, similarity analysis (Primer®), was used to investigate the correlation between immunoassay results and individual congener distributions. It revealed the highest correlations to be between prominent congeners in the Aroclor 1254 mixture and supports the conclusion that PCB ELISA

results are affected by the degree of chlorination. It did not, however, identify any specific substitution pattern to be more highly correlated.

4.4.3 Conclusions

ELISA can be used to measure "total" PCBs in hydrophobic extracts following removal of lipids and non-polar solvent.

The accuracy of PCB ELISA measurements can be maximised by grouping samples with a common source of PCB contamination and by using an appropriate technical PCB mixture as the calibration standard. The reactivity of the polyclonal antibody used allows the detection of Aroclor 1248, 1254 and 1260, with a good degree of agreement. In the present study, where environmental samples were contaminated with PCB patterns similar to that of Aroclor 1254, consistent results are reported.

Although cGC-ECD affords the capability to quantify individual congeners of differing reactivity and toxicity, the data reported indicates that ELISA analyses of mussel tissue offer a rapid general indication of the level of contamination. Even though Soxhlet extraction and partial clean-up is necessary to remove lipids, ELISA is not as time consuming or expensive as GC analyses. Because the same sample is used and extracted for both ELISA and GC-ECD analyses, initial ELISA screening can be used to identify samples appropriate for chromatography. After sample preparation, twenty quantitative ELISA analyses (in duplicate) can be obtained in less than 2 hours. The procedure described, which involves Soxhlet extraction and partial purification, is not, however, suitable for adaptation to "on-site" monitoring of PCBs.

4.5 PAH/PAH metabolites in crab urine

4.5.1 Introduction

Biological monitoring data are often essential for exposure assessments. Polycyclic aromatic hydrocarbons (PAHs) occur ubiquitously as complex mixtures in the environment and there is very strong evidence that some of them are carcinogenic to organisms (Geraci and Aubin, 1990; Di Giulio *et al.*, 1995; Baird, 1999). Generally, environmental exposure of organisms is assessed by monitoring their environment (sediment/soil, water and air). Biomonitoring, however, can provide an assessment of the integrated uptake through all exposure routes. This is important since only the

bioavailable chemical is likely to be assimilated/concentrated in tissues, body fluids and excreta. Owing to the complexity of biological samples, however, complex analytical protocols are traditionally used for analyses, and are often time consuming, labour intensive, and expensive.

Biomarkers of exposure can measure either the concentration of contaminants in body fluids or tissues of exposed individuals (Jongeneelen, 1997). Since PAHs comprise a mixture of compounds, biomarkers can either address the whole group of PAH isomers or specific/single PAHs (Jongeneelen, 1997). A variety of biomarker techniques have been used to investigate PAH exposure, e.g. urinary PAH-metabolites, DNA-adducts, cytochrome P450, and analytical measurements using immunochemical or chromatographic methods. Specific hydroxy-PAHs (e.g. 1-OH-pyrene) in urine (Levin *et al.*, 1995; Blackburn *et al.*, 1999) (Strickland and Kang, 1999; Jongeneelen, 2001) and immunoassays using monoclonal antibodies to detect specific DNA-adducts (Casale *et al.*, 1996; Bentsen-Farmen *et al.*, 1999) provide single PAH-markers. ^{32}P-post-labelling assays can detect total aromatic DNA-adducts, giving a measure of exposure to "total" PAH (Jongeneelen, 1997; Marafie *et al.*, 2000; Koganti *et al.*, 2001). Quantification of various PAH in urine has been suggested to be useful biomarker, either after 'reversed metabolism' (enzymatic hydrolysis) to parent PAH (van Schanke *et al.*, 2001) or by determining a variety of hydroxylated-PAH (Mannschreck *et al.*, 1996; Grimmer *et al.*, 1997; Angerer *et al.*, 1997).

Immunochemical methods, such as enzyme-linked immunosorbent assay (ELISA), have been routinely used for monitoring of human exposure (Knopp, 1995; Biagini *et al.*, 1995; Mastin *et al.*, 1998; Knopp *et al.*, 1999; Striley *et al.*, 1999; MacKenzie *et al.*, 2000). Only recently ELISA techniques have been tested on body fluids and excreta of aquatic organisms (Fillmann *et al.*, *In Press b*). Polyclonal ELISA can provide a "total" PAH measure without the need for enzymatic hydrolysis (Fillmann *et al.*, *In Press b*). This allows contributions from both pyrolytic and petrogenic sources to be assessed. Compared to conventional chromatographic methods, ELISA is rapid, easier and less expensive to use, can afford comparable limits of detection, and is easily adapted for use in the field (Mastin *et al.*, 1998). By integrating ELISA with urine analyses, an elegant (non-destructive) measure of exposure is afforded. Cross-reactivity and kinetics of metabolism and excretion, however, require consideration and testing.

In the present study, which includes laboratory exposure experiments and field investigations, the effectiveness of ELISA for measuring PAH metabolite levels in the urine of exposed aquatic crabs (*Carcinus maenas*) is assessed by: a) evaluating matrix effects associated with the urine; b) assessing cross-reactivities of the ELISA antibodies with parent PAH and their metabolites (1-OH-pyrene and 9-OH-phenanthrene); c) investigating sensitivity and limits of detection; d) determining whether immunoassay results correlate with more conventional analytical techniques (UV-fluorescence), and e) identifying the main urinary pyrene metabolites produced by *Carcinus maenas* using high performance liquid chromatography (HPLC). A commercially available immunoassay kit was selected for urinary biomonitoring (PAH RaPID Assay®, Strategic Diagnostics Inc., Newtown, PA). This kit was originally designed to measure PAH in water.

4.5.2 Results and Discussion

When using biological monitoring techniques, to effectively assess exposure of an organism to a chemical, the metabolism and excretion of the compound needs to be considered. The approach adopted in this research uses controlled experimental dosing utilising different quantification procedures and includes identification of metabolites. Environmentally exposed crabs from clean and contaminated site are then investigated to assess whether the presence of metabolites or differences in their concentrations can be measured by ELISA.

4.5.2.1 Analytical Performance

Fluorescence Spectrometry. The fluorescence spectrometry was calibrated against the major metabolites 1-OH-pyrene and 9-OH-phenanthrene. Linearity within the concentration range of 0-200 $\mu g\ L^{-1}$ exceeded 0.99 for both compounds. The limit of detection (LOD) of the fluorescence assay (defined as the average blank signal ± 3SD) was 6.2 ± 0.5 $\mu g\ L^{-1}$ for 1-OH-pyrene and 6.8 ± 1.7 $\mu g\ L^{-1}$ for 9-OH-phenanthrene. Blanks revealed negligible fluorescence. With analyses of the urine, for practical purpose (urine volume) and to negate matrix effects, samples were diluted at least 1:20 for pyrene and at least 1:40 for phenanthrene exposed crabs.

Immunoassay. The linearity of standard calibration curves of pyrene and phenanthrene analysed by ELISA was > 0.97. The method detection limit (MDL) for the PAH RaPID Assay® defined as the concentration giving 90% B/Bo was 0.92 ± 0.08

μg L^{-1} (phenanthrene), 24.5 ± 2.7 μg L^{-1} (9-OH-phenanthrene), 9.09 ± 0.76 μg L^{-1} (pyrene) and 8.82 ± 0.96 μg L^{-1} (1-OH-pyrene) (Table 4.5). The coefficient of variation (%CV) for repeated analyses for a single sample assay was 12 ± 5% (n = 8), similar to variations with conventional analytical techniques. A 1:20 dilution was found to be required to minimise matrix effects (see below), thus providing practical environmental MDLs 20-fold higher than these concentrations quoted for the standards.

Table 4.5. Reactivity of parent PAHs and metabolites in the ELISA (n = 3)

	MDL[a] (μg L^{-1})	50% B/Bo[b] (μg L^{-1})	Cross- reactivity (%)
Phenanthrene	0.92 ± 0.08	28.7	100
9-OH-phenanthrene	24.5 ± 2.7	655.2	4.4
Pyrene	9.01 ± 0.76	280.6	10.2
1-OH-pyrene	8.82 ± 0.96	290.9	9.9

[a] MDL – method detection limit (90% B/Bo)

[b] 50%B/Bo – is the concentration required to inhibiting one-half of the colour produced by the negative control.

Table 4.5 shows the cross-reactivity and 50% B/Bo (IC_{50}) values (concentration required to inhibit one-half of the colour produced by the negative control) determined for phenanthrene, 9-OH-phenanthrene, pyrene and 1-OH-pyrene using PAH RaPID Assay®. The ELISA proved to be highly sensitive to phenanthrene (indicated by low 50% B/Bo values), but less so for pyrene and monohydroxy metabolites. The better performance for phenanthrene is explained because the immunoassay antibodies were raised against phenanthrene (Waters *et al.*, 1997a). Table 4.6 shows a comparison of 50% B/Bo values for other PAHs (taken from the manufacturer's documentation). These results indicate that the ELISA will detect most PAH (albeit with differing sensitivities) potentially affording an effective monitor of exposure to environmental PAHs.

Undiluted human urine interferes with the ELISA to seriously hinder its sensitivity and dynamic range (Mastin *et al.*, 1998; Knopp *et al.*, 1999; MacKenzie *et al.*, 2000). In order to investigate matrix effects for the PAH ELISA assay, aliquots of diluted control crab urine (1:5 to 1:80) were analysed by the ELISA. A representative plot of the urine samples is shown in Fig. 4.8. Even though differences in absorbances (or B/Bo) were

evident in undiluted or slightly diluted urine samples, at dilutions equal or greater than 1:20 matrix-induced differences were minimal (Fig. 4.8).

Table 4.6. Specificity (cross-reactivity) of parent PAHs and metabolites in the ELISA. Taken from the manufacture's insert (RaPID Assay®)

	MDL[a] ($\mu g\ L^{-1}$)	50% B/Bo[b] ($\mu g\ L^{-1}$)
Phenanthrene	0.93	21.9
Fluoranthene	0.43	6.3
Benzo(a)pyrene	0.67	9.2
Pyrene	0.27	10.2
Chrysene	0.53	10.4
Anthracene	0.72	14.6
Indeno(1,2,3-c,d)pyrene	1.04	36.2
Benz(a)anthracene	1.02	37.8
Fluorene	2.19	46.8
Benzo(b)fluoranthene	1.21	72.1
Acenaphthylene	13.3	595
Benzo(k)fluoranthene	1.02	697
Acenaphthalene	17.2	915
Benzo(g,h,i)perylene	19.6	>1330
Naphthalene	86.5	>1330
Dibenzo(a,h)anthracene	34.2	>1330

[a] MDL – method detection limit (90% B/Bo)

[b] 50%B/Bo – is the concentration required to inhibiting one-half of the colour produced by the negative control.

4.5.2.2 Performance of ELISA

4.5.2.2.1 Laboratory exposure experiments

Response to exposure. To evaluate whether or not crabs are useful in biomonitoring PAH exposure it is necessary to investigate the dose-response relationship. It is also important to understand the kinetics of metabolism and excretion. Time- and dose-response experiments for *C. maenas* demonstrate that urinary levels of pyrene "equivalents" reached a maximum after 48 hrs (Fig. 4.9). Urinary levels proved to be dose-dependent for both phenanthrene (Fig. 4.10) and pyrene (Fig. 4.11). A simple regression analysis of mean urinary levels over the range of exposure concentrations produced a correlation coefficient of above 0.8 for both parent PAHs.

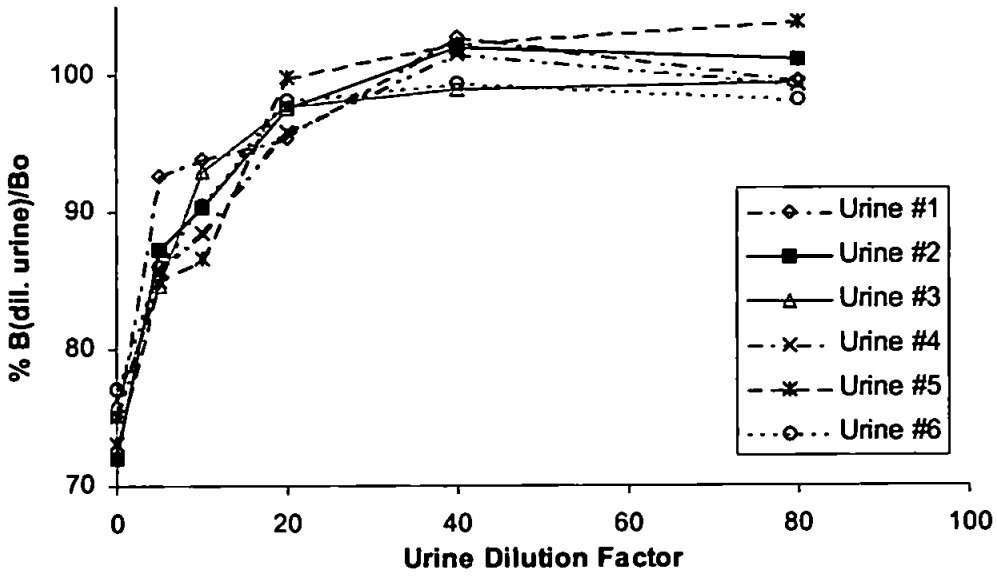


Figure 4.8. Matrix effects of crab urine on the PAH RaPID Assay® ELISA. B/Bo is the absorbance observed for a sample or standard divided by the absorbance at the zero standard)

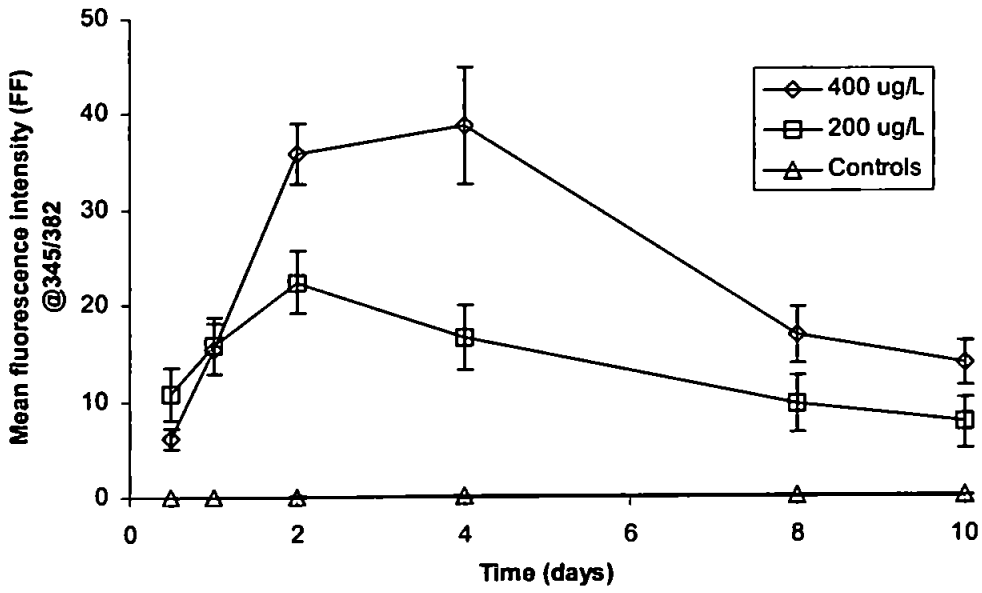


Figure 4.9. Fluorescence intensity (FF) measured in the urine of shore crabs exposed to pyrene for up to 10 days. Peak concentrations are reached between 2 and 4 days

Fluorescence spectra. Fixed excitation wavelength fluorescence (FF) analyses of urine samples from pyrene exposed crabs produced three distinct peaks on the emission spectrum, with the largest at approximately 382nm (Fig. 4.12). In phenanthrene exposed crabs urine a broad fluorescence in the emission range of 350-400nm resulted (Fig.

4.13). These peaks are conspicuously absent from control samples (Figs. 4.12 and 4.13). Synchronous excitation/emission fluorescence spectrometry (SFS) reduced these peaks to a single emission band, with a large sharp peak around Ex340/Em382 nm in pyrene exposed samples and around Ex300/Em354 nm in phenanthrene exposed samples (not shown). Once again, controls lacked these peaks. The position of the dominant emission peaks seen in FF and SFS for pyrene exposed samples is shifted approximately 5nm from that of the largest peak seen in the 1-OH-pyrene standard spectra (~Em387 nm) (Fig. 4.12). The unresolved "shoulder" to the main peak (exposed sample) is interesting since it indicates (together with the main peak) the presence of compounds with potentially similar properties of 1-OH-pyrene, possibly pyrene conjugates. Parent pyrene is almost certainly absent from the urine since its characteristic synchronous peak at 372 nm (wavelength difference of 37nm) is not present in the urinary spectra of exposed crabs. According to Ariese *et al.* (Ariese *et al.*, 1993), the emission spectrum of conjugated 1-OH pyrene (e.g. pyrene-1-glucuronide) is blue shifted by 5nm. The results above might indicate that *C. maenas* can transform pyrene and phenanthrene following exposure and excrete them in the urine as more water-soluble metabolites/conjugates.

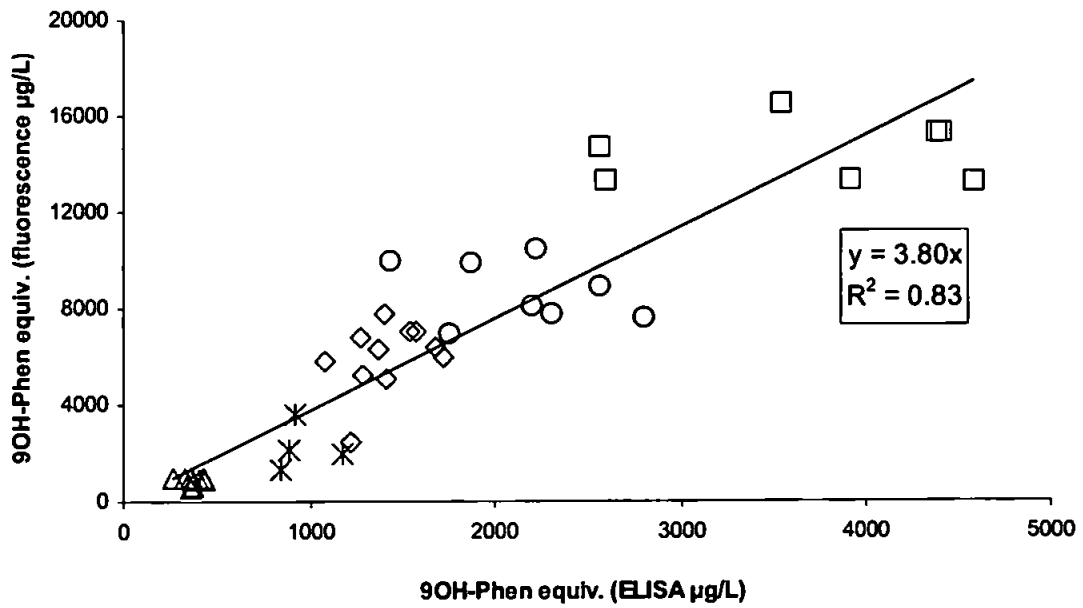


Figure 4.10. Correlation between PAH RaPID Assay® ELISA and $FF_{252/357}$ analyses of phenanthrene metabolites in crab urine. Exposure concentrations: (□) 200 µg L^{-1}, (○) 100 µg L^{-1}, (◇) 50 µg L^{-1}, (☂) 25 µg L^{-1}, (Δ) 0 µg L^{-1}

From previous studies, it is known that aquatic organisms are capable of metabolising PAH. Primary and conjugated metabolites of pyrene, phenanthrene and benzo(a)pyrene were identified in bile and urine of vertebrates and invertebrates (Solbakken *et al.*, 1980; James *et al.*, 1988; James *et al.*, 1991; Ariese *et al.*, 1993; Li and James, 1993; Law and Biscaya, 1994; Aas *et al.*, 1998; James and Boyle, 1998). Marine crustacean studies have shown that hepatopancreatic and other organs microsomes contained cytochrome P450 (Li and James, 1993; James and Boyle, 1998), which can metabolise PAH. PAH metabolism studies in shore crab (*C. maenas*) have shown that benzo(a)pyrene is rapidly metabolised to primary and conjugated metabolites (McElroy and Colarusso, 1988; Lemaire *et al.*, 1993).

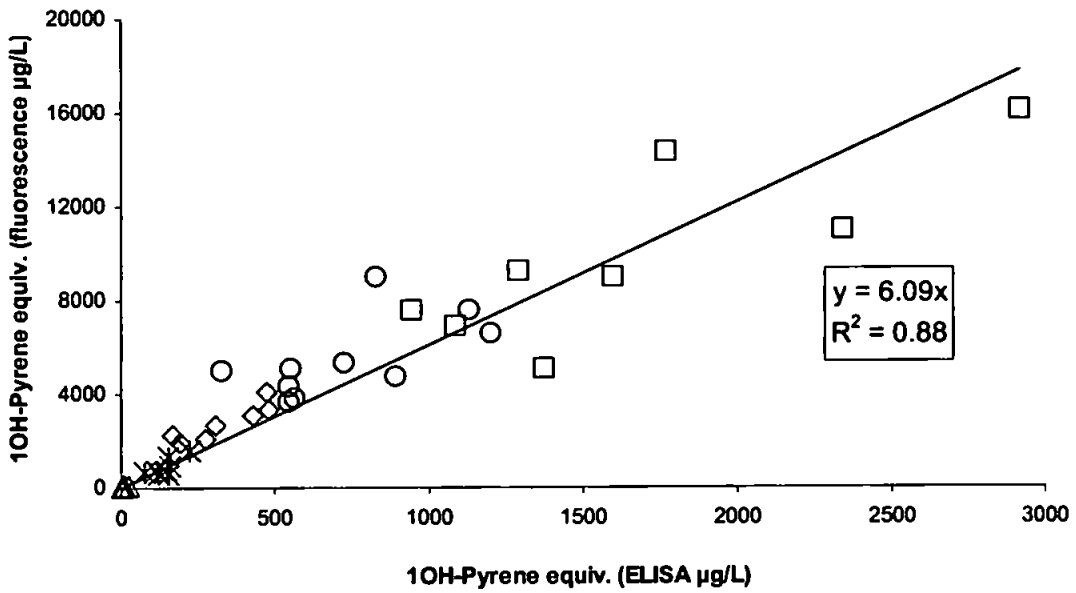


Figure 4.11. Correlation between PAH RaPID Assay® ELISA and $FF_{345/382}$ analyses of pyrene metabolites in crab urine. Exposure concentrations: (□) 200 µg L^{-1}, (○) 100 µg L^{-1}, (◇) 50 µg L^{-1}, (☂) 25 µg L^{-1}, (Δ) 0 µg L^{-1}

HPLC analysis. The fluorescence data (described above) indicate strongly that the pyrene is being metabolised. For this reason, HPLC analyses of urine samples were undertaken to investigate the metabolites. From the literature, the initial metabolite is thought to consist mainly of 1-hydroxypyrene, which is subsequently conjugated into Phase II metabolites (Stroomberg *et al.*, 1996; Stroomberg *et al.*, 1999). Our results concur with this and indicate conjugation into three major metabolites. A typical chromatogram is shown in Fig. 4.14. Two of the conjugates were identified as pyrene-1-

glucoside and pyrene-1-sulfate (Fig. 4.14). The identity of one other conjugate is still unknown but Stroomberg and co-workers, who have found the same metabolite in isopods, have, using electrospray mass spectrometry, established that it has an m/z ratio of 467 (Stroomberg *et al.*, *Submitted*). Evidence of a malonate and a glucoside functional groups on a pyrene moiety has been obtained (Stroomberg, personal communication). The other two peaks present in the chromatogram could not be identified (~17.6 / 18 min, and 24.2 min, Fig. 4.14), but from their fluorescence properties are possibly pyrene or pyrene-like structures. None of the metabolites observed coeluted with pyrene-1-glucuronide, although the peaks at 17.6 / 18 min were marginally close to it. In vertebrates, the preferred carbohydrate cosubstrate is UDP-glucoronic acid, giving rise to glucuronides. In crustaceans, however, the preferred cosubstrate is UDP-glucose, forming the glucosides (James, 1987; Li and James, 1993; Livingstone, 1998). Finally, the last peak in the chromatograms was parent pyrene. Although not quantified, the pyrene peak was always negligible (Fig. 4.14). The relative distributions of conjugates show that pyrene-1-glucoside and pyrene-1-'conjugate' dominated (Fig. 4.15). Comparing the relative amounts of 1-OH-pyrene and its conjugates, it was found that an average of 38.9% is present as pyrene-1-glucoside, 9.7% as pyrene-1-sulfate, 47.7% as the unknown conjugate and 3.7% as non-conjugated 1-hydroxy-pyrene. The fact that the amount of 1-OH-pyrene is rather small in comparison to the pyrene conjugates indicates a high conjugation rate of 1-OH-pyrene (Stroomberg *et al.*, 1999). These ratios indicate that glucosidation is more efficient than sulfation.

The metabolite pattern detected at high exposure concentrations, however, might not necessarily reflect baseline metabolic patterns. Exposure to high levels of pyrene might involve all available metabolic pathways, thus producing many different metabolites. Comparing conjugate patterns in experimentally exposed and in environmentally exposed organisms, it can be seen that most conjugates are formed under both exposure conditions although the relative levels of metabolites are different (Figs. 4.15 and 4.16).

Compared to PAH metabolism studies on benzo(a)pyrene in shore crab (*Carcinus maenas*) (Lemaire *et al.*, 1993), the number of metabolites formed following pyrene exposure is limited to one major intermediate (1-hydroxypyrene), which restricts the number of final conjugates.

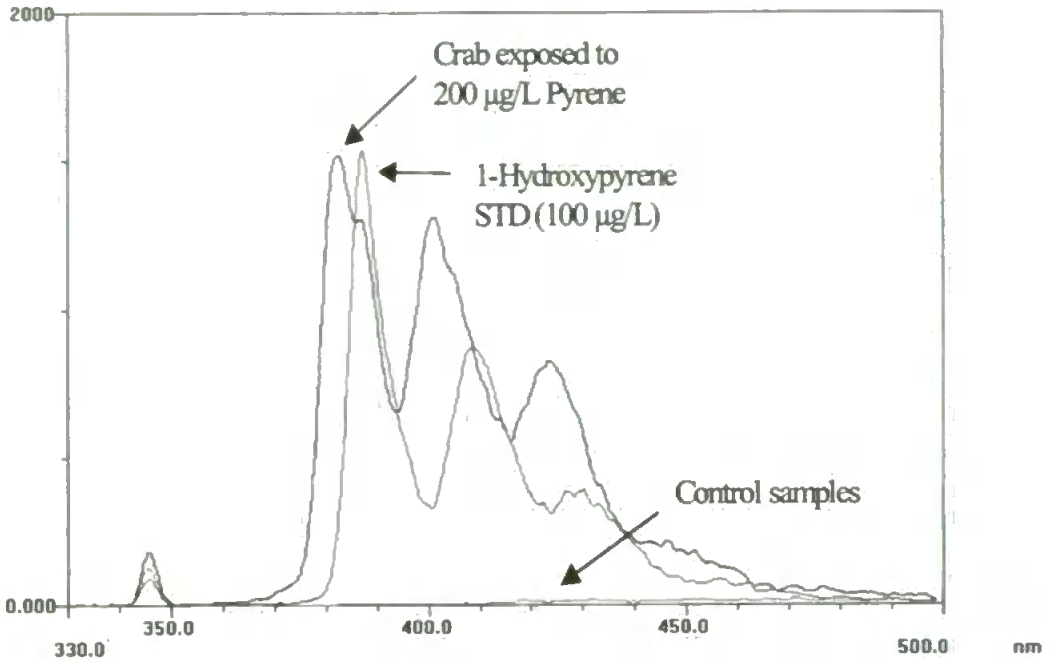


Figure 4.12. Fixed excitation wavelength fluorescence ($FF_{345/382}$) spectra of urine from crabs exposed to pyrene

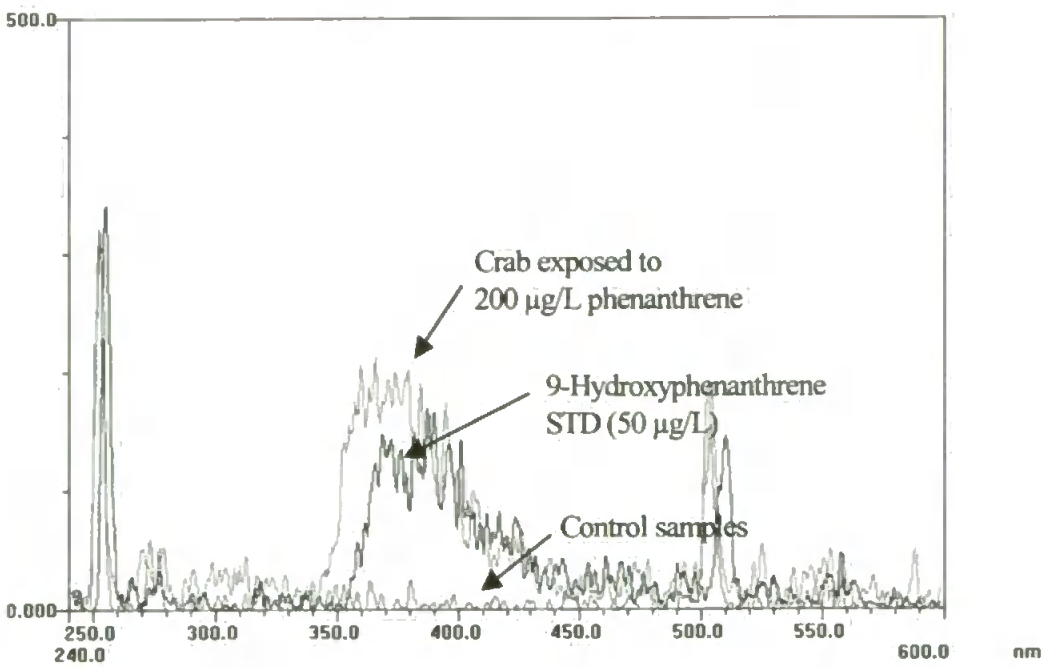


Figure 4.13. Fixed excitation wavelength fluorescence ($FF_{252/357}$) spectra of urine from crabs exposed to phenanthrene

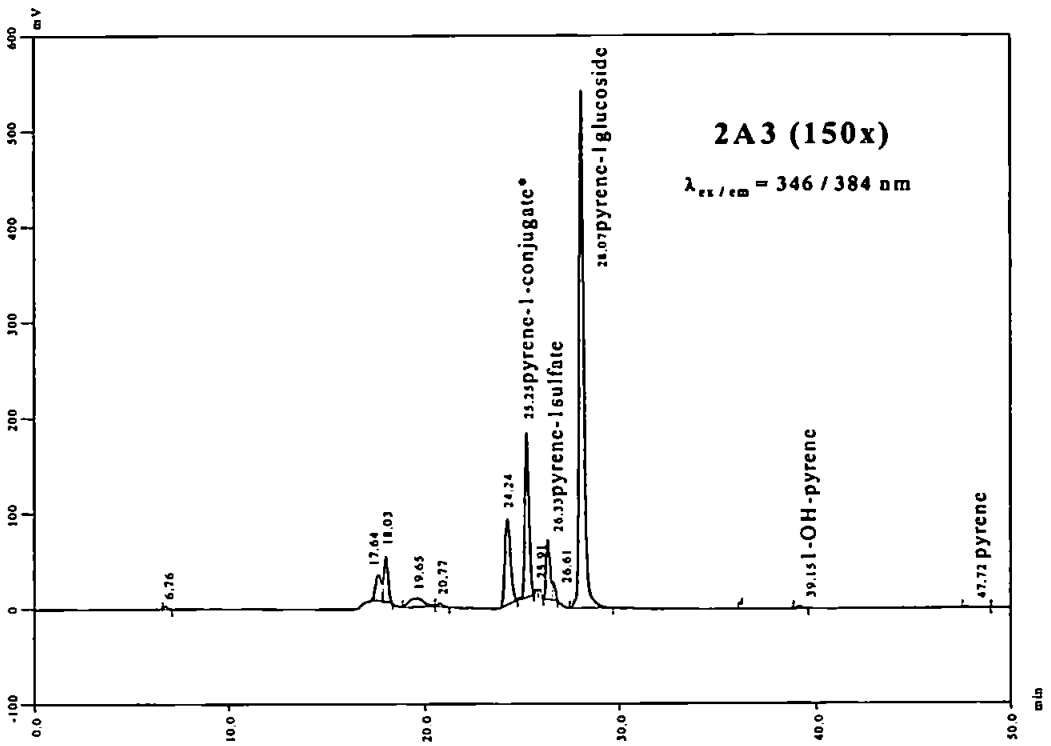


Figure 4.14. HPLC-fluorescence chromatogram ($\lambda_{ex/em}$ = 346/384 nm) of urine from crabs exposed to 200µg L^{-1} pyrene. *pyrene-1-'conjugate' has not been identified

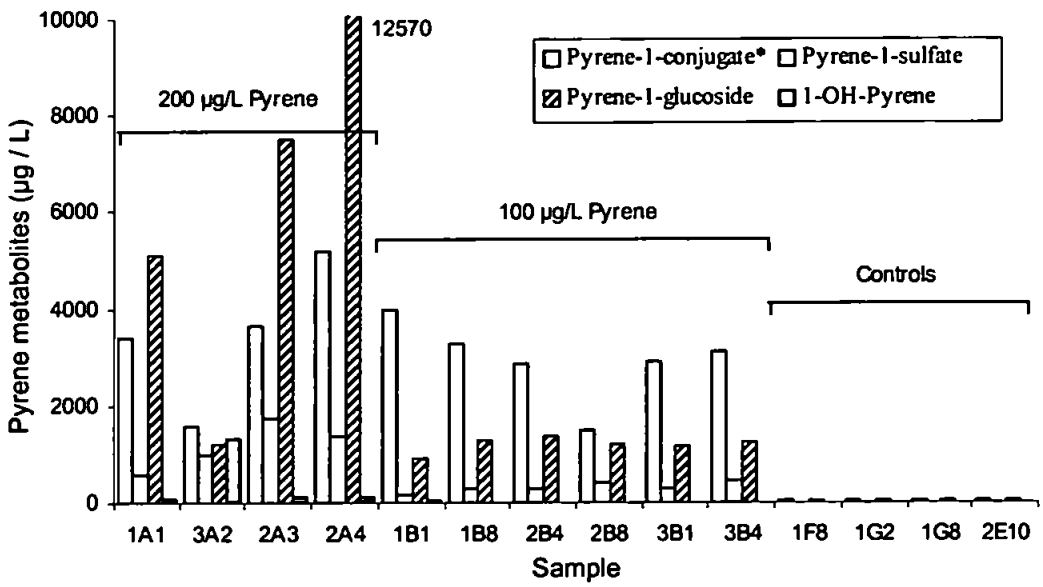


Figure 4.15. Concentrations of 1-OH-pyrene and conjugates (ng μL^{-1}) in urine from crabs exposed to pyrene (200 and 100 µg L^{-1}) and control. *pyrene-1-'conjugate' has not been identified

Comparison of ELISA, fluorescence and HPLC results. Comparison of results from the immunoassay and fluorescence techniques shows a high correlation for urine of crabs exposed to phenanthrene ($r^2 = 0.83$; n = 40) and pyrene ($r^2 = 0.88$; n = 42) (Figs. 4.10 and 4.11). ELISA results, however, were consistently lower than those obtained by fluorescence by a factor of 0.26 (9-OH-phenanthrene equivalents) and 0.16 (1-OH-pyrene equivalents) (Figs. 4.10 and 4.11). With polyclonal antibodies, reactivities differ between chemical structures such as the diverse metabolites and conjugates discussed in the previous section. It is therefore feasible that the PAH conjugates present in the crab urine samples are less reactive than the parent PAH used to raise the antibody.

PAH levels derived from HPLC results (sum of the 4 main pyrene metabolites/conjugates) show very good agreement with the ELISA ($r^2 > 0.94$) and fluorescence ($r^2 > 0.91$) data (Fig. 4.17). This affords strong validation for the "rapid" assessment techniques.

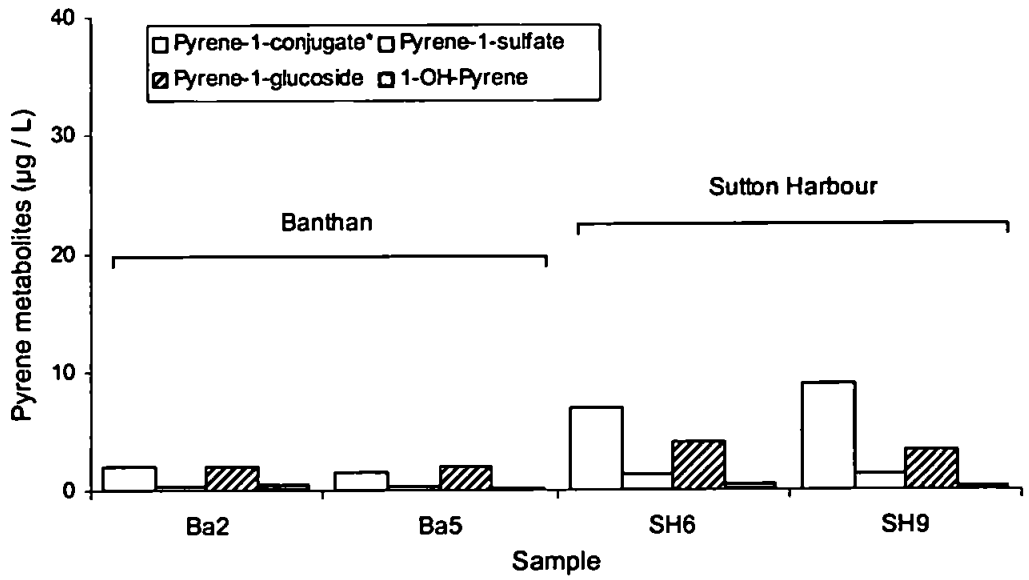


Figure 4.16. Concentrations of 1-OH-pyrene and conjugates (ng μL^{-1}) in urine from crabs taken from clean (Bantham) and contaminated (Sutton Harbour) sites. *pyrene-1-'conjugate' has not been identified

The results above provide evidence that exposure of crabs to PAH can be usefully assessed by analyses of their urine. Whilst absolute levels may be difficult to determine, both ELISA and fluorescence appear to provide a rapid and useful measure of exposure. However, it is important to extend the assessment from experimental systems to the field.

4.5.2.2.2 Environmental samples

To test the applicability of the urinary analyses to assess exposure of crabs to PAH under field conditions, crabs from clean and contaminated environments were investigated. Results are shown in Fig. 4.18 and demonstrate that urine analyses can clearly identify crabs from the differing environments. Variations in concentrations from Sutton Harbour (the contaminated site) are, however, large.

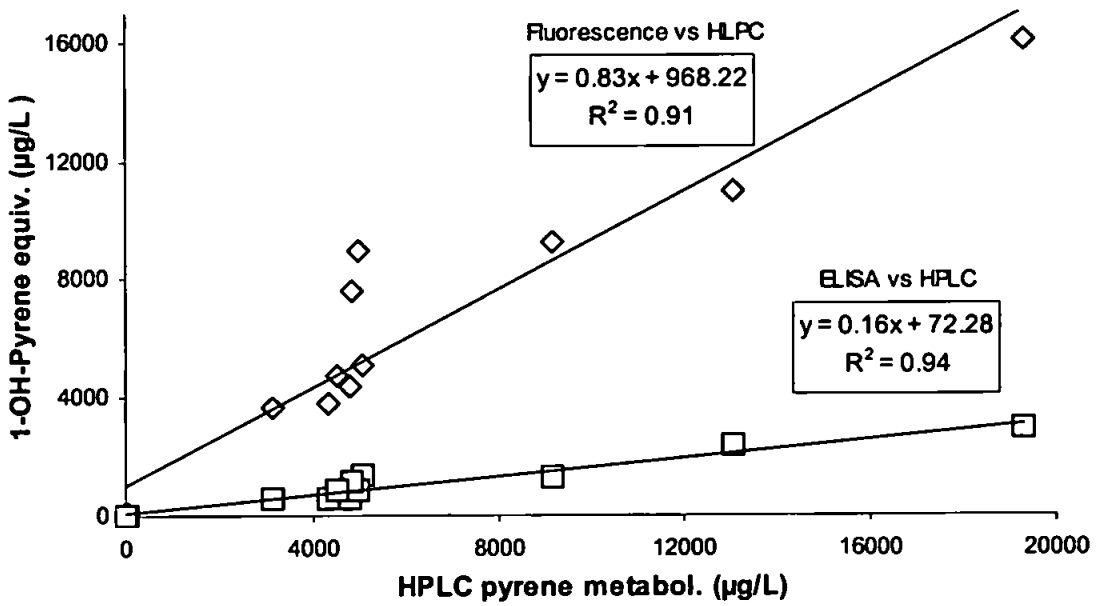


Figure 4.17. Correlations between PAH RaPID Assay® ELISA and HPLC results (□) and $FF_{345/382}$ and HPLC results (◇) of pyrene metabolites in crab urine

Comparison of the ELISA and UV-fluorescence results show that both techniques detected PAHs (mainly petrogenic) contamination in urine with good agreement (r^2 = 0.83; n = 15) (Fig. 4.18). The fluorescence spectra for the contaminated site showed a broad fluorescence characteristic of naphthalene/phenanthrene wavelengths (Fig. 4.19). In some samples, weak fluorescence at pyrene wavelengths (not shown) was also recorded. Samples taken from the clean site contained no such peaks (Fig. 4.19). To further characterise the PAH mixture, extracts were also subject to synchronous fluorescence spectrometry (SFS). HPLC/Fluorescence analyses were run on selected extracts to identify and quantify any pyrene metabolites/conjugates present in the environmental samples. Results from both Bantham (clean) and Sutton Harbour (contaminated) showed very low concentrations (close to the limits of detection of the method) (Fig. 4.16). Indeed, concentrations of PAH metabolites/conjugates approached

the levels in laboratory control samples. From the fluorescence results, however, mainly to 2-3 ring PAHs were shown to dominate the environmental PAH with only very small concentrations from 4 ring congener such as pyrene.

These data demonstrate that both the ELISA and fluorescence analyses of the crab urine can differentiate organisms from contaminated areas.

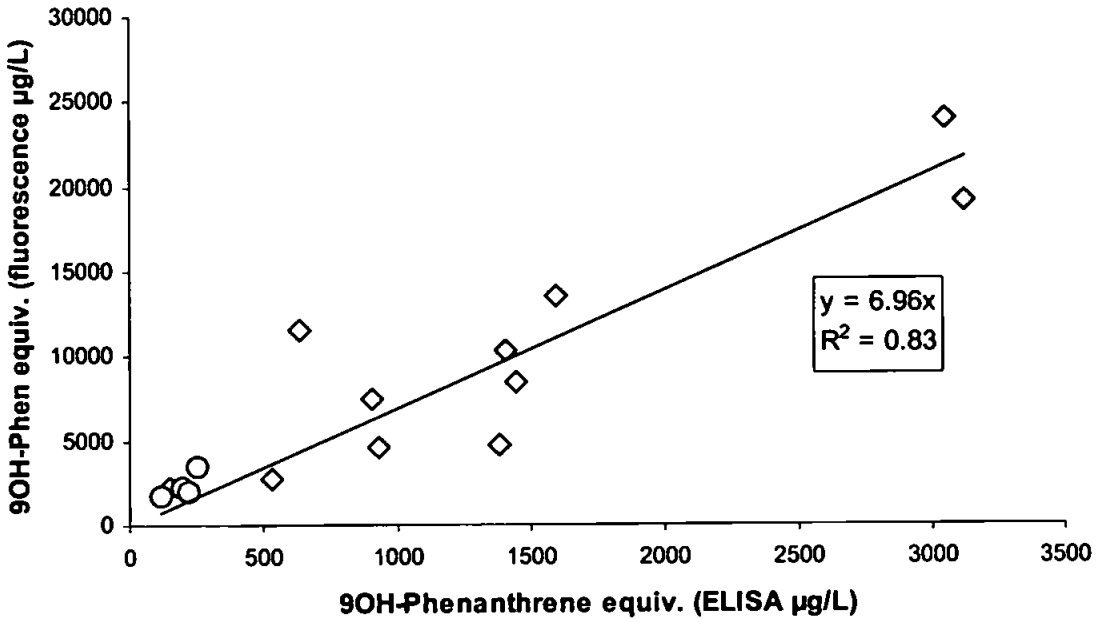


Figure 4.18. Correlation between PAH RaPID Assay® ELISA and SFS (42 nm diff.) analyses of PAH type metabolites in urine taken from environmentally exposed crabs. (●) Bantham (control) and (◇) Sutton Harbour (contaminated) - Southwest England

4.5.2.2.3 Interindividual variability of urinary PAH metabolites

Urinary PAH data showed considerable interindividual variability both within experimentally and environmentally exposed populations. In some cases, levels of urinary PAHs were 3-4 times higher in some individuals compared to others, despite being exposed to the same concentrations of parent PAH. Interindividual variations relating to biological differences are difficult to assess. Whilst this might be easier to explain with field populations, where variations in dietary, pre-exposure to xenobiotics and contaminant concentrations within sites is inevitable, it is more difficult to explain in a seemingly homogenous group of organisms (similar size, sex, and moulting stage) kept under experimentally controlled conditions. This might partly be explained by interindividual variations in absorption, metabolism and/or excretion of PAHs. Variations in the rate and degree of metabolism will also be influenced by the level of

induction and efficacy of the appropriate P450 enzymes, possible pre-exposure to xenobiotics (Carr and Neff, 1988) and subtle differences in moult stage between crabs (Mothershead and Hale, 1992). Concerning field populations, interindividual variations in PAH metabolite levels in aquatic organisms (fish bile) have been previously reported (Krahn *et al.*, 1986; Lin *et al.*, 1994).

It is feasible that the variability in data might be better characterised with larger sample numbers (Siwinska *et al.*, 1998). In the absence of previous data concerning variability in crabs exposed to contaminants and experimental constraints the present design was considered optimal for a preliminary investigation. Other scientists have promoted normalisation of urinary concentrations to other components to allow for fluctuations related to dilution (Jongeneelen *et al.*, 1987). These studies, however, relate to human investigations and, whilst theoretically very appealing, no current research has identified potential candidate compounds in crustaceans. Conversely, some authors believe this normalisation procedure has little effect on the results (Kanoh *et al.*, 1993; Kang *et al.*, 1995; Levin *et al.*, 1995; Aas *et al.*, 2000).

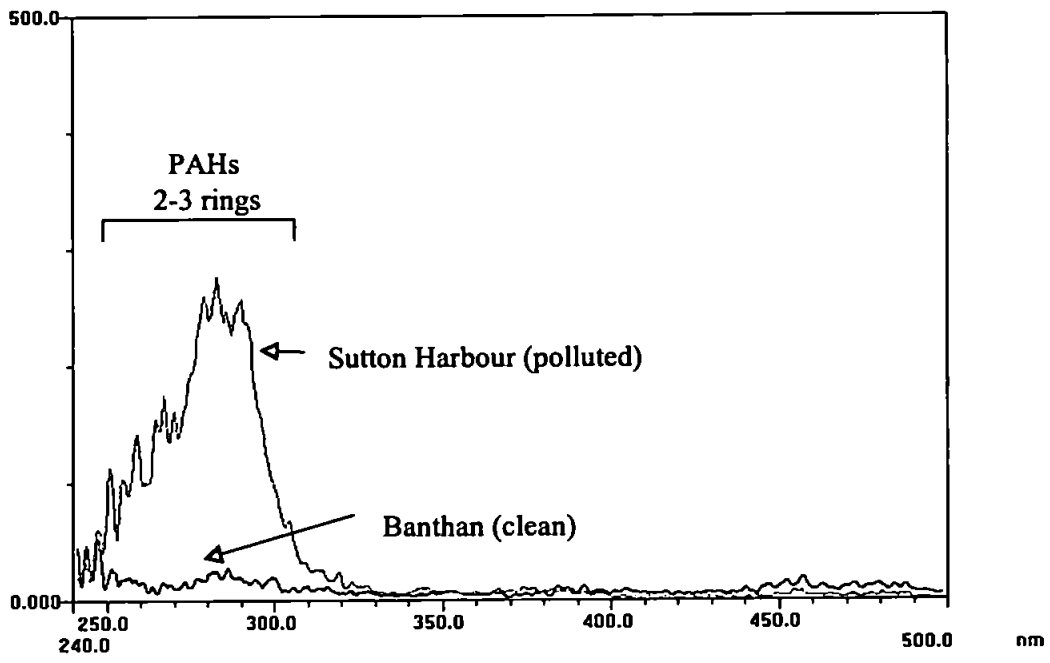


Figure 4.19. Synchronous excitation/emission fluorescence spectra ($SFS_{\Delta\lambda\ 42nm}$) of crab urine taken from environmentally exposed (Sutton Harbour) and control (Bantham) sites

4.5.3 Future research

With recent developments into new antibodies, it is now possible to selectively detect major metabolites, e.g. the phenolic glucoronides (Staimer *et al.*, 2001). This will afford greater sensitivities in future research and will also enable focussing on the most relevant metabolites.

Because metabolic oxidation of PAHs is responsible for the formation of toxic and carcinogenic intermediates (Livingstone, 1994), the determination of the level of metabolism by an organism can improve the assessment of risk. Whilst the present technique offers a most useful extension to monitoring parent/metabolite compound levels in organisms, it still retains some constraints regarding the limit between levels and effects (Kurelec, 1993). Concurrent ecotoxicological investigation can bridge this gap.

4.5.4 Conclusion

The reported results demonstrate that analysis of crab urine can enable PAH exposure to be detected.

Both the ELISA and fluorescence techniques described are adequately sensitive to the metabolites to provide a useful measure of exposure.

The ELISA tested is fairly insensitive to urine matrix effects, and a simple dilution ($\geq$ 1:20) is the only sample preparation required. With this method, 100 samples can be run in duplicate (along with standards) per day.

The major metabolites of pyrene (as determined by HPLC) were shown to be pyrene-1-glucoside, pyrene-1-'conjugate' and pyrene-1-sulfate.

Good correlations were obtained in the measurements of contaminants by the ELISA, fluorescence and HPLC techniques.

Preliminary studies demonstrate significant differences in the composition of PAH and their metabolites between crab populations sampled from contaminated (Sutton Harbour) and control sites.

Analyses of crab urine offers an inexpensive, rapid, non-destructive method to provide a measure of bioavailable contaminants taken up via all assimilation routes.

Chapter 5

5 Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassay

5.1 New Bedford Harbour Case Study

5.1.1 Introduction

Environmental managers are constantly faced with having to determine the extent of environmental contamination and identify localities and habitats most at risk. Environmental protection agencies in most developed countries achieve this aim and protect the environment using a combination of legislation and enforced chemical and biological monitoring programmes to check compliance. A wide range of ecotoxicological tools are available for detecting and assessing aquatic pollution (Depledge and Hopkin, 1995; USEPA, 1996; Anderson *et al.*, 2001). These include the monitoring of community characterisation to quantify changes in the abundance and diversity of species, sediment chemistry and tissue residue analysis to determine the bioavailability and bioaccumulation of contaminants and the use of biomarkers which relate quantifiable molecular, cellular or physiological measures in a dose or time dependent manner to the degree of dysfunction that the contaminant has produced (Peakall and Shugart, 1992).

When these procedures are combined and implemented in structured survey programmes, they can be used to identify which pollutants are responsible for environmental degradation, to identify sites at risk and to track the progress of remedial action. In recent years, individual biomarkers have been incorporated into research programmes which have highlighted the need for remedial action in a number of different aquatic ecosystems, e.g. the Venice lagoon (Lowe *et al.*, 1995) and the Black Sea Mussel Watch programme (Moore *et al.*, 1999). Only recently have suites of biomarkers begun to be included in routine management protocols (Burgeot *et al.*, 1996; Nasci *et al.*, 1998) and their combination with chemical analyses used to link chemical exposure and biological response *in situ* (Nasci *et al.*, 1999; Sole, 2000; Michel *et al.*, 2001). The widespread implementation of this approach has so far been limited, partly because complex instrumental methods can make detailed studies of contaminated sites expensive

and time-consuming, but also by a lack of understanding of how biomarkers can be incorporated into legal instruments.

In response to these practical and cost limitations, suites of rapid, robust biological methods have been developed in the laboratory for the detection of exposure to chemicals, sublethal biological damage and organismal health in marine biota which require only basic laboratory equipment and can be performed by personnel with non-specialist laboratory skills (Depledge, 2000). These biomarkers have been used together with commercial enzyme-linked immunosorbent assays (ELISAs), which use antibodies to measuring the presence of diverse environmental contaminants in soil and water samples (see Chapter 2 and 4). This so-called RAMP (Rapid assessment of marine pollution) approach has undergone preliminary testing in Brazil, Costa Rica and Vietnam where it has shown considerable promise. Recently we have applied these ELISAs to biological matrices including mussel tissue extracts (see Section 4.4) (Fillmann *et al.*, *Submitted a*). Complementary extraction procedures for hydrophobic contaminants have been developed and refined, and comparisons performed with more conventional chromatographic techniques. Close correlations have been reported between ELISA and chromatography for total PCBs in tissue extracts (see Section 4.4).

The aim of this study was to determine the viability of using the RAMP approach to characterise the relationship between anthopogenic contaminant levels, toxic damage and adverse health effects in the ribbed mussel *Geukensia demmissa* from sites in and around New Bedford Harbour (Massachusetts, USA). The estuary is a Superfund Site on the US EPA National Priorities List due to sediment heavily contaminated by heavy metals (Pruell *et al.*, 1990) (Ho *et al.*, 1997), polychlorinated biphenyls (PCBs) (Garton *et al.*, 1996) (Ho *et al.*, 1997) and other organics including polycyclic aromatic hydrocarbons (PAHs) (Stoffers *et al.*, 1977; USEPA, 1996). The ribbed mussel *Geukensia demissa* inhabits the upper tidal area. Bioaccumulation factors for organic pollutants are in the region of 10^5 for bivalves (Niimi, 1996) and this, coupled to relatively low metabolic transformation rates (Moore *et al.*, 1989) and a sessile, filter-feeding lifestyle make these bivalves useful bioindicators of organic hydrocarbon pollution. The biomarkers and chemical methods applied are listed in Fig. 5.1. The testing regime included biomarkers of cellular (cell viability, lysosomal integrity) and physiological (heart rate, condition index) status as well as measures of genotoxicity (micronucleus formation) and immunotoxicity (spontaneous cytotoxicity). The PCB and PAH levels of tissue extracts

were analysed by commercial ELISA and GC-ECD, GC-FID and GC-MS to afford a comparison between the different methods. Tissue metal levels were measured by ICP-MS. Exposure to metals was also assessed by determining metallothionein induction. Both simple and multi-variate statistical methods were used to describe the relationship between the biological and environmental variables. The results of this research has been submitted to the Environmental Science and Technology (Galloway *et al.*, *Submitted*).

Table 5.1. Biomarkers and chemical procedures selected and applied in this study

	Method
Biomarker	Neutral red retention time
	Spontaneous cytotoxicity response
	Cell viability
	Methallotionein analysis
	Heart rate monitoring
	Micronucleus detection
	Condition index
ELISA	"total" PCBs (RaPID Assay®)
	"total" PAHs (RaPID Assay®)
Analytical Chemistry	PCBs
	PAHs
	Metals

5.1.2 Results

5.1.2.1 Chemical analysis of tissues and extracts

The results of chemical analyses of metal residues in mussel tissues for each site are shown in Fig. 5.1. There was a discrete pattern of different metal concentrations for each of the sites which in all cases was lower than the US EPA (USEPA, 1996) results reported for sediments from the same areas (Table 2.2).

The results of total PCB values detected in extracted mussel tissues by immunoassay are shown in Fig. 5.1. Our results are consistent with levels and congener patterns previously reported for New Bedford Harbour (Pruell *et al.*, 1990; Lake *et al.*, 1995; Ho *et al.*, 1997). The results were correlated to GC-ECD analysis of the same samples (n=16) to give a correlation coefficient of $r^2 = 0.95$ with ELISA results lower than GC- ECD by a factor of 0.83 (see Fig. 4.3 and Section 4.4). The results for GC- ECD were not corrected

for procedural recovery, calculated to be ≥ 80% using the internal standard, as a similar recovery estimate was not available for ELISA. The samples were processed in largely the same manner for both analytical procedures, differing only in the final stages where ELISA samples were solvent exchanged and diluted and GC samples were further cleaned-up and fractionated using Florisil®. The level of recovery is therefore assumed in this instance to be similar for the two methods. The highest levels of PCBs (96.2 ± 5.8 μg g^{-1} dry weight) were seen unambiguously at site 4 ($p \geq 0.000$) with all four sites statistically different from each other for both ELISA and GC-ECD results. This represents a pollution gradient in tissue contamination of >100-fold between control and upper harbour sites and of around 20-fold between the upper harbour and Buzzard's Bay.

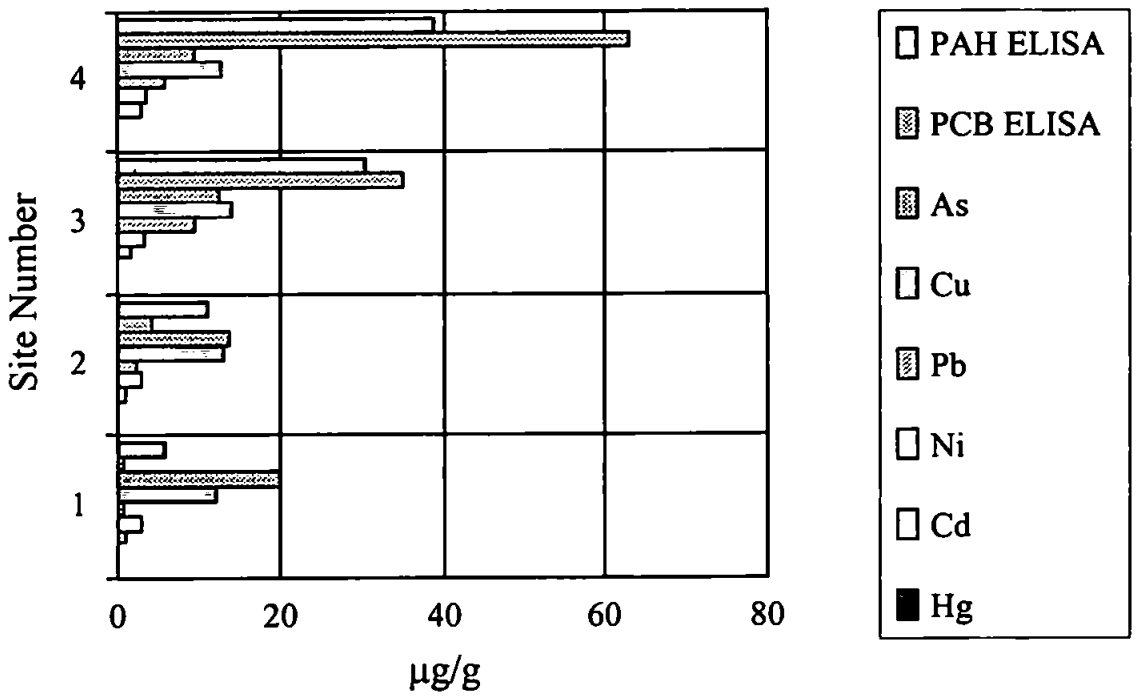


Figure 5.1. Summary of tissue chemical residue analysis according to site of collection. Results are expressed as μg g^{-1} dry wt tissue. Metals were analysed by IPC-MS, PCBs were analysed by PCB RaPID Assay® ELISA (results expressed as Aroclor® 1254 "equivalents"), PAHs were analysed by PAH RaPID Assay® ELISA (results expressed as phenanthrene "equivalents")

The relative toxicity of different PCB congeners can be explained in part by their ability to elicit dioxin-like effects. The toxic equivalency factor (TEF) proposed by Smith *et al.* (1990) for coplanar PCBs provides a consensus of toxicity for each congener relative to the most toxic congener, 2,3,7,8-TCDD. These values have been used to

calculate a Toxic Equivalent (TEQ) value for each site based on the tissue concentration of non-ortho, mono-ortho and di-ortho-PCBs present. The resulting TEQs varied from 0.034 to 3.02 ng TEQ g^{-1} dry wt and are shown in Table 5.2. Whilst these figures relate to vertebrate animals, no equivalent studies have been undertaken for invertebrates.

Table 5.2. Non-, mono- and di-ortho-polychlorinated biphenyl (PCB) congeners concentrations in *G. demmissa* tissue extracts and toxic equivalent concentrations (TEQs)

	PCB congener concentration (ng g^{-1} dry weight)			
PCB	Site 1	Site 2	Site 3	Site 4
		Non-ortho-PCBs		
PCB 77	1 (0.2)	97 (11.6)[a]	39 (5.8)	5.5 (1.1)
PCB 81	<0.3	5.3 (0.6)	2.2 (0.3)	0.3 (0.05)
PCB 126	0.05 (0.01)	4.8 (0.6)	2 (0.3)	0.3 (0.05)
PCB 169	<0.05	<0.05	<0.05	<0.05
		Mono-ortho-PCBs		
PCB 60	2.9 (1)	436 (52)	177 (26)	25 (4.8)
PCB 105	9.6 (2.3)	682 (56)	467 (85)	83 (12)
PCB 114	1.3 (0.3)	222 (22)	109 (21)	14 (4.6)
PCB 118	29 (20)	4270 (670)	2093 (211)	280 (18)
PCB 156	<0.19	44 (27)	43 (30)	5.6 (4.2)
PCB 157	<0.14	9.7 (1.2)	3.9 (0.6)	0.6 (0.1)
PCB 167	2.5 (0.6)	344 (50)	165 (16)	22 (1.1)
PCB 189	<0.05	<0.05	0.3 (0.3)	0.1 (0.1)
		Di-ortho-PCBs		
PCB 128	11 (3)	369 (61)	279 (62)	56 (14)
PCB 138	30 (5.5)	2907 (347)	1179 (175)	165 (32)
PCB 158	0.3 (0.6)	74 (14)	29 (22)	3.9 (2.8)
PCB 170	0.7 (0.3)	119 (47)	56 (30)	6.4 (3.7)
PCB 180	1 (0.9)	306 (96)	127 (63)	17 (9)
TEQ[b]	0.034	3.02	1.44	0.22

[a] Mean (standard deviation) of samples from four organisms.

[b] Calculated as the sum of TEQs for individual congeners using toxic equivalent factors proposed by Smith *et al.* (1990).

As it was possible that hydrocarbon compounds were also present in the samples, the remaining (second fraction) tissue extracts prepared for PCB analysis were used for analysis of PAH levels and the results are presented in Fig. 5.1. Although the analytical protocols and fractionations were optimised for PCB quantification, PAH were also analysed. Analytical recoveries for most PAH exceeded 75 ± 12%. For those compounds

falling outside of this performance, quantification was not undertaken. Linear regression analysis between GC-FID "total" PAH (resolved + unresolved components) and ELISA "total" PAH (phenanthrene "equivalents") for the 16 samples gave a correlation coefficient of $r^2 = 0.54$ with some discrepant results for some samples (Fig. 5.2). PAHs were present in samples from all sites with an increasing gradient of concentrations from site 1 (2.06 ± 1.8 $\mu g\ g^{-1}$ dry wt) through to site 4 (38.7 ± 35.4 $\mu g\ g^{-1}$ dry wt). All four sites were significantly different from each other (Kruskal-Wallis, $p \leq 0.01$). It was evident from the GC-FID analysis that sites 3 and 4 contained elevated quantities of aromatic unresolved complex mixtures (UCMs).

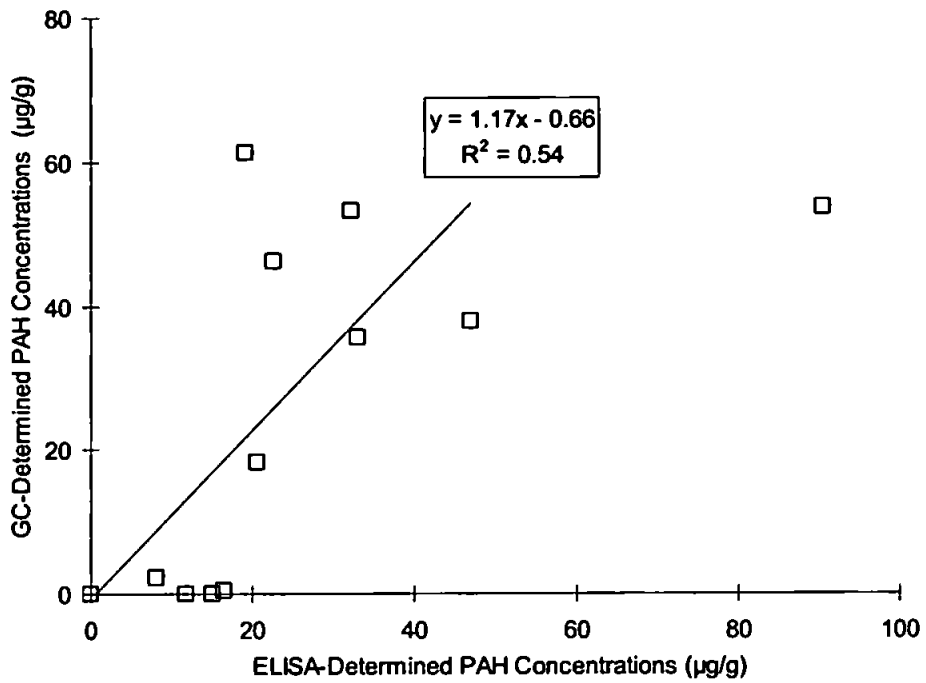


Figure 5.2. Correlation between PAH concentrations in field-contaminated samples of New Bedford Harbour. GC-determined PAH concentration - Σ resolved and unresolved PAH compounds; ELISA-determined PAH concentration – phenanthrene "equivalents"

5.1.2.2 Biological responses

The results for each of the biomarkers were initially analysed individually to determine the statistical significance of differences between sites. These results are summarised in Fig. 5.3. Significant differences were seen between sites for heart rate, spontaneous cytotoxicity response, cell viability and the presence of micronuclei (Manova: p values = 0.000, 0.002, 0.000 and 0.000) All of these exhibited increasingly detrimental effects from sites 1-4. There was no significant difference between sites for

condition index or for whole body metallothionein levels. Neutral red retention time revealed a trend of decreasing lysosomal stability between sites 1-4, but this did not reach statistical significance.

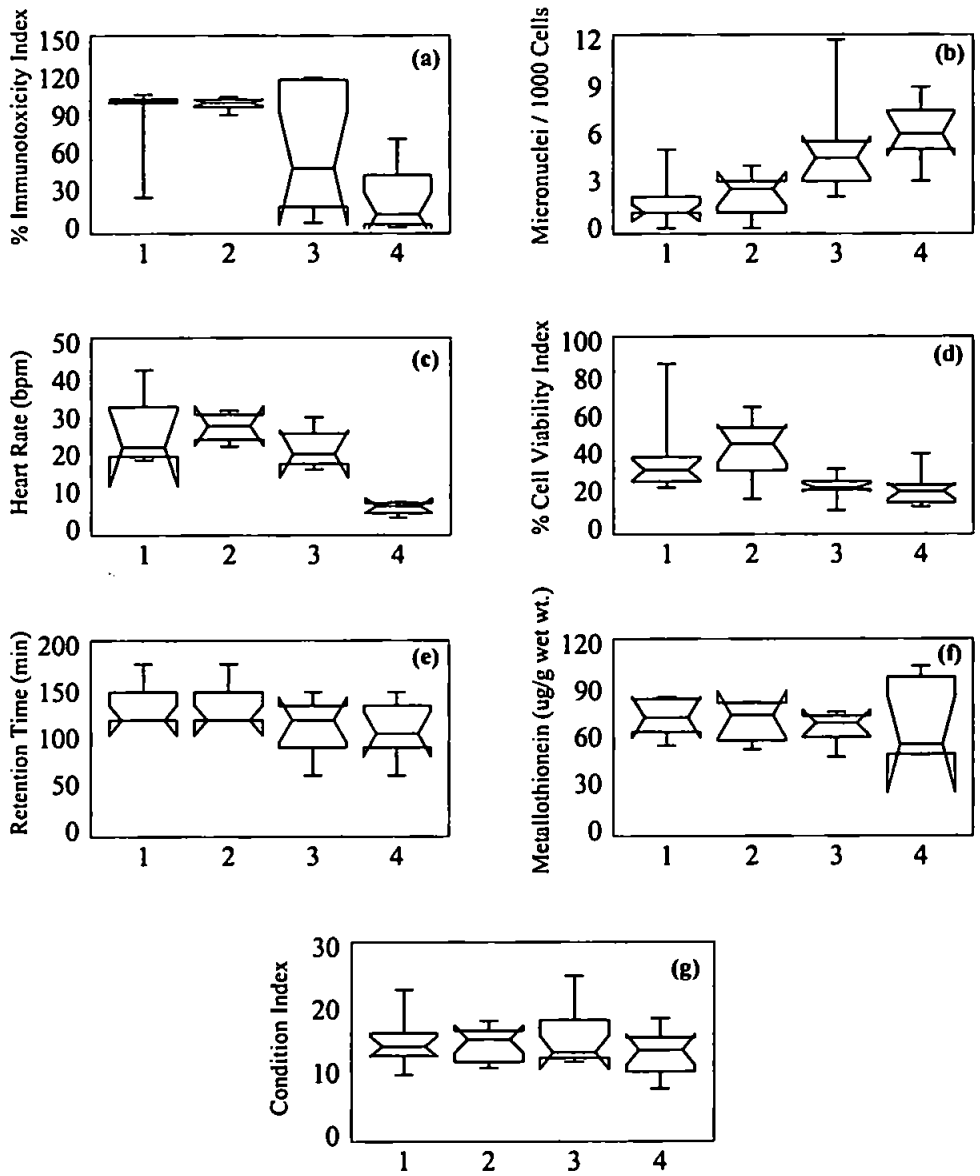


Figure 5.3. Summary of biomarker responses according to site of collection. (a) immunotoxicity measured by the spontaneous cytotoxicity assay and expressed as an index relative to the control (b) micronucleus number per 1000 cells, (c) heart rate as beats per minute (d) cell viability assessed using neutral red retention expressed as a relative index (e) lysosomal stability expressed as the neutral red retention time (f) metallothionein, $\mu g\ g^{-1}$ wet weight and (g) condition index as the ratio of whole tissue weight as a function of shell length

5.1.2.3 Multivariate analysis

In order to detect and describe differences between the different biomarkers between sites more fully, the results were subjected to a multivariate analysis of variance (Manova). An assumption was made that the within-group distributions of variables were multivariate normal with the same variances and covariances in each group. The likelihood ratio statistic Wilks' lambda was used to test for significance (Sparks, 2000). The analysis revealed that all sites differed, although it was not possible to distinguish which of the biomarkers was most responsible for the variation.

Significant differences were again evident for spontaneous cytotoxicity, cell viability, heart rate and micronucleus formation ($p \leq 0.04$, ≤ 0.04, ≤ 0.002, ≤ 0.001). Sites 1-2 (outside the breakwater) and sites 3-4 (inside the breakwater) tended to be grouped together. This was evident for all of the parameters tested except heart rate, in which sites 1-3 were grouped and site 4 was significantly different from the others.

The relationship between the biological and chemical variables was then studied using multivariate canonical correlation analysis, an ordination procedure which can be viewed as a canonical form of principal components analysis (PCA). In ordination techniques such as PCA, the aim is to replace the original, usually large set of variables by a much smaller set of derived variables which still retain most of the relevant information. Ordination thus tries to approximate the complex pattern of the full data set in a few dimensions. In canonical correlation analysis, the variables measured are divided into two sets and a linear combination of the first set of variables is created that maximises correlation with a linear combination of the second set. Thus canonical correlation analysis extracts the correlation between the two sets of variables and concentrates it in a few new pairs of variables (Sparks, 2000); in contrast to PCA which takes the variability of a single set of variables and concentrates it in a few new axes. Table 5.3 shows the first two pairs of axes and the corresponding correlations (the canonical correlations) between the axes in each pair, treating the biomarker results as one set of variables and the immunoassay results as the other set of variables. In the first pair of axes, the biomarkers axis aligned a strong positive correlation with micronucleous number and a negative correlation with heart rate and neutral red retention time. The chemical axis gave a positive coefficient for PCBs and PAHs. The correlation between these two axes was 0.959, indicating a highly significant relationship. This can be interpreted to mean that

mussels with high levels of PCBs and PAHs in their tissues had increased levels of micronucleus formation, lower heart rate and decreasing lysosomal stability.

No correlation was distinguishable between any of the biomarkers and tissue metal levels when evaluated individually by linear regression or when subjected to canonical correlation analysis with or without organic contaminants.

5.1.3 Discussion

This study has provided an illustration of how a suite of biomarkers at the biochemical and physiological level coupled with rapid chemical analysis using immunoassays can provide a detailed picture of the existence of stress situations.

Despite the high levels of metals, particularly Cu, Cr, Cd and Zn previously measured in sediments in the estuary (Table 2.2), there were no statistical differences between tissue metal concentrations between the sites and nor did any of the results fall outside archived normal concentration ranges for closely similar bivalves of similar size collected from uncontaminated areas (NOAA, 1987; Fowler, 1990). The average metallothionein levels for organisms collected from each site varied between 66-72 $\mu g\ g^{-1}$ total body wet weight and showed no statistically significant differences between sites. These values are comparable to the reported average digestive gland tissue concentration of metallothionein of around 80 $\mu g\ g^{-1}$ wet weight in mussels (*M. galloprovincialis*) of similar size collected from uncontaminated sites and subjected to the same procedure (Viarengo *et al.*, 1997). Therefore the mussels in New Bedford Harbour had neither accumulated significant quantities of metals, nor had they evidence of a biological response to the presence of metals. This illustrates how difficult it can be to predict the bioavailability of heavy metals that may accumulate in water or sediments. A proportion of the metal may be strongly bound in dissolved complexes or onto sediment surfaces, or in organic films surrounding particles. This speciation of metals can be extremely difficult to follow or predict either qualitatively or quantitatively (Salomons *et al.*, 1995).

The PCB concentration was standardised against total body dry weight, following the recommendation of Bergen *et al.* (2001) that the standard method of normalising organism PCB concentrations to total lipid may not be appropriate as a routine practice for organisms with low total lipid content. The good agreement achieved between the ELISA method and GC-ECD ($r^2 = 0.95$) demonstrate the suitability of this method (see Section 4.4). The reasons for the slight negative bias in ELISA results could be

attributable to slight differences in the congener pattern between the samples and that of Aroclor® 1254, against which ELISA was raised (see Section 4.4).

Table 5.3. Canonical correlation analysis of the relationship between seven biomarkers and two chemicals. The significance level for correlation 1 was $p \leq 0.002$.

	axis 1	axis 2
canonical correlations	0.959	0.777
Biomarkers		
spontaneous cytotoxicity	-0.012	0.389
cell viability	0.125	-0.566
condition index	0.295	-0.056
lysosomal stability	-0.384	0.734
micronucleus	0.714	0.074
metallothionein	-0.095	-0.376
heart rate	-0.255	-0.655
Contaminants (immunoassay)		
"total" PCBs	0.700	1.037
"total" PAHs	0.407	-1.183

The PAH RaPID Assay® ELISA is raised and calibrated against the three-ringed phenanthrene. Results are presented as "total" PAH (as phenanthrene "equivalents") which will include biogenic and anthropogenic aromatic hydrocarbons. Comparison of "total" PAH obtained by GC-FID and ELISA analyses of extracted tissue samples gave relatively comparable results (Fig 5.2). The correlation between ELISA and GC-FID analyses also revealed some samples, particularly from sites 3 and 4, which gave anomalous results between the two methods. This may have resulted from the relative cross-reactivity with the antibody of the different PAH constituents present at the different sites. The relative distributions of PAH compounds measured here by GC-FID was in agreement with previous studies, being dominated by parent compounds with lesser amounts of the alkylated homologues (Pruell *et al.*, 1990; Lake *et al.*, 1995).

Quantification by ELISA revealed significant contamination of mussel tissues with PCBs and PAHs representing a pollution gradient across the four study sites. The TEQ calculated as the sum of TEQs for individual congeners maintained an identical rank order to the total PCB burden revealing that the proportion of toxic congeners across the sites did not vary. The gradient for PCBs extending from inner to outer harbour and

continuing into Buzzard's Bay and West Island covers two orders of magnitude (120-fold), whilst that for PAHs is around 6-fold.

The scale and extent of the organic contaminant gradient was mirrored by the biological deterioration identified in the mussels collected from across the four sites. This contrasts again with the studies reviewed by Sole (2000) in which the effects of PAHs and PCBs on the xenobiotic metabolising system and the antioxidant defence mechanism of mussels were evaluated in four coastal European sites. The results from these studies were inconsistent, with low levels of induction of the enzyme systems relative to background levels. This reflects the incomplete understanding of molluscan xenobiotic pathways which still hinders interpretation of such approaches. In the present study, a holistic approach to the choice of biomarkers has been made, to reflect the multiple mechanisms of toxicity and ensuring physiological impairment that possible follows exposure to combinations of contaminants. Although not all biomarkers revealed differences between sites, the combination of effects seen at the molecular, cellular and whole organism level provided compelling evidence of stress in the contaminated mussels.

The biomarker which correlated most strongly with organic contaminant load was the micronucleus assay. Cytoplasmic micronuclei of reduced size associated with the main cellular nucleus are formed at the end of cell division and provide evidence of DNA breakage and spindle dysfunction during cell division associated with exposure to genotoxins (Mersch and Beauvais, 1997). In mussels, cell division of normal haemocytes does not usually take place in the open circulatory system (Ratcliffe and Rowley, 1981) where direct exposure to genotoxic agents might be expected to occur. This would imply that exposure has taken place during cell development within the tissues of the mussels, in accord with the high level of bioaccumulation of PCBs and PAHs evident from the tissue residue analysis. In a previous study which compared micronucleus formation in the clam *Mya arenaria* collected from New Bedford Harbour with a relatively pristine area (Martha's Vineyard), a significant increase in micronucleus formation was seen for the New Bedford Harbour clams (Dopp *et al.*, 1996), which was attributed to exposure to genotoxins. Although no measurement of tissue chemical residues was made, the assumption was that PCB contamination was the main causative agent. Increased micronucleus frequency has also been associated with PCB concentrations in feral fish (Hose *et al.*, 1987). Mammalian toxicological studies have in general concluded that

PCBs are not mutagenic or genotoxic but are efficient tumour promoters, results which have been verified by carcinogenic studies on trout (Silberhorn *et al.*, 1990). PCBs were not important factors in the initiation of hepatic lesions but had a promotional effect when present with other chemicals (Pandey *et al.*, 1995). PCBs, in common with other hydrocarbon compounds, can induce increased expression of cytochrome P450 monooxygenase activities in many species, which in turn can catalyse the metabolic activation of PAHs to form deleterious DNA adducts. Although the exact pathways of biotransformation by P450 related pathways remains incompletely understood in mussels, elevations in mixed function oxidase activities following hydrocarbon exposure are well documented (Moore *et al.*, 1989) and several, such as benzo(a)pyrene hydroxylase, have been used as biomarkers of hydrocarbon exposure (Michel *et al.*, 2001). Both bulky aromatic DNA adducts and 8-oxo-7,8-dihydro-2'-deoxyguanosine, a product of oxidative DNA damage have been identified directly in the tissues of the mussel *Mytilus galloprovincialis* following exposure to benzo(a)pyrene (Akcha *et al.*, 2000), confirming this as a mechanism of toxicity. Thus the toxicological effects of PCBs and PAHs on the New Bedford Harbour mussels have been augmented by their joint presence. The concentrations of aromatic UCMs at sites 3 and 4 may also have contributed to the toxic effects; monoaromatic UCM components have recently been linked to toxicity responses and adverse health in exposed mussels (Rowland *et al.*, 2001).

Synergism between the contaminants present may be used to explain the marked decrease in immunocompetence and viability of haemocytes evident at the two most heavily contaminated sites. Both PCBs and PAHs induce a broad spectrum of immunotoxic effects in molluscs including decreased cell viability, altered cytology and reduced phagocytic ability (Galloway and Depledge, 2001). The spontaneous cytotoxicity response is elicited in mussel haemocytes by direct cell contact with allogeneic cells and the release of lytic pore forming factors (Hubert *et al.*, 1997). Perturbation to the cell membrane or to cellular energy metabolism and ATP production are both likely to affect the reaction. It is of relevance that *in vitro* testing regimes revealed that mixtures of PCBs and/or congener class (specifically, non-coplanar congeners) may be more highly immunotoxic than individual planar and ortho-coplanar congeners alone (Stack *et al.*, 1999). The practical significance of this for laboratory testing regimes is evident when sites such as New Bedford Harbour, containing mixtures of congeners and other hydrocarbons are considered.

At the whole organism level, the physiological effect of exposure was illustrated by a marked bradycardia. At high PCBs and PAHs concentrations, depression of respiration rate may be a result of partial valve closure or the narcotization effect on ciliary action, both of which may reduce ventilation rate and thus oxygen availability (Moore *et al.*, 1989). This type of non-specific narcosis could also contribute to a reduction in energy acquisition through decreased ciliatory movement and neuro-muscular function. It is noteworthy, therefore, that there was no obvious effect on the growth of the mussels as measured by somatic tissue weight relative to length. Although this morphometric measurement has proven a useful indicator of growth in some studies (Krishnakumar *et al.*, 1994), there is no allowance made for alterations in shell thickness or shape which might better be detected by including a measure of shell volume in the equation.

Taken together, the suite of biological and chemical assays used in this study has provided a rapid indication of the extent of contamination and biological deterioration of *G. demmissa* in the New Bedford Harbour area. The relative tolerance of bivalve species to the toxic effects of PCBs and PAHs (Gilbertson, 1989) associated with their low level of biotransformation of these compounds has tended to suggest that the consequences of contamination are of more concern to organisms at higher trophic levels. However, the picture of genotoxic and immunotoxic damage and physiological impairment revealed here confirms the deleterious effects of organic contamination and is in accord with earlier estimates made by Nimmo (1974) that concentrations of PCBs of >25 $\mu g\ g^{-1}$ in tissue would be likely to compromise growth and survival in aquatic invertebrates. Likewise, the threshold value of 10-80 $\mu g\ g^{-1}$ "total" PAH estimated by Hellou (1996) above which biochemical and physiological effects might occur is confirmed as a realistic limit.

The approach outlined has permitted the detection of environmental contamination and adverse biological effects rapidly, cheaply and (for the biological assays) relatively non-destructively. This is suggested as a useful initial approach to the hierarchical process of risk assessment to complement information regarding vulnerable ecosystems, contaminant discharges and planned developments. Data regarding the extent of contamination and biological degradation can then inform and guide planning of a more detailed programme to identify specific chemicals of concern prior to instigating regulatory and remedial action. In the face of continued human and financial resources, this approach represents a practical means of protecting the environment.

Chapter 6

6 Summary, Conclusions and Future Research

6.1 Summary

To assess the significance of pollutant releases into the environment it is necessary to determine both the extent of contamination and the biological effects they give rise to. This research is based on a tiered system, which commences with conventional analytical chemistry (gas chromatography), followed by the development, evaluation and application of rapid and simple immunochemical techniques and, finally, the integration of chemical and biological markers to assess pollution.

6.1.1 Conventional analytical chemistry to evaluate contamination

The first objective was to carry out and evaluate a range of contamination assessments based on conventional analytical chemistry analyses using gas chromatography. A study was performed to appraise the extent of contamination of the Black Sea, which has been widely perceived as being heavily contaminated. There are little reliable data on contaminants being discharged, or to their environmental concentrations in this region (Mee, 1992; EEA, 1995).

To investigate the extent of contamination of the Black Sea, surficial sediments were taken from throughout the region and were analysed by gas chromatography (GC-ECD/MS).

6.1.1.1 Persistent Organochlorine Residues

Concentrations of HCHs at sites influenced by the Danube delta were shown to be among the highest recorded on a global basis (up to 40 ng g^{-1} dry wt). The ratio between the α- and γ-isomers was relatively low indicating contamination through the use of lindane as an agricultural pesticide. Concentrations of DDTs (0.06-72 ng g^{-1} dry wt) and PCBs (0.06-72 ng g^{-1} dry wt) were not especially high in comparison to levels reported from other regions throughout the world. The DDE/DDT ratio was, however, low indicating fresh inputs and hence current usage of DDT within the Black Sea area.

6.1.1.2 *Petroleum and Polycyclic Aromatic Hydrocarbons*

The European Environment Agency (EEA, 1995) and Mee (1992) have expressed concern regarding severe contamination (particularly by oil) in areas subject to riverine discharges, navigation routes and ports. To investigate, hydrocarbons in surface sediments from several areas were analysed by GC-FID/MS. Results indicate that levels of petroleum hydrocarbons (2 to 300 $\mu g\ g^{-1}$ dry wt "total" hydrocarbons) are generally comparable to those encountered in the Mediterranean and are lower than concentrations reported for highly contaminated areas. Highest concentrations of "total" hydrocarbons (>100 $\mu g\ g^{-1}$ dry wt) were associated with discharges from Odessa, Sochi and the River Danube. Chronic/degraded petroleum was the major contributor at these sites. Samples from the Ukrainian coastline were comparatively clean (<10 $\mu g\ g^{-1}$ dry wt "total" hydrocarbons). Major contributions of fresh oil (as indicated by $\Sigma n\text{-}C_{14\text{-}34}$) occur through the River Danube. Concerning "total" PAH, concentrations (7 to 638 ng g^{-1} dry wt) compare to relatively unpolluted locations in the Mediterranean and are much lower than levels reported for polluted estuaries. Both pyrolytic and petrogenic PAH were identified in most samples, although petroleum derived PAH were dominant at Sochi and pyrolytic sources were prevalent in the Bosphorus region. The absence of a correlation between "total" hydrocarbons and PAH ($r^2 = 0.04$) indicated different primary sources for the two. These results are in general agreement with Wakeham (1996) and Maldonado *et al.* (1999) who reported only moderate contamination by hydrocarbons.

6.1.1.3 *Sterol Markers to Assess Sewage Contamination*

With a growing population density in coastal urban centres within the Black Sea basin (at least 171 million people, about 81 million in the Danube basin), the need for disposal of sewage containing faecal wastes is substantial. Coprostanol (5β(H)-cholestan-3β-ol) together with other steroids have proven to be a successful indicator of sewage pollution (Goodfellow *et al.*, 1977; Readman *et al.*, 1986a; Readman *et al.*, 1986b; Grimalt *et al.*, 1990; Venkatesan and Kaplan, 1990; Leblanc *et al.*, 1992; Chalaux *et al.*, 1995). For elucidating the present status of sewage contamination, faecal sterols were measured in surficial sediments from several areas along the Black Sea coast. The concentration of coprostanol in the samples ranged from 1 to 5400 ng g^{-1} dry sediment. Differences of 1-2 orders of magnitude were observed between sediments contaminated and uncontaminated areas. Compared to many other regions throughout the world, levels of coprostanol were generally low (<500 ng g^{-1}). Conversely, there was evidence of gross sewage

contamination at Sochi (up to 5400 ng g^{-1}) and adjacent to the Danube delta (2600 ng g^{-1}). 5β/(5β+5α) isomeric ratios confirm sewage contamination together with 5β/(5β+5α) isomeric ratio of cholestan-3β-ol against cholestan-3-one. In the Bosphorus, the highest values were found near shore, with values decreasing with increasing distance seawards. In addition, elevated 5α(H)-stanol/Δ^5 stenol ratios in particulate matter at the oxic-anoxic interfaces in the water column probably relate to *in situ* microbial conversion of sterols to stanols (Wakeham, 1989).

6.1.2 Evaluation, development and application of immunoassay techniques

Hence, conventional analytical chemistry can raise substantial and useful information and, in addition, identify contamination relating to a broad range of important pollutants. The protocols used in these assessments are labour intensive and time consuming. For this reason the next phase in this research was to develop, evaluate and apply rapid and simple immunochemical techniques. It was envisaged: a) to provide an independent evaluation of the performance and suitability of commercially available immunoassay kits; b) to evaluate and validate commercial enzyme-linked immunosorbent assays (ELISAs) for screening of contamination in water and sediments; c) to adapt and evaluate the effectiveness of ELISA techniques in measuring contaminant levels in the biological tissues/fluids, and; d) to develop new applications of ELISA techniques in accordance with the "Rapid Assessment of Marine Pollution" (RAMP) programme. Basically, four distinct applications of immunochemical techniques have been presented. Conventional gas chromatographic techniques were utilised to validate the rapid ELISA techniques.

6.1.2.1 Semi-volatile hydrocarbons (BTEX) in groundwater

Initially, an ELISA kit (BTEX RaPID Assays®) designed to detect semi-volatile hydrocarbons (BTEX) in contaminated groundwater was evaluated. Determination of BTEX in environmental matrices typically involves several steps (e.g. purge and trap GC). To avoid complex procedures, immunoassay techniques have recently been directed towards measuring environmental contaminants (Waters *et al.*, 1997b). The goal was to investigate the performance of the immunoassay for the detection of benzene, toluene, ethylbenzene and xylene (BTEX) as tracers for gasoline contamination in groundwater. Results were validated against GC-FID/PID. The case study described arose as a result of leakage from storage tanks in an urban petrol station in Rio de Janeiro city. Results obtained from chromatographic and immunoassay analyses were in good agreement (r^2 =

0.997) and provided a clear delineation of the extent of contamination. The ratio of $[BTEX]_{ELISA}$ / $[BTEX]_{GC}$ did, however, show more scatter at the lower concentrations probably because of less discrimination between BTEX and other hydrocarbons at lower concentrations when competition for binding sites increases.

6.1.2.2 Petroleum hydrocarbons and PAHs in sediment

Next, a laboratory study was conducted to evaluate the performance of two immunoassay-based analytical methods for "total" petroleum hydrocarbons and polycyclic aromatic hydrocarbons. Two commercially available ELISA kits (BTEX and carcinogenic PAH RaPID Assays®) were evaluated for quantification of hydrocarbons in estuarine sediment samples, including reference materials. The BTEX RaPID Assay® was employed to analyse aliphatic and small aromatic hydrocarbons and the c-PAH RaPID Assays® was employed to analyse polycyclic aromatic hydrocarbons (> 3 aromatic rings). Results were validated by comparison with gas chromatographic analyses (CG-FID/MS). The immunoassay data is generally in good agreement with the chromatographic data obtained. However, BTEX and c-PAH RaPID Assay® ELISA cannot strictly be used as a quantitative method for determination hydrocarbons in environmental sediments, since the antibodies used bind differently to different hydrocarbons/PAHs. An estimate of "total" hydrocarbons contamination is achieved by summing measurements from both assays.

6.1.2.3 PCBs in mussel tissue

The adaptability and applicability of ELISA to measure PCB levels in mussel tissues was then tested. Biological monitoring data is often essential to provide a measure of exposure to biologically "available" contaminants. Results from PCB analyses of mussel tissue extracts by immunoassay (PCB RaPID Assay®) and conventional GC-ECD were described and compared. Mussels from natural populations with diverse concentrations of PCBs, mussel tissue fortified with technical Aroclor 1254 and a certified reference material were included. Whilst primarily designed for analyses of polychlorinated biphenyls (PCBs) in water, our goal was to apply the kit to analyse biological tissues of exposed invertebrates (mussels). A strong correlation was achieved between "total" PCBs quantified by both techniques (r^2 = 0.95, n = 27). Immunoassay results, however, exhibited lower values compared to GC-ECD. A reduced antibody response, due to differences in the congener composition between the mussel extracts and Aroclor 1254

(used to raise and calibrate the ELISA), provides the most likely explanation for this difference. Similarity analysis showed high correlations between the most prominent congeners in Aroclor 1254 and immunoassay results. This analysis did not, however, identify a specific chlorine substitution pattern to which the immunoassay preferentially responded. Whilst GC-ECD affords the capability to quantify individual congeners of different reactivity and toxicity, the data reported do indicate that immunoassay offers a rapid and inexpensive alternative method for estimation of "total" PCBs at environmentally significant levels. It is, however, necessary to remove extraneous lipids to reduce matrix effects in the immunoassay.

6.1.2.4 PAH/PAH metabolites in crab urine

In a fourth application of immunochemical techniques, ELISA (PAH RaPID Assay®) and fluorometric techniques were applied to quantify PAH metabolites in crab urine as a measure of exposure. Whilst immunoassays have been extensively applied to evaluate environmental contamination, to date (outside of human medicine) they have rarely been used for the analysis of biological fluids. These media are important because pollutants such as polycyclic aromatic hydrocarbons (PAHs) and their metabolites become concentrated in tissues, body fluids and excreta thereby offering a measure of exposure to biologically available contaminants. Such analyses can provide a non-destructive tool for monitoring exposure. Urine of experimentally exposed crabs was sampled and analysed by ELISA and UV-fluorescence. Good correlations were recorded between results from the techniques used ($r^2 = 0.83$ for phenanthrene and $r^2 = 0.88$ for pyrene). Matrix effects were overcome by dilution (≥ 20-fold) of the samples. However, cross-reactivity of the ELISA for pyrene and hydroxy-metabolites was lower than phenanthrene. HPLC analyses indicated that conjugate PAH metabolites were dominant in urine of crabs exposed to pyrene. Urine samples from crabs collected at clean and contaminated sites were also analysed by ELISA and UV-fluorescence. In samples from a polluted harbour, both techniques detected PAHs (mainly petrogenic) contamination in the urine. Good agreement ($r^2 = 0.83$; $n = 15$) was achieved between the techniques.

6.1.3 Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassay

Finally, a combination of biological markers and chemical assays were use to provide assessments of the relationship between anthropogenic contaminant levels, toxic damage

and adverse health effects in selected invertebrates from a pollution gradient. Here a combination of quick, simple to perform and inexpensive biomarkers and chemical immunoassays were used to assess the exposure to and effects of pollutants on the ribbed mussel (*Geukensia demmissa*). Samples were collected from sites in and around New Bedford Harbour (Massachusetts, USA). Significant differences in polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbon (PAH) tissue residues were detected between sites using both a RaPID Assay® ELISA and GC-FID/MS. The agreement between both techniques was good ($r^2 \geq 0.95$). Only small differences were observed in metals concentrations (Cu, Cd, Pb, As, Hg, Ni) between sites. Biomarkers of immunocompetence (spontaneous cytotoxicity), genotoxicity (micronucleus formation), cell viability and heart rate were significantly different between the selected sites. No significant differences were observed in lysosomal integrity, metallothionein or condition index. Multi-variate canonical correlation analysis indicated an association between micronucleus formation, heart rate, and PCB and PAH concentrations. Whilst causality cannot be conclusively stated, covariability with PAHs and PCBs is reported.

6.2 Conclusions

Conventional analytical chemistry provides a definitive measure of individual compounds which can be very useful for comparative purposes (e.g. to compare degrees of chlorination and legislation, etc.). It does not, however, afford any measure of the availability of the contaminants nor effects associated with their presence in the ecosystem. In spite of being very sensitive, conventional analyses are generally difficult to execute, are time consuming and expensive.

Rapid assays (ELISA and fluorescence) have within this research shown good correlation with the more conventional techniques. They do not provide, however, a truly definitive measure. The responses relate directly to the reactivity/fluorescence of the analytes in the test sample relative to a reference compound. Responses/reactivities vary with composition. Also, there are limitations on the range of antibodies available in the case of immunoassays. Problems with matrix interactions are common and increase when complex environmental matrices such as tissues are used. In such cases simple extraction procedures are not sufficient and more elaborate clean up procedures are needed. Development of the crab urine method (either by ELISA or fluorescence) strikes a useful compromise with matrix complexity and shows very good potential for future use in

environmental assessment. Finally, the portability of ELISA enables application under field conditions, making this technique a very convenient screening technique.

The advantages associated with the application of biological markers relate to their capability to differentiate between a contamination and a "pollution status", where biological effects are induced. In addition, they can provide information on pollutants which are not or cannot be measured. It is estimated that several million organic compounds have already been synthesised by man. About 250,000 new chemical formulations are created per year of which 300-350 reach the stage of commercial production and up to a third of their production may find their way into the environment (Stumm and Morgan, 1996). Clearly, not all can be screened and the application of simple biomarkers, which are easy and rapid to use, can fill this gap. However, many biomarkers provide highly variable results that are not definitive. Because the response obtain by the biomarkers is usually specific to species (or class of organisms), for a general assessment of environmental quality it is necessary to apply a broad range of biomarkers at different levels of biological organisation. This is essential to cover possible effects from a wide range of chemicals. Even if alterations are detected, often the response cannot be directly related to a specific compound (or class of compounds) (e.g., TBTs in dog whelks, herbicides, etc.). Clearly, a combination of chemistry and biological is desirable.

6.3 Future Research

Within the conclusions, both the advantages and disadvantages of individual assays and techniques are identified and discussed. A combination of conventional analyses and rapid chemical and biological assays appears to offer the best approach for environmental scientists, risk assessment managers and policy makers.

Efforts should continue into the development and implementation of immunoassays to provide comparatively easy, rapid and inexpensive assessments of contamination. With the broad range of potential contaminants, broad-based assays offer advantages as they cover classes of pollutants. For example, class-selective immunoassays for the measurement of glucuronides in human urine can aid evaluation of human exposure to complex mixtures of xenobiotics (Staimer *et al.*, 2001). A similar class-selective immunoassay for glucosides and/or sulfates in invertebrate urine would help assess

exposure of aquatic organisms to complex PAH mixtures (see Section 4.5) and provide a measure of stress.

Immunoassay technology needs to be improved in terms of field portability, sensitivity and cost to strengthen its position as a rapid analytical assessment technique. Many immunosensor developments relate to the elements and mechanisms used for the detection of the physico-chemical changes produced by the biological interactions. Improvements in the development of measurement devices/systems (e.g. real time measurement) complement these. For example, infrared biosensors, solid-phase fluoroimmunoassay and optical transducer chips (and combination thereof) have been described. Chromatographic (or flow) immunoassay has also received attention recently (Hage and Nelson, 2001). The direct detection of analytes as they are desorbed from immunoaffinity columns is the simplest approach that can be used in chromatographic immunoassays. Potential advantages of this flow-based immunoassay include their speed, precision, ease of automation, and ability to be coupled to other analytical methods (Hage, 1999). Techniques in this category include immunoaffinity chromatography, high-performance immunoaffinity chromatography, flow injection immunoanalysis, flow immunoassays, immunodetection, and immunoextraction.

Another novel and selective methodology is the use of molecular imprinted polymers (MIPs) (Murray and Fish, 1997; Katz and Davis, 2000). MIPs show antibody-like affinities toward the template analyte. Molecular imprinting aims to create solid materials containing chemical functionalities that are spatially prepared by interactions with imprint (or template) molecules during the synthesis process. Subsequent removal of the imprint molecule leaves behind sites for the recognition of the imprint molecules. These are well suited for applications such as separations, chemical sensing and catalysis. Applications of these methods presently remain in the early stages of development (Katz and Davis, 2000).

Because metabolic oxidation of contaminants (e.g. PAHs) is responsible for the formation of toxic and carcinogenic intermediates (Livingstone, 1994), the determination of the level of metabolites by an organism can improve the assessment of risk. Whilst immunoassays offer a most useful extension for monitoring parent/metabolite compound levels in organisms, it does not, however, measure effects. Concurrent ecotoxicological investigation (integrated approach) can bridge this gap.

Whereas it is clearly recognised that changes at the population/community/ecosystem levels of biological organisation are the ultimate concern, they are often too complex and far removed from the causative events to be of use in developing tools for the early detection and prediction of the consequences of environmental stress. For this reason exposure studies often focus on the molecular/biochemical, cellular and physiological changes within an individual. These alterations will induce environmental perturbations, which potentially can affect higher levels of organisation.

Within this study, work has concentrated on the application of simple biomarkers and responses to afford rapid assessment of exposure. Many other biomarker techniques have been described in the literature and often relate to complex biochemical assessments. The combination of chemical and biological assessments, in the present research, has proven of great benefit, but also it identified the need to utilise broad combinations of biomarkers at different levels of organisation to investigate environmental degradation.

Proteomic assays offers excellent prospects in future environmental monitoring and diagnostics since proteins control most processes in living organisms. Because protein compositions are selectively affected by any stressor they can act as the basis for early diagnosis of exposure or diseases (-, 2000; Evans, 2001). Although theoretically extremely complex it is envisaged that array developments will simplify diagnoses.

Finally, translation of the chemical and biological/ecological data into useful risk assessment still needs to be improved.

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