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Evaluation of Techniques For The Biomonitoring of Pollutants in Members of The Ulvaceae

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EVALUATION OF TECHNIQUES FOR THE BIOMONITORING OF POLLUTANTS IN MEMBERS OF THE ULVACEAE

by

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ABSTRACT

Evaluation of techniques for the biomonitoring of pollutants in members of the Ulvaceae

by Rebecca Schild

This study involved the development and evaluation of a number of biomonitoring techniques for use with members of the Ulvaceae, *Enteromorpha intestinalis* (L.) Link, and *Ulva lactuca* (L.). The techniques included both previously established ones and those which required development. The two main procedures developed for these algae were the neutral red retention technique (making use of a vital stain) and the ion leakage Health Index (based upon electrolyte leakage). Following optimisation both were used to produce quantitative structure-activity relationships (QSARs) for a series of n-alcohols using log K_{ow} as the physicochemical parameter. The resultant QSARs were statistically indistinguishable with line equations of -0.88 log K_{ow} + 2.87 and -0.95 log K_{ow} + 2.83 respectively, with r^2 values of 0.98, suggesting that both reflect the same non-specific narcotic effect on membrane integrity. However the neutral red retention technique was less useful than the Health Index with low reproducibility and is therefore unsuitable for use as an environmental biomonitor.

The effects of other compounds with specific modes of action were established using the ion leakage technique, the high toxicity of the antifouling compound tributyltin being reflected in the Health Index. Further development of the ion leakage technique involved inductively coupled - plasma mass spectrometry (ICP/MS) in the identification of ions lost during leakage. Synergistic toxicity was investigated exposing the algae to UV light and anthracene, simulating photo-induced toxicity. No photo-induced toxicity was observed although UV exposure did reduce the Health Index.

An environmental assessment was carried out for algae from clean sites and organically and inorganically polluted sites, comparing the Health Index with other measures of health and bioaccumulation data. A long term study of a clean site showed the algal Health Index to be influenced by seasonally dependent physical parameters, but reflected reduced health in algae exposed environmentally to organic pollutants. Algae exposed to long term heavy metal pollution in the Fal Estuary had 'normal' Health Indices, however chlorophyll fluorescence induction techniques did highlight a reduced photosynthetic efficiency in these algae.

The ion leakage technique has potentially a wide application in field and laboratory based biomonitoring and direct toxicity studies. Chlorophyll fluorescence analysis was also shown to be useful although it requires further investigation and evaluation.

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CHAPTER 1

INTRODUCTION

1.1 Ecotoxicology and Biomonitoring

Biomonitors, or biological monitors, are organisms which are utilised in the regular surveillance and possible quantification of pollutants within a given environment (After Martin and Coughtrey, 1982). The use of biomonitors therefore relates not to species composition but to uptake and incorporation of contaminants and the subsequent effects that such exposure has on the organism. In this study members of the Ulvaceae *Enteromorpha intestinalis* (L.) Link, and *Ulva lactuca* (L.) are thus employed.

Biomonitors are tools by which the fate and effect of environmental contaminants can be traced and evaluated. This type of study falls within the realm of ecotoxicology which has only in recent years been accredited as a discipline distinct from toxicology and ecology (Butler, 1984). As a subject area it therefore has a broad base, encompassing a chemical toxicity approach with the often subtle effects such pollutants exert on the natural biota.

The term ecotoxicology was first used by Truhaut in 1969 (Moriarty, 1983) as a natural extension of toxicology to the ecological effects such compounds have when released into the environment. Since then the term has been redefined countless times and until today there is no conclusive definition. One of the most useful remains that of Butler (1984) who considered ecotoxicology as 'a study of the effects of released pollutants on the environment and on the biota that inhabit it'. For the purpose of this present thesis this definition will be adopted.

The ultimate aim of the ecotoxicologist is to determine the effects of pollutants on the

structure and function of intact ecosystems or communities (Forbes and Forbes, 1993). To achieve this it is necessary to be able to anticipate how compounds will act on release into the environment and to assess what influence these released substances will have, or are having, on ecological systems. Tests designed to anticipate chemical fate are by nature general and laboratory based. However those designed to assess chemical effect often relate to particular circumstances and may require studies based both in laboratories and also in the receiving ecosystem. Some of the procedures employed to achieve this are regularly used and defined legally; however many have no legal base but are still used routinely.

Calow (1993) identified a number of performance criteria for ecotoxicological tests:

- All good procedures have in common the need to be relevant to the particular ecosystem in question, and be able to identify whether there is stress or disturbance within that ecosystem.
- All tests should be reproducible, regardless of when or where they are carried out. Such standardization being imperative for scientific and legal credibility.
- The tests should be reliable and not over-demanding.
- Tests should be repeatable and sensitive, to ensure any variation in response is statistically significant and not due to noise in the system.

It is inevitable that these criteria will not all be met concomitantly but, bearing in mind that it is the protection of the ecosystem which need be given priority, the emphasis on each criterion must depend on the ultimate aim of any particular study.

The type of test carried out can be *quantal* in nature (e.g. live or dead) or *continuous* (e.g. growth or reproduction). The majority of tests to date deal with quantal data, especially

for acute, lethal toxicity tests (Calow, 1993). However there is a new and necessary movement towards continuous testing, identifying and quantifying sublethal responses. Since the majority of pollution situations involve chronic, long term, low concentration exposures it is necessary that this type of approach be developed. Despite this there will always be a need for simple tests for use by industry and government to assess the toxicity of the hundreds of new chemical substances produced each year. This may not be ideal, but its inevitability leaves the onus with the academic community to produce such tests which also fulfil the criteria listed earlier (Forbes and Forbes, 1993). As stated by Depledge (1993)

> 'The vast majority of ecotoxicologists are engaged in this (i.e. developing practical tools for environmental administration). Whilst this is understandable, it does not permit us to neglect basic research in ecotoxicological science which will one day enable us to gain an understanding of how pollutants perturb ecosystems and to recognise both short and especially, long term undesirable changes'.

Organisms, both plants and animals, have been used for many years to assess the effects of pollutants, both individually and as bioindicator species (i.e. on a presence/absence basis). It has long been recognised that community structure changes in response to pollution exposure. Indeed an early account of the use of macroalgae as indicators of pollution was contained in a report of a correlation between sewage pollution in estuaries and the extensive sheets of *U. lactuca*, now referred to as 'green tides' (Burrows, 1971). Similarly for many years now, fish, plants and mammals have been analysed for trace metal accumulation and organochlorine pesticide residues. The list is as extensive as the problem. Risk assessment has also been used to determine the fate of chemical substances in the environment and the effects these substances, both original compounds and products of degradation, have on the biota. One vehicle used in this area of research exploits the similar properties of chemicals in the prediction of potential toxicity, referred to as

structure-activity relationships, SAR's or QSARs (quantitative structure-activity relationships). Probably the best known approach is that of actual toxicity testing, where organisms are exposed to lethal or sublethal concentrations of compounds and their response measured either qualitatively or quantitatively. To achieve this, parameters such as death, growth rate, photosynthesis/respiration rate and spore/larval survival are used. Monitoring studies and ecological assessments are also employed to determine at what concentrations contaminants are present in water, soil or the biota, and the concentrations at which changes begin to occur in individuals or community structure. The diversity of the problem of pollution monitoring and management makes it necessary to adopt a diverse solution, one that integrates bioaccumulation studies with toxicity testing, and one that provides a broad indication of the effects of such pollutants on a suite of organisms.

It is not possible for individual studies to encompass all the above approaches and it is therefore imperative that each investigation provide a complete picture in itself, relating any results to the environmental situation. This also implies an awareness of each organism's role within an ecosystem, and knowledge of an organism's normal 'health', without which abnormality can not be ascertained. This study aims to do this primarily for the macroalga *E. intestinalis*.

1.2 Macroalgae as biomonitors

Throughout history the marine environment has suffered abuse, oceans having been treated as massive pollution sinks into which contaminants will dilute and disperse. However it has become all too clear that the capacity of oceans to absorb and neutralise pollutants is not limitless. Marine pollution entered public awareness in the early 1960's with the Torrey Canyon oil spill which resulted in widespread destruction of the local marine environment. Oil pollution is only one of many marine contaminants; others include

sewage, trace metals and pesticides (Abel and Axiak, 1991), which are often far less obvious, but their effects may be just as devastating.

It has been recognised for many years that macroalgae can be used to indicate pollution. However their use has generally been neglected on the basis of their insensitivity to pollutants, despite their being important primary producers within the marine biota. Within the last 20 years more attention has been paid towards their use as biomonitors and as biological indicators of pollution (Thursby et al., 1985). The use of macroalgae in such studies have recently been extensively reviewed by Fletcher (1991) and prior to this by Levine (1984). A number of studies exist in which algal community structure has been used to indicate pollution, with changes in species diversity and biomass being recorded (Burrows, 1971; Hirose, 1978; Lavery et al., 1991). Macroalgae have also been used as biomonitors of heavy metal pollution (Phillips, 1994) and as experimental test organisms in laboratory based studies (Fletcher, 1991).

1.2.1 Macroalgae as test organisms

Until recently most toxicity studies have involved the use of field collected material, but an increasing number of researchers are now successfully maintaining stock cultures of test algae, although tnis requires both time and expertise. One such use of cultured algae involves *Champia parvula*, a red alga, which is utilised by the U.S. Environmental Protection Agency (EPA) in toxicity testing for its sensitivity to pollutants. Use of cultured algae has also led to the greater use of different life stages, the relative sensitivity of different stages being determined. For example Scanlan and Wilkinson (1987) used the spermatozoa and eggs of *Fucus spp.* in toxicity testing, and spore germination and rhizoid regeneration in *E. intestinalis* have similarly been investigated (Fletcher, 1989; Scanlan and Wilkinson, 1987). The latter species is of particular interest as a ship-fouling alga, spore

settlement of algae being the preferred test for antifouling studies (Fletcher, 1989). Additionally, in many cases young spores and germlings have proved to be the most sensitive life stage and are therefore the most suitable for use (Fletcher, 1991). However, the necessity for culture procedures and the time scales involved in such techniques mean that this approach is not always viable.

In the majority of tests the effects on growth, reproduction, respiration and photosynthesis have been measured. Many growth parameters have been used, each depending on the macroalgal morphology and mode of growth. These include increase in length, mass, surface area and volume (Fletcher, 1991). Cell viability tests have also attracted attention, vital and mortal stains being used to assess the percentage viability of tissue following pollution exposure (Alexander and Wilkinson, 1987). Measurements of membrane integrity have also been employed in toxicity testing through the use of ion leakage techniques (Axelsson and Axelsson, 1987), and in the assessment of frost damage (Frazer et al., 1988). Despite the variety of algal species and tests used there is to date no standard strategy with regard to the use of algae in toxicity testing. Although in the United States there is a move towards the use of algae (both micro and macro) in the setting of standards for allowable contaminants their use falls more within the realm of toxicology than ecotoxicology (Thursby et al., 1993).

Within the literature there are few references to the use of macroalgae in toxicity testing and biomonitoring of organic pollutants (e.g. Amico et al., 1979; James et al., 1987; Hsaio et al., 1978; Cross et al., 1987; Maroni et al., 1993), most studies having been carried out for trace metals. Both Amico et al. (1979) and Maroni et al. (1993) analysed for organochlorine pesticide residues finding them present at low concentrations. Maroni et al. (1993) reported that the level of accumulation depended upon: species; the area in

which growth occurred; the time of year of collection; the physical condition of the fronds; the lipid content of the algae. The accumulation levels of these organic pollutants are low with respect to other organisms but this does not negate the study, rather it highlights the need to know how all organisms respond to different pollutants in order that risk might be evaluated.

1.2.2 Macroalgae as biomonitors of heavy metals

Aquatic macrophytes accumulate significant amounts of trace metals from water, biomagnifying the metals within their tissues and thus providing information with regard to exposure. The species most commonly utilised belong to the green and brown macroalgae, although red algae and even seagrasses have been used. Laboratory based and field based studies have been carried out to show how macroalgae respond to trace metal exposure, and it has been shown that there is a relationship between tissue concentration and metal concentration. A thorough review of the use of macroalgae as biomonitors of trace metal pollution has recently been compiled by Phillips (1994).

Macroalgae fulfil most of the requirements of a trace metal biomonitor. They are sedentary, relatively abundant, accumulate metals from the water column and integrate fluctuations in metal levels over time, metals being lost only slowly from the organism (Say et al., 1986). It is also helpful if biomonitors of the marine environment can tolerate brackish water, as can a number of macroalgae, e.g. *E. intestinalis*. Many species are also available throughout the year which is desirable, as is easy identification.

Those species which have been used predominantly are *Laminaria digitata*, *Ascophylum nodosum*, *fucoids*, *Ulva spp.* and *Enteromorpha spp.* and their use has been validated by numerous studies (Phillips, 1994). One of the earliest was the use of *Laminaria digitata*

by Bryan (1969) where uptake of heavy metals into the alga was shown to be proportional to the water concentration of those metals. One of the most comprehensive studies to be carried out in the United Kingdom was an evaluation of the use of *Enteromorpha spp.* as an indicator of metal contamination, a study commissioned by the Department of the Environment (Say et al., 1986).

Generally it is concluded that macroalgae are good biomonitors of metal contamination, but it is necessary to bear in mind a number of factors which influence metal accumulation and subsequent analysis. These include sampling position, the presence of epiphytic organisms (including bacteria), the possibility that uptake may be from the sediment in addition to the water and the variability of metal uptake into different parts of the algae due to difference in growth rate. It therefore also follows that there will be a seasonal variation in the rate of metal uptake. The metal species present can also impose an effect on the levels of different trace metals incorporated into the algal thallus as at high concentrations metal-metal interaction can cause displacement and competition for binding sites within the algae. Physical parameters influencing uptake include salinity, temperature and light intensity (Phillips, 1994).

1.3 Thesis aims and objectives

The aim of this thesis is to explore the use of members of the Ulvaceae as biomonitors of pollution. As stated earlier, biomonitors are organisms which are used both for the purpose of assessing contaminant toxicity, and for the purpose of establishing the levels of environmental exposure to toxicants via incorporation within the algal thallus. The emphasis here will be upon the development and application of techniques designed to measure physiological responses and effects, as well as achieving a better knowledge of the levels of toxicants to which these algae are exposed. Effort has been made to establish

techniques which can be used in *effects-based* monitoring of organic toxicants, this being an area presently lacking in information. To optimise the techniques and the results gained by them, QSARs have been utilised with the aim of validating the techniques and relating sensitivity to other organisms. They also introduce a diagnostic aspect where toxicant mode of action is concerned. In addition environmental analysis has been carried out to allow a fully integrated picture to be drawn with regard to the alga and its ecological niche. This has been achieved for two areas, one in which organic pollution is suspected and one known to be highly contaminated with trace metals.

Members of the Ulvaceae, *U. lactuca* and *E. intestinalis*, have been chosen as the test organisms because of their abundance and distribution. They are also very tolerant of a variety of environments, from high salinity rock pools to almost freshwater estuaries. In the main, *E. intestinalis* has been utilised throughout this study. This alga has a wide distribution, is opportunistic and thrives under eutrophic conditions. It is also a marine fouling organism, undermining the efficiency of many immersed structures, causing both structural damage and economic loss (Fletcher, 1989). It has been shown to be a good biomonitor of trace metal pollution (Say et al., 1986), and its tolerance of a broad range of environmental conditions suggests that it might also be a useful biomonitor of organic compounds and their effects. Being such a tolerant species implies a possible high degree of insensitivity to pollutants which may reduce its capacity for use as a biomonitor. However this does not necessarily reduce its value as an effects-based biomonitor.

E. intestinalis, a Chlorophyte macroalga, has a thallus comprised of a hollow tube bounded by a single layer of cells. Attachment is by rhizoidal branches from which fronds arise; these can be branched or unbranched. Fronds generally increase in width from base to mid thallus, the length of each varying from 1 cm to 1 m, and up to 3 cm or more broad. Cells

are characteristically irregular from the base to the mid-thallus region, with occasional small patches of 10-12 cells being arranged regularly within the mid-thallus. They are polygonal, rectangular or rounded and contain a single hood-shaped chloroplast situated at the upper end of the cell; generally there is one pyrenoid, occasionally two. The photographs, Plate 1 and 2, are of two different species of *Enteromorpha, E. intestinalis* and *E. prolifera*. They have been included in order to demonstrate how identification of *E. intestinalis* is achieved microscopically. Macroscopically *E. prolifera* which has a similar tubular thallus can be confused with *E. intestinalis*, but as shown here the cell arrangement of this alga is very regular in comparison, cells at the base forming longitudinal rows, and those within the mid-thallus region forming either longitudinal or transverse rows (Burrows, 1991).

E. intestinalis shows an isomorphic alternation of haploid and diploid generations. Reproductive sporangia form at the terminal regions of the thallus from normal vegetative cells, identifiable by the light coloured senescent tips. The two types of sporangia arise from separate plants, the *gametophyte* producing sexual, biflagellate, spindle shaped gametes and the *sporophyte* producing asexual, pearshaped zoospores. Released gametes have distinct sexes, the male or '=' gametes being smaller than the female or '+' gametes. On mixing the two types of gametes pairing occurs and on settlement a round zygote is formed (Fletcher, 1989). Development and subsequent growth rates are rapid.

During experimentation microscopic analysis was employed to ensure the species being used was *E. intestinalis* as it is notoriously difficult to identify. Experimental trials carried out with other *Enteromorpha* species did not reveal widely varying results, although further tests would have to be carried out to evaluate whether these could be used in the place of *E. intestinalis*.

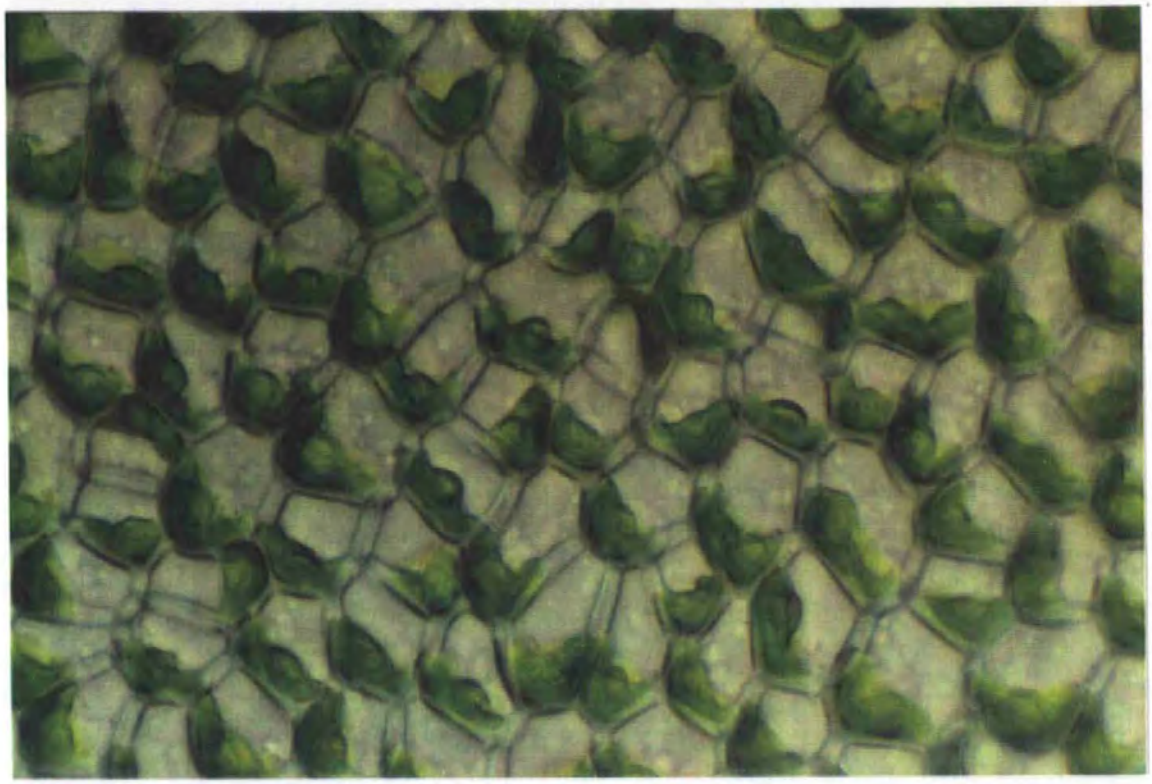


Plate 1: *E. intestinalis*, surface view of thallus in the basal region showing irregularity of cells. Mag x 700.

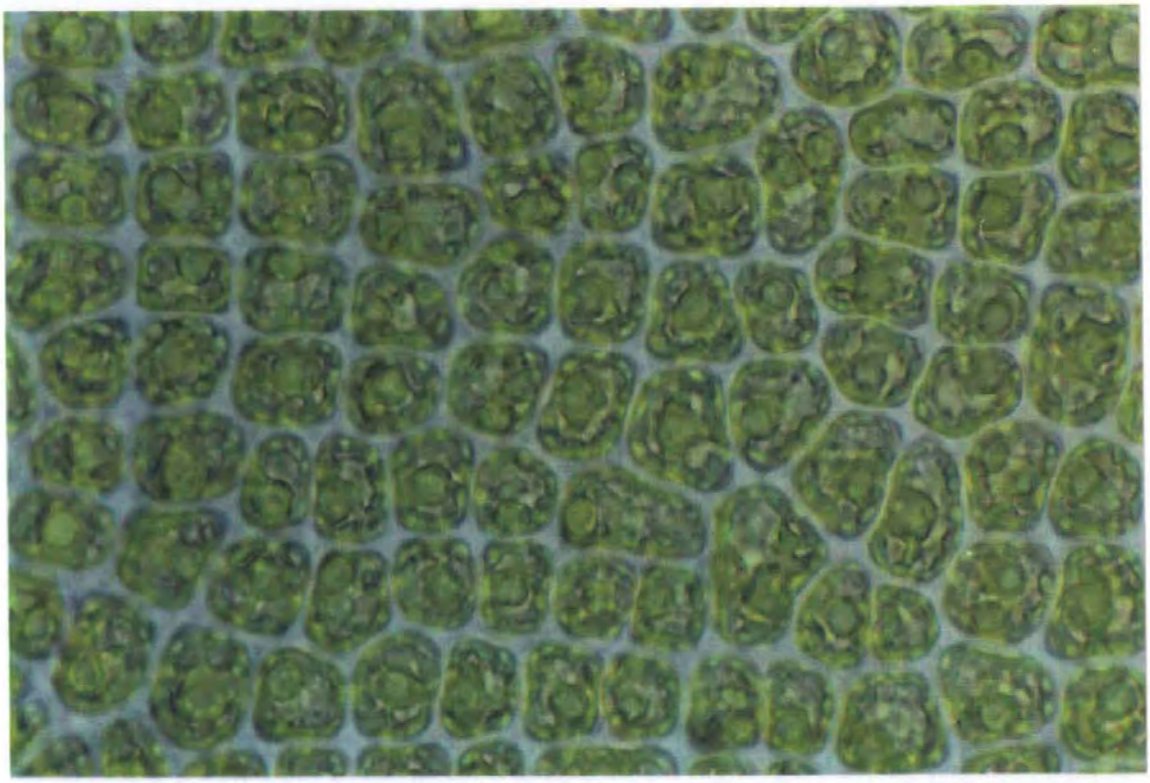


Plate 2: *E. prolifera*, surface view of thallus in the basal region showing regularity of cells. Mag x 700.

CHAPTER 2

EVALUATION OF NEUTRAL RED RETENTION

2.1 Introduction

During recent years the use of vital and mortal stains in the assessment of cell viability has grown in popularity. These dyes have been used to assess cell viability (Saga, 1989) and dispersal distances in macroalgae (Kendrick and Walker, 1991), and the effects of pollution exposure on a number of organisms, including fish cells (Dierickx and Van De Vyver, 1991; Babich and Borenfreund, 1987), unicellular algae (Herman et al., 1991; Gilbert et al., 1992) and macroalgae (Alexander and Wilkinson, 1987).

Marine unicellular algae and macro-algae have been utilized in the investigation of metal contamination (Wong et al., 1982; Say et al., 1986), but less work has been carried out concerning organic compound toxicity. Past research in this area has concentrated on the use of microalgae which are generally sensitive biomonitoring organisms but difficult to use for environmental monitoring. Macroalgae, having the advantage of fulfilling many of the criteria required of a biomonitor (Say et al., 1986), have potential as environmental monitors if reproducible techniques can be applied to them. Some methods have been developed and applied to the field situation (Scanlan and Wilkinson, 1987; Fletcher, 1991). Dye uptake is one such technique and has been shown to be efficient in measuring viability in cultured animal (Babich and Borenfreund, 1987) and plant (Gilbert et al., 1992) cells using microplate readers. Less specialised optical equipment will, however, suffice (Herman et al., 1991). Vital stain uptake can also be assessed in a semi-quantitative fashion by visual observation under a microscope (Alexander and Wilkinson, 1987), and using this technique intact thalli can be examined.

Previous investigations into the most suitable vital stain to use with macroalgae suggest that neutral red provides the most reproducible results (Saga, 1989; Alexander and Wilkinson, 1987). This chapter describes how the neutral red dye extraction approach of Babich and Borenfreund (1987) has been applied to the thallus of the intertidal macroalga *E. intestinalis* which was investigated by Alexander and Wilkinson (1987). The stain taken up and retained by the frond is extracted and assayed spectrophotometrically, rather than microscopically (the method used by Alexander and Wilkinson, 1987). Extraction from large pieces of thallus thus increases precision by taking into account the behaviour of more cells than can practically be measured by direct microscopic observation.

The procedure, initially developed by P. Cotsifis (1992), was calibrated by establishing a quantitative structure-activity relationship (QSAR) with a series of non-specific narcotic alcohols. Chemical substances acting by non-specific narcosis are common and abundant environmental contaminants (Abernethy and Mackay, 1988).

2.2 Method

2.2.1 Collection of algae

E. intestinalis was collected from rock pools within the intertidal region of Wembury Beach. The algae were placed in plastic bags and returned to the laboratory where they were washed in artificial seawater (Instant Ocean, obtained from J & K Aquatics Ltd) of salinity 33 ppt, and placed in buckets of aerated Instant Ocean of the same salinity. They were left under light frames with a photosynthetically active radiation of 120 $\mu E/m^2/s$ in a constant temperature room held at 16°C and allowed to equilibrate for at least 12 hrs. Identification was carried out by microscopic examination after each collection. This was achieved using a Vanox Olympus microscope with a x40 objective, (see Plates 1 and 2 in the introduction). Algae were held in the laboratory for a maximum period of two weeks,

and were discarded before this if chlorosis was observed.

2.2.2 Timecourse of neutral red uptake

Preliminary experiments were carried out to determine the time course of the neutral red uptake and to select a suitable incubation time.

A neutral red solution was prepared by adding 30 mg of neutral red (Gurr, microscopy materials - 90% pure) per litre of seawater. After vigorous agitation and adjustment to pH 7.0 using dilute acetic acid, the product was filtered through a 0.45 μm Millipore membrane filter to remove any particulates. The resultant dye concentration was typically 8 mg/l and was assessed using a Pye Unicam SP8-100 UV/VIS spectrophotometer.

The algae, *E. intestinalis*, were laid on blue laboratory paper and air dried for 30 min at room temperature. Samples of 200 mg were weighed out and exposed to 100 ml aliquots of neutral red solution held in 500 ml bottles at 15 ± 1°C. The samples were left up to 5 hrs, each time interval being represented by 3 replicate samples.

Following exposure, all the samples were removed from their bottles and washed quickly in artificial seawater to remove any dye from the surface of the algae. The dye was then extracted by grinding each sample, using a pestle and mortar, with 10 mls of a 50% ethanol - 1% glacial acetic acid solvent and approximately 2 g of fine acid washed sand. The resultant homogenate was centrifuged at 3000 rpm for 3 minutes and the absorption of the supernatant measured at 540 nm against a solvent blank. The amount of neutral red was calculated using a calibration curve, compiled using standard solutions of neutral red prepared in 50% ethanol - 1% glacial acetic acid, and expressed in terms of the amount of neutral red taken up per gram (wet weight) of seaweed.

2.2.3 Determination of alcohol toxicity

The toxicity tests were carried out on the homologous series of n-alcohols from methanol to octan-1-ol but also including decan-1-ol. Alcohols of 95-99.8% purity were obtained from various companies; methanol, propan-1-ol, pentan-1-ol, hexan-1-ol and heptan-1-ol from British Drug House (BDH), butan-1-ol and octan-1-ol from SIGMA, ethanol from Rathburn and decan-1-ol from Aldrich. Solutions of the alcohols were prepared in Instant Ocean and adjusted to pH of 7.0, to correspond with the neutrality of the dye. A minimum of five linearly distributed concentrations of each alcohol was used. The maximum concentration of each alcohol tested was determined by published solubility values (Schultz et al., 1990). These values were quoted for freshwater at 25°C and, to estimate the solubility under the experimental conditions of 33 ppt salinity and 15°C, they were halved.

Samples of air dried *E. intestinalis*, weighing 200 mg, were placed in 500 ml bottles containing 330 ml of alcohol solution, each concentration being tested in triplicate. A "background absorbance" control was also set up where the algae were exposed to seawater only. Incubation was for 24 hrs at 15°C, illuminated from the side with a photosynthetically active radiation of 15 $\mu E/m^2/s$. Screw capped bottles were used to minimise volatilisation of the alcohols.

After the period of incubation, the alcohol solutions were replaced by a solution of neutral red, prepared as described earlier. Alcohols were added to this to maintain the particular exposure concentration. The pH of the solution was readjusted to 7.0 with dilute acetic acid before use. The algae were left in the neutral red solutions for 3 hrs under the same conditions as before. At the end of 3 hours all the samples were rinsed quickly in seawater, then the neutral red taken up was extracted into the solvent (50% ethanol - 1% glacial acetic acid) and the absorption measured as described above.

From each experiment a subsample of air-dried algal fronds was retained for wet weight/oven-dry weight determination (dried for 24hr at 80°C, to constant weight). This measurement was replicated on at least 5 samples.

The EC_{50} for each alcohol was taken as the concentration at which 50% of the control stain uptake value was taken up by the frond. This value was calculated from the linear regression equations for the alcohols investigated. At very high concentrations, beyond the EC_{50} value for the alcohol, excess breakdown within the frond was seen, affecting neutral red uptake and the absorption. When this occurred the higher data points were omitted from the calculation of the EC_{50} and the lower points relied upon.

The wet weight/dry weight ratios determined for each experiment showed an approximate 3-fold variation for this parameter. It was observed to have no effect on the dye uptake within experiments, or on the EC_{50} data as evidenced in Figures 10 and 11.

2.3 Results

2.3.1 Uptake of neutral red

The degree of variation between the amount of neutral red taken up by separate samples of seaweed is presented in Figure 1. The experiment was carried out over five hours. After one hour the amount of variation, represented by the 95% confidence limit, was $\pm 248 \cdot 5$ μg/g and this decreased as time progressed to a minimum of ± 93 μg/g after three hours. Samples left for longer periods showed an increased degree of variability, with values ranging from $\pm 618 \cdot 5$ to ± 492. The maximum rate of uptake occurred during the first three hours, with evidence of a plateau between 3 and 4 hours. However, the rate appeared to increase again up to 5 hours.

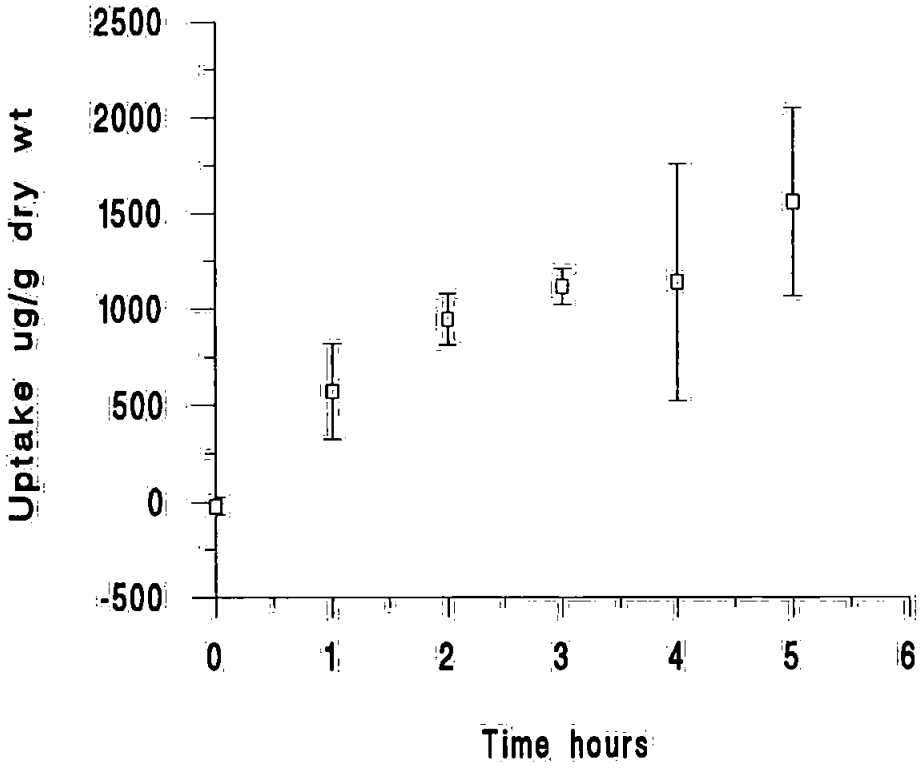


Figure 1: Uptake of neutral red over time, showing 95% confidence limits for the mean of each point.

2.3.2 Toxicity measurements

For alcohols methanol to octan-1-ol the amount of neutral red taken up by the algae decreased linearly as the concentration of alcohol increased. The results obtained were plotted and used to calculate a linear regression from which the EC_{50} was taken, that is, the concentration needed to reduce the neutral red uptake by 50%. Figures 2 to 9, show the concentration response curves for methanol through to octan-1-ol. Table 1 shows the actual neutral red uptake values for each alcohol at each concentration and Table 2 lists the alcohols used with their resultant EC_{50}'s, and the correlation coefficient (R^2) for each regression line. The EC_{50} data was plotted against both the carbon number and the octanol/water partition coefficient, creating two good quality linear QSARs. Figures 10 and 11 present these two relationships.

In an homologous series of compounds, toxicity, expressed as exposure concentration, normally increases until the molecules achieve a certain size, above which toxicity declines or disappears. In aquatic systems, one plausible reason for this 'cut-off' effect is that the solubility of the compound in water declines below the concentration required to produce the toxic effect (Donkin et al., 1991). The cut-off point may therefore be determined by plotting aqueous solubility and toxicity against an appropriate molecular parameter and establishing the point at which the two lines intersect (Lazarev, 1944; cited by Lipnick and Filov, 1992). This is illustrated for our data in Figure 12, where carbon number is the molecular parameter used. Here figures are taken from Schultz et al. (1990), which were for freshwater maintained at 25°C. However our experiments were carried out at 15°C and involved saltwater of salinity of 33 ppt. Therefore the solubility of the alcohol is reduced by a factor which we estimate to be approximately 50%. The graph below shows the cut-off point for the unadjusted data to be C=11. Using our estimated factor of 50% reduced solubility the cut-off point would be at > C=8, octan-1-ol, so that above this number of

Table 1: The amount of neutral red taken up by *E. intestinalis*, including standard errors, following exposure to various concentrations of alcohol.

Methanol	Concentration (mM)	0	3	31	313	1250	2188	3125
	Uptake (μg/g dry wt)	411±37	357±15	395±4	344±6	294±24	249±17	203±12
Ethanol	Concentration (mM)	0	2	22	217	870	1522	2174
	Uptake (μg/g dry wt)	441±62	454±17	416±31	484±19	228±16	174±9	141±4
Propan-1-ol	Concentration (mM)	0	2	17	167	500	(1000)	(1667)
	Uptake (μg/g dry wt)	514±68	387±62	609±60	259±33	111±57	(184±11)	(136±12)
Butan-1-ol	Concentration (mM)	0	41	81	122	162	(203)	(243)
	Uptake (μg/g dry wt)	230±15	157±9	140±12	114±10	94±9	(185±10)	(141±10)
Pentan-1-ol	Concentration (mM)	0	11	23	34	45	57	68
	Uptake (μg/g dry wt)	153±16	127±17	138±5	103±12	82±15	96±15	83±10
Hexan-1-ol	Concentration (mM)	0	3.9	7.8	11.8	15.7	19.6	
	Uptake (μg/g dry wt)	1739±96	1546±110	1214±77	624±59	521±3	367±47	
Heptan-1-ol	Concentration (mM)	0	0.86	1.72	2.59	3.45	4.31	5.17
	Uptake (μg/g dry wt)	785±22	565±49	668±35	691±51	430±78	330±27	216±11
Octan-1-ol	Concentration (mM)	0	0.31	0.62	0.92	(1.23)	(1.54)	
	Uptake (μg/g dry wt)	184±22	147±13	129±20	120±11	(174±25)	(176±32)	

NB. Brackets around values refer to those points which were not included in the EC_{50} calculation. This was because at high concentrations there were signs of chlorophyll degradation and breakdown of cellular components within the frond, resulting in abnormal absorbances.

carbons no effect of reducing neutral red uptake should be seen. When a further experiment using decan-1-ol (C=10) was carried out this theory was confirmed.

Table 2: Resultant EC_{50} and R^2 values for neutral red uptake in *E. intestinalis* with exposure to an homologous series of alcohols.

Alcohol	Number of carbon atoms	Log K_{ow}[a]	EC_{50} (mM)	Log EC_{50}	R^2 value
Methanol	1	-0.77	3214	3.507	0.93
Ethanol	2	-0.31	1396	3.145	0.88
Propan-1-ol	3	0.25	302	2.480	0.76
Butan-1-ol	4	0.88	135	2.130	0.91
Pentan-1-ol	5	1.56	71	1.851	0.83
Hexan-1-ol	6	2.03	16	1.204	0.95
Heptan-1-ol	7	2.41	3.9	0.591	0.80
Octan-1-ol	8	2.97	1.3	0.114	0.92

[a] K_{ow} is the octanol/water partition coefficient; values from Schultz et al., (1990).

2.4 Discussion

2.4.1 Timecourse of neutral red uptake

The accumulation of neutral red was very uniform after 3 hrs, but after 4 hrs or more the data became highly variable. Similar observations were made by Alexander and Wilkinson (1987) who used microscopy to observe uptake of neutral red into cells of the thallus. After 24 hours the cells showed clear evidence of toxicity due to the stain and were incapable of being restained. A 3 hr exposure period was therefore chosen for

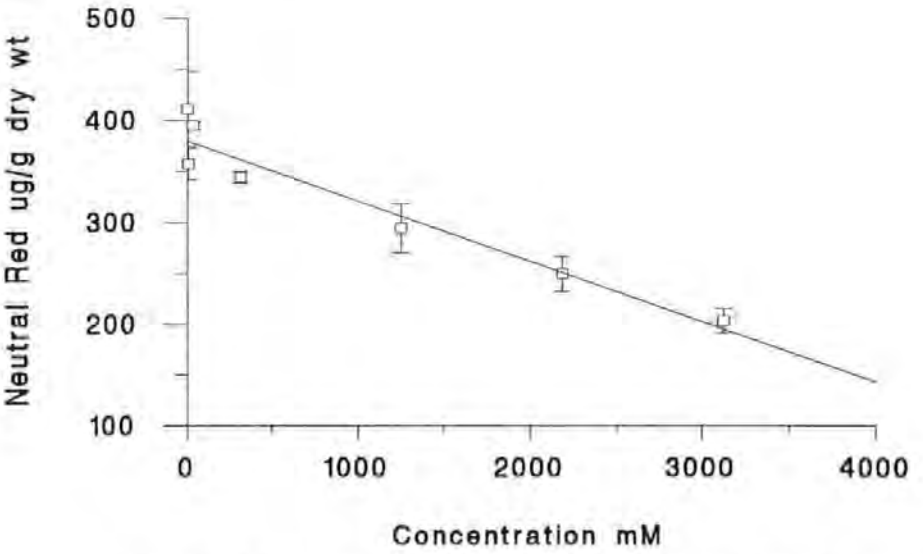


Figure 2: Concentration response graph for methanol, showing regression line for the mean of each point and standard errors. Resultant EC50 = 3214 mM.

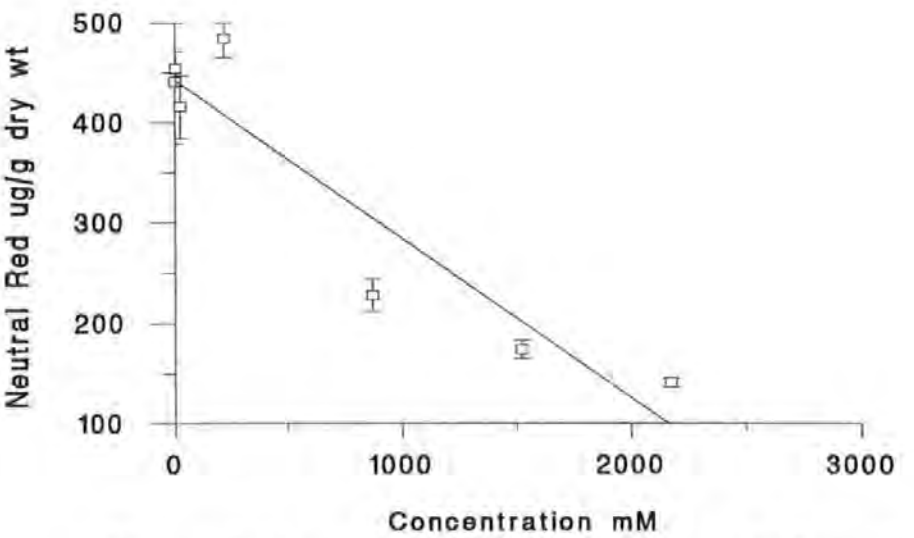


Figure 3: Concentration response graph for ethanol, showing regression line for the mean of each point and standard errors. Resultant EC50 = 1396 mM.

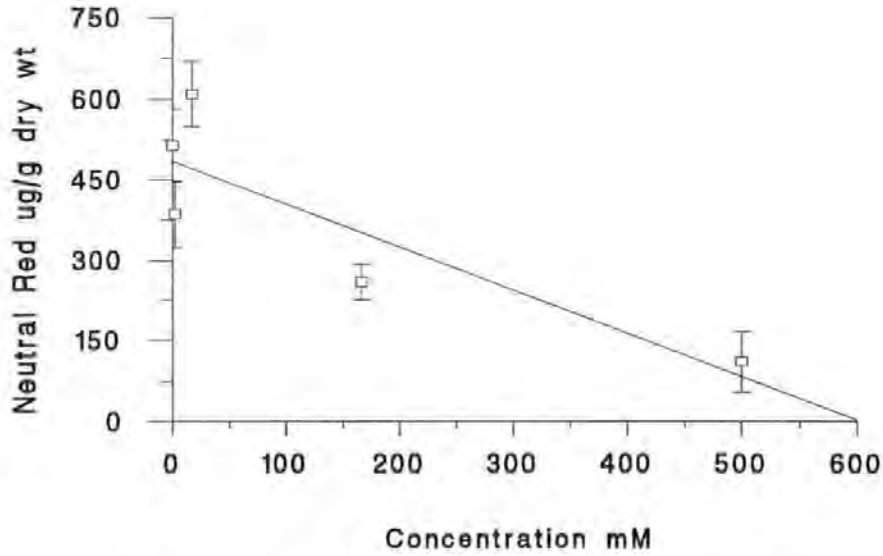


Figure 4: Concentration response graph for propan-1-ol, showing regression line for the mean of each point and standard errors. Resultant EC50 = 302 mM.

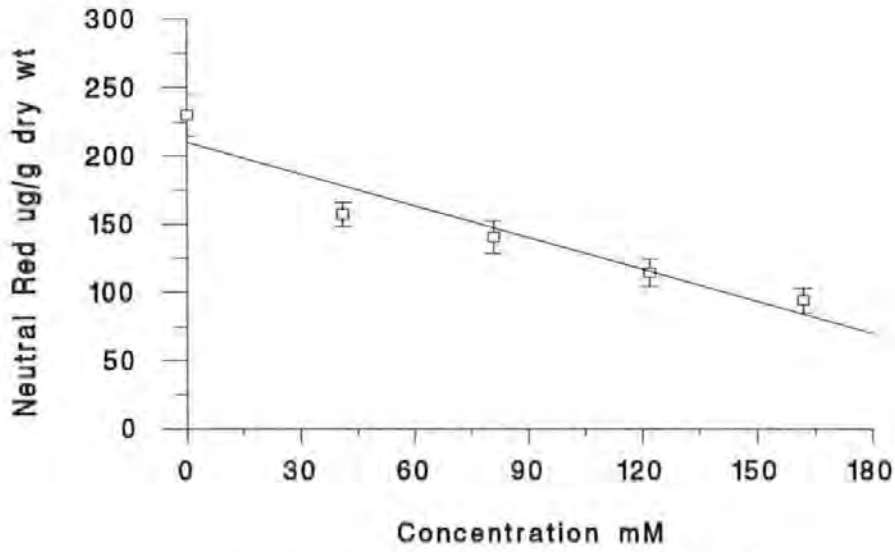


Figure 5: Concentration response graph for butan-1-ol, showing regression for the mean of each point and standard errors. Resultant EC50 = 135 mM.

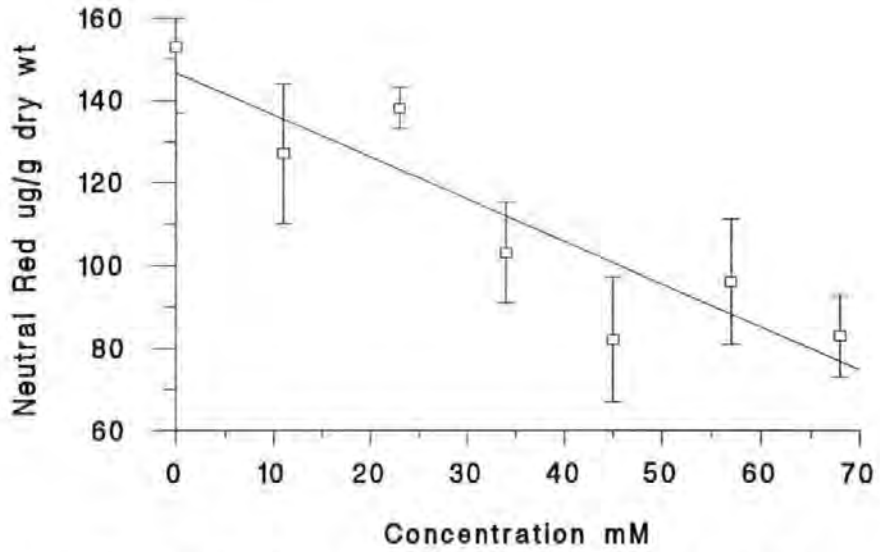


Figure 6: Concentration response graph for pentan-1-ol, showing regression line for the mean of each point and standard errors. Resultant EC50 = 71 mM.

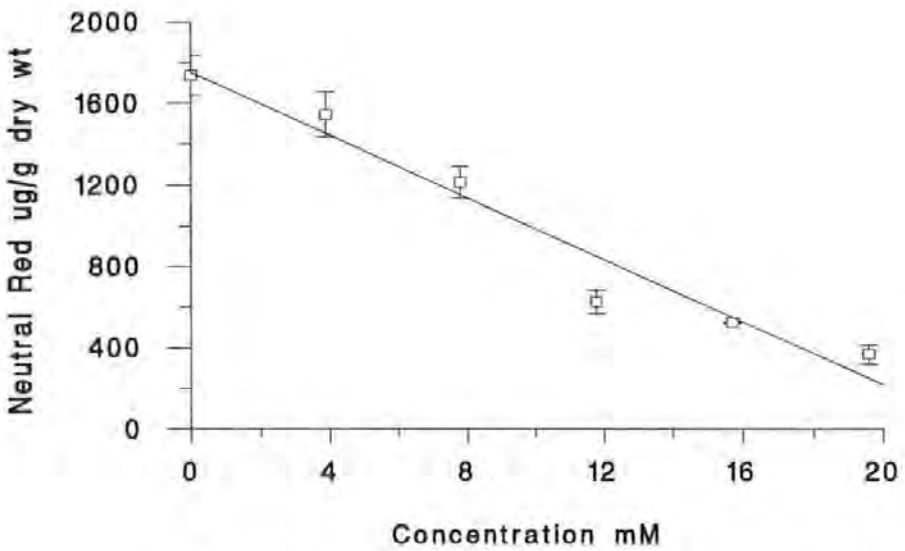


Figure 7: Concentration response graph for hexan-1-ol, showing regression line for the mean of each point and standard errors. Resultant EC50 = 16 mM.

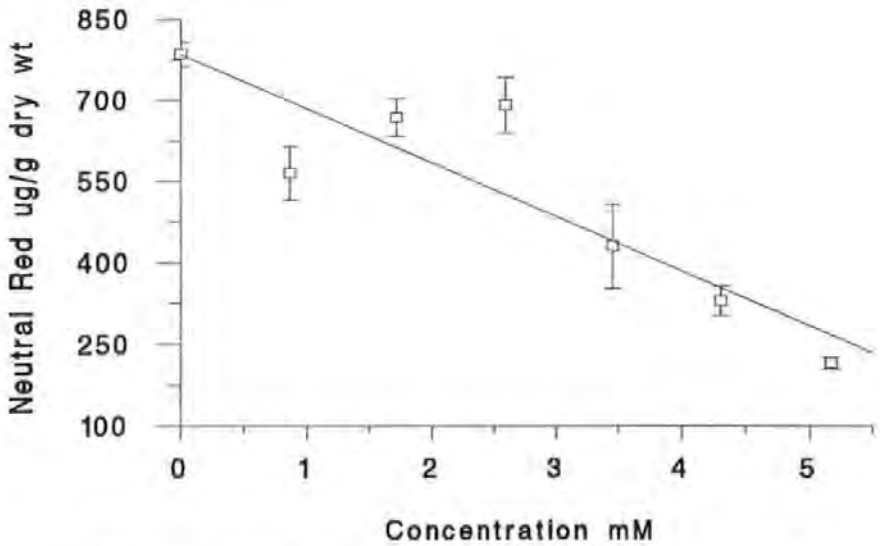


Figure 8: Concentration response graph for heptan-1-ol, showing regression line for the mean of each point and standard errors. Resultant EC50 = 3.9 mM.

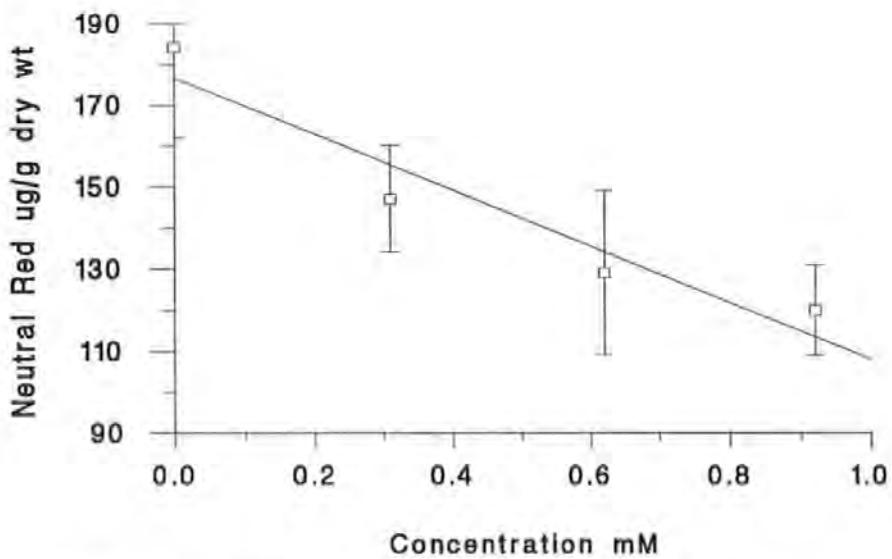


Figure 9: Concentration response graph for octan-1-ol, showing regression line for the mean of each point and standard errors. Resultant EC50 = 1.3 mM.

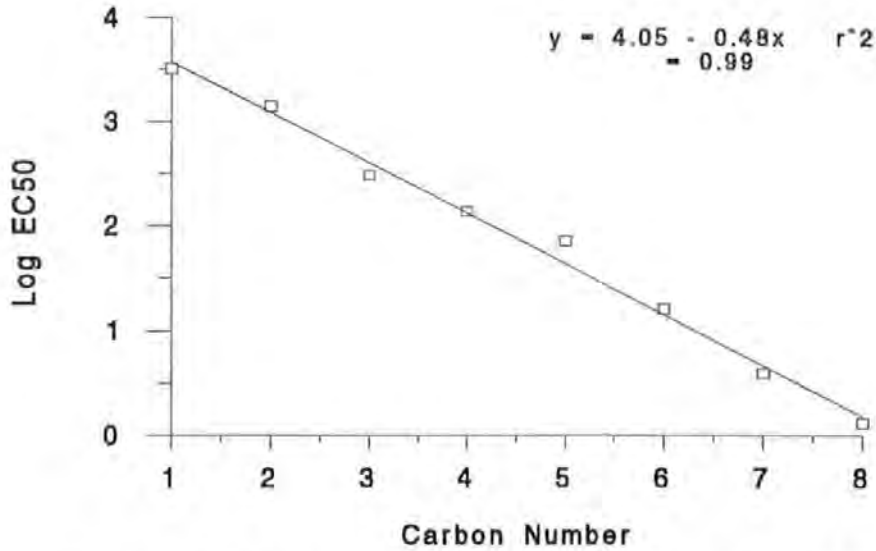


Figure 10: Relationship between log EC50 and carbon number.

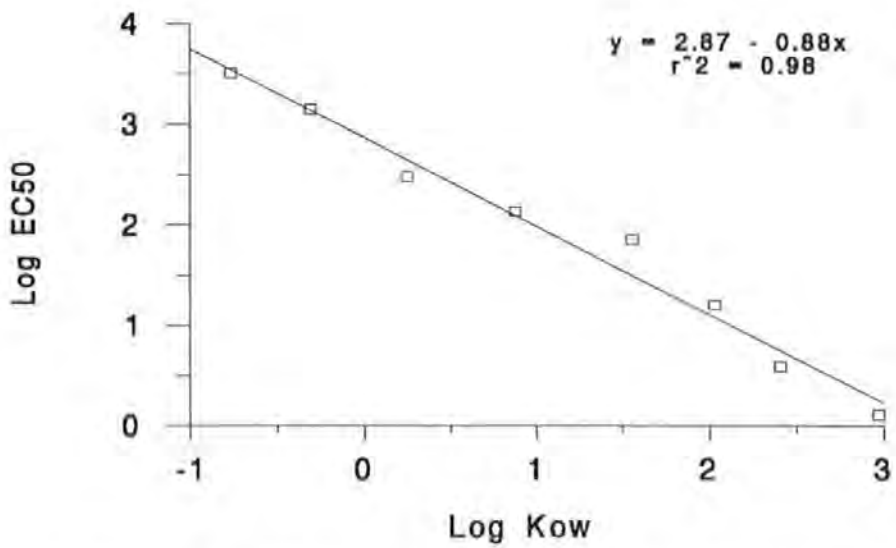


Figure 11: Relationship between log EC50 and log Kow.

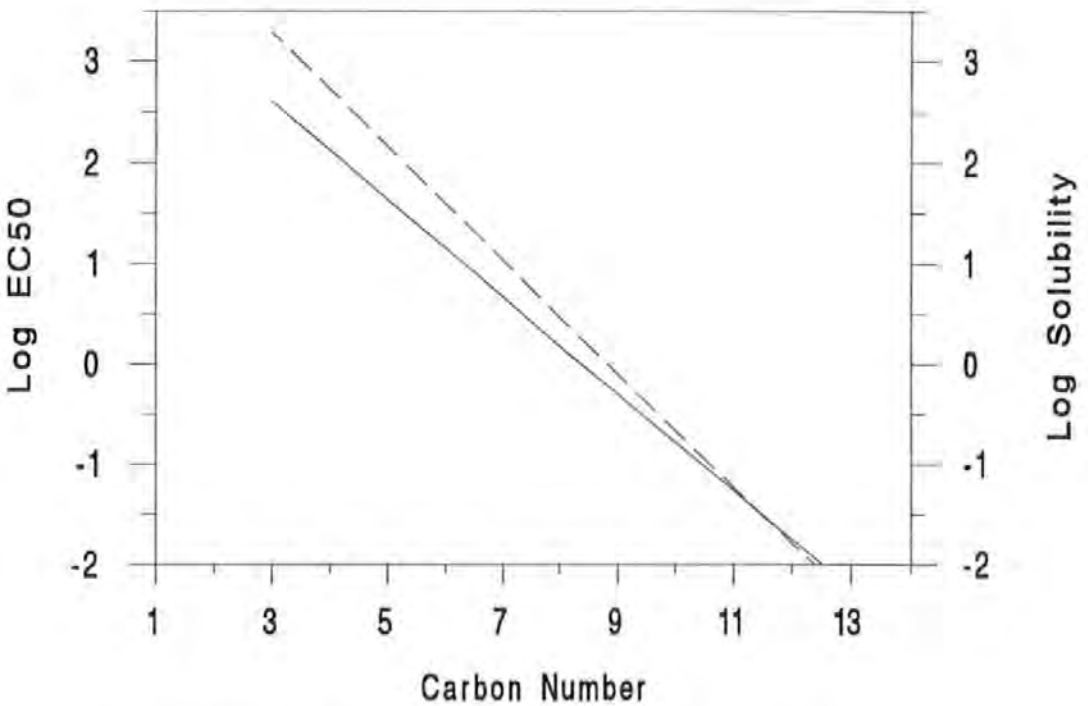


Figure 12: Estimated cut-off point for effect of alcohols on neutral red retention in E. intestinalis based upon solubility of alcohols in freshwater at 25 deg.C. Where Log solubility = dashed lines and Log EC50 = solid Lines.

our toxicity experiments. Babich and Borenfreund (1987) also found 3 hrs to be optimal for cultured animal cells. Alexander and Wilkinson (1987) have stressed the importance of controls in stain-retention procedures. The most important control in our procedure was live untreated *E. intestinalis*, which permitted correction for the small absorbance at 540 nm due to natural products, such as chlorophyll, extracted from the alga.

It was not possible to find a satisfactory killing treatment which eliminated neutral red uptake because all treatments investigated, including boiling and exposure to 4% formaldehyde, resulted in an increased neutral red uptake. The same effect was observed for very high concentrations of alcohol. However at sublethal levels of toxicity, neutral red uptake following alcohol exposure gave a clear concentration-response relationship. Lowe et al. (1992) observed that in Dab, neutral red was taken up and retained in the lysosomes of healthy fish, the rest of the cytoplasm remaining clear. When cell damage was induced by exposure to fluoranthene, the neutral red entered the cell and became distributed throughout the cytoplasm and was indicative of severe lysosomal membrane damage. Similar observations have been made with *E. intestinalis* (Schild unpub.) where, following cell death, neutral red was seen to accumulate within the cell and cell walls leading to increased absorbances on extraction. The use of dead controls was therefore nonsensical, and brings into question whether under such circumstances neutral red can be treated as a vital stain.

At sublethal levels, alcohol exposure gave clear, linear, concentration-response relationships, and in some cases substantially reduced neutral red uptake (see Figure 7, hexan-1-ol concentration response graph). Additional controls were therefore unnecessary. Indeed, Alexander and Wilkinson (1987) suggested using 95% ethanol (with the addition of 2,4-dinitrophenol) as a killing treatment.

2.4.2 Alcohol toxicity QSAR

The linear QSARs between the toxicological response (EC_{50}) and carbon number or log K_{ow} (Figures 10 and 11), both have very high correlation coefficients, 0.99 and 0.98 respectively, with the log K_{ow} line having a slope approaching 1. These plots are characteristic for non-specific narcotic alcohols (Hansch et al., 1989). The figures also demonstrate a remarkably close relationship between our data and that for the same group of compounds obtained by applying the method of Babich and Borenfreund (1987) to cultured fish cells (Dierickx and Van De Vyver, 1991). At least for non-specific narcotics these two methods could be used interchangeably.

For both animals and plants, the technique is rather insensitive when compared with procedures used successfully for monitoring in the environment. For example the EC_{50} for the inhibition of ciliary feeding in mussels is in the low μM region for hydrocarbons with similar hydrophobicity to octan-1-ol (Donkin et al., 1991). Laboratory studies investigating the effect of normal aliphatic alcohols on the unicellular freshwater alga *Chlorella vulgaris* indicate that growth in this organism is between 2 and 5 times more sensitive a parameter than neutral red retention by *Enteromorpha spp.* (Ikemoto et al., 1992). A similar sensitivity ratio was also reported by Herman et al., (1991) in their study of hydrocarbon toxicity to the freshwater alga *Selenastrum capricornutum* using a fluorescein stain retention assay. Babich and Borenfreund (1987) suggested that the neutral red retention assay may reflect damage to the lysosomal membrane. However, Lowe et al. (1992), by looking at rates of neutral red loss from the lysosomes observed under a microscope, has produced a procedure which is apparently more sensitive than the method of Babich and Borenfreund (1987). This suggests that the whole cell procedures reflect stability of the plasma membrane which may be less sensitive than the lysosomal membrane. Indeed, the EC_{50} values obtained for alcohols using the whole-cell neutral red procedure are of the

same order as those required to cause visible morphological abnormalities in the cell (Babich and Borenfreund, 1987).

In an homologous series of compounds, the toxicity of the higher molecular weight homologues to aquatic organisms is often restricted by their insolubility in water. This effect can be accentuated in temperate marine systems by low water temperatures and high salinities. Both these parameters can reduce the solubility of organic compounds and hence their bioavailability through the aqueous route. This is illustrated by Figure 12, where, using values for solubility in freshwater at 25°C the cut-off point would be above C=11. Under the conditions we used this is decreased and our predicted toxicity cut-off point is reduced to molecules with carbon numbers greater than 8. This is a rather low cut-off point in comparison to sublethal responses in freshwater systems at >20°C, where effects have been reported up to dodecan-1-ol in algae (Ikemoto et al., 1992) and tridecan-1-ol in protozoans (Schultz et al., 1990). Cut-off phenomena in relation to the neutral red assay require further investigation in order to define more precisely the limits of responsiveness of the technique.

A reproducible procedure has been established for measuring the retention of neutral red stain by the benthic macroalga, *E. intestinalis* and its utility demonstrated by establishing a quantitative structure activity relationship for normal aliphatic alcohols. The technique is sensitive enough for laboratory testing of potentially toxic organic compounds and the establishment of their respective EC_{50}'s. However, the level of reproducibility between experiments is so low that it is not possible to establish a 'normal' response. Because of this the method is not considered to be suitable as an environmental monitor, c.f. Chapter 5.

CHAPTER 3

EVALUATION OF ION LEAKAGE

3.1 Introduction

Ion leakage techniques have been used for measuring stress in plant tissues since the method was first proposed by Dexter et al. in 1932. Initially designed to detect frost damage it has also been employed to show the effects of high temperature and dehydration. Early published accounts used total electrolyte (ion) leakage into a bathing solution. Following recognition that ion content can vary between samples it was proposed by Stuart in 1939 that the ion leakage should be expressed as a percentage relating initial leakage to total leakage. The test consisted of placing the tissue segment in distilled water for a set period of time followed by removal and either autoclaving or freezing in liquid nitrogen with subsequent replacement into distilled water to extract all remaining ions. The electrical conductivity was measured for each solution and the percentage leaked initially calculated in relation to the total number of ions present within the tissue. Flint et al. (1967) then devised a damage index which mathematically related the % leakage from treated and untreated tissues adjusting the values to a scale of 0 to 100, the higher the percentage, the higher the damage. In a recent publication Whitlow et al. (1992) propose two new methods which take account of both the relative water content of the tissue and the leaf thickness, rejecting the damage index as being inadequate to assess membrane stability. The points they make are important with respect to relative water content and tissue thickness and should be acknowledged. However, algae such as *E. intestinalis* or *U. lactuca*, which are largely undifferentiated and of one or two cell layers thick, show very little variation throughout the thallus. This makes such an approach unnecessary, provided it is not claimed accurately to model physiological changes.

An ion leakage method has been employed by Axelsson and Axelsson (1987) to quantify environmental effects on *Laminaria spp*, the first reported use with macroalgae. Their method is very simple and does not make use of the damage index but relies on the basic % leakage. Despite their utilisation of algae with thick thalli they were able to show that with increased exposure to certain pollutants, loss of ions increased, producing concentration response curves with acceptably small standard deviations. Additionally they were able to employ considerably shorter incubation times than have been used for higher plants and simply boiled the algae to extract all remaining ions following the initial incubation.

This technique therefore showed potential for development for use with *E. intestinalis* because this algae is composed of largely undifferentiated cells, is principally one layer thick, and leakage should not be influenced by thallus morphology. This technique also provides a very useful comparison to that described in the previous chapter where a vital stain, neutral red, was used to assess membrane damage due to a series of n-alcohols. To aid comparison the algae was exposed to the same series of alcohols, and in addition to a number of other organic compounds with specific modes of action. These compounds were the herbicides diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCMU), and diquat (1,1'-ethylene-2,2'-dypyridine) which have modes of action linked to photosynthesis (Duke, 1990), 2,4-dichlorophenol (2,4-DCP) an uncoupler affecting both mitochondrial respiration and photosynthesis (Tissut et al., 1987) and the antifouling compound tributyltin (TBT) which is also an uncoupler but has additional effects at the cellular level (Snoeij et al., 1987).

Further development of the technique involved analysis of the leakage samples to establish the nature of the ions leaked. This utilised inductively coupled plasma - mass spectrometry

(ICP/MS) which is a highly sensitive method used to detect and quantify many different ions.

3.2 Methods

3.2.1 Collection of algae

E. intestinalis was collected from rock pools within the intertidal region of Wembury Beach. The algae were placed in polythene bags and returned to the laboratory where they were briefly washed in Instant Ocean of salinity 33 ppt, and placed in buckets of aerated Instant Ocean of the same salinity. They were left under light frames of 66 $\mu E/m^2/s$ in a constant temperature room held at 16°C and allowed to equilibrate for at least 12 hrs. Before use microscopic examination was carried out to confirm the identification of the algae.

3.2.2 Development of the technique

Following the method of Axelsson and Axelsson (1987) the initial leakage and boiling times were determined. Their method involved the exposure of *Fucus* and *Laminaria* spp. to various treatments followed by analysis, and can be summarised as follows:

Each algal sample was removed from the test or control solution and placed into sea water of salinity 33 ppt to equilibrate for between 10 min and 1 hr. To remove the sea water from the fronds each piece was placed in 100 ml distilled water for 2 sec and then transferred to a beaker containing 50 ml distilled water. After 4 min the alga was transferred into a screw cap bottle, also containing 50 ml distilled water, the first 50 ml water sample being retained and referred to subsequently as sample 1. The bottle containing the alga was next placed in a boiling water bath for 5 min, and after this period the alga was removed and the water retained as sample 2. The resultant conductivity of the

two water samples, 1 and 2, was measured and the ion leakage expressed as a percentage of the conductivity of sample 1 divided by the total conductivity. Thus the whole technique is independent of the weight of the sample used.

To establish whether this method required adaptation when applied to the Chlorophyte macroalga *E. intestinalis* two experiments were carried out. Firstly the boiling time which allowed the maximum amount of ions present to be lost was ascertained. One g samples of algae were weighed out, having been damped off on laboratory tissues prior to weighing, and washed as described earlier then placed into 250 ml, glass, screw cap bottles containing 50 ml distilled water. These were placed in a boiling water bath and left for either 2, 4, 6, 8 or 10 minutes, after which the algae were removed and the conductivity of each solution measured using a Corning model 220 conductivity meter. Three samples were used for each boiling time. The following results were obtained:

Table 3: The use of different boiling times to achieve optimum ion leakage from 1 g samples of *E. intestinalis*

Boiling Time (min)	2	4	6	8	10
Conductivity (mean ±SE)	849 ±123	768 ±22	726 ±50	695 ±10	752 ±38

As there was no significance in the difference between each time used, a boiling time of 5 min was adopted which would ensure that all ions were leaked.

Secondly, it was necessary to select a leakage time for the initial ion leakage into sample 1. This was the time required to give the maximum difference between two references, a heat treated sample and a healthy one, in terms of ion loss. To achieve this two sets of

algae were weighed out, one set was heat treated by boiling in Instant Ocean for 5 min, and then left for 30 min to equilibrate in Instant Ocean (33 ppt), whilst the other was left as an untreated control. Ion leakage tests were carried out for both sets of algae as detailed earlier, but for each set different samples of algae were left in the first 50 ml of distilled water for different lengths of time, i.e. to produce sample 1. These were 2, 4, 6, 8 and 10 min. A 5 min boiling time was used in all cases to give sample 2. It was unnecessary to leave the untreated algae to equilibrate prior to testing as they were already in Instant Ocean of the same salinity. The percentage Health Index for each individual sample was calculated using the conductivities of samples 1 and 2, and the relationship $2/(1+2) \times 100$ calculated to allow a low percentage to represent lack of health and membrane integrity, and a high percentage to represent good health. For *E. intestinalis* healthy algae typically gave a Health Index value of 60%, and dead algae a value of 20%. The results are displayed in Figure 13 and show the greatest difference between the two treatments to be for 2 min leakage. This was therefore adopted as the initial leakage time for use with *E. intestinalis*.

3.2.3 Finalised method

Prior to weighing, all algae were damped off using laboratory tissues to remove surface water. One gram samples were weighed out ready for incubation or immediate testing. Before testing each algal sample was washed for approximately 2 sec in 100 ml distilled water to remove any ions due to sea water. The alga was then placed in a beaker containing 50 ml distilled water and left for 2 min (sample 1). Following the initial leakage the alga was transferred into a screw cap bottle also containing 50 ml distilled water. This was placed in a boiling water bath and left for 5 min, after which time it was removed and the contents strained into a 100 ml beaker. The alga was discarded and the resultant solution retained (sample 2). The conductivity of samples 1 and 2 were measured and the

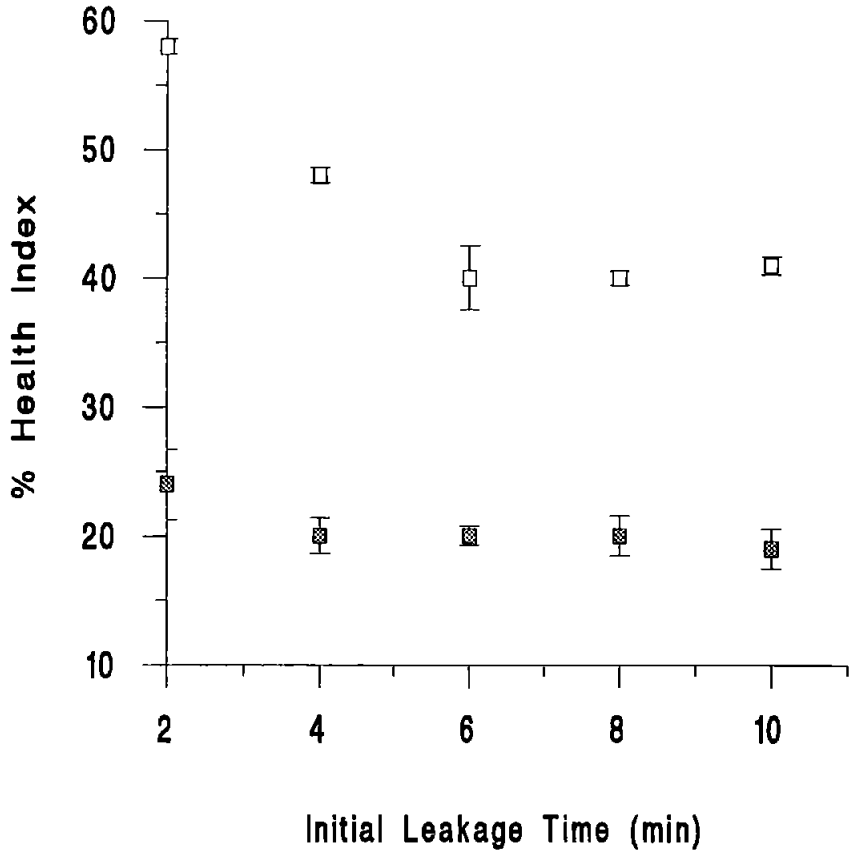


Figure 13: Difference in Health Index, with varied initial leakage times, for untreated (empty squares) and heat treated (filled squares) algae.

Health Index calculated using the following equation:

$$\% \text{ Health Index} = 100 \times \frac{\text{(Conductivity of ions lost on boiling)}}{\text{Conductivity of all ions lost}}$$

or, % Health Index = 100 (sample 2/(sample 1+2))

3.2.4 Salinity experiments

Two experiments were carried out to establish the effect of changing salinity on the algae and Health Index. This was achieved by exposing the algae to various salinities for 24 hr and assessing membrane integrity via ion leakage. In the first experiment the algae were tested immediately after exposure, in the second the algae were transferred to vessels containing Instant Ocean of salinity 33 ppt, following exposure and allowed to equilibrate for 15 min prior to the ion leakage test being carried out.

One g samples of *E. intestinalis* were exposed to a range of salinities from 0 ppt to 100 ppt made up using different amounts of Instant Ocean. Each sample was placed in a 500 ml screw cap jar containing 330 ml of exposure solution, each salinity being tested in triplicate. Incubation was for 24 hrs at 17°C, with photosynthetically active radiation of 15 $\mu E/m^2/s$. Following incubation the ion leakage technique was carried out on the algae, either with, or without, equilibration in normal salinity Instant Ocean.

3.2.5 Toxicity tests

Concentration-response curves were produced for a number of toxicants in artificial seawater, essentially as described in Chapter 2, Section 2 and Section 3.2.3 and Schild et al. (1993; 1995). The toxicants used were a series of n-alcohols from one to eight carbon atoms; herbicides diuron and diquat; an uncoupler of photosynthesis and mitochondrial

respiration - 2,4-DCP; the antifouling compound TBT, which is an uncoupler with additional effects.

The solutions were prepared in Instant Ocean (salinity 33 ppt) in all cases except for TBT for which a stock solution was prepared in methanol, aliquots of which were added to the vortex in a vigorously stirred 1000 ml of artificial seawater. The final methanol concentration was 80 mg/l, considerably below the level known to have a toxic effect on the algae (Schild et al., 1993; and Chapter 2). Exposure was for 24 hrs in all cases and ion leakage was carried out immediately following incubation, equilibration not being necessary as all exposures were carried out at the same salinity.

Octan-1-ol/water partition coefficients (K_{ow}) were used to derive quantitative structure activity relationships and to help interpret the results. K_{ow} values were obtained from Jaworska and Schultz (1993) for the alcohols, Howard (1991) for diuron and diquat, Shannon et al. (1991) for 2,4-DCP and Tas (1993) for TBT.

3.2.6 Growth Studies

An experiment was set up to investigate the relationship between growth and ion leakage. In outline, the experimental design involved taking samples of algae of known wet weight, exposing them to a range of butan-1-ol concentrations and leaving them to grow over a period of 6 days. Dry weights were measured at the beginning and end of the experiment. Wet weights were measured every other day when solutions were also changed. At day 6 half the samples (n=12) were dried to give final dry weight and the other half (n=12) were used for Health Index determination.

E. intestinalis fronds were dried on laboratory tissues to remove as much surface water as

possible. Samples of 0.5 g were weighed out accurately and introduced into 500 ml bottles containing 330 ml of control or alcohol solution, each treatment having four replicates. The bottles were then covered with glass crystallising dishes to allow light penetration, but simultaneously reducing volatilisation of the alcohol. Six algal samples were weighed into porcelain crystallising dishes and placed in an oven at 80°C, dry weight being determined after 24 hrs (c.f. Chapter 2). The exposure bottles were incubated in a Fisons environmental cabinet at 16°C with illumination of 45 $\mu E/m^2/s$ for 16 hrs/day. On day 2 and day 4 all samples were removed from the cabinet and the wet weight of the algae determined following damping off. On replacement new solutions were added. On day 6 wet weights were measured and two samples from each treatment were dried, while ion leakage was carried out on the remaining two.

3.2.7 Copper toxicity on *Ulva lactuca*

The effects of copper on *U. lactuca* was assessed using ion leakage (data from Wendy Bishop, Honours Project, University of Plymouth, 1994). Following the procedure detailed in section 3.2.2 the method was adapted for use with this alga and an initial leakage time of 4 min was selected, with a boiling time of 5 min. Each sample consisted of three, 39 mm discs cut randomly from the frond using a cork borer.

Concentrations of copper sulphate were made up in Instant Ocean from a stock solution to give 0.0008, 0.004, 0.04 and 0.4 mM. 15 ml of each concentration was placed in each of four petri dishes, along with an Instant Ocean control. Three discs of algae were then added to each dish and incubated for 24 hrs in a growth room held at 16°C, with a 16 hr photoperiod of 63 $\mu mol/m^2/s$. Following incubation the health of the algae were assessed using ion leakage.

3.2.8 Further developmental work: ICP/MS and Flame Photometry

Inductively coupled plasma - mass spectrometry was used to identify which ions were lost during leakage in healthy and alcohol treated algae (*E. intestinalis*). To achieve this 1 g samples of algae were prepared, washed briefly as described earlier, and placed into beakers containing 25 ml distilled water. These were left for 30 sec and then the algae were transferred into other beakers also containing 25 ml distilled water. This procedure was repeated twice to give four, 25 ml samples, giving a total leakage time of 2 min. At this point the algae were transferred into bottles containing 50 ml distilled water and placed in a boiling water bath for 5 min. The algae were discarded following boiling and all the water samples retained for ICP/MS analysis. This procedure was carried out for healthy, untreated algae and for algae exposed to 122 mM butan-1-ol for 24 hr with incubation environment as detailed in section 3.2.4. This concentration was chosen as it is the calculated 24 hr EC_{50} for this alcohol. The control algae were treated identically to those incubated with butan-1-ol. The samples were stored in acid washed polypropylene vessels (10% nitric acid), acidified to 2% nitric acid, and refrigerated until used. Analysis was carried out on a V. G. Plasma Quad PQ2 TurboPlus ICP/MS and the ions present measured both semi-quantitatively using Pulse Counting (PC), and quantitatively using Extended Dynamic Range (EDR). Correction was made for the different volumes. An internal standard of indium, yttrium, uranium and rhodium (all 50 ppb) was added to each sample for comparison.

It was not possible to record data for potassium or sodium ions using the ICP/MS because of interference with the carrier gas, argon. To achieve data for these ions a Corning flame photometer (model 410) was used. Standards supplied by Corning were utilised to check for the linear concentration range and the samples were diluted as necessary.

3.3 Results

3.3.1 Salinity experiments

The two experiments conducted in order to investigate the effect of salinity on the alga gave two distinct responses which depended upon whether or not equilibration in a standard seawater solution (33 ppt) was carried out prior to ion leakage. Where no equilibration occurred the Health Index achieved was exceptionally high for algae incubated in seawater of low salinity, i.e. zero and 5 ppt, with Health Indices of 98% and 84% respectively. The Health Index then followed a slow decline reaching a minimum of 29% at a salinity of 100 ppt. When equilibration was allowed, the response was much reduced, exposure to all salinities resulting in algae with Health Indices between 47% and 63%, equivalent to no detectable membrane damage. Algae incubated in sea water of 20 ppt had a significantly higher Health Index than all other salinities tested, indicative of an adaptation. This data is presented as Figures 14 and 15.

3.3.2 Toxicity tests

The Health Index data are presented in Table 4 for the all the alcohols tested, TBT and 2,4-DCP. The concentration response curves (Figures 16 to 24) for the alcohols and 2,4-DCP show how increasing concentration causes a sigmoidal decrease in the Health Index. When algae were exposed to TBT the Health Index declined in an approximately logarithmic relationship (Figure 25). Curves were fitted using the Maximum Likelihood Programme statistical package. The fitted curves are displayed in Appendix 1. The range of data was defined by the maximum or control value and the minimum value achieved with high concentrations of toxicant, where increased concentration gave no further decline in Health Index. The range was relatively constant between tests and typically fell between 50-60% for control values, and $\leq$26% for minimum values, equivalent to the boiled controls for this algae. The log EC_{50} data obtained for the alcohols tested (see Table 5)

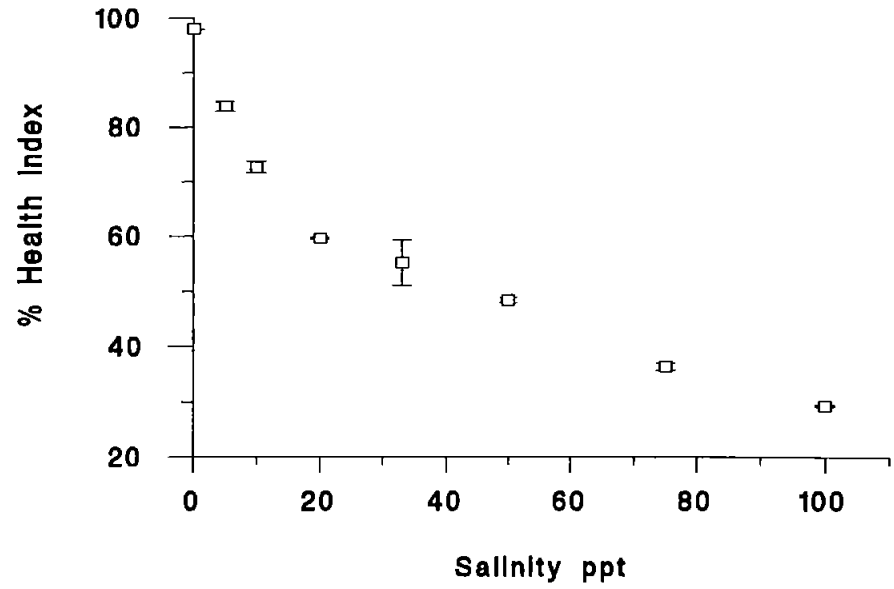


Figure 14: Health Index, with standard errors, for algae exposed to various salinities, measured without equilibration.

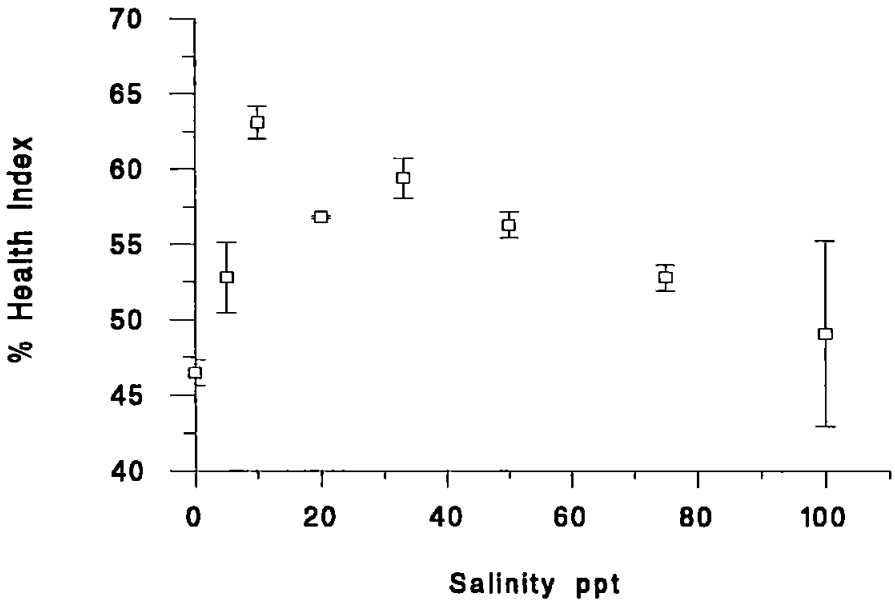


Figure 15: Health Index, with standard errors, for algae exposed to various salinities, measured after equilibration.

Table 4: The calculated Health Index for *E. intestinalis*, including standard errors, following exposure to various compounds.

Methanol	Concentration (mM)	0	1563	3125	4688	6250	7813	9375
	Health Index (%)	56.0±0.9	48.6±2.9	37.9±0.3	22.6±2.5	26.3±1.6	21.9±4.7	20.6±1.4
Ethanol	Concentration (mM)	0	435	870	1304	1739	2174	
	Health Index (%)	57.0±1.9	59.1±0.6	50.9±2.7	52.4±4.9	26.6±1.1	28.8±2.7	
Propan-1-ol	Concentration (mM)	0	333	667	1000	1333	1667	
	Health Index (%)	51.9±1.5	30.6±2.7	12.4±0.6	12.9±0.5	15.1±0.6	16.9±3.4	
Butan-1-ol	Concentration (mM)	0	40.5	81.1	122	162	203	243
	Health Index (%)	52.6±2.1	49.7±0.4	53.0±2.5	41.7±0.2	25.0±0.6	17.7±1.0	24.9±1.7
Pentan-1-ol	Concentration (mM)	0	11.4	22.7	34.1	45.5	56.8	68.2
	Health Index (%)	49.6±0.1	49.3±0.1	52.1±1.7	47.7±1.0	46.8±0.1	22.1±1.0	21.1±1.8
Hexan-1-ol	Concentration (mM)	0	3.92	7.84	11.8	15.7	19.6	
	Health Index (%)	50.9±2.4	32.0±1.0	16.1±0.3	16.0±1.2	17.1±1.0	17.5±1.9	
Heptan-1-ol	Concentration (mM)	0	0.86	1.72	2.59	3.45	4.31	5.17
	Health Index (%)	51.7±1.6	48.0±1.2	47.3±1.6	44.4±3.3	26.0±5.6	18.5±0.6	18.3±0.7
Octan-1-ol	Concentration (mM)	0	0.31	0.62	0.92	1.23	1.54	
	Health Index (%)	55.9±1.7	52.4±1.5	49.7±5.1	37.6±5.8	24.1±3.7	20.8±4.7	
TBT	Concentration (μM)	0	0.15	0.31	0.62	0.92	1.23	1.54
	Health Index (%)	60.8±1.7	49.3±1.1	36.5±1.1	29.4±0.8	25.9±2.0	25.0±1.1	24.6±0.5
2,4-DCP	Concentration (mM)	0	0.5	1.0	1.5	2.0	3.0	4.0
	Health Index (%)	54.3±1.7	47.3±2.1	22.2±2.0	17.7±1.0	18.6±0.6	19.3±1.1	18.7±0.6

were plotted against the log K_{ow} producing a good linear QSAR. The equation is as follows:

$\text{Log } EC_{50} = -0.95(\pm 0.06) \log K_{ow} + 2.83 \qquad n = 8, r^2 = 0.98$

See also Figure 26.

Results for herbicides diquat and diuron are shown in Figures 27 and 28. Neither had an effect on ion leakage at the maximum concentrations tested. For diuron this was 0.1 mM which is close to its solubility limit in seawater.

Table 5: Log EC_{50} Health Index values for *E.intestinalis* with exposure to various compounds.

Compound	Log K_{ow}	EC_{50} (mM)	Log EC_{50}
Methanol	-0.77[a]	2934	3.47
Ethanol	-0.31[a]	1418	3.15
Propan-1-ol	0.25[a]	310	2.49
Butan-1-ol	0.88[a]	134	2.13
Pentan-1-ol	1.56[a]	50.2	1.70
Hexan-1-ol	2.08[a]	3.88	0.59
Heptan-1-ol	2.41[a]	3.11	0.49
Octan-1-ol	2.97[a]	0.95	-0.02
2,4-DCP	3.08[b]	0.68	-0.17
TBT	4.15[c]	0.00025	-3.6

[a] K_{ow} (octanol/water partition coefficient) values from Schultz et al., (1990).

[b] K_{ow} value from Shannon et al., (1991).

[c] K_{ow} value from Tas, (1993).

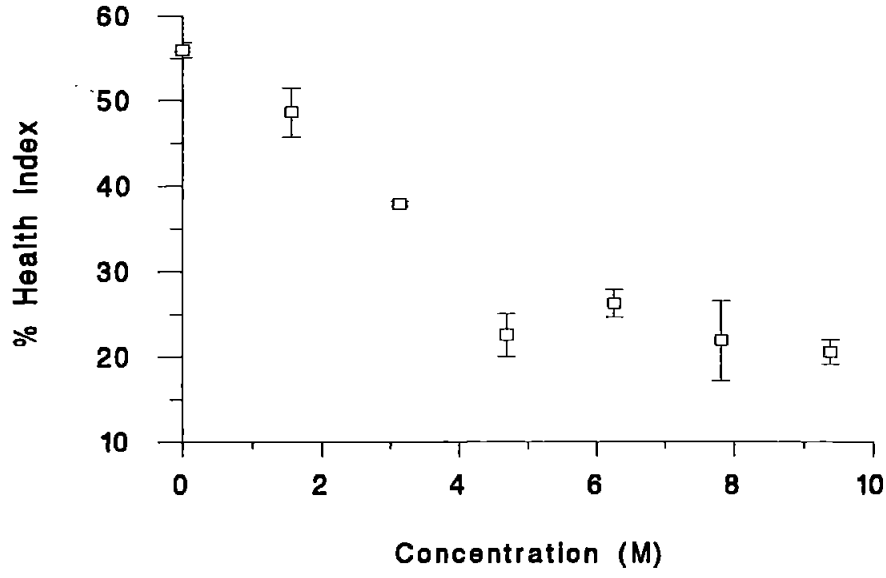


Figure 16: Concentration response curve for methanol, showing standard errors. Resultant EC50 = 2934 mM.

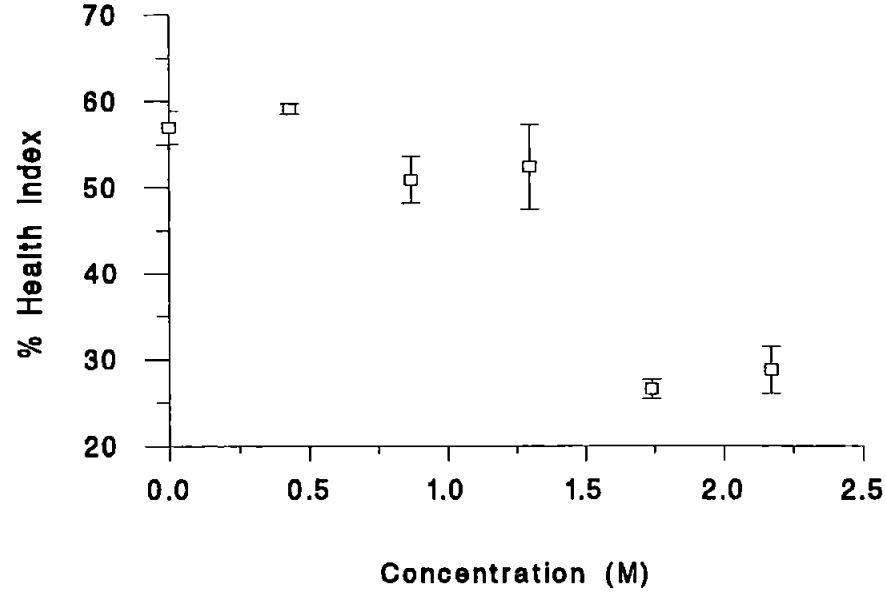


Figure 17: Concentration response curve for ethanol, showing standard errors. Resultant EC50 = 1418 mM.

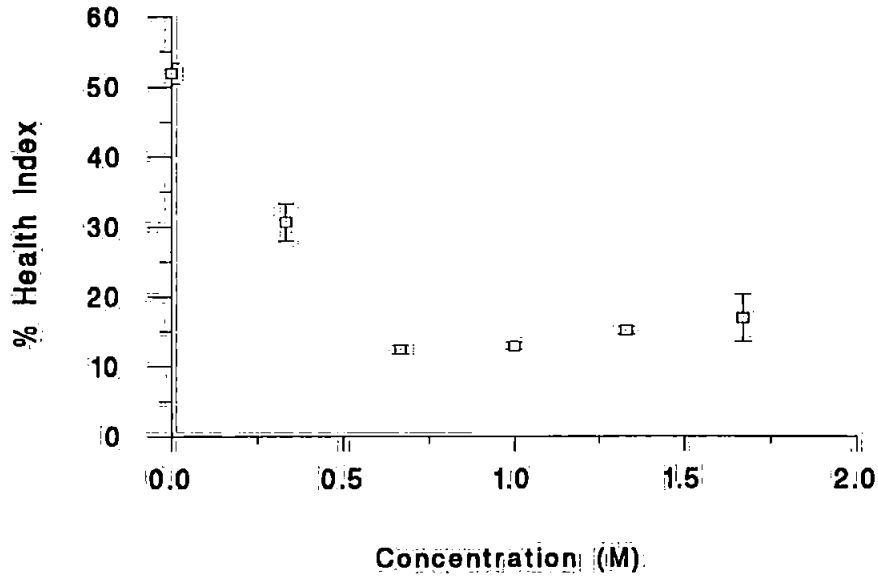


Figure 18: Concentration response curve for propan-1-ol, showing standard errors. Resultant EC50 = 310 mM.

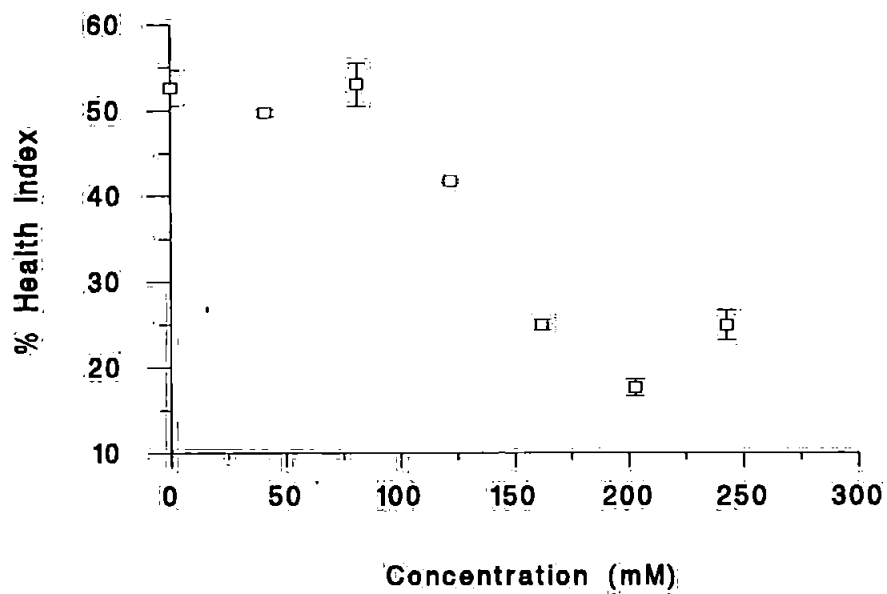


Figure 19: Concentration response curve for butan-1-ol, showing standard errors. Resultant EC50 = 134 mM.

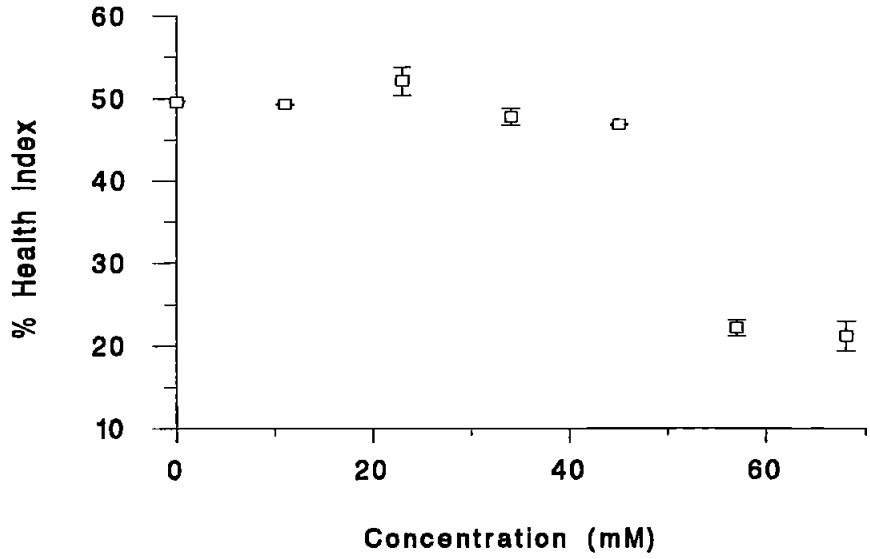


Figure 20: Concentration response curve for pentan-1-ol, showing standard errors. Resultant EC50 = 50.2 mM.

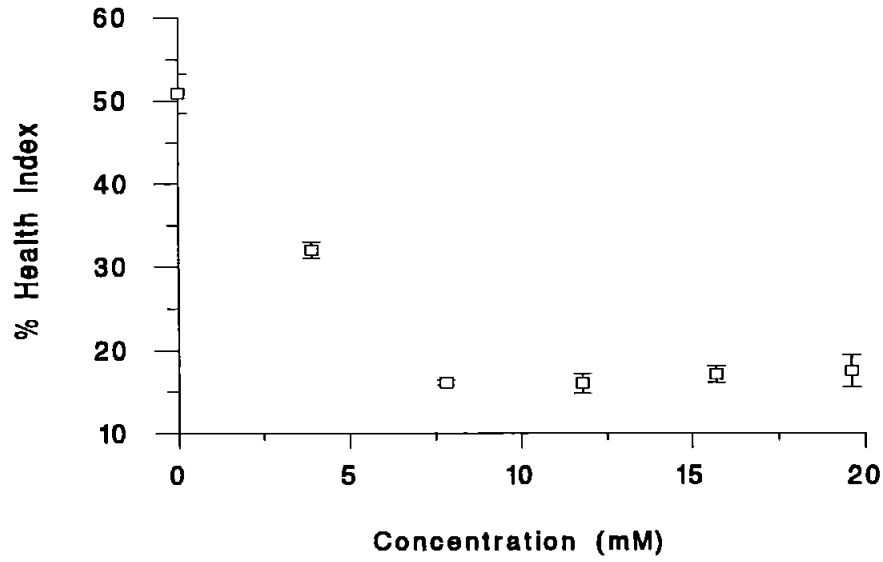


Figure 21: Concentration response curve for hexan-1-ol, showing standard errors. Resultant EC50 = 3.88 mM.

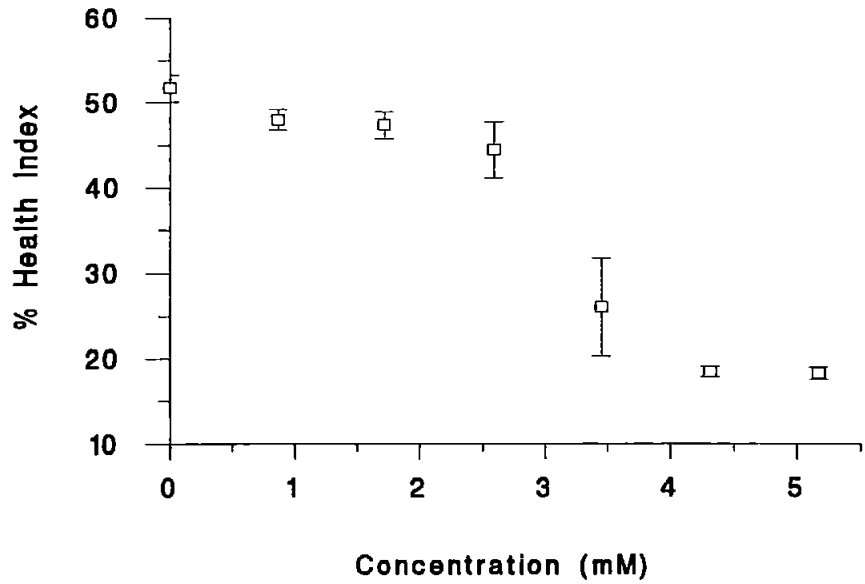


Figure 22: Concentration response curve for heptan-1-ol, showing standard deviations. Resultant EC50 = 3.11 mM.

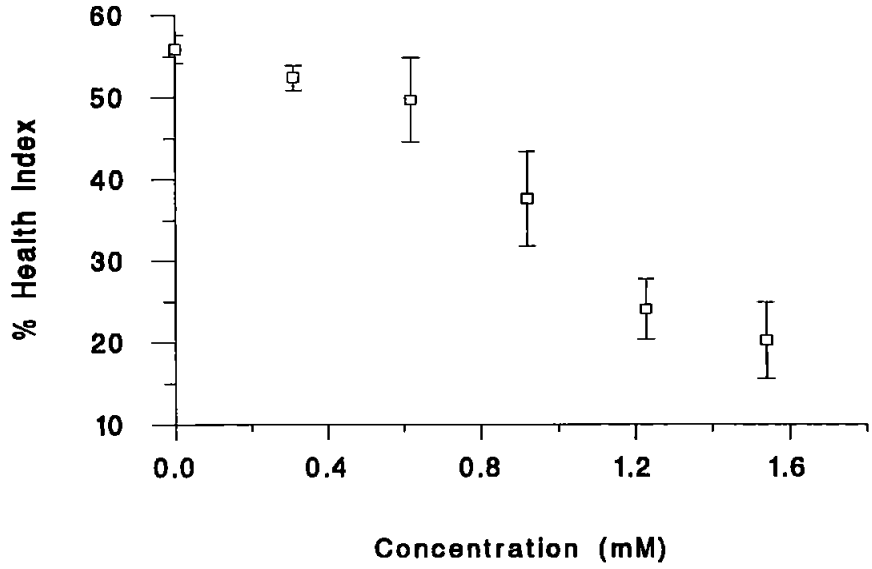


Figure 23: Concentration response curve for octan-1-ol, showing standard errors. Resultant EC50 = 0.95 mM.

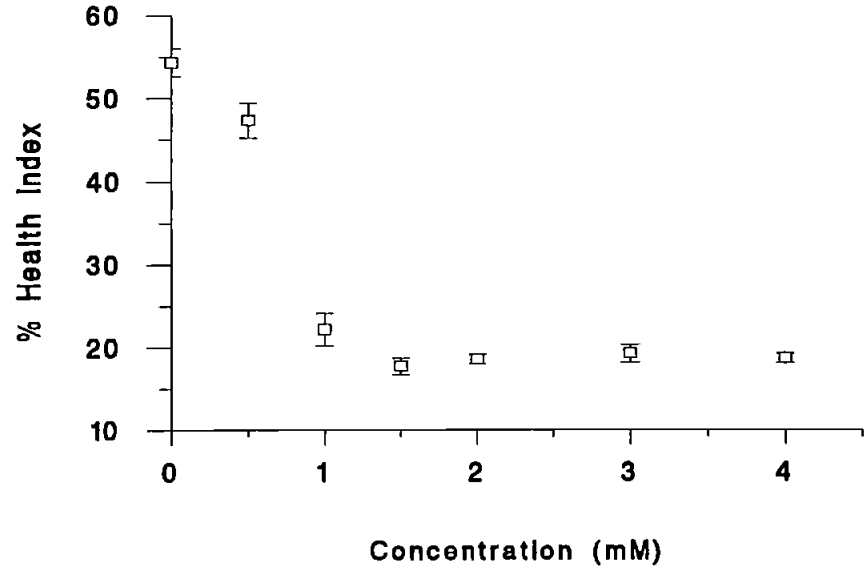


Figure 24: Concentration response curve for 2,4-dichlorophenol, showing standard errors. Resultant EC50 = 0.68 mM.

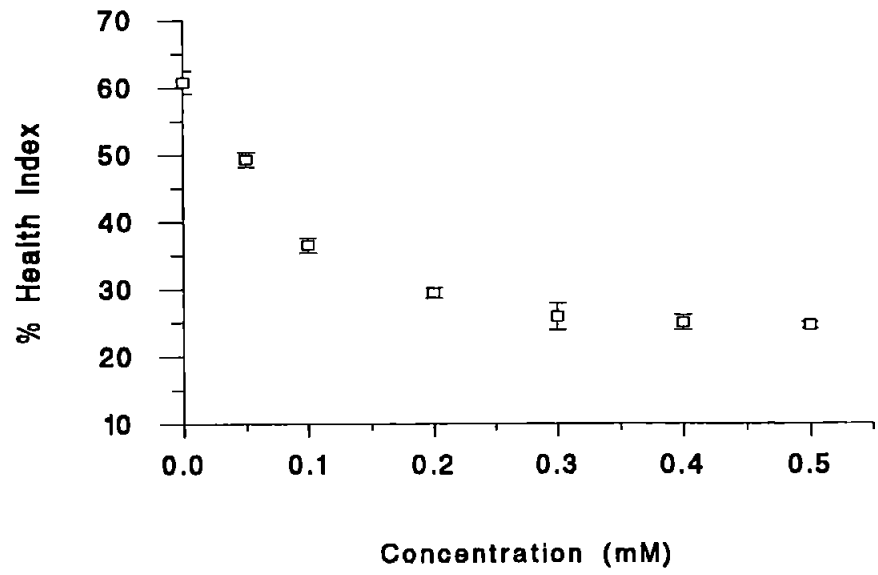


Figure 25: Concentration response curve for tributyltin, showing standard errors. Resultant EC50 = 0.00025 mM.

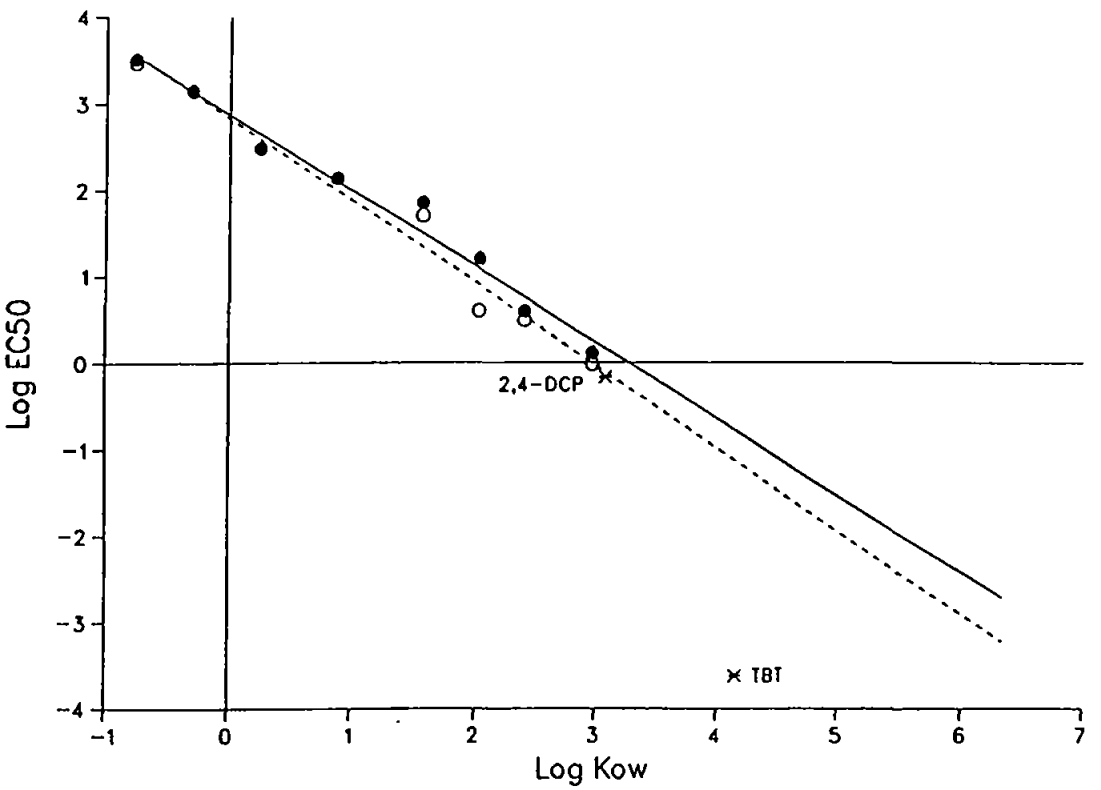


Figure 26: Quantitative structure-activity relationship for the effect of narcotic alcohols on neutral red retention by (●, ----), and ion leakage from (Health Index, ○, - - -) *E. intestinalis*. Also included are data for tributyltin and 2,4-dichlorophenol (though these are excluded from the regression line).

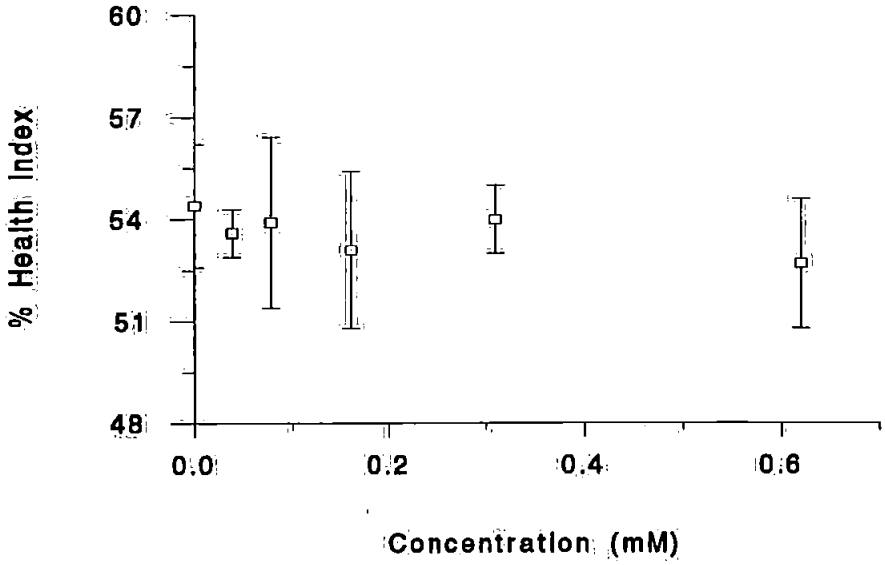


Figure 27: Concentration response curve for diquat, showing standard errors.

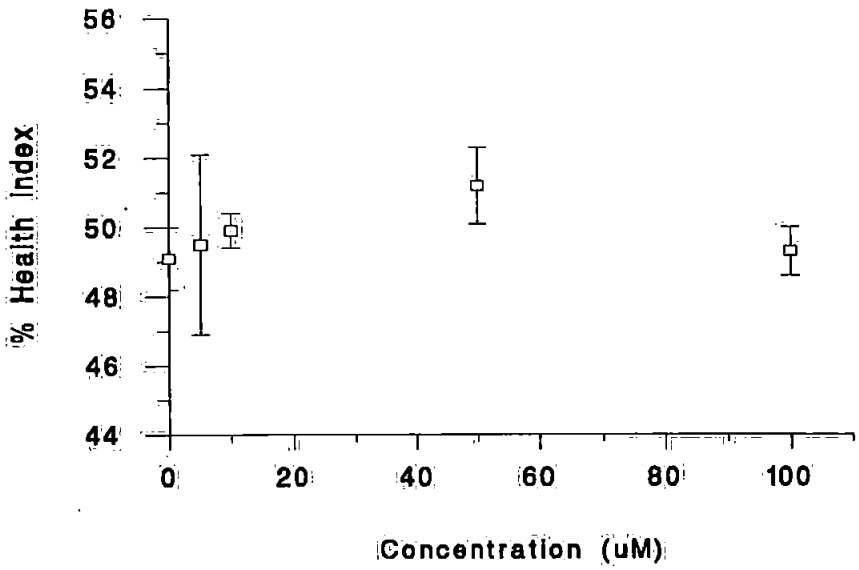


Figure 28: Concentration response curve for diuron, showing standard errors.

3.3.3 Growth studies

The wet weights of the algae measured at the beginning and during the 6 d butan-1-ol exposure did not significantly increase in all but the samples exposed to 54 mM, Figure 29. The weight of the control samples fell initially and then remained constant, whilst the other four fluctuated around the start weight of 0.5 g.

The equivalent dry weights of the algae measured initially and at the end of the experiment revealed little increase in the control samples but greater increase in those exposed to 14 and 27 mM butan-1-ol. At concentrations greater than these the dry weights had fallen to levels below the control (Figure 30). When this data is expressed in terms of dry weight/wet weight ratios a similar relationship is observed (Figure 31).

The Health Index data presented in Figure 32 reveals no increased ion leakage as a result of exposure to concentrations of 27 mM and below. Beyond this the Health Index fell to a minimum of 20 for 122 mM which is equivalent to the calculated 24 hr EC_{50} for butan-1-ol. The 144 hr (6 d) EC_{50} for butan-1-ol is lower than this at 74 mM, see Figure 32.

3.3.4 Copper toxicity on *Ulva lactuca*

As a result of exposure to 0.4 mM copper sulphate a slight decline was observed in the Health Index achieved for *U. lactuca*. No significant effect was seen for algae exposed to lower concentrations. See Figure 33.

3.3.5 ICP/MS

Using pulse counting to achieve semi-quantitative results yielded a large data set, some relating to ions whose biological functions are not known. A more quantitative measurement was carried out using the extended dynamic range, although unfortunately

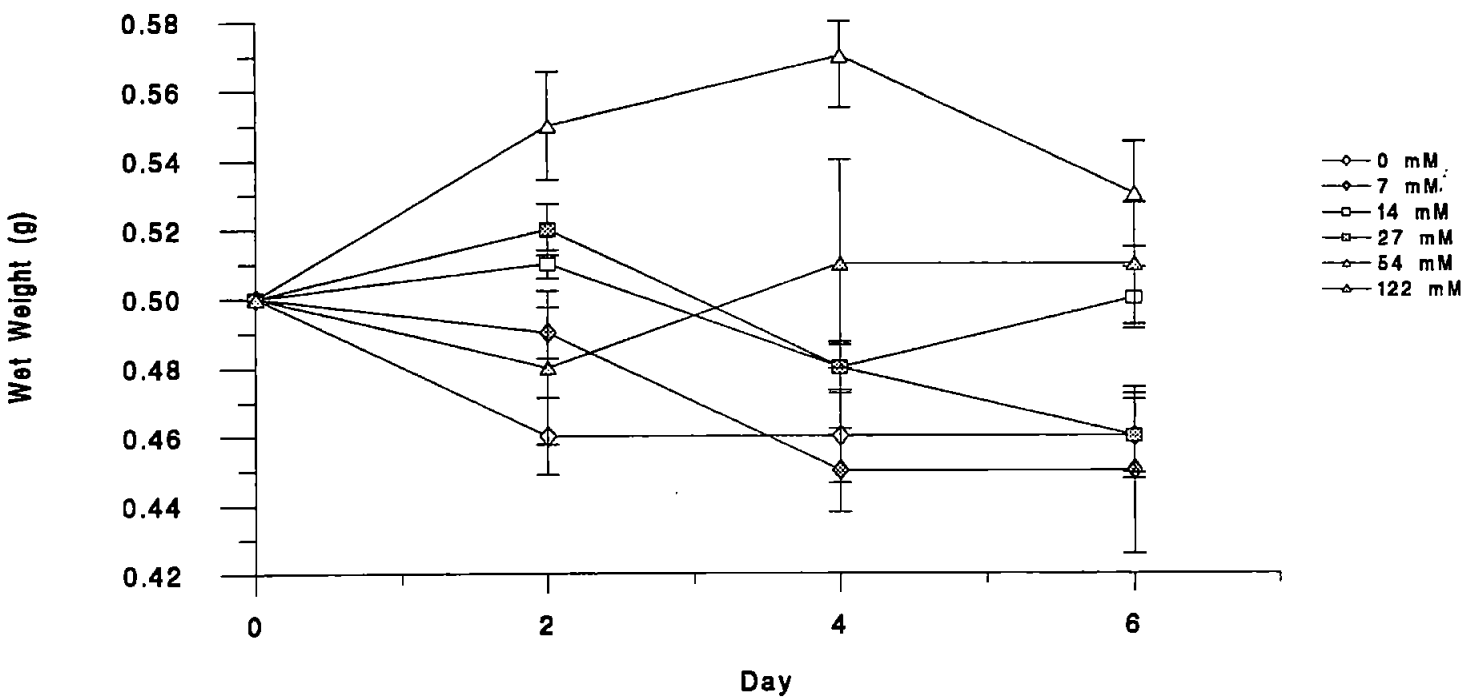


Figure 29: Wet weights of algae at beginning of, and during exposure to various concentrations of butan-1-ol. Showing standard errors.

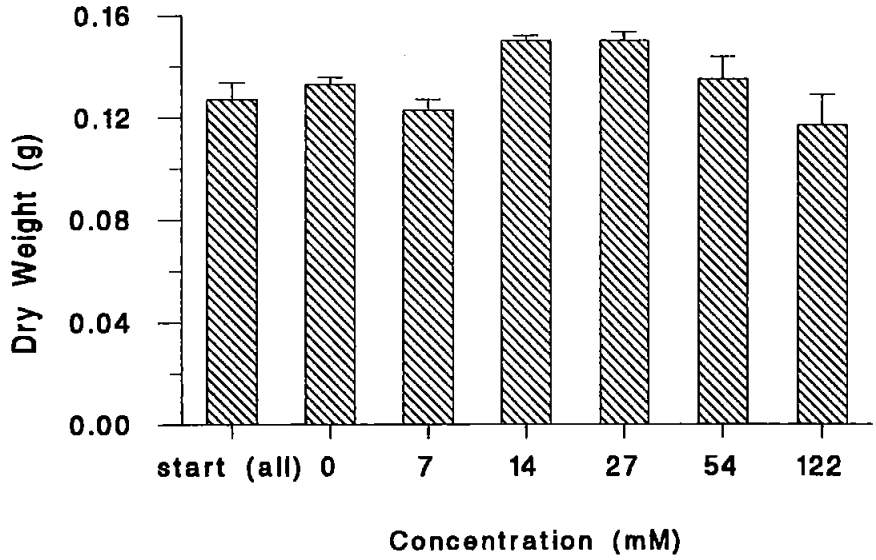


Figure 30: Dry weights of algae samples at start of, and following, exposure to butan-1-ol for six days. Including standard errors.

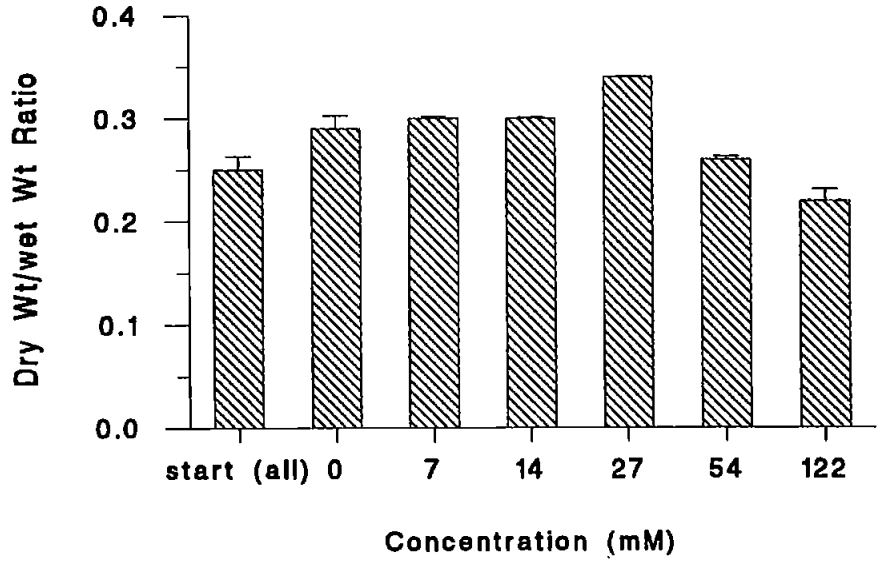


Figure 31: Dry weight to wet weight ratios for samples of algae at start of, and following, exposure to butan-1-ol for six days. Including standard errors.

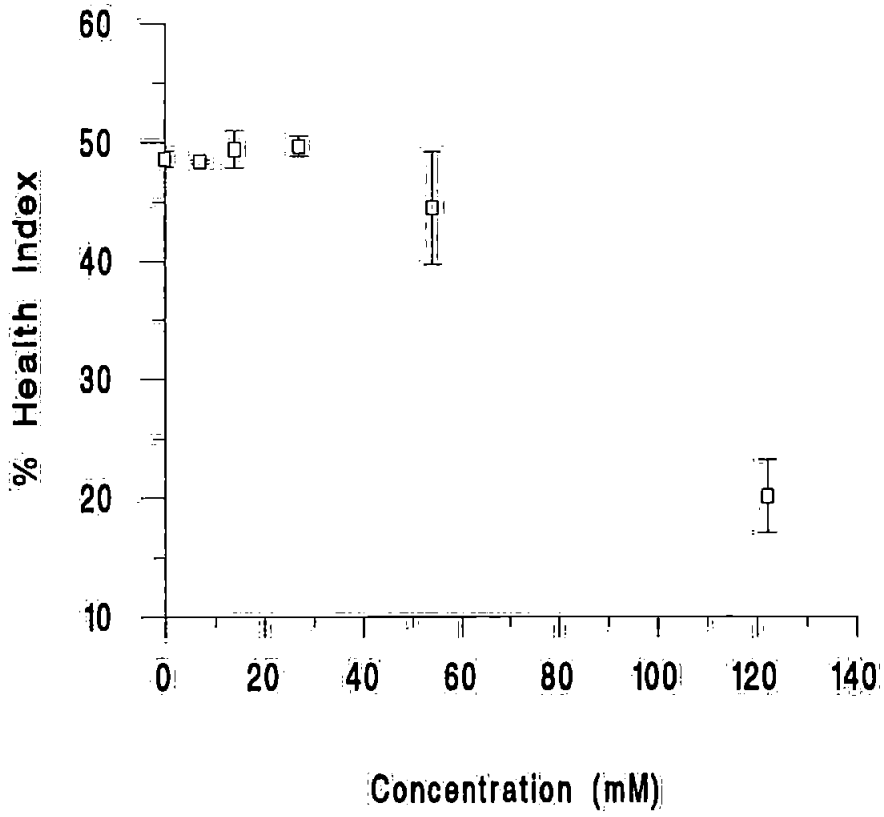


Figure 32: Health index for algae following a six day exposure to butan-1-ol, showing standard errors.

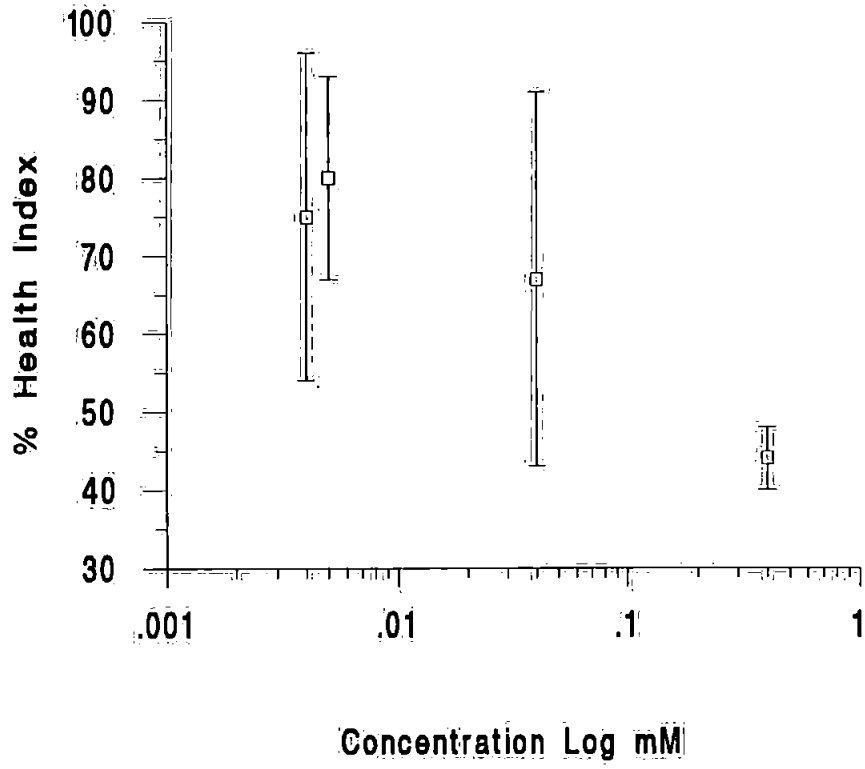


Figure 33: Concentration response curve for copper ions, showing standard deviations. Data from Bishop (1994).

this restricted the number of different ions which could be looked at. Only these results are presented, see Figures 34 to 46. Unfortunately a fault during ICP/MS analysis caused only ppm to be recorded rather than ppb for the butan-1-ol treated samples and consequently data was lost. However, an insight into the nature of some ions leaked has been achieved. Also presented is data for potassium and sodium ions achieved using a flame photometer (Figures 47 and 48).

Using the extended dynamic range results were obtained for a number of elements, eleven of which have known functions in either algae or higher plants. Other elements are not included here as they did not contribute significantly more than those presented. The data for these 11 elements is displayed as both the amount of ion present in each sample and as cumulative data. Three different types of loss dynamics were observed which broadly fall into the following classes:

Type 1. Ions are lost rapidly throughout the first two minutes, with less being lost during the final boiling. Elements that fall into this category include chromium and vanadium. Chromium (Figure 36) is lost at a similar rate in both the untreated and butan-1-ol treated samples, but on boiling less remained to be lost from those exposed to the alcohol. A similar pattern is seen for vanadium (Figure 44) with less of the ion being lost in total from the samples treated with butan-1-ol. The flame photometer data for sodium reveals ion loss with the same dynamics, with no significant difference between the two treatments.

Type 2. The second pattern of loss is similar to the first but the proportion of ions retained until boiling is far greater. Calcium, iron, magnesium, boron and potassium fall into this category (Figures 35, 40, 41, 34 and 47 respectively). The loss of calcium and boron was

Figures 34 to 48: Legend to X axis.

a, b, c and d refer to the ion content of 4 distilled water samples (a-d) following ion leakage of a single sample of alga transferred from a to b, to c, to d. The alga was left for 30 s in each solution prior to transfer.

Boil refers to the ion content of the distilled water sample in which the alga had been boiled for 5 min following initial leakage (see a-d above).

NB. For the purpose of these graphs ppb = μg/l, and ppm = mg/l.

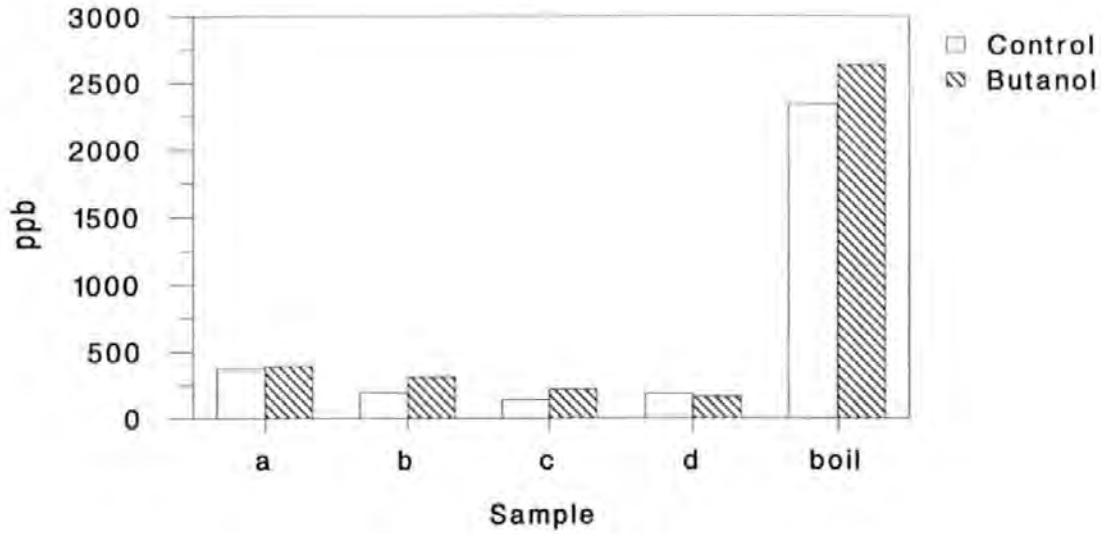


Figure 34a: ICP/MS analysis of ion leakage samples - Boron.

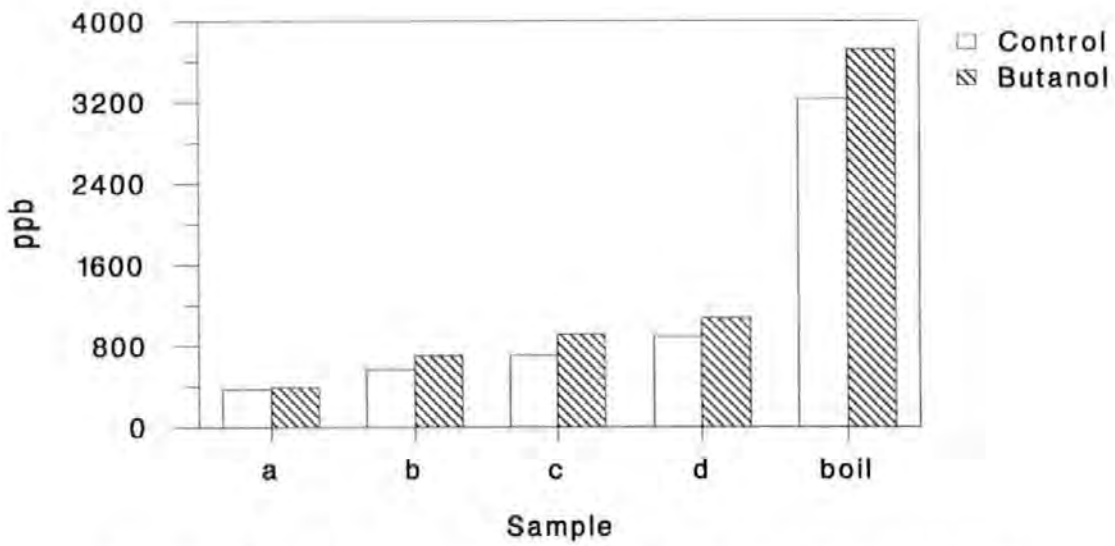


Figure 34b: ICP/MS analysis of ion leakage samples, cumulative data - Boron.

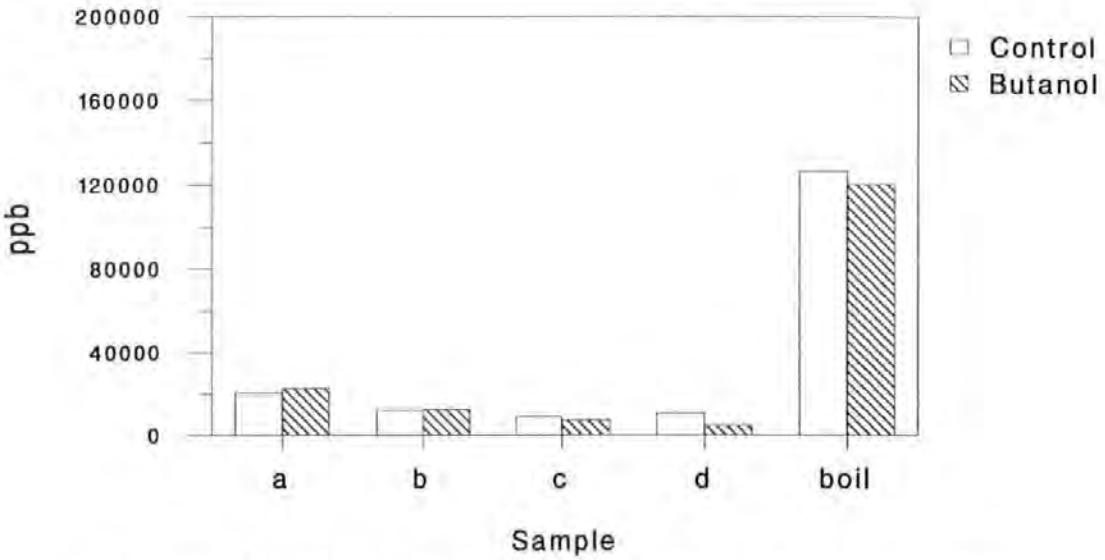


Figure 35a: ICP/MS analysis of ion leakage samples - Calcium.

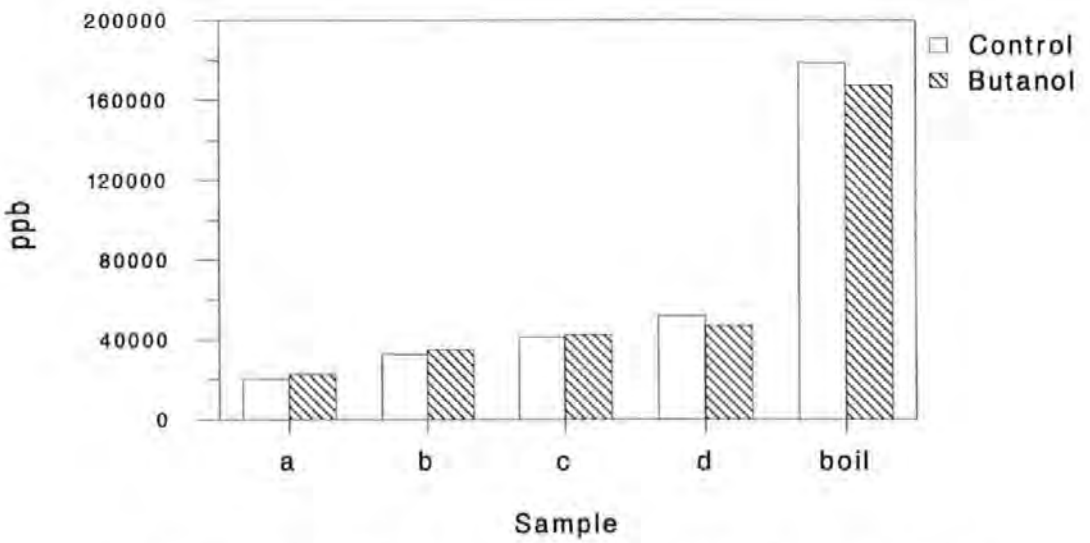


Figure 35b: ICP/MS analysis of ion leakage samples, cumulative data - Calcium.

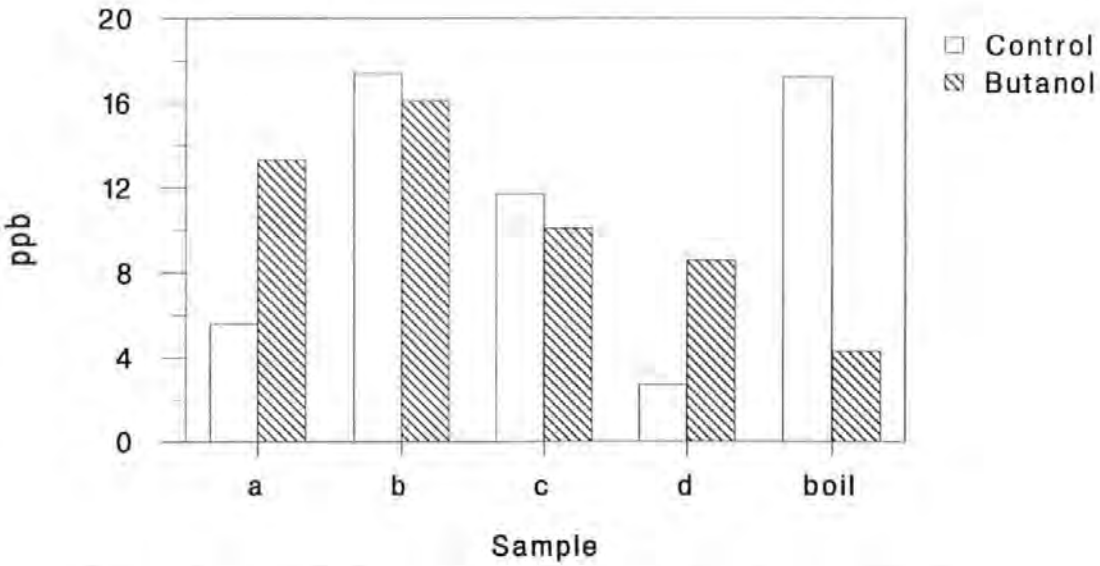


Figure 36a: ICP/MS analysis of ion leakage samples - Chromium.

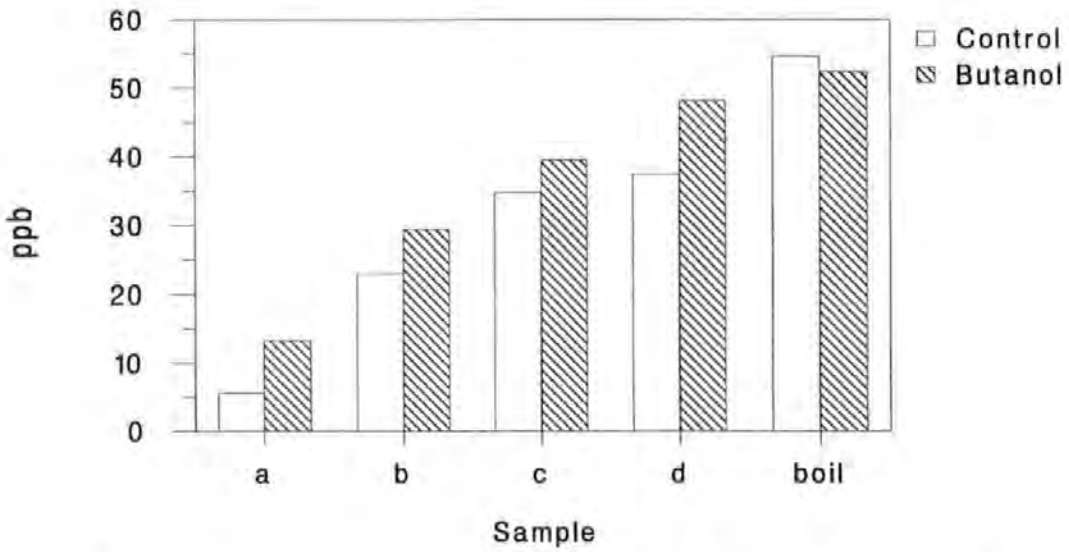


Figure 36b: ICP/MS analysis of ion leakage samples, cumulative data - Chromium.

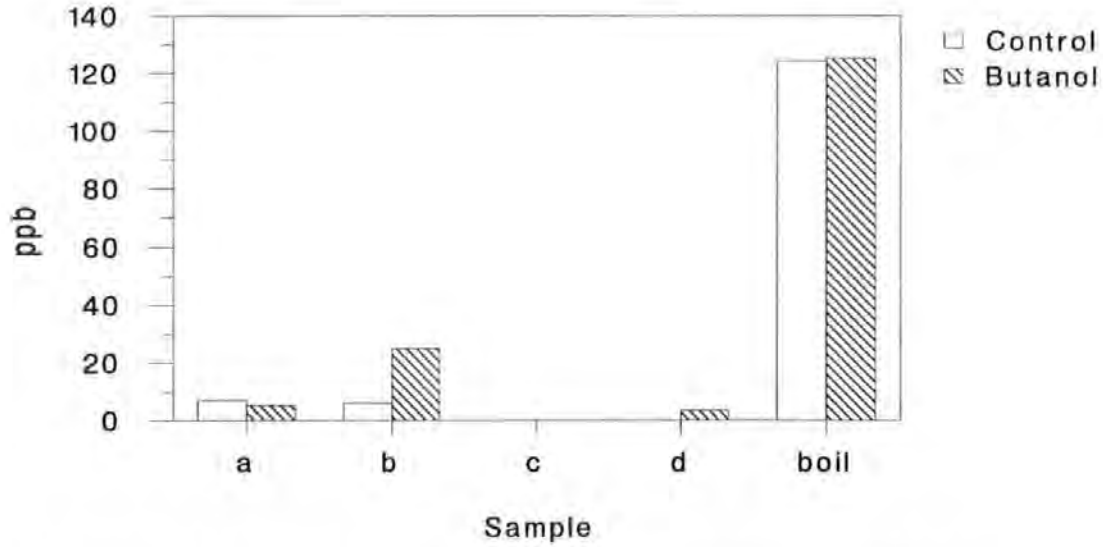


Figure 37a: ICP/MS analysis of ion leakage samples - Copper 63.

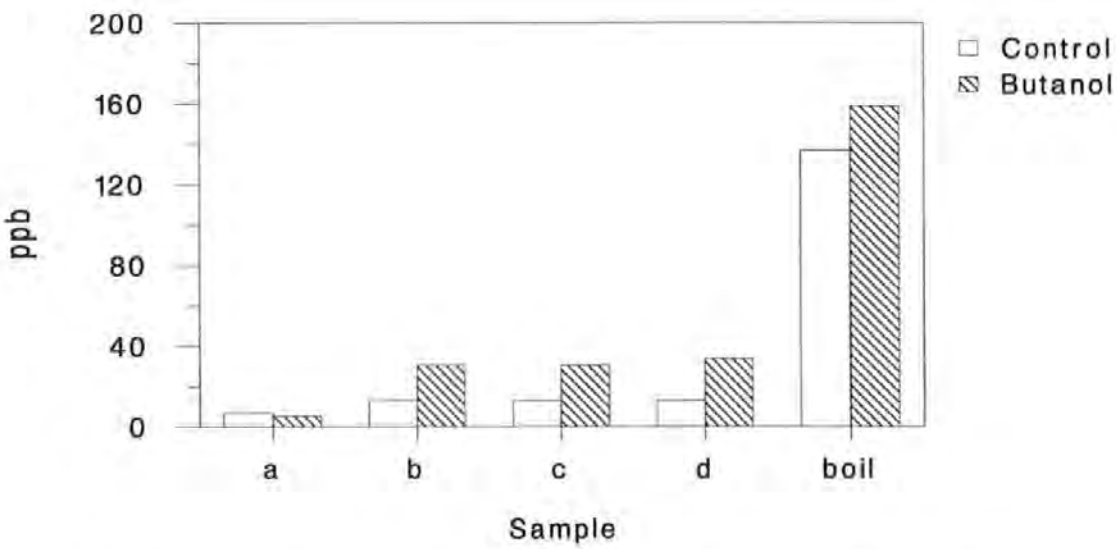


Figure 37b: ICP/MS analysis of ion leakage samples, cumulative data - Copper 63.

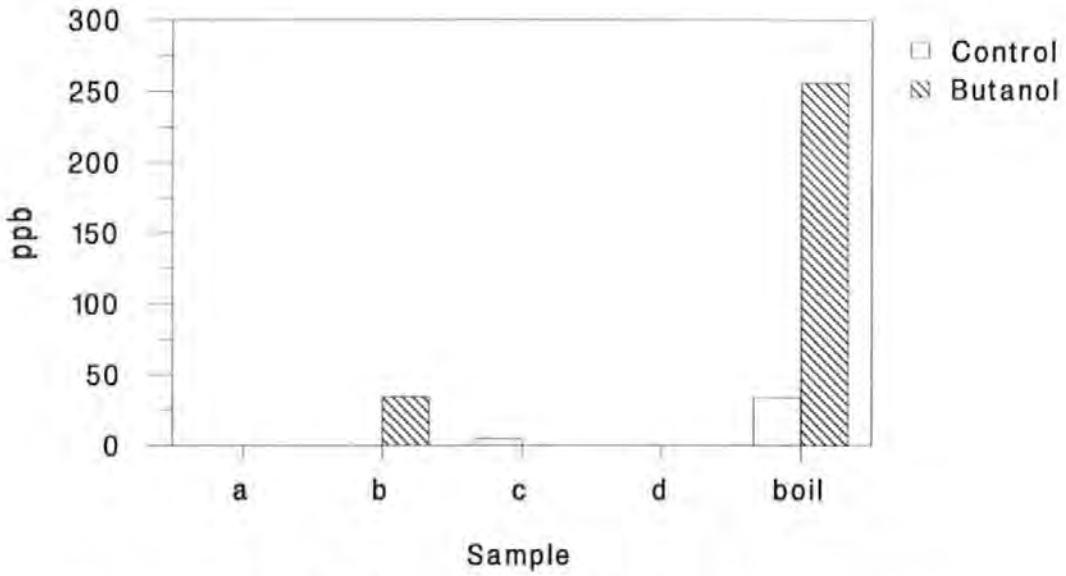


Figure 38a: ICP/MS analysis of ion leakage samples - Copper 65.

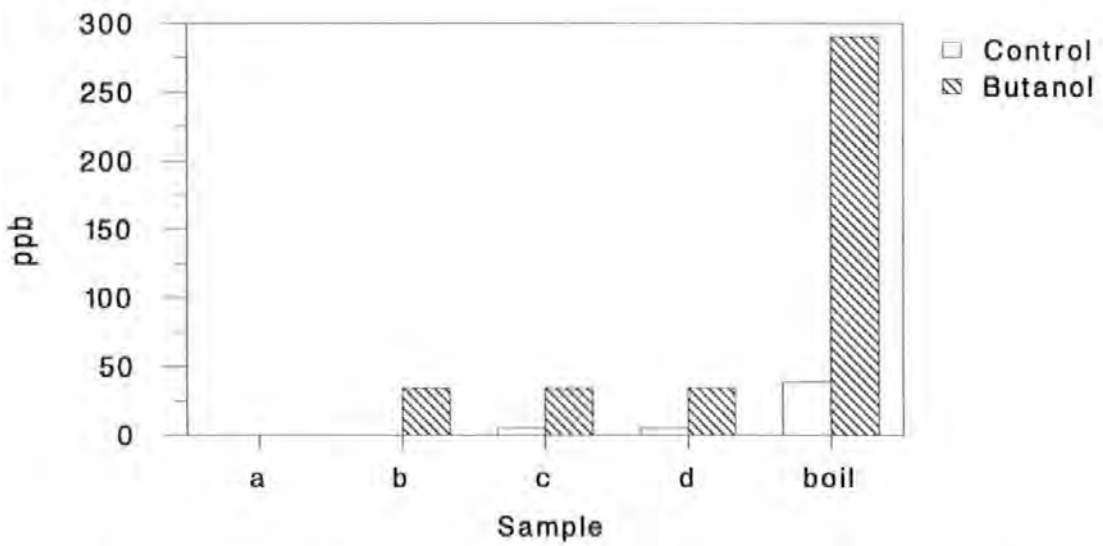


Figure 38b: ICP/MS analysis of ion leakage samples, cumulative data - Copper 65.

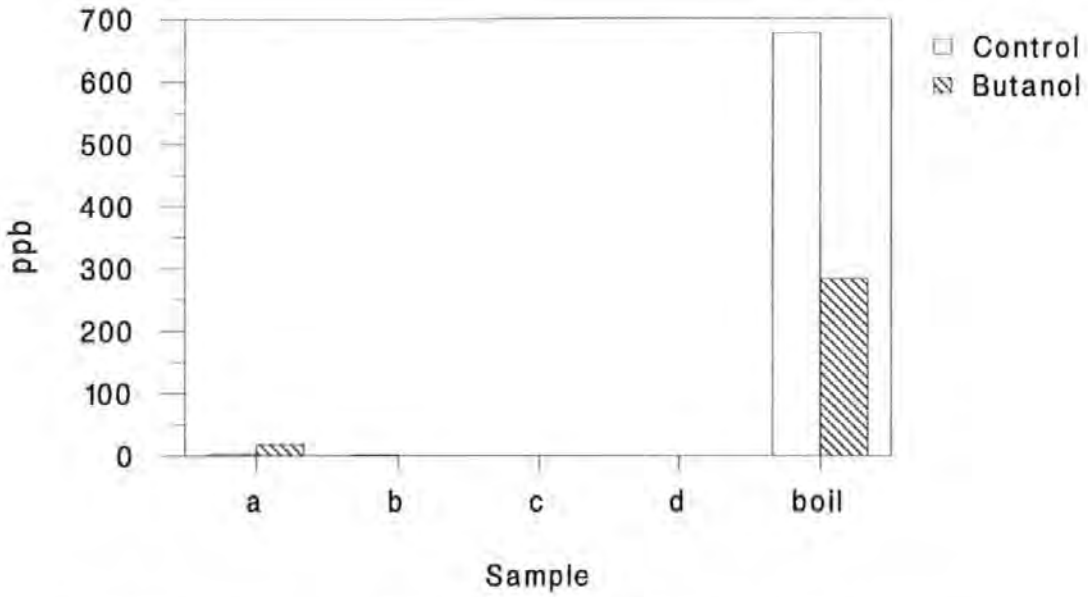


Figure 39a: ICP/MS analysis of ion leakage samples - Iodine.

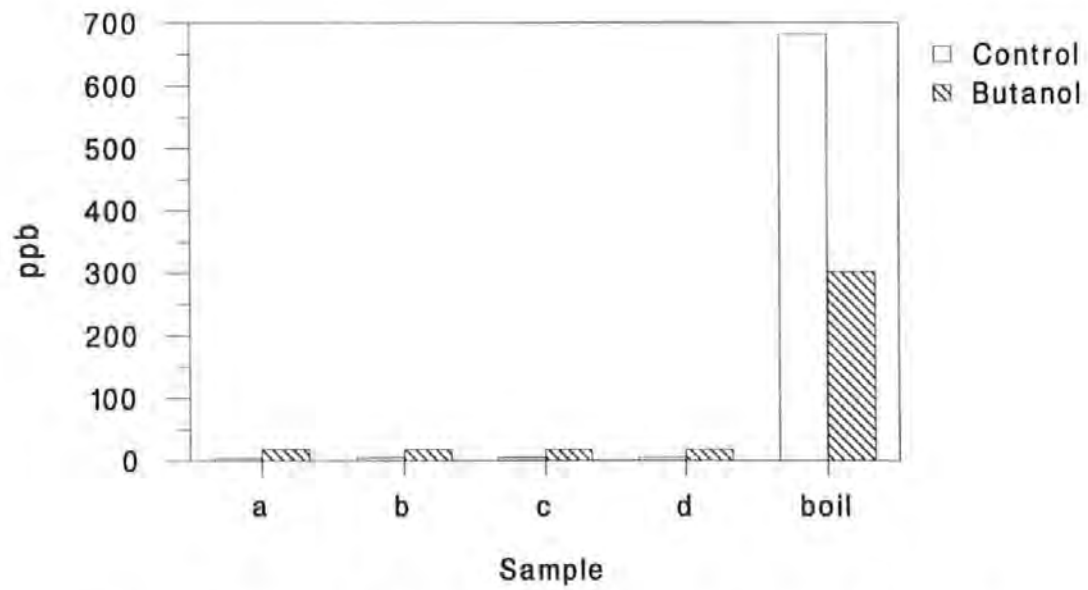


Figure 39b: ICP/MS analysis of ion leakage samples, cumulative data - Iodine.

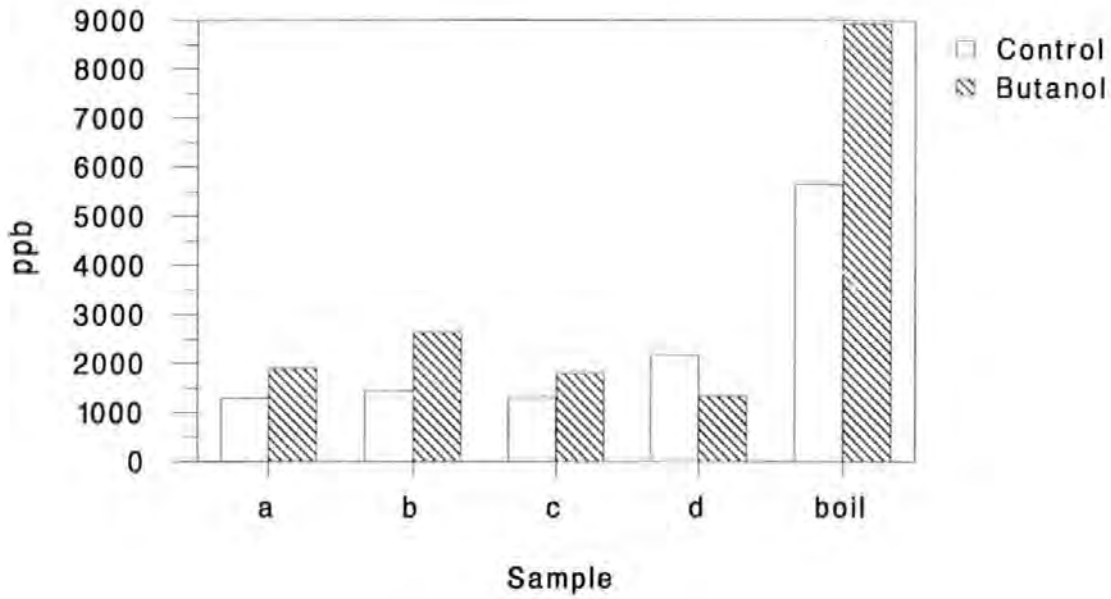


Figure 40a: ICP/MS analysis of ion leakage samples - Iron.

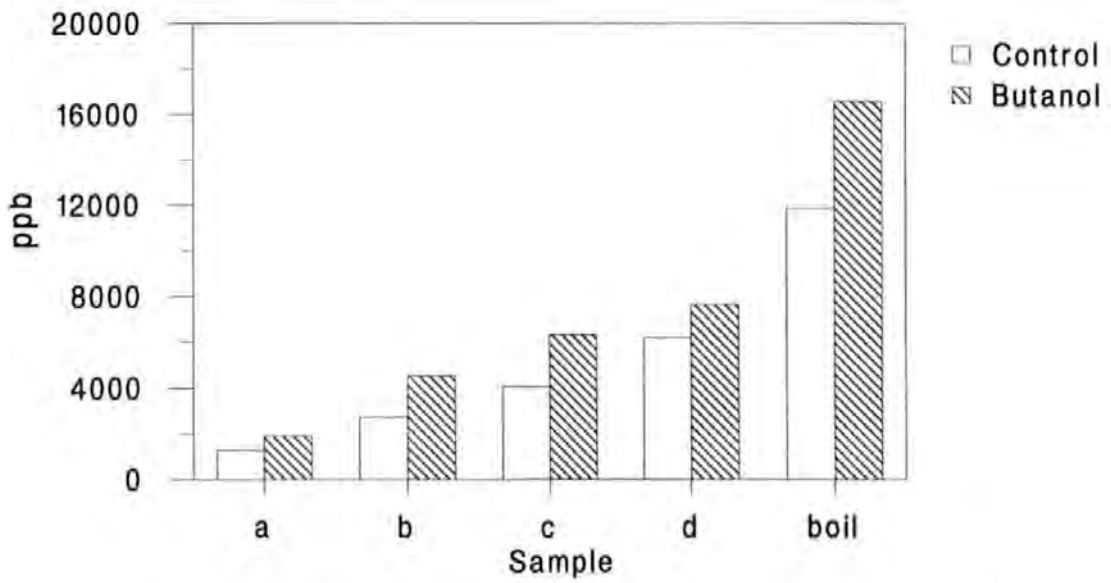


Figure 40b: ICP/MS analysis of ion leakage samples, cumulative data - Iron.

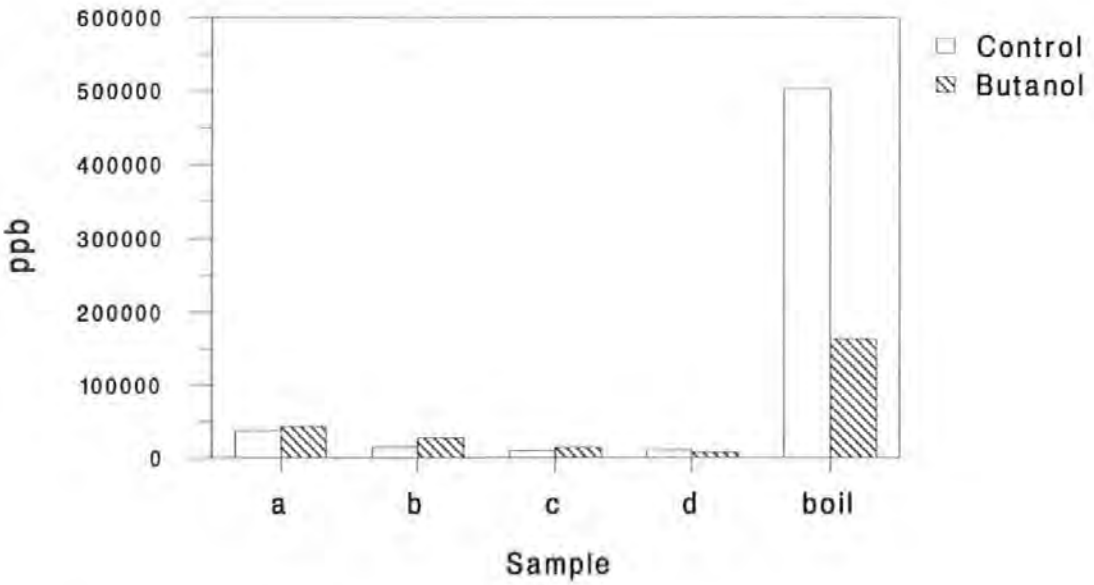


Figure 41a: ICP/MS analysis of ion leakage samples - Magnesium

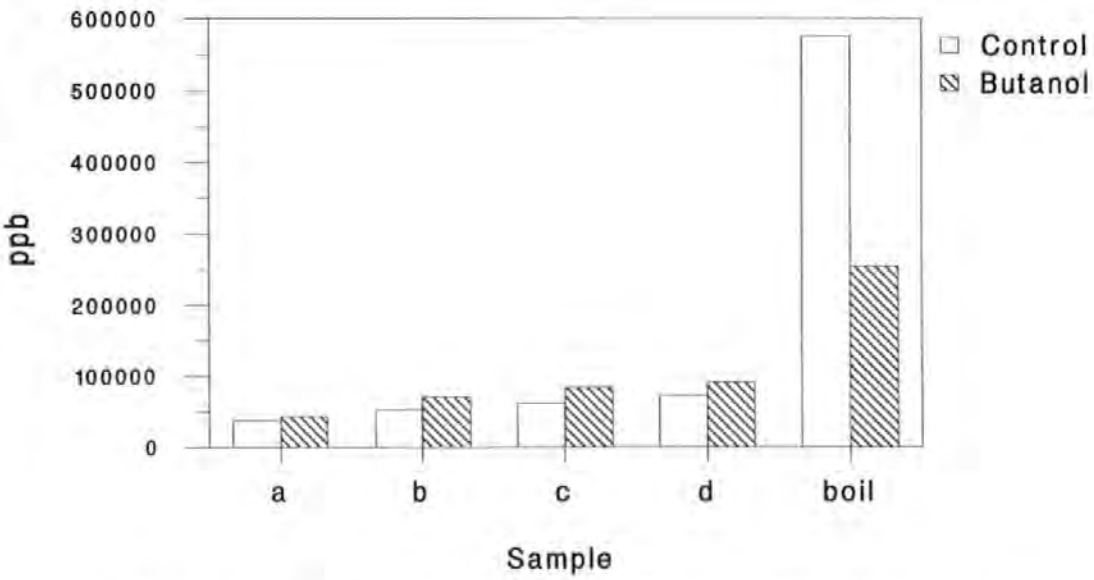


Figure 41b: ICP/MS analysis of ion leakage samples, cumulative data - Magnesium.

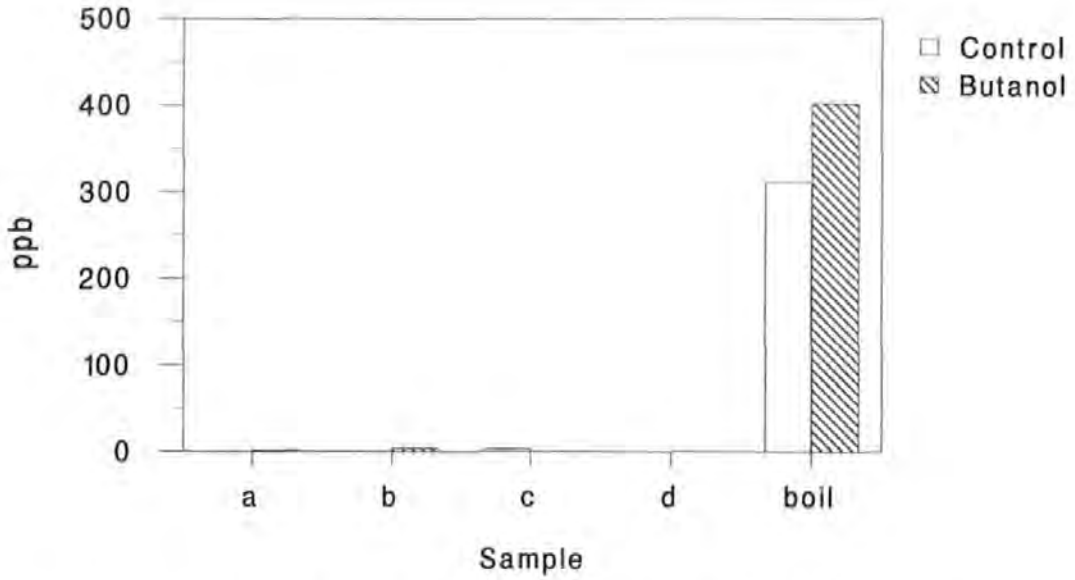


Figure 42a: ICP/MS analysis of ion leakage samples - Manganese.

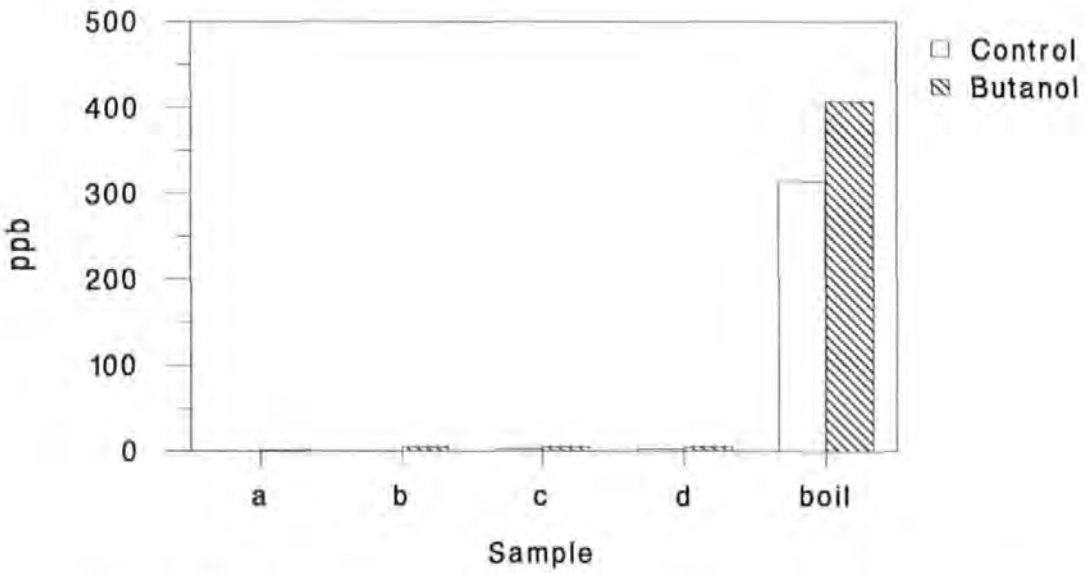


Figure 42b: ICP/MS analysis of ion leakage samples, cumulative data - Manganese.

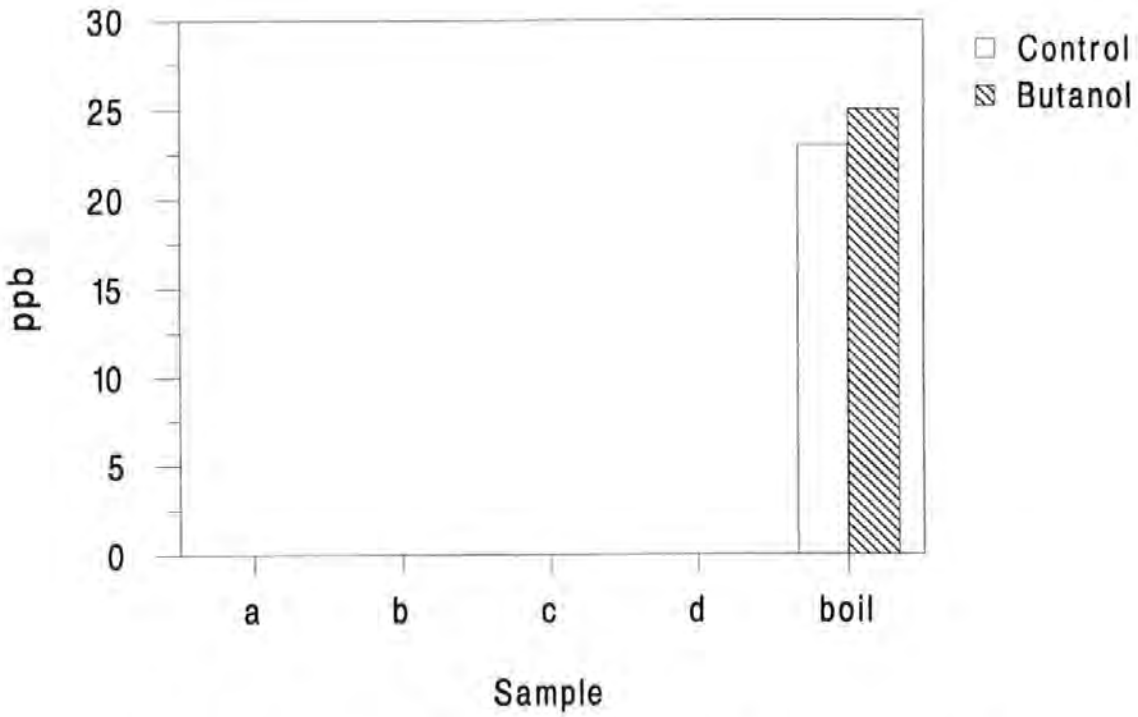


Figure 43: ICP/MS analysis of ion leakage samples - Rubidium.

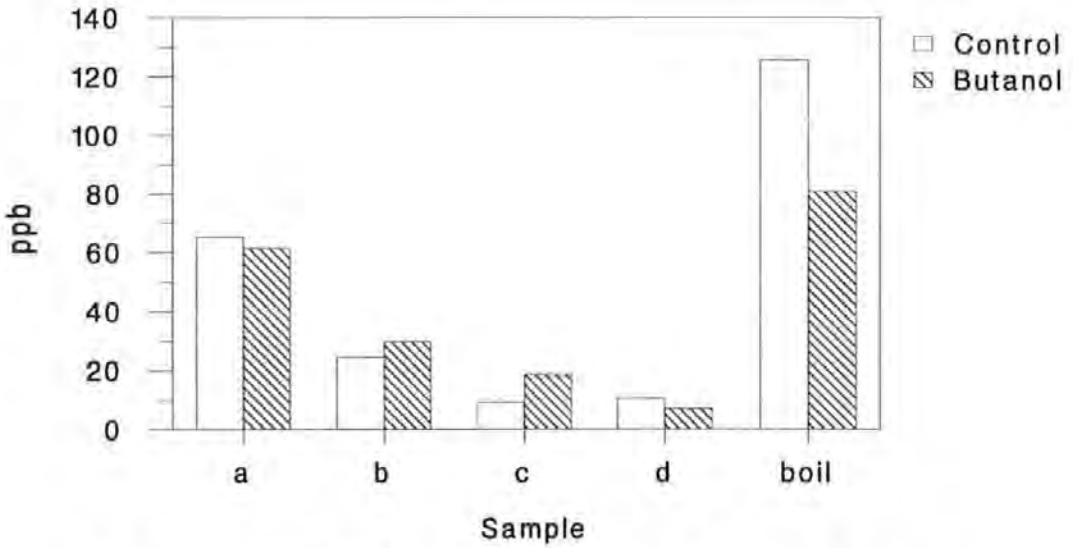


Figure 44a: ICP/MS analysis of ion leakage samples - Vanadium.

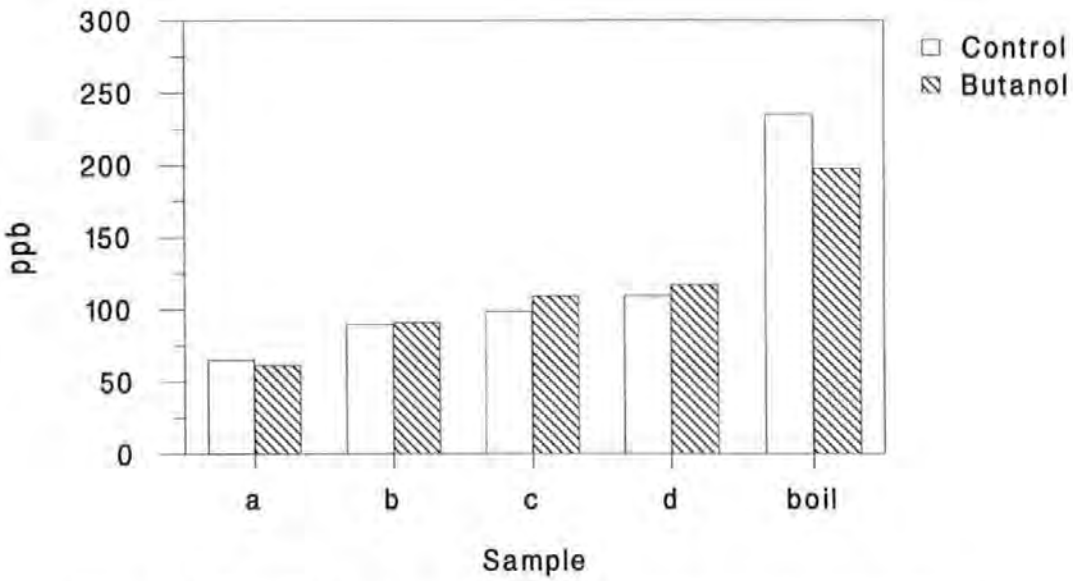


Figure 44b: ICP/MS analysis of ion leakage samples, cumulative data - Vanadium.

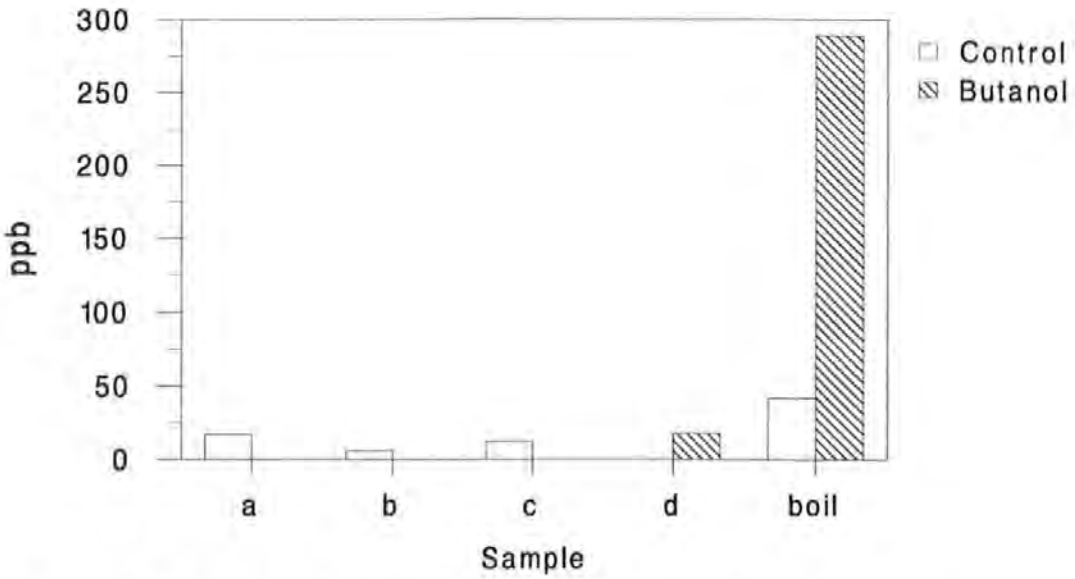


Figure 45a: ICP/MS analysis of ion leakage samples - Zinc 66.

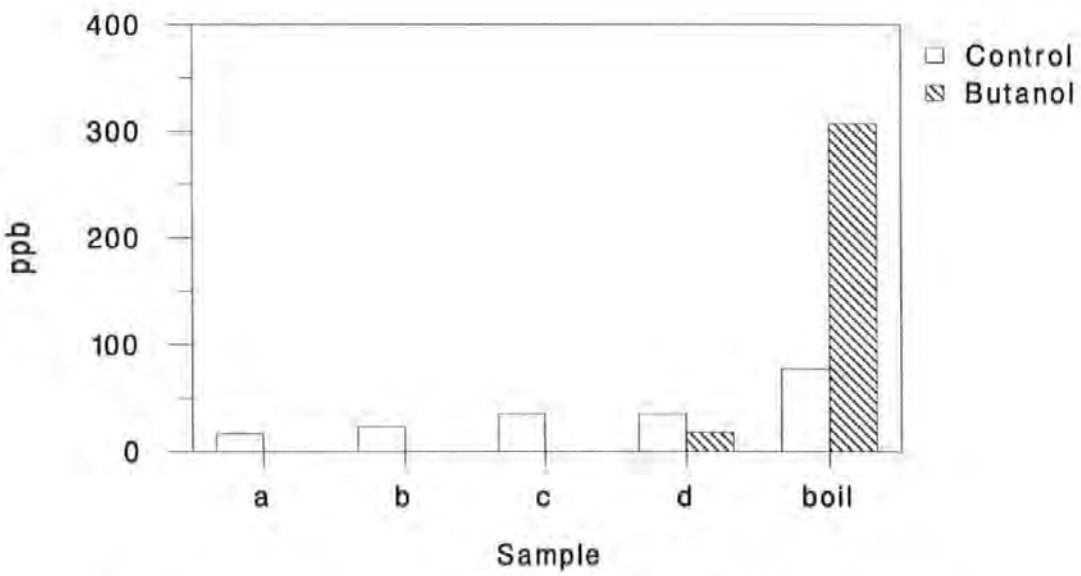


Figure 45b: ICP/MS analysis of ion leakage samples, cumulative data - Zinc 66.

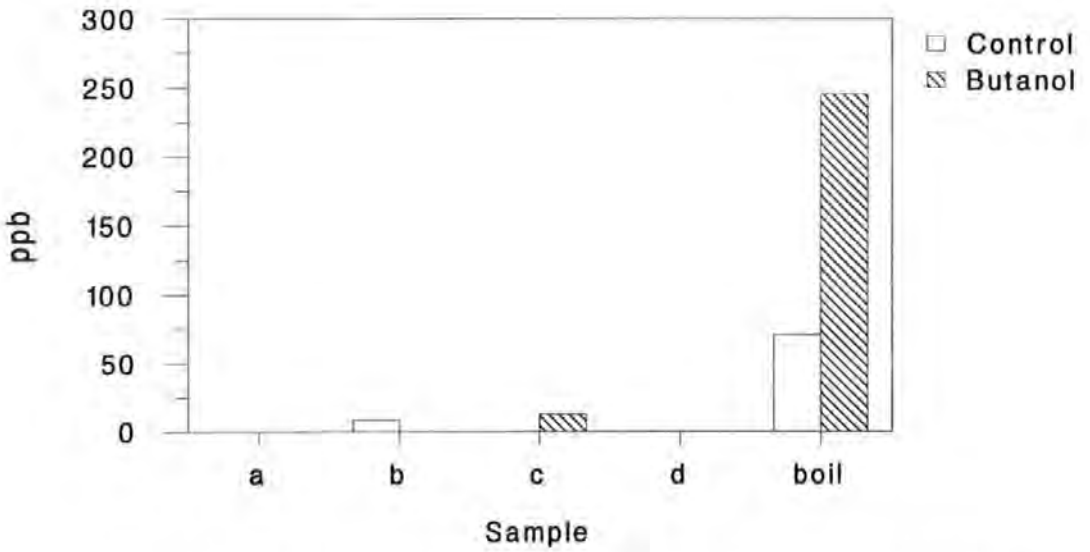


Figure 46a: ICP/MS analysis of ion leakage samples - Zinc 68.

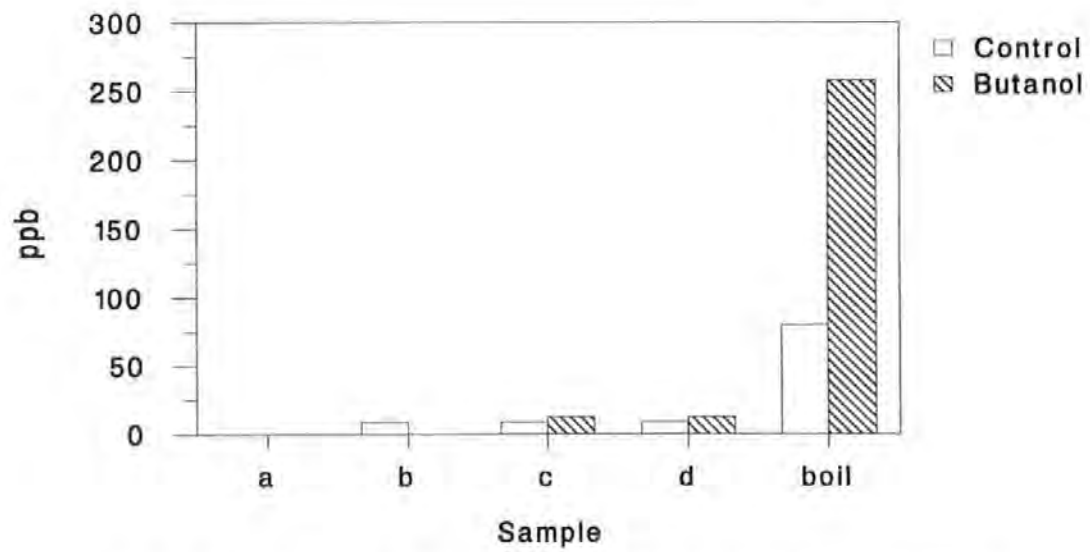


Figure 46b: ICP/MS analysis of ion leakage samples, cumulative data - Zinc 68.

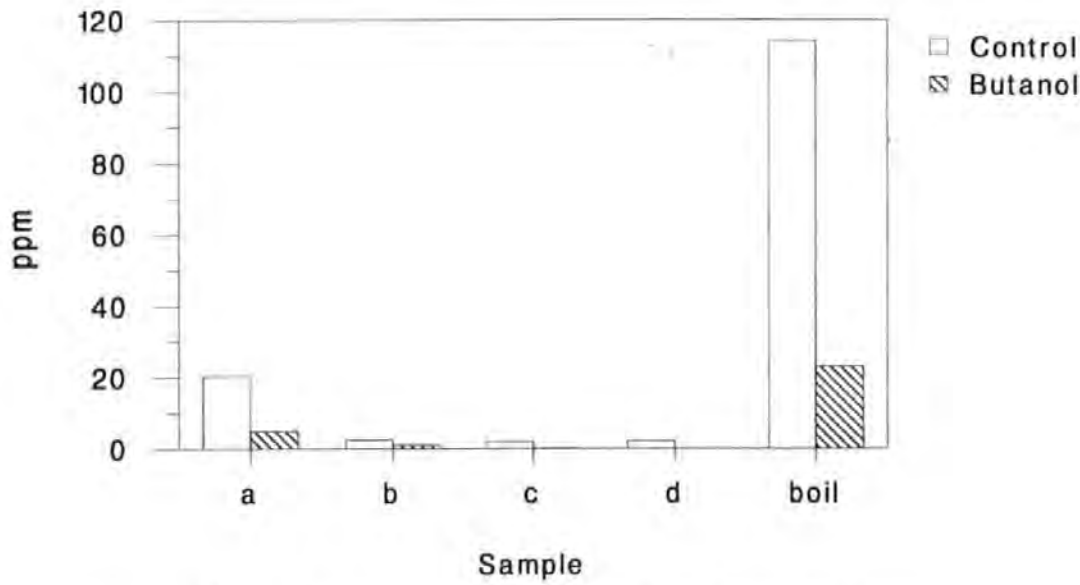


Figure 47a: Potassium ion content of ion leakage samples.

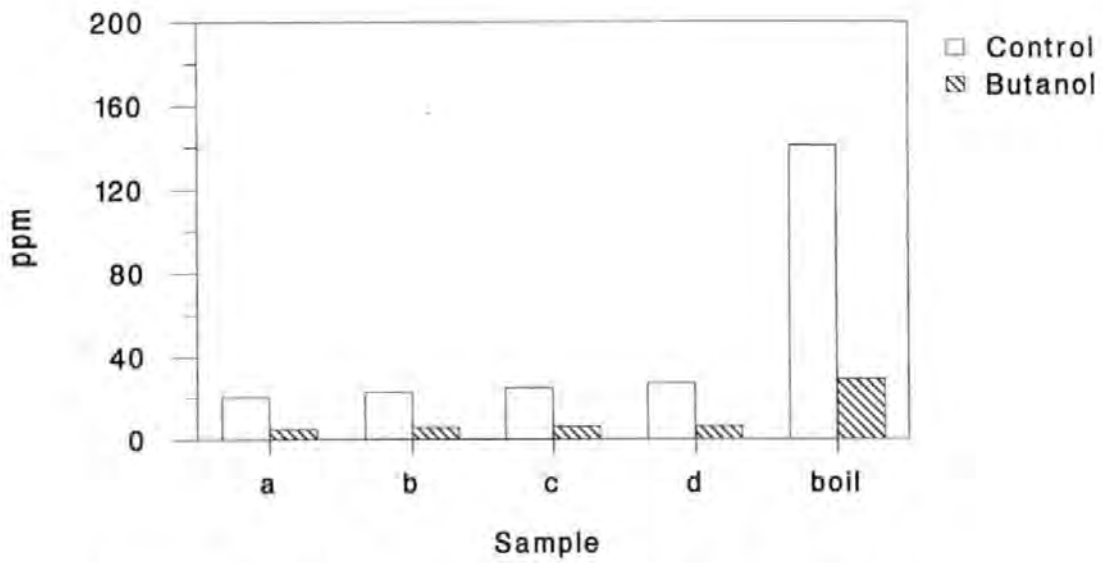


Figure 47b: Potassium ion content of ion leakage samples, cumulative data.

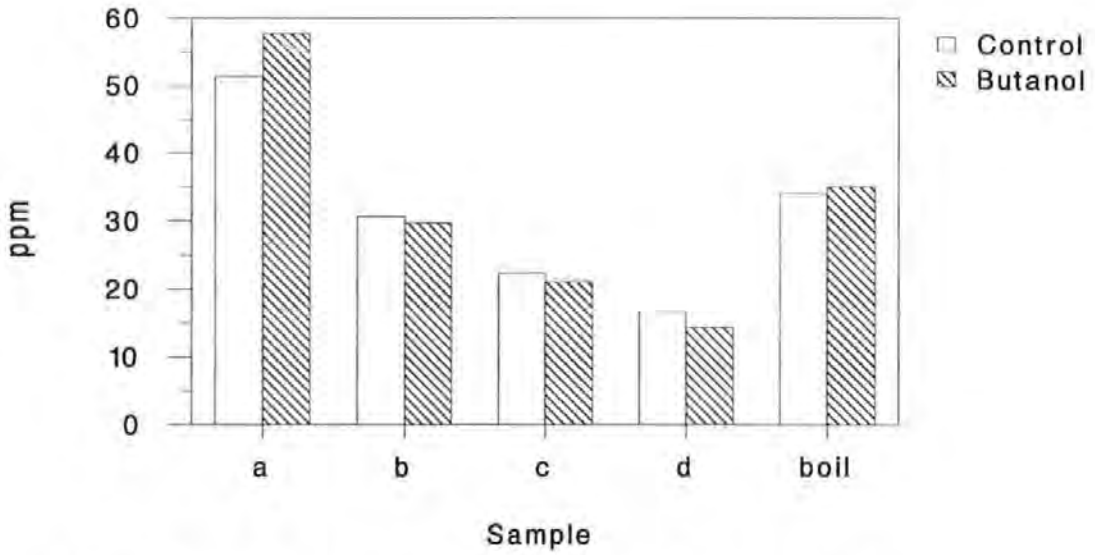


Figure 48a: Sodium ion content of ion leakage samples.

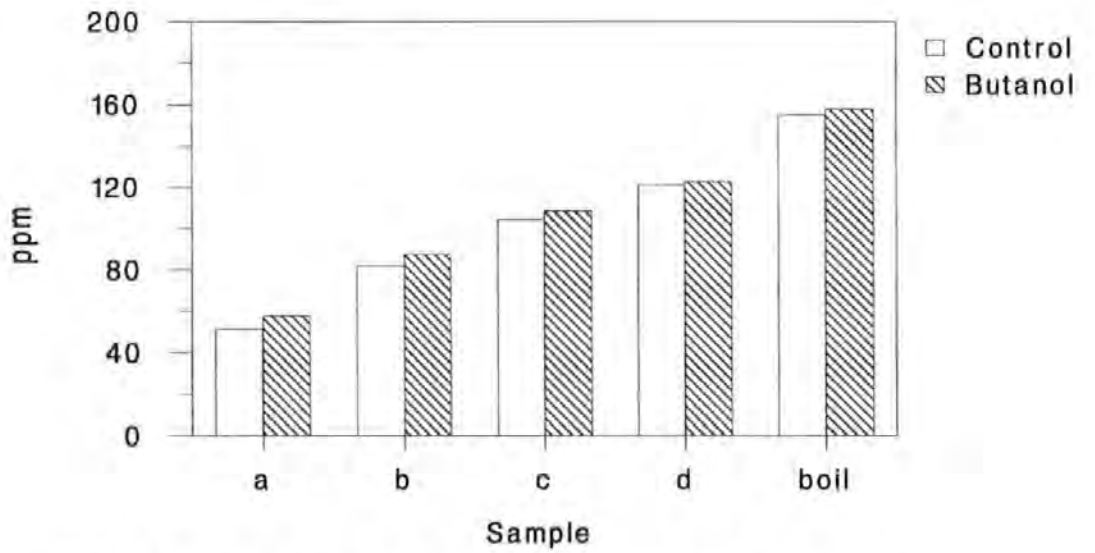


Figure 48b: Sodium ion content of ion leakage samples, cumulative data.

not affected by the addition of butanol, but the leakage of magnesium was increased prior to boiling with much less of the ion being present in the boiled sample. The total amount of potassium lost from the treated samples was approximately 80% less than from the control samples and the majority of this was only lost on boiling. Iron showed an increased leakage following butan-1-ol exposure, especially in the final boiled sample.

Type 3. Ions are very strongly bound with little or no leakage into water, the majority of ions being lost on boiling. This pattern of ion loss is seen for rubidium, zinc (66 and 68), copper (63 and 65), iodine and manganese (Figure 43, 45 and 46, 37 and 38, 39 and 42 respectively). No rubidium loss from the algae was detected prior to boiling and loss was not affected by the addition of alcohol. In all the other examples butan-1-ol had an effect on the loss dynamics. For zinc 66 and 68, and copper 65 loss in the control samples was relatively low but total loss from the samples exposed to butan-1-ol (including loss on boiling) was greater than in the control. This was also true for copper 63, but there was less difference in the total ion loss following the two treatments. Iodine and manganese are typical of this third pattern with very little loss into the water samples, a to d. This loss is then much greater in the final boiled sample. For iodine the effect of the alcohol is to decrease the total amount lost, however for manganese a slight increase is seen.

3.4 Discussion

The ion leakage technique was easily adapted for use with the macroalga *E. intestinalis* and has proved useful in the assessment of membrane damage due to toxicants. It is both quantifiable and diagnostic. The Health Index, derived as a result of the ion leakage test and quoted throughout this text, relates only to the species of alga tested, it is not intended to allow direct translation between species.

3.4.1 The ion leakage technique

Adaptation of the method from that of Axelsson and Axelsson (1987) was simply achieved, little change being required. The boiling time for sample 2 was maintained at 5 min as there was no significant difference in the conductivities of the resultant solutions, demonstrating that all removable ions were lost. At time 2 min the standard error was very high however which may be attributed to incomplete leakage. With slightly longer boiling the standard errors were much lower and made it possible to confirm that all ions had been leaked.

The selection of an initial leakage time was carried out to establish the optimum resolution for the method. For each different species used this will be different. The resolution of the method is dependent on the % Health Index obtained for healthy samples compared to that of unhealthy or dead samples. Observation of Figure 13, representing this data, shows that for *E. intestinalis* the best resolution, or difference between the two extremes, was observed for 2 min leakage into sample 1. After longer leakage, the % Health Index for the healthy samples fell, although throughout, that obtained for the boiled samples remained relatively constant. This reduction however in turn reduces the resolution of the technique and therefore a leakage time of 2 min was adopted. In comparison with *Laminaria* this is a shorter time and also a smaller resolution. Axelsson and Axelsson (1987) achieved Health Indices equivalent to 80-90% for healthy algae and between 20-30% for boiled controls. This is a greater difference between the two controls than is seen in *E. intestinalis*. However, in practice, the Health Indices for *E. intestinalis* were very consistent and therefore this more restricted range was acceptable. As a rapid method for measuring harmful effects on common macroalgal species this technique is easily adapted from one species to another and consequently has a wide range of applications. It remains a very simple technique whereby rapid answers can be achieved with good reproducibility.

In their evaluation of this technique for use with higher plants Whitlow et al. (1992) pointed out that ion leakage cannot be claimed to assess membrane stability or membrane permeability as it does not take into account membrane activity or electrical gradients. Although very important, I do not believe their observation invalidates this method, even if it can only be used to reflect general membrane integrity it remains a useful diagnostic tool. Their second misgiving about the technique, and the one they overcame by developing an alternative calculation, was that the ion leakage equation does not take into account leaf or thallus thickness and relative water content. In applying this technique to *E. intestinalis* whose thallus is essentially composed of a single layer of undifferentiated cells, concern about this observation need not be so great. However it must undoubtedly be relevant when using algae such as *Fucus* or *Laminaria* which have thick thalli composed of differentiated cells.

3.4.2 Salinity

The two approaches used for investigating the effects of various salinities yielded very different results, see Figures 14 and 15. When no equilibration was carried out the Health Index appeared to show the algae to be healthiest at low salinities and least healthy at higher salinities. *E. intestinalis* is known to be able to adjust to changes in salinity (Black and Weeks, 1972), and the algae tested were taken from a population subjected to frequent changes, with salinity ranging from approximately 10 ppt to 35 ppt (see Chapter 5 for environmental data). Within this region it may therefore be expected that the Health Index of the algae would be high. The raw data however reveals a very different relationship. At the low salinities the initial sample contained few ions, an outcome reflected in lower than normal conductivities, whereas the boiled samples had higher conductivities which were approximately the same for all the salinities tested. With the algae incubated at high salinities giving initial samples of high conductivities this led to wide variations in the

Health Index (between ~100% and ~30%). This rapid loss of ions to the water can be attributed to the first and second phases of osmotic acclimation where osmotic stress results in a direct impact on the cellular water potential, and ions are subsequently lost or taken up (Kirst, 1989). In non-equilibrated samples the contents of the cells vary widely according to the salinities to which they were exposed. Therefore on initial leakage this is reflected by the amount of ions present within the cell available to be lost. These appear to fall into two categories however, those ions which are rapidly lost to allow for acclimation (i.e. those lost initially), and those which are somehow maintained or retained within the cell to allow for normal function (i.e. those lost when boiled). The latter proportion may consist of specific ions such as K^+, the adjustment of which occurs as phase two of osmotic acclimation (Kirst, 1989) and are due to selective permeability changes. When the algae were allowed to equilibrate for 15 min, ions were again taken up to adjust to the new salinity increasing the number of 'exchangeable' ions and thus resulting in Health Indices within the range associated with normal healthy algae. This indicates that the changes in salinity were not detrimental to the normal function of the cellular membranes but rather point towards good salinity tolerance and fast osmotic adjustment. The effect of salinity is significant with respect to the ion leakage technique and one which must be taken into account when doing laboratory work. All algae were routinely equilibrated in Instant Ocean for a minimum of 12 hr prior to use in laboratory based experiments described in this thesis. However, observation of Figure 14 reveals that only the extreme salinities yielded Health Indices outside the 'normal' range for healthy algae. *E. intestinalis* is known to survive under very low salinities, although Figure 15 suggests this could be stressful. But it can be concluded that the consequence of testing algae straight after collection would normally not be significant with regard to the calculated Health Index. Reference to the environmental data in Chapter 5 demonstrates that there was no relationship between the Health Indices of algae taken from rock pools

of different salinities, which were tested immediately after collection or when equilibration had taken place. Even so, in order to ensure the minimum of difference between samples and to reduce the effects of salinity stress it is important to ensure that equilibration has taken place prior to use.

3.4.3 Toxicity tests with non-specific and specific pollutants

Eight non-specific narcotic alcohols were used to construct a QSAR between the toxicological response (EC_{50}) and the log K_{ow} (Figure 26) with a high correlation coefficient of 0.98. The QSAR has a slope approaching 1 which is characteristic of non-polar narcotic alcohols (Hansch et al., 1989). The alcohols studied can be considered representative of a large number of environmentally important hydrophobic industrial chemicals (Donkin, 1994). Hutchinson et al., (1979) previously demonstrated that hydrocarbons increase the leakage of potassium, magnesium and photosynthetically fixed ^{14}C from a unicellular freshwater green alga, *Chlamydomonas angulosa*. The toxicity of these compounds was strongly correlated to their aqueous solubility which is also related to the K_{ow}, the descriptor used here.

Non-polar narcotics are toxic by direct molecular interaction with cellular constituents, not by induced chemical or metabolic changes. The influence of time therefore is restricted to bioaccumulation rather than effect. Our exposure time of 24 hr was chosen for consistency with the NR uptake studies in Chapter 2, which was selected to allow comparison with studies on NR uptake in animal cells by Babich and Borenfreund (1987). The thallus of *E. intestinalis* is a single cell thick so equilibration between the alga and toxicant solution should be rapid, as in animal cell cultures. As time taken to reach a steady state situation also positively correlates to the log K_{ow}, and the majority of compounds tested are only moderately hydrophobic, a steady state should be reached within 24 hr. This is supported

by the absence of any curvature in the QSAR.

The production of this QSAR using the ion leakage method yielded a line statistically indistinguishable from that produced using NR uptake (Figure 26). However this method being superior in ease of use it was employed to determine the response of the alga to other toxicants with specific actions. The two herbicides studied did not have any detectable effect on ion leakage although the concentrations used are known to inhibit photosynthesis in marine macroalgae (Schild unpublished observations). These concentrations have also been shown to severely inhibit photosynthesis in freshwater unicellular green algae (Schäfer et al., 1994). The primary mode of action of diuron is to block photosynthetic electron transport at photosystem II (Duke, 1990). At the low concentrations used however it seems that insufficient diuron was present to have a direct effect on the plasmalemma and therefore had no effect on ion leakage. Diuron has a log K_{ow} of 2.77, similar to that of octan-1-ol which required a concentration of 0.95 mM to produce a 50% change in the Health Index by a narcotic action. Induction of a similar narcotic effect with diuron would therefore be limited by the solubility of the compound, which at 25°C in fresh water is only 0.16 mM. Axelsson and Axelsson (1987) also exposed *Laminaria* to diuron and similarly found that it did not affect ion leakage. The same was found here for the herbicide diquat which also failed to have an effect on ion leakage in *E. intestinalis*. Diquat intercepts electrons at photosystem I, transferring them to molecular oxygen to form superoxide radicals which can damage membranes (Duke, 1990). It is likely that *E. intestinalis* has sufficient protection against such oxidative damage and thus is resistant to the attack, at least over the 24 hr exposure period. Diquat has a log K_{ow} of -3.05, and consequently very high aqueous concentrations would be required to produce a narcotic effect on the plasmalemma which could be detected via ion leakage.

The concentration response curve for 2,4-dichlorophenol (2,4-DCP), Figure 24, is sigmoidal, the compound having a similar effect on ion leakage as a nonpolar narcotic of equivalent hydrophobicity. For a compound of specific action this is a rather low toxicity which may be attributed to the ionisation of chlorophenols at the pH of seawater ($\simeq$ 8) which can cause a reduction in bioaccumulation (Saarikoski and Viluksela, 1982). Babich and Borenfreund (1987) obtained an EC_{50} of 0.51 mM for the action of 2,4-DCP on fish cells measured using NR retention, this is close to the value of 0.68 mM for the same compound on algae. An ionophore such as 2,4-DCP (Terada, 1990) can influence cellular membranes, and thus ion leakage, in two ways: firstly, by affecting the ion or charge gradients across the membrane; and secondly, because their impact on the mitochondria and chloroplasts can affect their ability to maintain any such gradients. However there is no evidence that this compound is acting as anything other than a nonpolar narcotic because of the nature of the response curve and also because the EC_{50} fits onto the QSAR line.

In contrast the concentration response curve for tributyltin (TBT), Figure 25, shows an approximately logarithmic decrease in Health Index with increase in concentration, with a greater toxicity than that of a nonpolar narcotic of equivalent log K_{ow}. Using a log K_{ow} of 4.15 for TBT the predicted EC_{50} would be 0.077 mM, which is far greater than that obtained at 0.00025 mM. A similarly high potency was observed for *Laminaria* species, EC_{50} of 0.00026 mM (Axelsson and Axelsson, 1987), and with NR retention by fish cells, EC_{50} of 0.00055 mM (Babich and Borenfreund, 1988). TBT like 2,4-DCP is an ionophore (Snoeij et al., 1987) and like the herbicides tested it can inhibit primary production in unicellular green algae at sub narcotic concentrations (Wong et al., 1982), but unlike these other compounds it is able to increase ion leakage at low concentrations. Our data suggests that this is due to a direct effect on the cellular membranes.

Comparison of this data with that detailed in the previous chapter, Chapter 2, and also with that obtained for fish cells (Babich and Borenfreund, 1987), indicates that both the NR and ion leakage techniques respond in a similar way to nonpolar narcotics and toxicants with specific modes of action likely to be influenced by plasmalemma function. Further study is necessary to establish the long term effects of herbicides on *E. intestinalis* as an increased exposure time would probably increase the toxicity response. However over the short exposure time selected the ion leakage technique appears to respond to compounds which cause disruption of membranes.

3.4.4 Growth studies: long term exposure to butan-1-ol

E. intestinalis exposed to butan-1-ol for the extended period of 6 days showed an increase in sensitivity to the chemical with respect to Health Index. This demonstrates that although equilibrium between the alcohol and alga may be reached within 24 hr of exposure, an increase in exposure leads to an increase in toxicity in the order of two fold.

In addition to the measurement of final Health Index for the algae, wet weights were measured throughout exposure in an attempt to relate this parameter to the Health Index. This was however not successful as Figure 29 reveals. Each sample point shows unacceptably large standard errors many of which overlap. At the beginning of the experiment all samples were weighed accurately to 0.5 g, but by the second weighing on day two the mass of these was reduced for some samples and increased for others. The only samples with a significantly different mass were those exposed to 54 mM butan-1-ol. The main source of variation is the method used for 'damping off' the algae prior to weighing. Because *E. intestinalis* has a hollow thallus it is very difficult to ensure that all surface moisture has been removed. Although a standard procedure was adopted the standard errors show that this was not sufficiently reproducible. The only conclusion that

can be drawn from this approach is that the algae exposed to 54 mM butan-1-ol significantly increased in mass in the first 4 days although a decrease, due to possible cell death, was seen when measured on day 6.

Dry weight measurements were more reliable than wet weights. Over the 6 day incubation period no significant increase in dry weight was seen in the control samples. This was surprising because the conditions were the same as those which resulted in optimal growth, in experiments not described here, where algae were incubated in petri dishes with similar ratios between mass of algae and volume of incubation solution. The lack of oxygenation of the vessels is likely to have caused growth limitation, but active aeration could not be carried out since it would have increased the volatilisation of the alcohol. The small increase in dry weight over the 6 days was not significant in the control samples but the average Health Index suggested that the samples were healthy. It is therefore possible to use this as a comparison for the samples exposed to butan-1-ol. At concentrations greater than 7 mM the algae showed an increase in dry weight to concentrations beyond 27 mM, where a general lack of growth was seen for algae exposed to 54 mM, and a decline in dry weight occurred after 6 days exposed to 122 mM. A possible explanation for these results is that in the absence of optimal conditions for growth, the alga is able to utilise the alcohol as an energy source, suggesting that it is a facultative heterotroph. This appears to be possible when alcohol concentrations are below the 6 day EC_{50} (i.e. 74 mM), but as the alcohol concentration increases and becomes toxic the mechanisms involved with utilisation can no longer function. An alternative interpretation is that membrane integrity fails and the necessary transport of substances across the membranes is unable to continue. At this point lack of growth can be attributed to either the inability to use the alcohol as an energy source or to the direct toxicity to the alga resulting in cell death. A similar pattern is revealed by calculating the ratio between the dry weight and the wet weight,

Figure 31. Alternatively the alcohol may be being used as a substrate by contaminating micro-organisms, resulting in the removal of the alcohol and subsequent reduction in exposure concentration. This does not however explain why exposure to 27 mM butan-1-ol resulted in an increased mass, but it is possible that higher concentrations are also toxic to the contaminating micro-organism. The increase in dry weight may alternatively, or additionally, be attributed to the production of mucus as a stress response. This was not observed however, and it would not be usual for *E. intestinalis* to show such a response.

Observation of Figure 32, the Health Indices, adds weight to the hypothesis that *E. intestinalis* is able to utilise butan-1-ol. No effect is seen on the ion leakage from the thallus for algae incubated with only low concentrations of alcohol, but as the concentration increases to toxic levels there is a significant decrease in the Health Index indicating cell membrane disruption. This sigmoidal type of response is typical of concentration response curves for nonpolar narcotics (see Figures 16 to 23). However during 24 hr exposure metabolism is less likely to be so significant, but utilisation of the alcohol, removing the toxicant from the alga's environment and providing a useful substrate, could be a possible explanation for why such a response is seen.

3.4.5 Copper toxicity

At the concentrations of copper tested there was no significant effect on ion leakage from *U. lactuca*. The concentrations used are higher than those known to have a wide range of effects on *E. intestinalis* (Reed and Moffat, 1983). In their study the effects on growth, rhizoid production, cell viability, photosynthesis, intracellular β-dimethylsulphoniopropionate (DMSP) concentration and intracellular K^+ concentration were measured. Although some of these tests were necessarily carried out over a longer period of 6 or 9 days those looking at the effects on viability, photosynthesis, DMSP and

K^+ concentration were measured daily. At the highest concentration used in their study, 9.6 μM, a significant decline was seen in all these parameters after 24 hrs. It must therefore be concluded that the alga was affected by the copper exposure in this study but any physiological changes were not detected by this method.

Copper is a trace element essential to all algae (O'Kelley, 1974), it is a constituent of many enzymes, and essential for photosynthetic function. It is present in plastocyanin, one of the photosynthetic electron transport proteins (Bidwell, 1979). However, it is highly toxic at higher than trace concentrations. Copper toxicity manifests itself in a range of effects as demonstrated by Reed and Moffat (1983), the most relevant to this present study being the loss of intracellular K^+. These researchers concluded that K^+ loss was likely to be due to membrane damage. In ship fouling algae which exhibit copper tolerance this loss of K^+ is not seen, suggesting some form of membrane protection. It is therefore surprising that copper does not cause an increase in general ion leakage from this alga. However Reed and Moffat (1983) measured only intracellular K^+ which is probably not measured when this technique is applied to sublethal Cu toxicity. The limited initial leakage time utilised when measuring the Health Index extracts only those ions easily exchangeable, which are most likely within intercellular spaces, unless great disruption of the plasmalemma has occurred. Over the short exposure time utilised in this experiment such damage probably does not take place. As demonstrated in the previous section ion leakage is increased by nonspecific effects where general membrane disruption occurs or where the toxicant has a direct action on the plasmalemma. It therefore seems unlikely that copper has such an effect despite causing a loss of intracellular K^+. This lack of effect of inorganic metal compounds on ion leakage was also reported by Axelsson and Axelsson (1987) who found that stannic chloride at concentrations ranging from 0.1 to 10 mg/l had no effect on *Laminaria saccharina*.

K^+ is only one of the ions which is lost during ion leakage (see Sections 3.3.5 and 3.4.6) and this study shows changes in the ion to be at the low ppm level, significantly less than others, e.g. Na, Mg and Ca. Unlike many ions it is concentrated against the concentration gradient (Black and Weeks, 1972) and the effects seen by Reed and Moffat (1983) may relate to a breakdown in the alga's ability to maintain this gradient, not to membrane damage. If this were so it may explain why it is not reflected in the initial leakage from the thallus and thus has little effect on the Health Index. The flame photometry results for K^+ in Section 3.4.6 suggest that the majority of this ion present within the algal thallus may alternatively be lost soon after initial exposure to toxicants. It is possible therefore that any changes in this ion may be swamped by other effects.

3.4.6 ICP/MS and flame photometry

The results obtained using ICP/MS analysis and flame photometry show that the ions present within the thallus are bound to different degrees reflecting their different cellular functions. The three loss kinetics seen can be attributed to the degree of binding of the ion, the function of the element within the cell and the site of binding, i.e. whether the majority of the element is bound inter- or intra- cellularly.

ICP/MS is a technique which can be used to measure a range of elements semiquantitatively or single elements quantitatively. Here the technique has been used to produce semiquantitative data about a fairly small number of elements and those with known cellular functions will be commented upon. As mentioned in the results section a fault during analysis caused only ppm to be recorded rather than ppb for the butan-1-ol treated samples which has unfortunately led to the loss of some data. However, an insight into the nature of the ions leaked from the algae during this technique has still been achieved.

The first type of response seen, rapid and steady ion loss, occurred for three elements, chromium, vanadium and sodium. This type of loss suggests that these elements are exchangeable, held very loosely and therefore easily leaked. It is possible that the majority of the ions are not present within the cell membrane but either adsorbed onto the thallus or cell surface (though not lost through washing), or present in intercellular spaces or constituents of the cell wall, referred to as the cell wall free space (Black and Weeks, 1972). The initial rapid loss from this free space is equivalent to phase 1 of osmotic acclimation (Kirst, 1989) and in healthy algae with intact membranes it is probably the source of the majority of ions present in the pre-boiled ion leakage samples. The second, slower phase, is therefore considered to be exchange with the non-free space, or osmotic volume of the cell. Unlike phase 1 this is an active process (Black and Weeks, 1972) and may not contribute significantly to the elements recorded as being present in the pre-boiled samples in this study. That sodium is rapidly lost is a reflection on its role in osmotic acclimation where the element is initially taken up rapidly due to extracellular adsorption or lost due to active efflux. Rapid change in salinity due to submersion in distilled water would therefore be expected to eliminate this ion. Vanadium and chromium both have roles related to photosynthesis, though the precise nature of their functions is not known. Both appear to be present in the cell wall free space, though at much lower concentrations than sodium. All three ions were also present in the boiled sample, their origin therefore being intracellular, either bound as constituents of enzymes or other proteins, or from the vacuole. The effect of butan-1-ol on the loss of these ions is not conclusive, but it is possible that more rapid loss of chromium resulted. This is a surprising result as the butan-1-ol concentration used was chosen as being the calculated EC_{50} for this alcohol, and would therefore be expected to cause greater membrane damage. With no faster ion leakage being observed this would appear not to have happened.

The second pattern of loss is very similar to the first, but the rate of initial loss is reduced with a greater proportion being lost only on boiling. This suggests that these elements are present at lower concentrations within the cell wall free space than are 'type 1' elements. Of these elements boron and iron have metabolic functions and are therefore incorporated in other cellular constituents. Magnesium is a major constituent of chlorophyll and also an enzyme cofactor and activator of cellular reactions, it is therefore present bound within the chloroplasts, and in an available form within the cell. Calcium is involved with cell membrane maintenance, and in some algae, but not *E. intestinalis*, in calcification (Borowitzka, 1977). It is present at high concentrations within the cell and this, incorporated with its presence within cellular membranes, is a possible explanation for it showing this type of response. Potassium also falls within this category. Potassium, like sodium, is primarily involved with ionic relations and is actively taken up and passively lost as a result of changing salinity in algae (Black and Weeks, 1972). As loss is a passive rather than active process, it is slower and more gradual than was seen for sodium. Hence the majority of the ion present was only lost on boiling, not prior to this. The effect of butan-1-ol on potassium leakage is to reduce the total ion present in all solutions. In higher plants it is assumed that both sodium and potassium are the major ions contributing to ion leakage. Loss neither of boron nor calcium was affected by exposure to the alcohol. Iron leakage was increased slightly by the alcohol treatment but, like potassium, magnesium leakage was lower following treatment. This may be due to leakage into the treatment solutions prior to ion leakage determination. With hind sight these solutions should have also been analysed.

In the final category are ions which are very tightly bound intracellularly and are therefore not lost significantly prior to boiling. These elements are copper 63 and 65, iodine, manganese, rubidium and zinc 66 and 68. The role of copper within the cell is metabolic,

being essential for photosynthesis and as an enzyme cofactor (Bidwell, 1979). Similarly manganese acts as an enzyme cofactor and is vital to oxygen evolution (Bidwell, 1979). Zinc is an activator for dehydrogenase enzymes and is involved in protein synthesis enzymes in higher plants. It is essential to algae where it is likely to have similar roles (O'Kelley, 1974). These three elements therefore have functions which relate to cell metabolism and are present in tightly bound forms intracellularly. Rubidium has been shown in some algae to be able to substitute for K^+ in ionic relations and in *Porphyra* it has been shown to have similar kinetic properties (Reed and Collins, 1981). However Ritchie (1988) found it was a poor analogue for K^+ in experiments with *U. lactuca* and considering its strong binding in this experiment I suggest that it is not utilised in this way by *E. intestinalis*. Iodine is concentrated by many seaweeds, incorporated in iodo compounds, though its actual role is not understood. Presuming that iodine is complexed, its general lack of leakage is understandable. The effects of butan-1-ol on the loss kinetics of these ions again vary from element to element. Exposure to the alcohol had no effect on rubidium or copper 63, but resulted in decreased leakage of iodine. Conversely addition of butan-1-ol increased the total amount of ions lost from the alga for copper 65, manganese and zinc. Although this seems improbable it is likely that a certain proportion of all elements present within the frond and cell are never leaked even on boiling. Addition of the alcohol may therefore cause breakdown of the integrity of some cellular constituents and bring about an increased release of certain elements. Alternatively the very low concentrations of these elements in the test solutions may simply have resulted in inaccuracies during detection, and interference is known to occur between some elements and even the carrier gas in ICP/MS analysis, although elements with known interferences were avoided. Certainly, such inaccuracies of detection could account for the difference in action seen for the two isotopes of Cu, the results being only semiquantitative. Hutchinson et al. (1981) showed that the leakage of manganese from *Chlamydomonas*

angulosa occurred quickly into the bathing medium following treatment with naphthalene, a hydrocarbon. This took place over a few hours and was concluded to be due to membrane damage, such a response being absent from control samples. They also showed that only a limited percentage of the manganese was lost at all, the rest being strongly bound in the chloroplast to photosystem II reaction centres. This also provides an explanation here, as the lack of loss in the butanol samples may be due to the loss of the majority of labile ions into the incubating media prior to ion leakage

This last investigation aimed to answer a number of questions. Firstly it was carried out to increase our understanding of the ion leakage technique and to gain knowledge about which ions contribute towards the conductivity measured. This has been as successful as the analysis techniques have allowed, and has revealed some interesting results with regard to the kinetics of loss. Secondarily this analysis was carried out in order to establish whether any single ions could be used as 'markers' indicating membrane disruption and thus acting as a selective way of detecting and interpreting ion leakage. The results are inconclusive at this stage as quantitative analysis of single elements needs to be carried out. The elements showing most promise are those which fall into the first two categories of ion loss. However the ideal response would be seen if an ion which had shown a type 2 response under normal conditions showed a type 1 response following exposure to pollutants. The only candidates are therefore chromium, magnesium and possibly vanadium, of which only magnesium has a well understood role as an essential element. The situation for potassium is very interesting because it appears that the majority of the ion is lost very quickly, even before ion leakage analysis was carried out. This could therefore provide an exceedingly sensitive tool, but, to make use of it a different approach would need to be developed.

CHAPTER 4

SYNERGISTIC STUDIES - PHOTOINDUCED TOXICITY

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAH's) are widely distributed in the environment, derived from natural and manmade sources. Initially this large group of compounds were not thought to be acutely toxic at environmental concentrations (Herbes et al., 1976), and environmental risk assessment concentrated on the reported carcinogenic, teratogenic and mutagenic properties of some of the larger PAH's (Neff, 1979). Knowledge that these compounds were photosensitive often lead to the avoidance of natural light during hazard assessment experimentation, and specialised lighting was utilised to prevent photodegradation of the parent compound (Oris et al., 1984). Following a change towards the concept of ecological relevance and pollution realism in predictive toxicity testing it was found that in the presence of sunlight, anthracene, a linear 3-ring PAH, was much more acutely toxic to a variety of aquatic organisms than had previously been demonstrated. Toxicity occurred at concentrations well within the limits of solubility (0.25 μM, fresh water, 25.3°C, Whitehouse, 1984) (Bowling et al., 1983; Oris et al., 1984). Subsequent to this discovery, many studies have been carried out for aquatic organisms, including green algae (Gala and Giesy, 1992), with a wide range of PAH's (Newsted and Giesy, 1987), and with a wide range of environmental conditions (McCloskey and Oris, 1991 and 1993).

PAH's are a class of compounds comprised of two or more fused benzene rings with occasional cyclopentane inclusions in the ring structure, or variously substituted alkyl side chains. Compounds in this class which are of environmental relevance range from those composed of two rings (e.g. naphthalene) to those of five or six rings (Mekenyan et al.,

1994). PAH's are produced naturally by plants and some are formed as a result of forest fires and volcanic activity. However, major sources of PAH's in surface waters are from oil spills, industrial processes, fossil fuel combustion and other pyrolytic processes (Neff, 1979). PAH's are extremely hydrophobic and consequently are only present at low concentrations in water but accumulate in sediments. Consequently they also bioaccumulate in organisms.

A particular hazard of PAH's to aquatic ecosystems results from photo-induced toxicity which is caused by PAH excited states in the presence of ultraviolet (UV) radiation in sunlight. The mode of action of photo-induced toxicity of PAH's is complex but is thought to relate to the formation of PAH free radical intermediates and the generation of singlet oxygen (Foote, 1968; Foote, 1976; McCloskey and Oris, 1993). This response is distinguished from the effects of UV irradiation alone by the type of effects seen and by the dependence on the presence of oxygen. The effects of photodynamic damage include membrane damage, mutagenesis, interference with metabolism, reproduction and many other processes (Foote, 1976).

UV exposure in the environment increases PAH toxicity through photoactivation and therefore in laboratory based experimentation this must be simulated. This approach has now been adopted by researchers and much data is available as to the toxicity of a wide range of PAH's to a number of aquatic organisms, including *Daphnia spp.* (Allred and Giesy, 1985; Newsted and Giesy, 1987), fish larvae *Pimephales promelas* (Oris and Giesy, 1987) and juvenile bluegill sunfish *Lepomis spp.* (McCloskey and Oris, 1991). However very little research has been carried out on photo-induced toxicity in green algae. In 1992 Gala and Giesy exposed the green alga *Selenastrum capricornutum* to what is now accepted as the 'model' PAH, anthracene. This exposure revealed that although less sensitive to

anthracene than are fish, the alga showed a marked decrease in specific growth rate when exposed to environmentally realistic levels of UV-A in combination with anthracene at concentrations well below the solubility limit. The same researchers in 1993 demonstrated that the presence of carotenoids in plants help to protect them from PAH toxicity. PAH's within estuaries and in marine environments must also pose a threat to macroalgae such as *E. intestinalis*, which this study has attempted to quantify.

The aim of this study was therefore to induce a photo-toxic response in *E. intestinalis*. The alga was exposed to anthracene, a three ring PAH. Uptake took place over 24 hours in the dark, followed by irradiation with photosynthetically active and UV-A wavelengths. The effect of this treatment on non-specific membrane damage was then assessed using the ion leakage Health Index for *E. intestinalis*. The amount of anthracene taken up by the algae was also measured by extraction using steam distillation, a method developed for the extraction of petroleum hydrocarbons (toluene to pyrene) from water and mussel tissues (Donkin and Evans, 1984). Analysis was carried out using fluorescence spectrophotometry.

4.2 Methods

4.2.1 Development of the technique

Initially it was necessary to develop a method to measure the concentration of anthracene in solution.

Anthracene has a low solubility in fresh water at 25°C (0.25 μM, Whitehouse, 1984) which is further lowered to 0.095 μM (calculated from Whitehouse, 1984) by the experimental conditions necessary in this experiment, i.e. seawater of 33 ppt held at 15°C. To establish the maximum concentration achievable a solution of anthracene was prepared in Instant Ocean, using acetonitrile (Sigma-Aldrich, HPLC grade) as a solvent carrier. A stock

solution was made up dissolving 10 mg of anthracene in 100 ml acetonitrile. Using this to dose seawater a number of solutions were produced, the maximum amount being 300 μl of stock to 1 l Instant Ocean. This solution was stirred and allowed to stand in a dark place for a minimum of 2 hr to allow for solubilisation. All glassware was washed in decon and rinsed thoroughly in de-ionised water prior to use. The anthracene was then extracted from 100 ml of solution into 2 ml hexane, and repeated using a further 2 ml hexane. This process was carried out for two further 100 ml samples of the same anthracene concentration. The absorbance of the resultant hexane solution was measured on a UV/Vis spectrophotometer (Phillips PU 8720 UV/VIS Scanning Spectrophotometer) at 375.7 nm.

Comparison with a calibration curve revealed the maximum solubility to be approximately 0.056 μM, which was consequently adopted as the maximum concentration used.

The above method of direct water analysis was not utilised during the remainder of the investigation as further experiments carried out to assess the level of uptake of anthracene into the algae, i.e. loss of anthracene from the water, resulted in low concentrations and lead to highly variable readings which were further interfered with by pigments originating from the algae. It was realised that far greater accuracy could be achieved if the anthracene taken up by the algae was measured directly by steam distillation.

4.2.2 Steam distillation extraction/fluorescence analysis

The steam distillation apparatus consists of an assemblage of 'Quickfit' glassware, comprised of a round bottomed flask, connected to a Dean and Stark water estimator, which is connected in turn to a straight water condenser. The whole apparatus is then placed in a heating mantle.

As the method had never been used for algae before, a trial run and recovery experiment was carried out, using the basic method but with algae 'spiked' with 5μg anthracene. Algae were collected from Plymouth Sound, 1 g samples were weighed, cut up and placed in six round bottomed flasks. Anthracene in acetone was added to half the flasks and the acetone allowed to evaporate. Steam distillation was then carried out.

The tissue was saponified using 10 ml of sodium hydroxide solution (160 g/l), a few anti-bumping granules were added along with 4 ml of isohexane and 400 ml distilled water. Low heat was then applied for 2 hrs. Before the heat was turned up the flasks were neutralised by adding 50 ml HCl (62.5 ml conc. HCl/l), and washed down with an equal volume of distilled water. The flasks were boiled for a further 2 hrs. After the apparatus had cooled the solvent layer was taken off using a pasture pipette, transferred to a clean tapered test tube and frozen overnight. On thawing, the solvent was carefully removed, made up to 5 ml and either analysed immediately or frozen as before.

A second extraction was carried out for each algal sample to ensure a high degree of recovery. This was done by adding a further 4 ml solvent to each flask and boiling for 4 hrs, removing and treating the solvent as in the first distillation.

The resultant solvent was analysed using fluorescence spectrophotometry on a Perkin Elmer Luminescence Spectrophotometer LS50B. An emission scan was employed, recording the intensity maxima at 376 nm (slit width 0) (Debber, 1983), using an excitation wavelength of 253 nm with slit width of 5 (Schwarz and Wasik, 1976).

On analysis the recovery rates were sufficiently high and reproducible, at 82.5 $\pm$ 7.5 %, to allow this method to be used for the quantification of anthracene taken into the algal

frond prior to UV exposure.

4.2.3 Experimental protocol for photo-induced toxicity of anthracene

Samples of *E. intestinalis* were collected from Wembury Beach and maintained as in section 2.2.1. One gram samples were weighed out and placed in 500 ml bottles, containing 330 ml anthracene solution of various concentrations (0, 2, 4, 6, 8 and 10 μg/l) each in triplicate and prepared as previously. The concentrations, including the control were prepared so that they all contained the same amount of acetonitrile (100 μl/l). After 24 hr dark exposure the algae were removed, blotted dry on blue laboratory paper, placed in individual sealable bags and frozen. Steam distillation extraction followed by fluorescence analysis was then carried out to establish the amount of anthracene taken up by the algae.

This method of exposure was repeated, with an additional control containing neither acetonitrile, nor anthracene. Once the algae had taken up the anthracene they were exposed to either photosynthetically active radiation (PAR) only or PAR and UV radiation. The samples of algae were removed from their exposure solutions and placed in 150 ml Instant Ocean in a crystallising dish. One full set of samples was placed into each of two clear perspex lined metal troughs and illuminated from below by three fluorescent tubes of PAR 42.3 μmol/m^2/s. The perspex lining of the troughs prevented the penetration of UV light. One trough was then covered to eliminate additional radiation, whereas the other was illuminated with 2 UV lamps each producing 574 μW/cm^2 UV-A (315 - 400 nm) and 20 μW/cm^2 UV-B radiation (280 - 315 nm) (measured using an MP200 Intellirod Meter).

Following 24 hrs continuous light the algae were removed and their Health Index measured as described in Chapter 3.

4.3 Results

4.3.1 Bioaccumulation of anthracene

The recovery rates averaged at 82.5%, sufficient for the quantification of anthracene uptake in algae. Following exposure anthracene was incorporated into the fronds and subsequently extracted successfully using the steam distillation technique, more than 90% of which was recovered in the first distillation. The amount of anthracene bioaccumulated by the algae was consistently lower than the maximum available, although this appeared not to relate to actual concentration of the exposure solution.

Table 6: The recovery of anthracene from the fronds of *E. intestinalis* following 24 hr exposure in the dark. (Algal weights shown are wet weights.)

Anthracene exposure (μg/330 ml)	Average anthracene uptake $\pm$ SE (μg/g algae) (n=3)	% Uptake of available anthracene	Bio-concentration factor (BCF)
0.00	0.011 $\pm$ 0.002	-	-
0.67	0.375 $\pm$ 0.026	56	185
1.33	0.894 $\pm$ 0.022	67	222
2.00	1.391 $\pm$ 0.013	70	230
2.67	1.386 $\pm$ 0.332	52	171
3.33	2.245 $\pm$ 0.039	67	222

The data is also graphically represented in Figure 49, showing the difference between the amount incorporated into the fronds in comparison with the maximum amount of anthracene to which the algae was exposed. The average BCF for all samples was

206 ±10 (SE).

4.3.2 Health Index measurements

Figure 50 represents the relationship between the two treatments, exposure to anthracene and UV radiation. The two UV treatments show distinct responses. Where PAR was used on its own the Health Index achieved for the algae was consistently higher than for those where PAR and UV radiation were used together, with the exception of 8 μg/l anthracene. The algae exposed to the UV radiation had significantly lower Health Indices and this response was consistent for each concentration including both controls. This therefore eliminates the effect of the anthracene or the solvent carrier, acetonitrile, and points towards mild toxicity or stress caused by the UV radiation alone. None of the treatments caused severe membrane damage and no photo-induced toxicity was observed.

4.4 Discussion

Uptake of anthracene into the marine alga *E. intestinalis* occurred during the 24 hr exposure period, accumulating between 52 and 70% of the anthracene added to the exposure water. Exposure to 3.33 μg anthracene resulted in the maximum uptake of 2.25 ± 0.04 μg/g wet weight with a BCF of 222. This BCF is comparable to that for the aquatic macrophyte *Myriophyllum spicatum* (Gobas et al., 1991), where a BCF of 174 was recorded for tetrachlorobenzene which has a log K_{ow} of 4.51. This is similar to that of anthracene which has a log K_{ow} of 4.54 (Miller et al., 1985). Anthracene had no adverse effect on the Health Index, and subsequent UV exposure did not cause an additional toxic effect. However the UV radiation had a mildly toxic effect on the algae. This was demonstrated by all the treatments, including the controls, having Health Indices which were statistically significantly lower than for those which had not been exposed to UV radiation. This relationship was confirmed by a two-way ANOVA (Statgraphics for

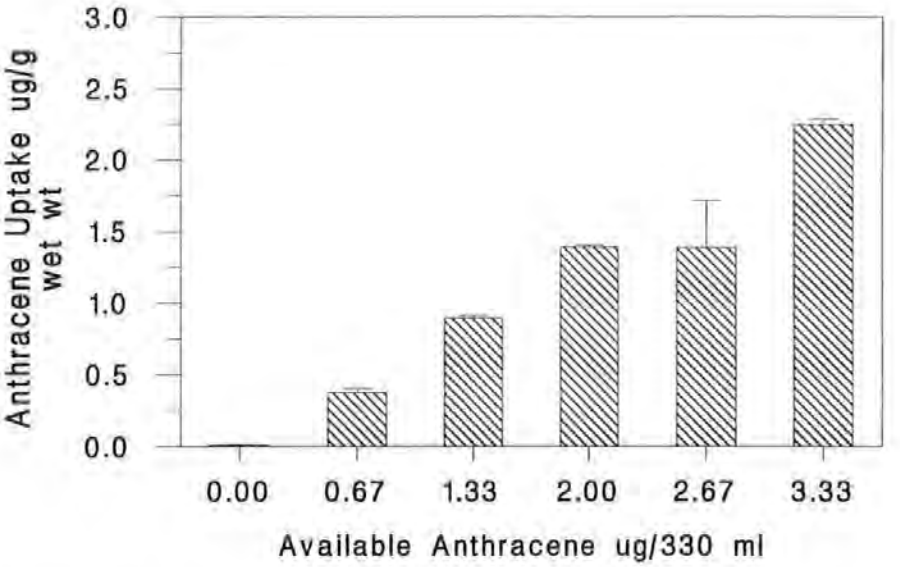


Figure 49: The bioaccumulation of anthracene into the fronds of E. intestinalis following 24 hr dark exposure, including SE.

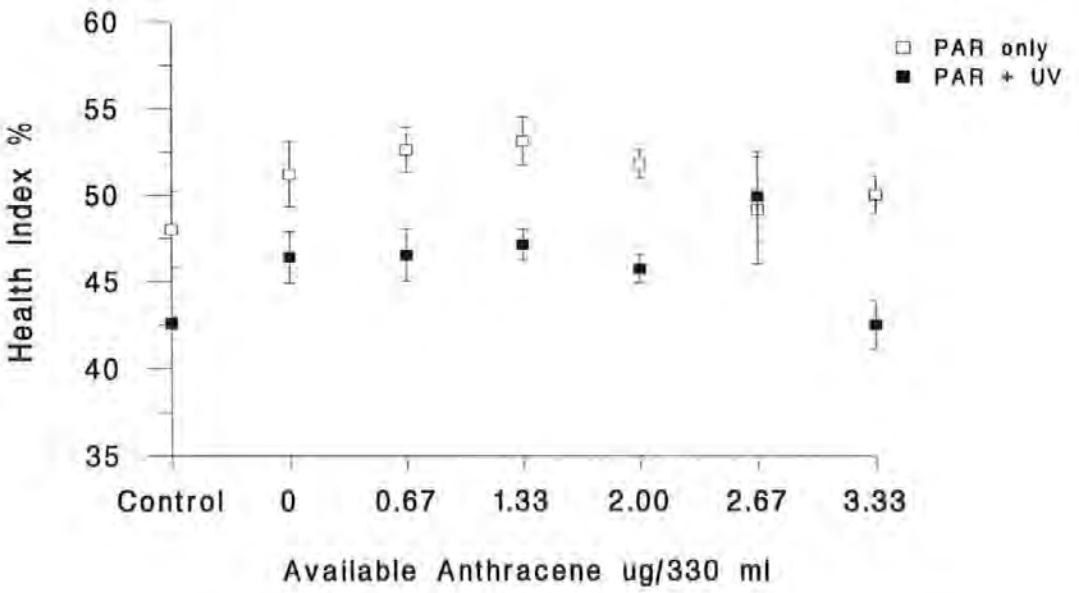


Figure 50: The combined effect of anthracene and UV-A and UV-B on the Health Index of E. intestinalis

Windows). These results indicate that this marine alga is less sensitive to anthracene in the presence of UV radiation than the green freshwater microalga *Selenastrum capricornutum* (Gala and Giesy, 1992), where a threshold value for the photo-induced toxicity of anthracene was between 1.5 - 3 μg/l, and the 24 hr EC_{50} ranged from 37.4 and 3.3 μg/l depending on UV intensity.

Anthracene, in common with all PAH's, has a low solubility in water (Whitehouse, 1984) which is further reduced by the conditions necessary in this study, designed to mimic the natural situation. It therefore appears that anthracene and PAH's with similar physicochemical properties may not pose an environmental threat to marine algae, though they are known to be toxic to marine invertebrates and fish at levels below their aqueous solubility (in marine systems) as these organisms are more sensitive than algae (Oris et al., 1984). The BCF must also be taken into consideration as organisms may be able to accumulate PAH's to levels which could prove toxic. However bioaccumulation only occurs if uptake rates exceed rates of depuration and metabolic degradation. Bowling et al. (1983) recorded that, following anthracene uptake, if the bluegill sunfish was allowed to depurate in the dark over 6 days no mortality was observed when it was subsequently exposed to previously toxic levels of UV radiation.

A survey on organisms within the Brisbane River Estuary in Australia recovered only low levels of anthracene from tissues. The mean concentrations of anthracene in bony bream, sea mullet and mud crab were 0.01, 0.004 and 0.0038 μg/g wet weight respectively (Kayal and Connell, 1995). These concentrations are equal to, and less than, those recovered from *E. intestinalis* before anthracene exposure (i.e. control algae) and it seems unlikely that they would have any toxicity to the organism at such concentrations. Oris and Giesy (1987) recorded a high BCF of 1354 following the exposure of fathead minnow larvae

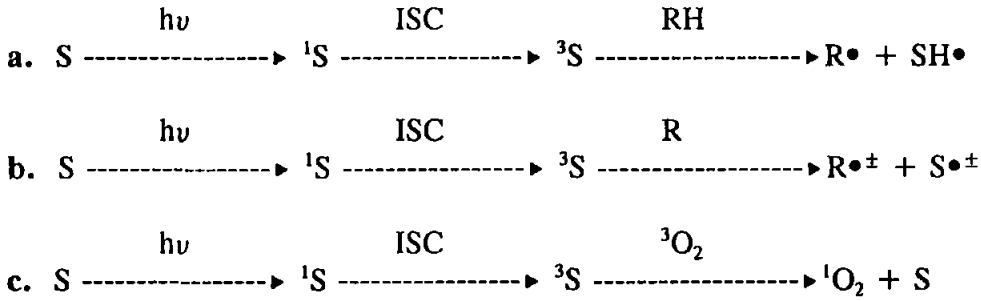
(*Pimephales promelas*) to water of nominal anthracene concentration 14.7 μg/l which resulted in a tissue concentration of 19.9 μg/g. At this concentration an LT50 of 15.75 hr was recorded with UV-B radiation of the same intensity as that used in this experiment. It may therefore be that anthracene present in low concentrations is not an environmental threat, although toxicity is largely dependent on the level of UV radiation (Allred and Giesy, 1985), and the BCF.

There are a number of explanations as to why no photo-induced toxicity was observed. In summary these are:

- The amount of PAH accumulated within the thallus was too small to cause a toxic effect, carotenoids also have an additional protective role, though not enough to protect against direct UV damage.
- The UV levels to which the alga was exposed were insufficient to produce a photo-induced toxic effect.
- The UV exposure time was not sufficiently long for a toxic photo-induced effect to be seen.

How much each of these explanations actually contribute to the results obtained in this investigation could be assessed experimentally in further work. It would be interesting to see whether increasing the exposure time could induce a photo-induced toxic response, or whether the amount of anthracene the alga is able to accumulate is simply too small. It is also possible that the level of UV radiation required to produce a photo-induced toxic response has a greater effect on the algae than it does on the anthracene. The amount of PAH accumulated appears to be the main variable to be investigated and could be increased by using larger exposure volumes or alternatively by using a flow through system. UV exposure of the PAH prior to uptake has also been shown to increase

subsequent toxicity (Ren et al., 1994). However, the presence of carotenoid pigments such as β-carotene in *E. intestinalis* may be sufficient to protect the alga from any photo-induced toxicity. Carotenoids are known to have a protective role in plants, efficiently quenching singlet oxygen (Foote and Denny, 1968). The photochemical reactions are initiated by light (hυ) which excites the sensitizer molecule, in this case anthracene. The first electronically excited species, the singlet species (^1S) is formed initially but has a very short lifetime ($\sim 10^{-11}$ s) and its energy is dissipated through fluorescence or by undergoing an intersystem crossing (ISC) to form a new excited species called the triplet sensitizer (^3S). The ^3S species has a longer lifetime than the singlet and undergoes further reactions, type I are redox reactions (Figure 51, a. and b.) and in type II reactions (Figure 51, c.), ^3S reacts directly with ground state O_2, a triplet species, to produce singlet oxygen $^1O_2$. This can be summarized in the following equations:



Where R and RH are reducing substrates.

Figure 51: The reactions involved in both type I (a+b) and type II (c) photosensitized oxidations.

Early experiments revealed that mutants lacking carotenoids, and plants where synthesis is inhibited, are readily killed by oxygen and light (Foote, 1976). This response was also observed by Gala and Giesy (1993) when the herbicide Fluridone was used to inhibit carotenoid synthesis in cultured *Selenastrum capricornutum*. The percent inhibition of ^{14}C-

bicarbonate incorporation due to the photo-induced toxicity of anthracene was found to be inversely correlated to the concentration of carotenoid pigment present. Since carotenoids are known to quench singlet oxygen (Foote and Denny, 1968) this observation also confirms that a type II, singlet oxygen, mechanism is involved in the *in vivo*, photo-induced toxicity of anthracene.

The fact that a type II response is the cause of the photo-induced toxicity may be an explanation as to why no effect was observed on the Health Index of the alga. Although membrane damage is amongst the effects seen in type II reactions, this is usually secondary to the oxidization of enzymes, RNA and other cellular macromolecules (Foote, 1976). Type I reactions are more likely to cause direct membrane damage as a result of the production of superoxides and hydrogen peroxide. By using some other toxicity parameter, such as photosynthetic efficiency, damage which was not detected using the Health Index might be revealed. The method used by Gala and Giesy (1992; 1993) measured cellular photosynthesis via the incorporation of ^{14}C-bicarbonate into the green alga *Selenastrum capricornutum*, and this method was sensitive to the photo-induced response giving a 24 hr EC_{50} of 3.3 μg/l at the highest UV-A intensity (375 $\mu W/cm^2$). However in their most recent paper Gala and Giesy (1994) used flow cytometric determination to assess the effects of anthracene on *Selenastrum capricornutum*. They concluded that this method was more sensitive than bicarbonate incorporation, the 28 hr EC_{50} for the stress index (SI), based on changes in cell fluorescence, being 16.1 μg/l anthracene at the more environmentally realistic UV-A level of 125 $\mu W/cm^2$. Both these UV-A levels are none-the-less lower than those used in our study, i.e. 574 $\mu W/cm^2$.

Chlorophyll fluorescence techniques have been employed by other researchers in the detection of UV-B toxicity to a number of species of macroalgae (including *E. intestinalis*)

(Larkum and Wood, 1993), and to soybean seedlings (Kraus et al., 1995). In both cases this technique revealed reduced photosynthetic efficiency, and a relationship between the level of UV-B exposure and reduced efficiency was observed. Both studies used marginally higher UV-B intensities than that to which the algae were exposed in our study (35 and 23 $\mu W/cm^2$ respectively), but, in comparison, the toxicity of this narrow waveband of light (280 - 315 nm) had a significant effect on the membrane integrity of *E. intestinalis* at our level of 20 $\mu W/cm^2$. Kraus et al. (1995) also utilised an ion leakage technique to assess membrane integrity in a comparable way to the Health Index, and they similarly revealed an increase in leakage following an 11 day UV-B exposure. It is therefore a matter of concern that the levels of UV-B exposure utilised in our study are only slightly higher than environmental levels recorded in southern Ontario (Canada), (7 to 12 $\mu W/cm^2$, Kraus et al., 1995) so effect on photosynthesis in the field seems likely. Algae also appear to be more sensitive to UV-B than higher plants as exposure of *E. intestinalis* to 35 $\mu W/cm^2$ resulted in a reduction of photosynthetic efficiency (Fv/Fm) from 0.73 to just 0.4 (Larkum and Wood, 1993) whereas the same parameter in soybean seedlings fell from 0.812 to 0.712, although this was still a significant reduction.

This technique was unable to reveal a photo-induced toxicity, but none-the-less the results obtained are of interest with regards UV-B toxicity. In the absence of increased toxicity of anthracene, due to the absorbance of UV light and subsequent photomodification of the substance, the chemical did not induce any detectable toxic response. Anthracene has a log K_{ow} of 4.54 (Miller et al., 1985) and for it to induce a non-specific narcotic effect a concentration of 0.06 mM would have to be achieved (see Ion Leakage QSAR). Anthracene's low solubility (0.097 μM, at 17°C and 33.1 ppt salinity, Whitehouse, 1984) prevents this and in the absence of an increase in toxicity due to photomodification the chemical does not cause any detectable damage.

CHAPTER 5

ENVIRONMENTAL POLLUTION ASSESSMENT TECHNIQUES

5.1 Introduction

Environmental assessment techniques have been developed in this project to evaluate the levels of pollution at a number of sites and to assess the relative health of algae taken from these sites. The approach was designed to integrate the effect of environmental parameters with the effect of different pollutants, specific and non-specific in action. To achieve this, one site, regarded as uncontaminated by organic and inorganic pollutants, was monitored throughout one year giving a set of environmental data and showing to what extent changing environmental parameters give rise to different Health Indices which indicate the relative health of the algae. To allow for comparison, algae were then sampled from sites contaminated by organic and inorganic (heavy metal) pollutants. Techniques developed to detect specific and non-specific effects were employed to establish how these pollutants with different modes of toxic action lead to variation in the response of the algae. Bioaccumulation studies were also carried out on these samples to verify the extent of contamination.

As discussed in Chapter 1 the use of macroalgae as biomonitors of pollution and comparisons between the health of the algae and the effects of pollutants have been investigated during the last three decades. Two recent reviews are available on past and current use (Fletcher, 1991; Phillips, 1994). Some of these methods use seaweeds as biomonitors of environmental contaminants (e.g. heavy metal bioaccumulation), and some present laboratory based toxicity tests, (e.g. use of vital stains and growth measurements) but there is little evidence of the two approaches being married to allow for *effects-based* biomonitoring. Only a few studies exist where physiological parameters such as growth

and photosynthesis have been employed in environmental monitoring programs (Hsaio et al., 1978; Reed and Moffat, 1983). In this chapter the different methods developed during previous toxicity studies, both in this project and by other researchers, are applied to the environmental situation in an attempt to overcome this lack of integration between biomonitoring and physiological response. These methods include the Health Index and neutral red toxicity tests and introduce the use of photosynthetic chlorophyll fluorescence induction kinetics. Growth, oxygen evolution and chlorophyll analysis were also used alongside extraction techniques both for organic and heavy metal contaminants.

Three clean sites have been utilised as controls for the purpose of environmental assessment. All are bathing beaches used recreationally and lie to the north of Plymouth, on the south coast of Devon. They include Wembury Beach (O.S. SX517484), from which algal material was also collected for toxicity studies, Thurlestone (O.S. SX660435) and Mothecombe (O.S. SX610473). To gain more insight into the impact of organic pollution on *E. intestinalis* a boating harbour, Sutton Harbour within Plymouth's Barbican was utilised. This harbour is frequented by both fishing and recreational boats and experiences contamination relating to such activity. To investigate the effects of long term metal contamination algae were sampled from the Fal Estuary. This estuary is situated in southwest Cornwall, an area which has a long history of metal mining. Mining activity has led to the contamination of sediments by trace metals where a concentration gradient has developed between the various creeks. A massive metal discharge in 1991 of acidic metalliferous water prompted the initiation of a number of investigations. These were designed to assess the influence of the high levels of metals on the meio- and macrobenthic community structure (Somerfield et al., 1994). A number of different heavy metals are present within the sediments and include copper, iron, manganese, lead, cadmium and zinc. A summary of the concentrations of these and other metals in sediment taken from

five different creeks within the Fal Estuary can be found in Somerfield et al., 1994, where a positive relationship between the meiofaunal community structure and heavy metal contamination was found. Past analysis of macroalgae (*Fucus spp.*) taken from Restronguet Creek, the most contaminated creek in the Fal Estuary, confirms that the metals present are taken up by the algae from the water and probably reflect the concentrations dissolved in the water (Bryan and Hummerstone, 1973).

5.2 Methods

The environmental assessment techniques used in this study are broken down into three separate investigations which will be dealt with individually.

5.2.1 Assessment of a clean area over time using ion leakage techniques

Environmental data for a rock pool area within the upper intertidal zone on Wembury beach was collected throughout a year from 07.06.93 to 07.06.94. In all cases collection of *E. intestinalis* was carried out at low tide and from approximately the same site. Five physicochemical parameters were recorded: The incident photosynthetically active radiation (PAR) was measured at water level using a PAR meter (Skye Instruments Ltd. SKP 200). Dissolved oxygen and temperature of the rock pool water were measured with a YSI model 58 dissolved oxygen meter. The salinity of the water was assessed using an ATAGO hand refractometer, and pH using a hand held probe (Whatman pH μ sensor). While collecting these data, samples of *E. intestinalis* were collected for the ion leakage Health Index to be carried out as detailed in Chapter 3. At the start of the investigation this was carried out immediately on return to the laboratory but from the beginning of September, 24 hr equilibration in Instant Ocean (salinity 33 ppt) was allowed prior to testing. During equilibration the algae were placed in buckets of aerated Instant Ocean of the same salinity, and left under an irradiance of 66 $\mu E/m^2/s$ in a constant temperature room held

at 16°C.

5.2.2 Assessment of clean and organically polluted sites in and around Plymouth

5.2.2.1 Collection of algae

An environmental survey was carried out within the Plymouth area. Three sites thought to have relatively low levels of organic pollution were chosen as controls and compared to one site potentially organically polluted. This latter site is in an urban setting and contains a large number of boats. The control sites were Thurlestone Beach, Mothecombe Beach and Wembury Beach, and the polluted site, Sutton Harbour in Plymouth (OS grid ref. SX486542). At the point of collection the salinity of the water was measured and samples of *E. intestinalis* collected and returned to the laboratory.

5.2.2.2 Assessment of health

Following collection, tests were carried out on the algae to assess their health and later analysis was undertaken to determine the content of polyaromatic hydrocarbons (1, 2 and 3^+ ring PAH's). Health was assessed using four different techniques: Neutral red retention (cf Chapter 2); Health Index (cf Chapter 3); growth and by fluorescence ratios using a plant efficiency analyser (PEA, Hansatech Ltd.).

5.2.2.3 Growth studies

Growth of the algae from each site was investigated using excised pieces of intercalary thallus which were left to grow for 7 days (Reed and Moffat, 1983). For each site nine 3 cm pieces of thallus were cut from separate non fertile fronds, placed into divided petri dishes flooded with Instant Ocean (33 ppt) and incubated overnight. No nutrients were added. The initial length of each piece was measured the next day using vernier callipers. Measurements were taken every other day until day 6. Following each measurement the

water in the dishes was changed. The dishes were incubated in an environmental cabinet at 16°C with illumination of 45 $\mu E/m^2/s$ for 16 hrs/day.

5.2.2.4 Chlorophyll fluorescence studies

The plant efficiency analyser (PEA) measures photosynthetic efficiency through kinetic changes in chlorophyll fluorescence. Thalli or leaves are placed into clips which exclude all light, are left to dark adapt for a set length of time, then light of a chosen saturating intensity is flashed onto the surface of the sample and the resultant chlorophyll fluorescence is detected giving a ratio Fv/Fm. The intensity of light and appropriate dark adaptation time were determined using the procedure suggested in the manual. It is important to establish the minimum time required for dark adaptation as this can vary according to the amount of ambient light to which the algae have been exposed prior to testing. Twenty clips were placed on individual pieces of thallus with the shutters closed. With light intensity at 100% the Fv/Fm ratio was measured for each piece in turn at 2 min intervals. The ratio reaches a plateau at the minimum satisfactory dark adaptation time. For *E. intestinalis* kept under standard laboratory conditions this was 6 mins. Having determined the dark adaptation time it was necessary to select the required saturating light level. Ten clips were placed over thalli with their shutters closed to exclude light. After 6 mins dark adaptation a measurement was taken for each sample using increasing light intensities, starting at 10% and rising to 100%. The Fv/Fm ratio reached a plateau at 70% and this was therefore chosen as the saturating light intensity. The efficiency of algae from Mothecombe and Thurlestone were measured for 8 pieces of thallus, originating from different specimens of algae, but as variation was very low between samples only 5 replicates were used for algae collected from Wembury and Sutton Harbour.

5.2.3 Extraction of organics

The presence of organic substances within the algal frond was investigated by extraction through steam distillation (Donkin and Evans, 1984). The basic technique is described in section 4.2.2. Samples of algae from each site were rinsed and patted dry in blue laboratory paper, placed in sealable plastic bags from which all air was expelled, and frozen until analysis could be carried out. For each distillation approximately 10 g of frozen algae was used and for each site the dry weight was determined (see Chapter 2). In all cases the condensed solvent (iso-hexane) containing the extracted organics was made up to 5 ml and measured without dilution on the luminescence spectrophotometer (Perkin Elmer - LS50B). Excitation wavelengths of 253 nm, 280 nm and 310 nm were used and the intensity of emission was measured at the specific wavelengths of 376 nm, 330 nm and 360 nm respectively (after Schwarz and Wasik, 1976; Jurgensen et al., 1981 and Hála et al., 1981 respectively). The slit widths were maintained at 5 for excitation and 0 for emission in all cases. For each excitation wavelength, emission between 300 or 320 nm and 500 nm was recorded. Four standard solutions of different organic compounds were used to give some indication of the relative importance of 1, 2 and 3 ringed compounds present in the extracts. These included: An anthracene solution, for which an excitation wavelength of 253 nm and specific emission wavelength of 376 nm were used; A 2 ring standard, 2,3-Dimethylnaphthalene, using an excitation wavelength of 280 nm and specific emission wavelength of 330 nm; A 3 ring standard, 1-Methylphenanthrene, using an excitation wavelength of 310 nm and specific emission wavelength of 360 nm; and a yellow fuel oil standard for which all the above excitation wavelengths and corresponding emission wavelengths were used. In addition to the four sites used previously samples of algae from Restronguet Creek in the Fal Estuary were also analysed. This was carried out in order to assess the extent of organic pollution to which the algae are exposed. In all cases the solvent used was iso-hexane.

5.2.4 Assessment of heavy metal polluted sites in the Fal Estuary

The health of *E. intestinalis* taken from the Fal Estuary was assessed via growth, Health Index (see Chapter 3), chlorophyll analysis and oxygen evolution. The bioaccumulated metals were analysed for each creek and three control sites, Thurlestone, Mothecombe and Wembury.

The algae were collected from five creeks within the Fal Estuary on 09.07.94, placed in polystyrene bags and returned to the laboratory in a cool box. On return the samples were washed in Instant Ocean to remove all surface sediment and identification was carried out. Those to be used in bioaccumulation studies were frozen at -16°C while those to be used for other analysis were placed in buckets of Instant Ocean (33 ppt) and maintained as described in Chapter 2. The five sites from which the algae were collected were Restronguet Creek (SW385810), Pill Creek (SW385827), Mylor Creek (SW356809), St. Just's Creek (SW361848) and Percuil Creek (SW343857).

5.2.4.1 Growth analysis

Growth studies were set up as previously described in section *5.2.2.3* although measurement was accomplished using the Image Analyser (Cambridge Instruments, Quantimet Q570). A program was utilised which automatically calculated the length of the frond at its longest point. To achieve this a standard template was placed onto a light box. A video camera suspended above then produced an image of the template on the monitor of the image analyser which was subsequently calibrated. Each frond was then placed onto the light box and the length measured automatically.

5.2.4.2 Chlorophyll analysis

The total chlorophyll *a* content of the algae was measured by extraction into 80% acetone

and spectrophotometric assay at 663 and 645 nm (Arnon, 1949). In each case 1 g of algae was ground with a pestle and mortar using a little acid washed sand and 10 ml 80% acetone. The resultant homogenate was then centrifuged at 1000 rpm for 2 mins and the supernatant carefully poured off and the volume recorded. The absorbance was then measured spectrophotometrically and the chlorophyll *a* content calculated using the following relationship:-

Chll *a* = (0.0127 x Abs at 663 nm) - (0.00263 x Abs at 645 nm)
- in g/l of 80% acetone

5.2.4.3 Oxygen evolution

Oxygen evolution was measured for Restronguet and St. Just Creeks using an oxygen electrode (Clandon Scientific, YSI model 53). Both evolution and dark uptake of oxygen due to respiration were measured allowing net photosynthesis to be calculated. The electrode was equilibrated for oxygen saturation at 18°C. Following calibration the membrane was checked and replaced when necessary. The chart speed was set to 1 cm/min. The algae were pressed together lightly to exclude any air bubbles within the thallus and placed into 50 ml Instant Ocean in the chamber. The chamber was covered with black plastic, left for 3 mins and the trace was then run for 10 mins. The plastic was removed and the chamber lit using a projector lamp at a distance of 40 cm, giving a PAR output of 45 $\mu E/m^2/s$. Again the apparatus was left for 3 mins and then the trace was run for 10 mins. Oxygen changes were calculated from the slope.

5.2.4.4 Bioaccumulation of heavy metals

The bioaccumulation of copper, zinc, manganese and cadmium was analysed using the following procedure (preparation and analysis was carried out by John Wedderburn, Analytical Chemistry Technician, Ecotoxicology):

Preliminary analysis was undertaken using the standard reference material (SRM) of *U. lactuca* (Community Bureau of Reference-BCR). A sample of SRM was frozen and then freeze dried for 24 hrs. Six 120 ml Teflon microwave vessels were acid washed in 10% v/v nitric acid for 48 hrs, rinsed with Milli-Q water and dried overnight in a laminar flow hood. Approximately 0.5 g freeze dried SRM was added to five vessels, one being left as a blank control. The SRM was pre-digested overnight by the addition of 5 ml concentrated nitric acid (Aristar) and 2 ml hydrogen peroxide (Analar). Teflon lids were placed loosely on the vessels which were then left in a laminar flow cupboard. Digestion was undertaken using a microwave MDS-2000 system using the following program:

Power (%)	20	40	60	80	100
Pressure (psi)	20	40	60	80	100
Time (mins)	2	2	2	2	2

Following digestion the vessels were removed and allowed to cool in a laminar flow cupboard for half an hour. Each was then vented to allow the release of gases produced as a by-product and to relieve the pressure. The above procedure was then repeated, after which each sample was made up to 25 mls using 0.1 M nitric acid. Standard solutions were made up from BDH 1000 ppm metal standard solutions again using 0.1 M nitric acid. Analysis of Cu, Zn, and Mn was then undertaken using Flame Atomic Absorption Spectrophotometry (FAAS) on a Varian SpectraA-400 Plus. Background correction for Zinc was not necessary. Cd analysis was undertaken using Graphite Furnace AAS but the results were uninterpretable as concentrations were below detection limits and the variation between samples was high. Following analysis, the percentage recovery of the stated standard values was found to be acceptable (between 86 and 87%) and analysis of field samples was then undertaken using the same digestion and analytical procedure, except for Cd analysis which was carried out on the Flame AAS where results were obtained, though

these were still unsatisfactory.

Table 7: % Recovery of metals from SRM material.

Vessel No.	Wt of SRM g	Copper conc ppm	% Recovery	Zinc conc ppm	% Recovery	Manganese conc ppm	% Recovery
1	0.5084	11.55	87.9	44.1	85.9	1847	85.9
2	0.5089	11.49	87.5	43.08	84	1828	85.1
3	0.5224	11.62	88.5	47.6	92.8	1835	85.3
4	0.5081	10.63	80.9	44.3	86.4	1838	85.5
5	0.5001	12.05	91.7	42.09	82.1	1884	87.6
6	Blank	-	-	-	-	-	-
Mean	-	-	87.3	-	86.2	-	85.9
SD	-	-	3.9	-	4.0	-	1.0

5.2.4.5 Copper ion exposure

To assess the level of acquired tolerance to copper and the nature of its effects on *E. intestinalis*, samples of algae from Restronguet Creek in the Fal Estuary and Wembury Beach were exposed to copper concentrations for 24 hrs and the effects were analysed using the modulated fluorimeter (OS-100 Opti-Sciences, Inc.) to give both yield and efficiency data. For efficiency data a dark adaptation time of 6 mins was used. Following this an ion leakage test was performed to give a Health Index (see Chapter 3 for method).

5.2.4.6 The pulse modulated fluorimeter

The pulse modulated light fluorimeter works in a similar way to the PEA meter, measuring chlorophyll fluorescence (see Section *5.2.2.4*). However, rather than using a single burst

of light the measuring light is switched on and off at high frequencies and a low intensity is used. Photosynthetic efficiency is established in a manner comparable to that employed by the PEA meter, except that Fo (the initial level of fluorescence emitted when the first stable reaction centre, QA (a quinone), is fully oxidised or 'open') is ascertained differently, through direct measurement rather than by mathematical extrapolation. This instrument also allows actinic light to be used, which is environmentally significant. The steady state fluorescence yield, Fs, is measured prior to a saturation pulse being triggered. This pulse gives the maximal fluorescence under these conditions, Fms, and the difference between these two values is taken as the 'Yield' of energy conversion. As this procedure is carried out under continuous illumination, clips capable of excluding light are not required. Instead the probe was clamped at a 45° angle, one inch above a cardboard shield in which the alga was rested. The whole apparatus was placed under a light frame of light intensity 42.3 $\mu E/m^2/s$ to ensure even lighting. In all cases a modulation of 255 was maintained and an autopulse length of 0.7 s used.

For algae from both Restronguet and Wembury the same experiment was carried out. Three 1 g replicates of algae were exposed to copper ion concentrations of 0, 100, 200, 300, 400 and 500 $\mu g/l$ in Instant Ocean made up from a standard of copper chloride in Milli-Q. The samples were incubated for 24 hrs at 15°C with PAR 15 $\mu E/m^2/s$. Following incubation photosynthetic yield and efficiency were measured using the OS-100 and afterwards ion leakage analysis was performed.

5.3 Results and Discussion

5.3.1 Assessment of a clean area over time using ion leakage techniques

The environmental data collected from Wembury and displayed in Figures 52 to 57 was compiled over one year and should thus reflect seasonal changes in the parameters

measured. Those parameters include the salinity, pH, temperature and dissolved oxygen (DO) of the rock pool water at the time of collection of the algae, the incident photosynthetically active radiation (PAR) and the calculated Health Index for the samples of *E. intestinalis* collected. Each parameter will be discussed individually and where applicable interactions will be commented upon. In contrast to most fresh waters the marine environment shows greater constancy, for example, in pH, temperature and DO (Abel, 1991). However, a rock pool lying in the higher intertidal region of a beach can experience great variation due to tidal action and consequent exposure, both of which are influenced by the tidal cycle, the weather and seasonal changes. This is demonstrated by the results.

5.3.1.1 Salinity

Throughout the year the salinity of the rock pool shows great variation ranging from 3 to 40 ppt. The seaweed present in this pool is therefore subjected to salinities outside the usual range for coastal seawater, which is normally between 30 and 35 ppt (see Lobban and Harrison, 1994). All sampling was carried out at low tide when the rock pool was exposed, so variation can be attributed to the weather and to the confluence of a stream which runs down the beach, the course of which changes constantly due to wave action on the shingle. From day to day the stream either by-passes the rock pool area entirely or feeds directly into the pools, causing the dilution of the rock pool water and reducing the salinity. Fresh water is less dense than saline and therefore floats on it, causing stratification. However these rock pools are shallow (maximum depth $\approx$ 50 cm) and when a series of measurements was made at various depths stratification was not observed.

The salinities recorded show a general trend with values being higher during the summer months and lower during the winter. High salinities during hot weather can be attributed

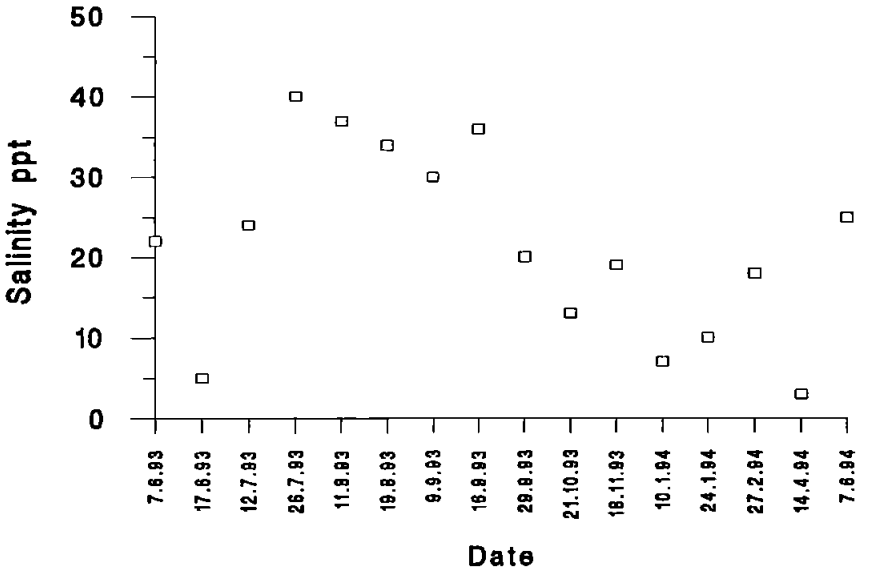


Figure 52: Salinity of rock pool water at point of collection of E. intestinalis on various dates during one year.

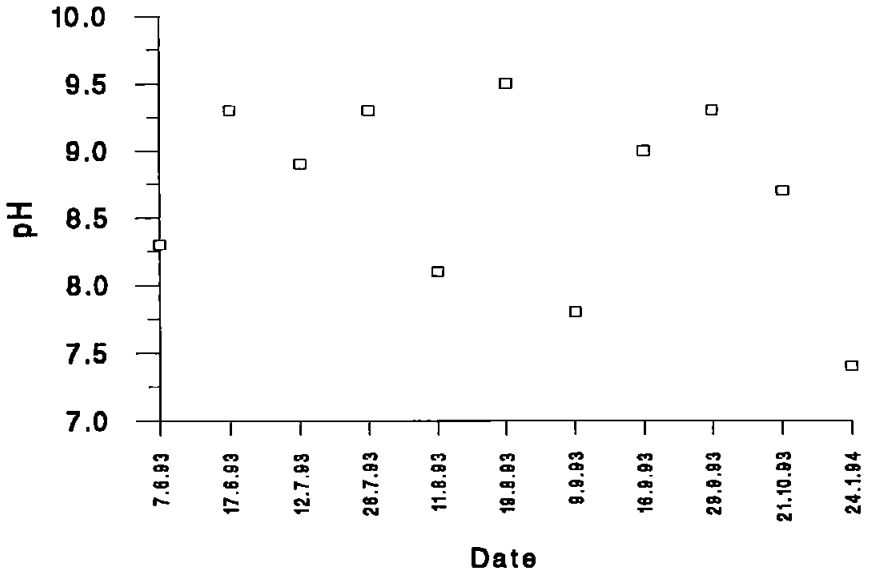


Figure 53: pH of rock pool water at point of collection of E. intestinalis on various dates during one year.

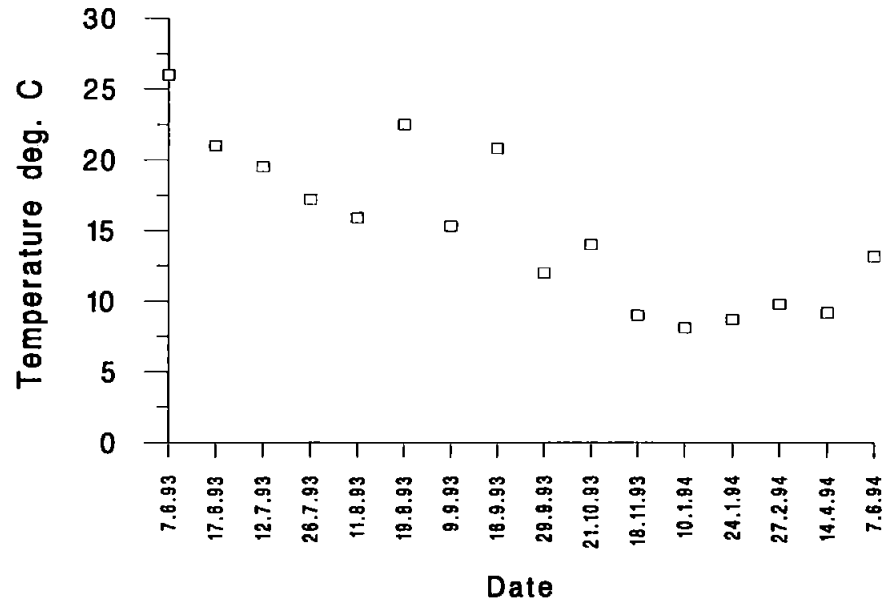


Figure 54: Temperature of rock pool water at point of collection of E. intestinalis on various dates during one year.

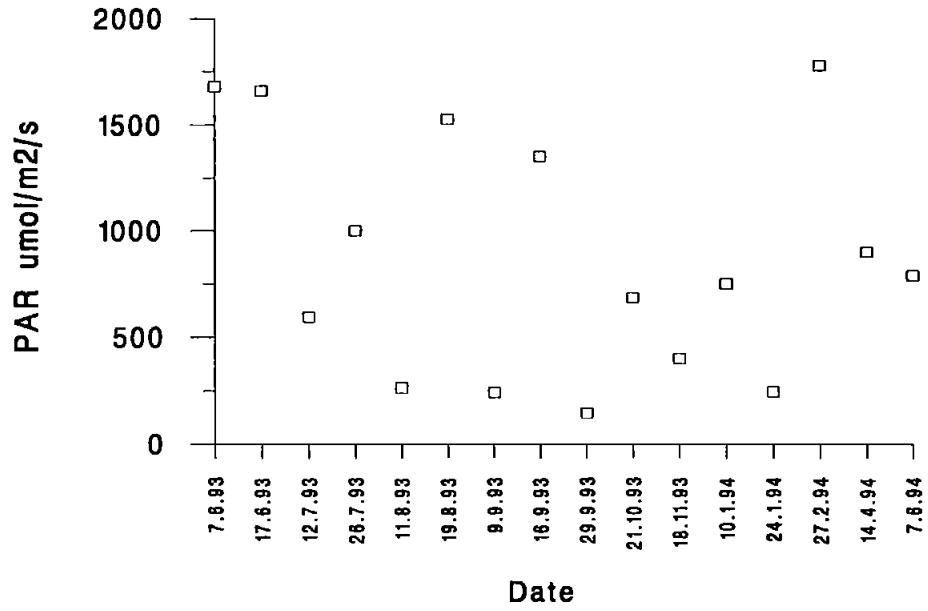


Figure 55: PAR intensity over rock pools at point pf collection of E. intestinalis on various dates during one year.

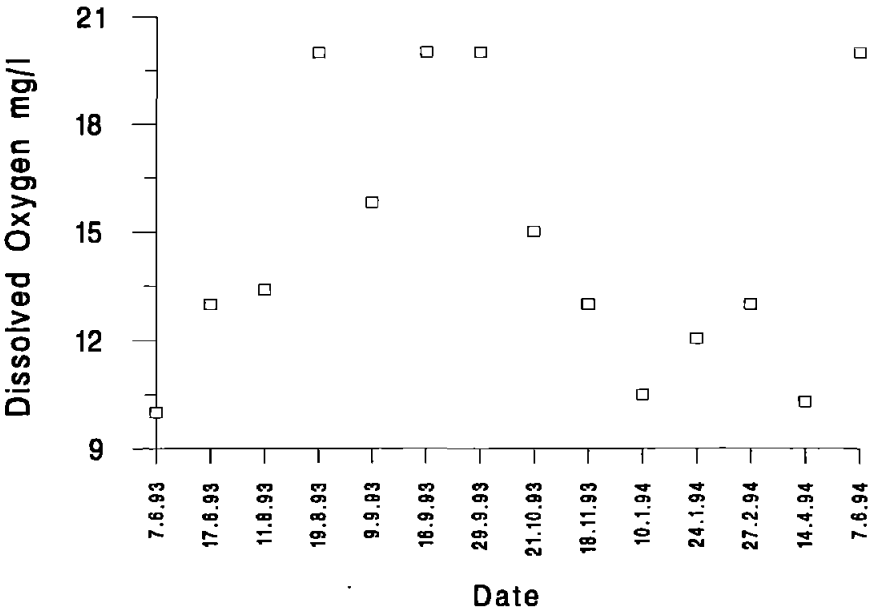


Figure 56: Dissolved oxygen content of rock pool water at point of collection of E. intestinalis on various dates during one year.

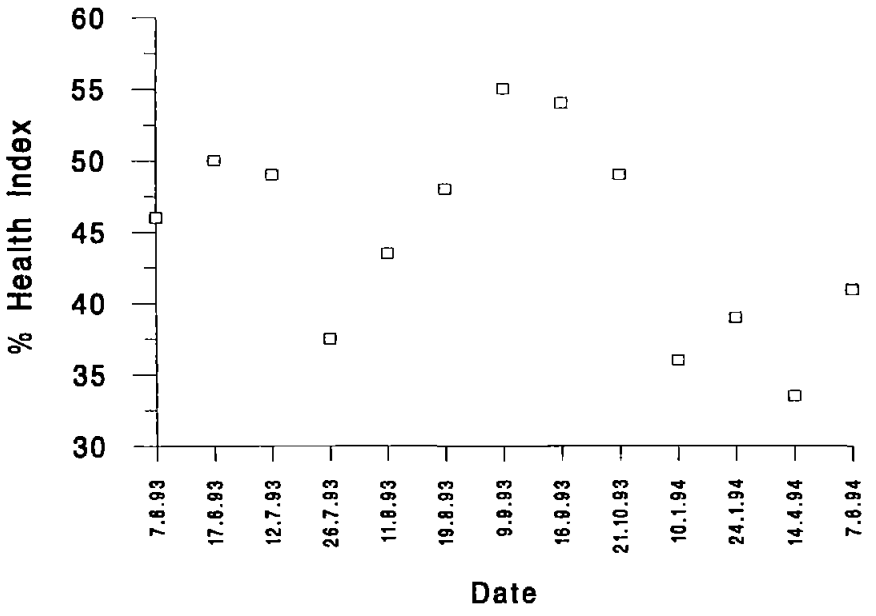


Figure 57: % Health Index of E. intestinalis collected from rock pools on various dates during one year.

to the evaporation of water from the exposed pools increasing the concentration of salts. This is a slow process and the effects are only seen because of the position of the pools in the mid- to high-intertidal zone (see Lobban and Harrison, 1994). This means that the exposure time of the pools is long, occurring 2 hrs after high tide until 2 hrs before the next, but varying according to the height of the tide. The large surface area to volume ratio exacerbates the tendency for evaporation to occur. Lower salinities are due either to a reduction in evaporation or to dilution by rain or by the behaviour of the fresh water stream. The greater amount of variation during the winter seems to relate mostly to the latter, the stream's course being changed repeatedly during storms. Not surprisingly concentration of salts does not normally occur at this time of year when the highest salinity recorded was 19 ppt on 18.11.93. Freezing of salt water concentrates salts within the remaining water body but during the mild winters experienced in south west Britain this does not occur. However the surface water on exposed algae was observed to freeze on a number of days during the winter. Freezing will kill most seaweeds but the presence of high concentrations of salts within the cytoplasm lowers the actual freezing point and provides protection against freezing of intracellular water (Lobban and Harrison, 1994). In addition the presence of high concentrations of salts on the outsides of cells causes water to be drawn out of the tissues causing desiccation and as a result further concentrating the cytoplasm. Mechanical damage of the cell by ice crystals is therefore largely avoided unless freezing is very rapid or the temperatures fall to -35 or below (Bidwell, 1979).

5.3.1.2 pH

The pH of the pool water ranged from 7.4 to 9.5. This is a considerable fluctuation compared to coastal water which in Wembury Bay ranges from 7.5 to 8.4 with an average of 8.1 (n=61) (NRA data for summers of 1992 to 1995). Figure 53 shows that in general

the pH of the pool is more alkaline than the coastal water, the average being 8.7. This increase in alkalinity is due to the algae photosynthesising, HCO_3^- ions being taken up and OH^- being produced by the frond. When exposed to air, intertidal seaweed use atmospheric CO_2 as their main source of inorganic carbon necessary for photosynthesis. So long as a layer of water covers the algal thallus, CO_2 is able to diffuse into this in sufficient quantity and uptake occurs (Beer and Shragge, 1987). However, when submerged, inorganic carbon in the form of CO_2 is not present in sufficient quantities so HCO_3^- ions are utilised instead. This ionic form is present in higher concentrations than the latter due to the high pH. Uptake in this form has however only been observed in a number of red and brown algae (Cook et al., 1986); in other algae, including *E. intestinalis* (*compressa*) (Beer and Shragge, 1987), uptake is still in the dehydrated form of CO_2 following extracellular conversion via carbonic anhydrase and/or acidification.

5.3.1.3 Temperature

The temperature of the rock pool water varies throughout the year with higher temperatures being recorded in the warm months from June to the end of September. The highest temperature, 26°C, was recorded in early June (07.06.93), the lowest, 8.1°C, in January (10.01.94). Fluctuations of as much as 7°C from one visit to the next demonstrate the high level of variation in environmental conditions to which rock pool dwelling organisms are subjected. During the summer months the temperature of the water is linked to the photosynthetically active radiation (PAR) when the shallow pools are warmed by the sunlight. During the winter however the PAR has less of an influence when the sun is at a low angle and the air and water temperatures are less. This relationship is presented in Figure 58. In February, when the highest PAR value was recorded (1778 $\mu E/m^2/s$), the water rose by less than one degree above that which had been recorded on a previous visit when the PAR was low (245 $\mu E/m^2/s$). Overall there is a statistically significant

relationship between temperature and PAR (P=0.038). The link between water temperature and PAR relies on the overall heating effect of the sun. When higher in the sky the light is absorbed by the water and is not reflected to such a degree. When the sun is low on the horizon during the winter much more light is reflected from the surface of the pools so the measured irradiance is much higher than the actual penetrating light.

5.3.1.4 Photosynthetically Active Radiation

The incident PAR available to the seaweed shows great fluctuation throughout the year. The higher values recorded mainly occurred between June and October, and the lower from October to June. However the highest value was recorded on February 27th and low levels were measured throughout the summer on cloudy overcast days. Physiologically PAR has a highly significant affect on the algae which are dependent on light for photosynthesis. As discussed earlier PAR also influences the algae by causing the water temperature to rise, increasing metabolic rates. The degree to which this occurs is dependent upon the angle of the sun in the sky, the level of turbulence and the amount of light scattering within the water column. The morphology of *E. intestinalis* is sheetlike, so all cells have the same theoretical ability to photosynthesise and although some clumping occurs where individuals grow very close to one another the scattering of light entering the water tends to equalise its intensity. The quality of light is affected by both scattering and absorption by suspended material in the water as well as reflection at the surface, so incident PAR can only be used as a guide to the actual quality and quantity of light available to the algae for photosynthesis. Furthermore the timing of high and low tides in relation to diurnal changes in irradiance has an effect on photosynthesis in intertidal seaweed species. Both are a function of the times of tides and also the change in day length. In many populations of *E. intestinalis*, some individuals not in rock pools are fully exposed as soon as the tide goes out and are then largely unable to

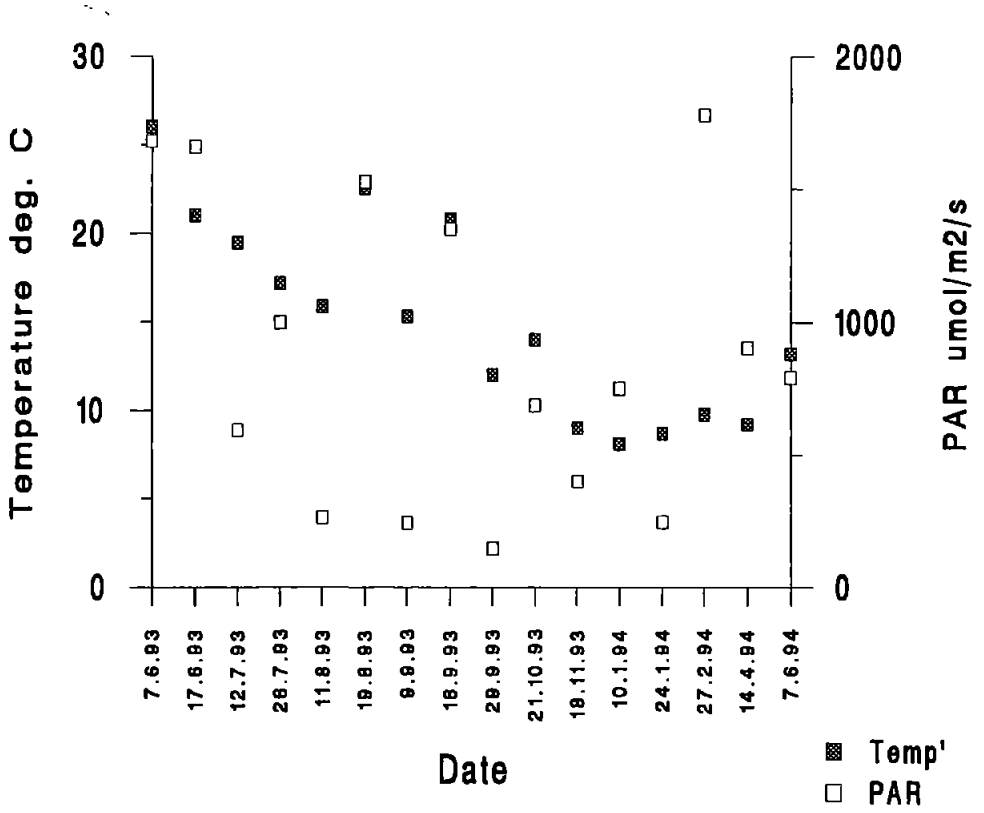


Figure 58: Relationship between temperature and PAR measurements collected on various dates during one year.

photosynthesise due to the restricted source of inorganic carbon as desiccation progresses (Beer and Shragg, 1987). If the tide is out for most of the day, a proportion of the population exposed could experience long periods when photosynthesis is not possible, affecting overall health. In addition, high irradiance when accompanied by high temperatures increases desiccation. This has a physiological cost resulting in a decline in growth rate related to a reduction in photosynthesis (Chapman 1966).

5.3.1.5 Dissolved Oxygen

Dissolved oxygen within the rock pool showed seasonal changes in line with temperature and to a lesser extent PAR. Levels of DO ranged from 10 mg/l to greater than 20 mg/l (the maximum recordable value), and were generally higher during the summer months in comparison to the winter months. In a controlled environment the solubility of oxygen in water decreases with rising temperature. However in these trials an opposite effect was recorded, but with such variation as to make statistical correlation unreliable. This trend is probably related to an increase in the photosynthetic rate of the algae present in the pool caused by the increase in water temperature. Interestingly, the amount of PAR available to the algae has much less of an effect on the DO. When 'regressed together' no relationship is found between the two. DO tends to be high when PAR is high, and certainly when PAR is sufficient to affect the temperature of the rock pool water this would seem to follow. Alternatively it may reflect an increase in light penetration. During the winter, when air temperatures are lower, PAR has less effect on both water temperature and DO.

5.3.1.6 Health Index

The Health Index of the algae was measured using two different approaches. During the first two months of the study, samples were tested immediately on return to the laboratory.

In September it was established in laboratory experiments that differences in the salinity of the incubating water gave rise to different Health Indices (cf Chapter 3) and thus it was decided that at least twelve hours equilibration in Instant Ocean of salinity 33 ppt should be allowed before testing. All measurements taken after this were therefore equilibrated. Despite this change in approach the range of Health Index values was not great, varying between 33.5 % and 55 %, where the higher values are indicative of the healthiest algae. The average Health Index for the year was 45 % with a standard error for the sample being $\pm 6.9\%$. No significant differences between results were seen for the different techniques. The measured Health Index shows a trend towards good 'health' during the summer and worse during the more challenging winter months.

Regression analysis between Health Index and all other parameters individually, and in multiple regression reveal no significant relationship between any of the parameters. By carrying out a stepwise multiple regression it was ascertained that the best model for the Health Index is given by a combination of DO, PAR and temperature, with exclusion of both salinity and pH which did not contribute significantly to the relationship. The equation of this relationship is:

$y = 0.64a - 0.008b + 1.12c + 24.6$, $(P = 0.038)$

where a = DO, b = PAR, c = temperature (°C) and 24.6 is a constant.

The absence of any quantifiable relationship between Health Index and salinity of the rock pool may be because *E. intestinalis* is known to be a good osmoregulator (Kirst, 1989). Changes in salinity usually occur slowly in the environment giving the algae sufficient time to adapt and avoid membrane damage. The two laboratory exposure experiments carried out to investigate the effect of different salinities on the Health Index of *E. intestinalis* (cf Chapter 3, Figures 14 and 15), exposed the algae to salinities ranging from 0 to 100 ppt.

In the first experiment the health of the algae was assessed using the ion leakage technique immediately following exposure. In the second a 30 min equilibration time was allowed following recommendations of Axelsson and Axelsson (1987). Where no equilibration occurred the Health Index values did not seem to relate to the health of the algae following exposure but rather to the total number of ions (reflected in the conductivity) present within the frond. This was directly related to the salinities of the solution. Where equilibration was allowed, the trend changed to one which gave more realistic Health Indices, reflecting membrane damage. This demonstrates that this algae can osmoregulate, as water and ions are rapidly lost to or taken up from the incubation media, a process which is affected by membrane damage.

In the main the rock pool salinity fluctuated between 40 and 10 ppt. These salinities would not be expected to result in significant membrane damage (cf. Figure 15), nor was equilibration following exposure to these salinities shown significantly to influence the calculated Health Index. However in Figure 52 the salinity of the rock pool on the 17th August 1993 and the 14th April 1994 was below 10 ppt. On the first date the Health Index was calculated, and allowing no equilibration, a relatively high value of 50% was obtained; On the 14th April 1994, following equilibration, a relatively low value of 33.5% was achieved. The latter value is closer to that expected than the former, demonstrating that equilibration is necessary to achieve an accurate result. On the whole this is not what was observed for the rest of the data because the rock pool salinities fell within the 'band' of salinities where ionic concentration has less effect on the resultant Health Index. Even so it is important that when measuring environmental samples equilibration must take place prior to ion leakage testing.

The task of the Health Index technique in this context is to determine what is normal and

abnormal for the algae in terms of health. Research is required to obtain information on the physiology and biochemistry of healthy organisms not exposed to any form of toxic pollution. Realistically this is difficult to achieve because each ecosystem contains negative influences, even if these are natural in origin. It must be attempted however if the biological significance of a technique is to be ascertained. Here the Health Index does show variation with time; though the influences on it are manifold and this study has not found any one parameter which appears to affect it more significantly than any other. Temperature, PAR, salinity and levels of desiccation will affect the overall health of the algae as all have profound effects on growth rate, the ability to photosynthesise and consequent reproductive success. Therefore to say that a population is healthy does not presuppose that this status is attained without significant cost to the organism. Growth measurements should be able to determine this but are inadequate because they are necessarily carried out under artificial conditions to which the algae must adapt physiologically and biochemically. Although statistically significant differences between control and treated organisms may be established this is only an indication that a change of some nature has occurred; it does not establish how deleterious this is to the organism. Statistical significance is, therefore, not the same as biological significance (Abel, 1991). The problem for toxicology lies in distinguishing the point at which statistical significance becomes biological significance, which is what the concept of the Health Index attempts to do. Interestingly the range of Health Indices achieved for these environmental samples are generally lower than those recorded in the toxicity studies carried out (see Chapter 3). It must therefore be concluded that a normal base line for algal health has not been achieved with respect to the Health Index.

In conclusion, this study has shown that the natural variation of the Health Index for algae at different times of year is low and is not significantly affected by salinity or pH. The

time of year when health was shown to be lower was during the winter and early spring. This general insensitivity to environmental factors, but responsiveness to physiological change suggests a potential for the Health Index to be used as a biomonitoring tool. This is further investigated below.

5.3.2 Assessment of clean and organically polluted sites around Plymouth

5.3.2.1 Health Index

The results of the Health Index for algae taken from the four sites investigated are presented in Figure 59. For each site the mean Health Index has been calculated, together with the standard error. The results show that in all cases the algae were healthy at the point of collection as all had average Health Indices between 68% and 55%. Thurlestone was the healthiest (68%) while Sutton Harbour was the least healthy (55%). This result suggests that algae from the harbour area are the most challenged, with a high environmental deficit to overcome. This result is statistically significantly different from the Health Indices achieved for algae taken from the relatively unpolluted sites. However the difference is small and the Health Indices are actually higher than any recorded for algae during the environmental assessment study detailed earlier in this chapter.

5.3.2.2 Neutral Red

The level of neutral red retention by the algae varied greatly from one site to the next. The results are listed below:

Wembury - 333 μg/g (dry wt)

Thurlestone - 930 μg/g (dry wt)

Mothecombe - 1550 μg/g (dry wt)

Sutton Harbour - 3877 μg/g (dry wt)

Please note that algae from Wembury and Thurlestone were tested using the same neutral

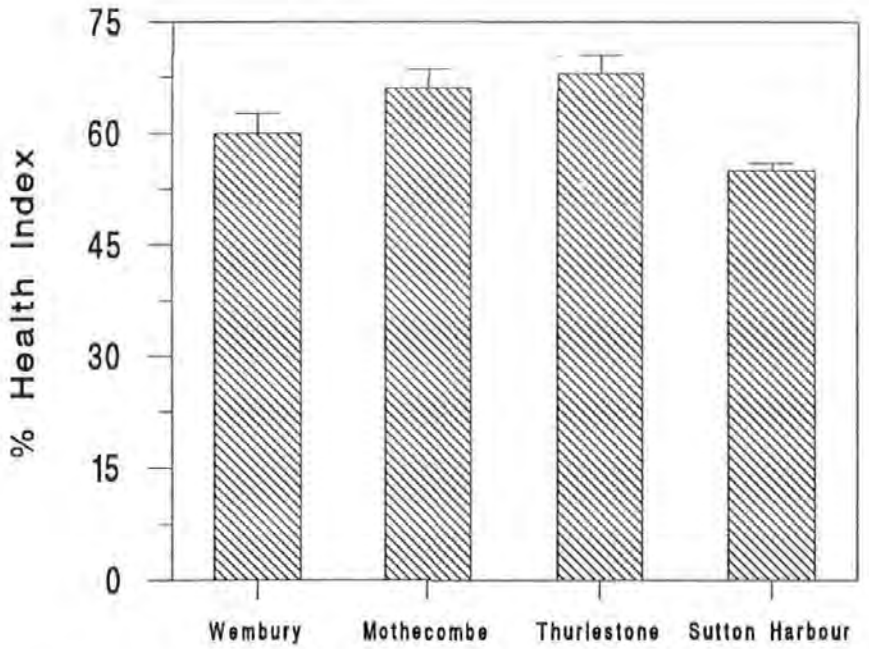


Figure 59: % Health Index of algae taken from four sites around Plymouth. Showing SE.

red solution, as were algae from Mothecombe and Sutton Harbour.

Wembury algae have the lowest neutral red retention in comparison to Thurlestone and both were low in comparison to Mothecombe and Sutton Harbour. The greatest retention was seen in the algae collected from Sutton Harbour.

Neutral red is a vital stain which has been used to measure the viability of animal cells (Babich and Borenfreund, 1987) and was used by Alexander and Wilkinson (1987) semi-quantitatively by microscopic observation of *E. intestinalis*. The degree of retention of the stain by the cells of the thallus might be expected to be proportional to the number of healthy cells if retention were by healthy cells only. However where cell damage has occurred accompanied by breakdown of cellular components within the frond abnormal retention was observed (Chapter 2 and Schild et al., 1993). This therefore demonstrates the major limitation of the technique, namely that it is impossible to draw conclusions from environmental retention data because of the high variation between experiments and the consequent lack of any base line. Where the technique has been used to detect changes due to particular treatments it is demonstrably reliable. The calculation of EC_{50}'s and subsequent comparison yields useful information. Under these circumstances when abnormal retention occurs during the production of a concentration response curve analysis of the data reveals the error and the points can be disregarded.

As indicated by neutral red retention the results present Thurlestone algae as being more healthy than Wembury. The second set of results can not be directly related to the first but the amount of stain retained indicates good health. This suggests that the algae from Sutton Harbour are in good health and in comparison Mothecombe algae are very poor, which conflicts with the data achieved using the Health Index. It must be concluded that the

technique is not suitable for use in environmental monitoring programmes and should only be used in laboratory based toxicity testing where clear base lines and controls can be built into the experimentation.

5.3.2.3 Plant (photosynthetic) Efficiency Analysis

The average efficiency (PEA) results for the four sites investigated all lie between 0.815 and 0.823 (see Table 8). There is no significant difference between any of the four algal populations. Thurlestone has the lowest standard error between samples, Mothecombe the highest. Sutton Harbour has the lowest average efficiency but this is not significantly lower than that obtained for Mothecombe.

Table 8: PEA readings (Fv/Fm) obtained for *E. intestinalis* from four sites near Plymouth.

Replicate	Wembury	Thurlestone	Mothecombe	Sutton Harbour
1	0.811	0.829	0.803	0.826
2	0.828	0.828	0.801	0.833
3	0.814	0.827	0.840	0.795
4	0.809	0.815	0.835	0.809
5	0.828	0.809	0.826	0.813
6	0.844	0.808		
7	0.813	0.818		
8	0.833	0.824		
Average	0.823	0.820	0.821	0.815
SE	0.013	0.008	0.018	0.015

The Fv/Fm ratio for all samples lies within the typical range for higher plants of 0.75 - 0.85 (Bolhàr-Nordenkampf and Öquist, 1993). This ratio has been shown to be proportional to the quantum yield of photochemistry of Photosystem II (PSII). The PEA meter uses chlorophyll fluorescence as an *in vivo* probe into photosynthetic function. When

a photon of red light is absorbed by a chlorophyll molecule enough energy is absorbed to move this molecule into the first excited state. This excited state lasts only a matter of nanoseconds during which time charge separation within the reaction centre takes place, the primary photochemical step. If this does not occur then the energy absorbed is given out either as heat or as fluorescence when the excited electron within the chlorophyll molecule returns to ground level. Essentially all fluorescence at room temperature originates from chlorophyll molecules associated with PSII. Fo represents the emission by the excited Chll *a* molecule in PSII when the first stable electron acceptor QA is fully oxidised, or open, awaiting primary photochemistry. For time-resolving fluorimeters such as the PEA meter Fo is calculated via mathematical extrapolation, whereas modulated fluorimeters obtain Fo by using a very low excitation light. At this point in time, Fo, the potential for use of the excitation energy is maximal. Fm represents the maximum fluorescence when all the reaction centres are shut, QA being fully reduced. Fv, the variable fluorescence, is the difference between the calculated value of Fo, and Fm. Fv/Fm therefore is proportional to the quantum yield of photochemistry, (Bolhàr-Nordenkampf and Öquist, 1993). These characteristic fluorescence induction kinetics are also termed the 'Kautsky' curve. For more detailed information on this see Bolhàr-Nordenkampf and Öquist (1993).

In higher plants PSII is sensitive to a number of stresses since all stresses affect the function of PSII directly or indirectly (Bolhàr-Nordenkampf and Öquist, 1993). Fluorescence can therefore be used as a tool for identifying stress responses under laboratory and field conditions. It can also be used in the screening of plant individuals for those best adapted to certain conditions. In this study the PEA readings show us that *E. intestinalis* has a similar fluorescence response to that of higher plants. Of all the marine algae the Chlorophyta contain the most photosynthetic pigments in common with higher

plants. However the relative quantities are different, with more Chll *b* and accessory pigments per unit of Chll *a* (Dring, 1986). When the same system was applied to the red algae *Gracilaria spp.* it was very difficult to get meaningful results, probably due to the very different pigment content (James Newman, unpublished work). The PEA meter can therefore be used with no adaptations for this green alga, making it an impressive, quick, efficient and simple method of analysis. The algae taken from the different sites are healthy, and even if stressed there is no adverse effect identifiable through fluorescence induction kinetics. The Fv/Fm ratio indicates that there is efficient energy capture by the open PSII centres, thus even if these populations are exposed to pollutants they are probably not exposed to high levels of PSII inhibitors.

5.3.2.4 Growth

The growth data are represented in Figure 60. In all cases length increased over time at variable rates. Wembury showed the most constant rate of growth of all the samples, with an increase of approximately 3% per day, equivalent to 0.9 mm/day. With a 20% increase by day 6 these algae were also the fastest growing, though not significantly faster than that from Mothecombe. The algae from Thurlestone grew fastest in the first two days of the trial and then showed a slightly slower growth rate till day 6. The rate of growth was less than that of Wembury algae though not significantly less than that from Mothecombe and Sutton Harbour. Mothecombe showed a fairly constant rate of growth throughout at a rate not significantly lower than Wembury, although on day 4 growth was faster. Algae taken from Sutton Harbour was slower growing than that from Mothecombe, greater than Thurlestone and approximately equal to Wembury.

Under the conditions set up in the Fisons growth cabinet growth should be optimal for this species of algae (personal communication with S. Lewis, University of Plymouth). Light

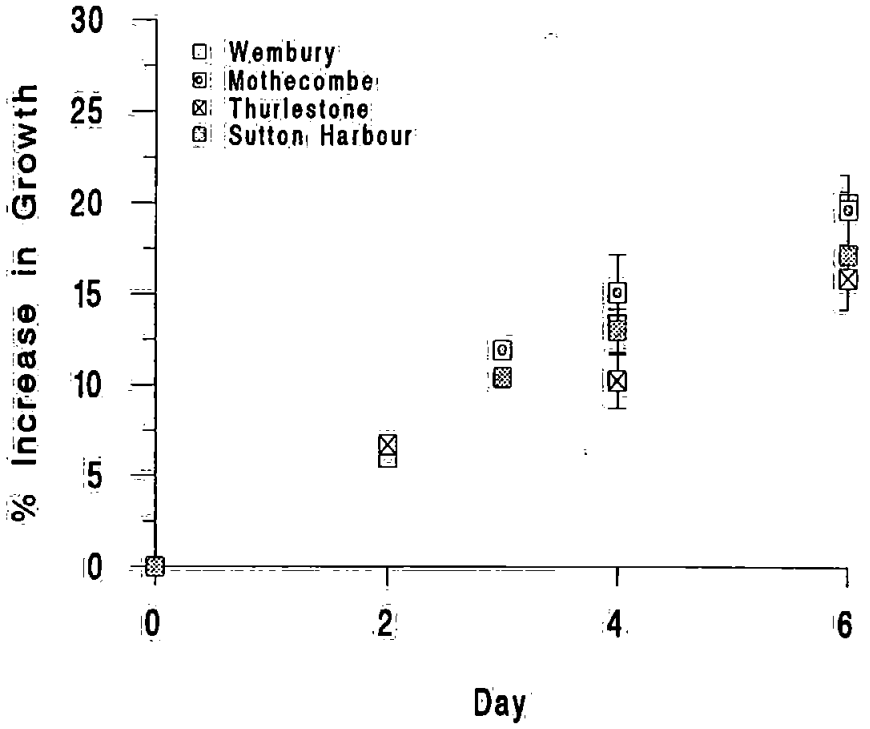


Figure 60: % increase in growth over a six day period of E. intestinalis taken from four different collection sites. Including standard error.

intensity and duration were adjusted to avoid sporulation of the excised pieces and, therefore, to increase the amount of energy invested in growth. Sporulation is invariably followed by death so must be avoided, and although not all the individual pieces did survive, sporulation was not the cause of death. Those samples which survived showed very similar increases in growth over 6 days, any contaminants present at the Sutton Harbour site having no long lasting influence on subsequent growth when compared with algae collected from the control sites.

5.3.2.5 Extraction of organic compounds

Steam distillation of the environmental samples into iso-hexane extracted measurable quantities of polycyclic aromatic hydrocarbons (PAH's), (see Tables 9, to 11). Algae from Sutton Harbour contained the most PAH's in comparison to the other four sites tested. All samples contained a similar mixture of chemical compounds showing similar excitation and emission spectra although the relative concentrations varied between samples. Variation between the two samples from each site was high in most cases and two distillations were required, the second distillation often resulting in extraction of similar quantities to the first. An emission scan of each distillate was carried out with excitation wavelengths of 253 nm, 280 nm and 310 nm. Because each organic compound has its own characteristic fluorescence different excitation wavelengths are used to allow identification. Excitation at 253 nm is specific for anthracene, a 3-ring aromatic compound (Schwarz and Wasik, 1976: Jurgensen et al., 1981) which is a common by-product of combustion. It is also known to be phototoxic to organisms (see Chapter 4). Excitation at 280 nm is used for the detection of hydrocarbons with two aromatic rings (e.g. naphthalenes, fluorenes, dibenzothiophenes) (Jurgensen et al., 1981) and excitation at 310 nm is used to identify compounds with three or more aromatic rings (e.g. Pyrene and related compounds) (Law et al., 1987). Tables 9, 10 and 11 display these results; all traces can be found in

Appendix 2.

Table 9: Fluorescence intensity data for polycyclic aromatic hydrocarbons extracted from algae by steam distillation into iso-hexane using an excitation wavelength of 253 nm and emission wavelength of 376 nm (optimal for anthracene).

Sample Origin		1st Distillation	2nd Distillation	Total	'Oil Equivalent'* μg/g wet wt
Sutton Harbour	1	4.84	7.17	12.01	34.41
	2	4.61	3.71	8.32	24.82
Thurlestone	1	0.24	0.11	0.35	1.02
	2	5.63	0.60	6.23	18.08
Wembury	1	0.48	0.75	1.23	3.57
	2	0.17	0.15	0.32	0.93
Mothecombe	1	2.52	1.43	1.95	5.66
	2	0.91	0.65	1.56	4.53
Restronguet	1	0.75	0.56	1.31	3.80
	2	0.17	0.20	0.37	1.07

*Oil equivalent concentration calculated from data for yellow fuel oil given below.

The fluorescence intensity for yellow fuel oil (1.48 μg/ml iso-hexane) was 0.51

Table 10: Fluorescence intensity data for polycyclic aromatic hydrocarbons extracted from algae by steam distillation into iso-hexane using an excitation wavelength of 280 nm and emission wavelength of 330 nm (optimal for naphthalenes).

Sample Origin		1st Distillation	2nd Distillation	Total	'Equivalent'* μg/g wet wt
Sutton Harbour	1	16.34	15.07	31.41	9.83
	2	16.07	11.75	27.82	8.71
Thurlestone	1	0.48	0.15	0.63	0.20
	2	0.46	0.33	0.79	0.25
Wembury	1	0.72	0.23	0.95	0.30
	2	0.65	0.14	0.79	0.25
Mothecombe	1	1.94	0.49	2.43	0.76
	2	0.83	0.40	1.23	0.39
Restronguet	1	0.42	0.37	0.79	0.25
	2	0.06	0.41	0.47	0.15

* Oil equivalent concentration calculated from data for 2,3-Dimethylnaphthalene given below.

The fluorescence intensity for yellow fuel oil (1.48 μg/ml) and 2,3-Dimethylnaphthalene (3.56 μg/ml iso-hexane) were 2.11 and 11.37 respectively.

Table 11: Fluorescence intensity data for polycyclic aromatic hydrocarbons extracted from algae by steam distillation into iso-hexane using an excitation wavelength of 310 nm and emission wavelength of 360 nm.

Sample Origin		1st Distillation	2nd Distillation	Total	'Equivalent'* μg/g wet wt
Sutton Harbour	1	10.90	7.78	18.68	334.88
	2	5.85	3.76	9.61	172.28
Thurlestone	1	0.12	0.18	0.3	5.38
	2	0.33	0.36	0.69	12.37
Wembury	1	0.14	0.19	0.33	5.92
	2	0.13	0.13	0.26	4.66
Mothecombe	1	0.91	0.66	1.57	28.15
	2	0.36	0.33	0.69	12.37
Restronguet	1	0.19	0.56	0.75	13.45
	2	0.09	0.26	0.35	6.27

* Oil equivalent concentration calculated from data for 1-Methylphenanthrene given below.

The fluorescence intensity for yellow fuel oil (1.48 μg/ml) and 1-Methylphenanthrene (146 μg/ml iso-hexane) were 0.21 and 8.1438 respectively.

Yellow fuel oil (diesel) was used as a multi-compound standard. Diesel fuel contains a range of aromatics of two and three rings. Naphthalene is an important component of the aqueous dispersion of fuel oil and crude oil (Stebbing et al., 1990) and pyrene is a major component of PAH's found locally in the River Tamar (Readman et al., 1982). Two single compound standards were utilised, 2,3-Dimethylnaphthalene a 2-ring aromatic and 1-Methylphenanthrene a 3-ring aromatic. The fluorescence intensities for these standards were used to calculate estimated concentrations for the algae samples.

The trace for yellow fuel oil excited at a wavelength of 253 nm showed no evidence of anthracene which has a very characteristic emission (see Trace 1, Appendix 2). Some of the PAH components of fuel oil fluoresce at this wavelength and the emission spectra show a very broad peak around 320 nm which tails off at 280 nm. Traces 2 to 5 in Appendix 2 show the emission peaks for the distillates from the environmental samples using an excitation wavelength of 253 nm and these are of a greater intensity and different shape from those of the fuel oil, suggesting the presence of compounds of another origin. The emission trace is not that of anthracene but could possibly be related compounds with similar excitation wavelength. Observation of Trace 4, the second sample, first distillation of the environmental compounds, reveals a compound very like anthracene extracted from the algae from Thurlestone. This is probably an artefact as it does not occur in any other sample and may have its origin in a carbon/anthracene deposit within the sample. Comparison of the estimated concentration of 'yellow fuel oil' type compounds within the algae show those taken from Sutton Harbour to be most contaminated. The concentrations are also on average as high or higher than the calculated concentration of anthracene taken up by the algae, per g wet weight, during exposure experiments (see Chapter 4).

Qualitative and semi-quantitative analysis of the two ring aromatic compounds was achieved using an excitation wavelength of 280 nm. Yellow fuel oil and 2,3-Dimethylnaphthalene were used as standards (Traces 6 and 7, in Appendix 2). The fluorescence trace for 2,3-Dimethylnaphthalene showed high emission between 320 and 370 nm, with three peaks within this region. The yellow fuel oil yielded a similar trace with high emission between 300 and 370 nm. This is characteristic of 2-ring PAH's (Debber, 1983: Hála et al., 1981) which make up a large proportion of the smaller PAH's in fuel oil. Traces 8 to 11 display the results achieved for the environmental samples. They show very similar trends to the standards with high intensity emission between 320 and

380 nm. The distillate from the algae collected at Sutton Harbour contained the most PAH's and it is likely that these have their origin in marine fuels and oil with which the harbour has been observed to be contaminated. 2-ring aromatic compounds were also extracted from algae collected elsewhere though at lower concentrations. Mothecombe algae contained more 2-ring aromatic compounds than Wembury, Thurlestone and Restronguet though more than eight times less than algae from Sutton Harbour.

Excitation at 310 nm for yellow fuel oil and 1-Methylphenanthrene showed very different emission traces. 1-Methylphenanthrene showed multiple peaks between 340 and 400 nm (Traces 12 and 13). The environmental samples all had peaks at approximately 310 nm (the excitation wavelength) and some emission around 340 - 380 nm (Traces 14 to 17). The traces obtained for the Sutton Harbour distillate had peaks between 320 and 380 nm. Peaks within this region are characteristic of 2, 3 and more ring PAH's and consequently this excitation wavelength is used in the routine sampling for dissolved and dispersed petroleum hydrocarbons in seawater (Intergovernmental Oceanographic Commission). The PAH's present in the algae do not correspond to those in fuel oil. However the fuel oil sample had not been weathered, which affects the composition, most volatile and biodegradable components being lost rapidly.

The results of the extraction of organic compounds show that all the algae samples had been exposed to various levels of organic pollution. The algae containing the most was that taken from Sutton Harbour. This is a possible explanation for why the Health Index (Figure 59) was slightly lower for the Sutton Harbour algae in comparison with the control sites, a response which has been shown to be due to reduced membrane integrity such as can be caused by exposure to non-specific narcotic compounds e.g. many organic compounds (Chapter 3; Schild et al., 1995). There were however no significant differences

in growth rate, and the Plant Efficiency Analysis (PEA) showed up no significant differences between any of the sites which suggests that the algae are not exposed to PSII inhibitors at toxic concentrations.

5.3.3 Assessment of heavy metal polluted sites in the Fal Estuary

Analysis of the algae sampled from five Creeks within the Fal Estuary revealed different exposures to metals, reflected in their bioaccumulation data, but only subtle differences in their physiological responses, which are detailed in Table 12 with growth and Health Index also being graphically presented as Figures 61 and 62.

Table 12: Physiological data for algae sampled from five Creeks within the Fal Estuary, Cornwall. Including standard errors (where available).

Creek	Growth % increase	Health Index %	Chlorophyll *a* g/l 80% acetone	Photosynthesis $\mu molO_2/min/g$
Restronguet	28	63 ±4.9	0.122 ±0.009	4.49 ±1.77
Mylor	28	62 ±5.5	-	-
Pill	25	65 ±4.4	-	-
Percuil	29	67 ±4.9	-	-
St. Just	20	70 ±0.6	0.107 ±0.018	4.23 ±1.14

The corresponding amounts of metals taken up by the algae from the water and sediments to which they were exposed during growth are set out in Table 13.

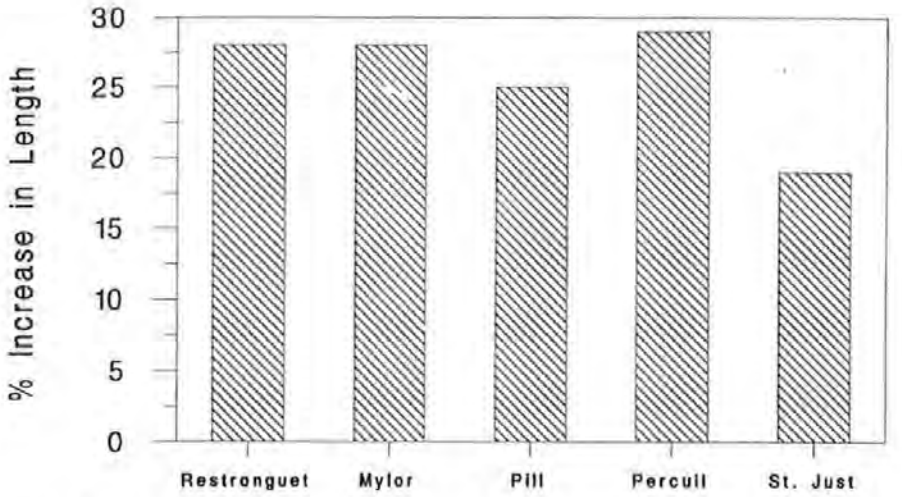


Figure 61: % Increase in length over 6 days, for algae sampled from five different creeks within the Fal Estuary.

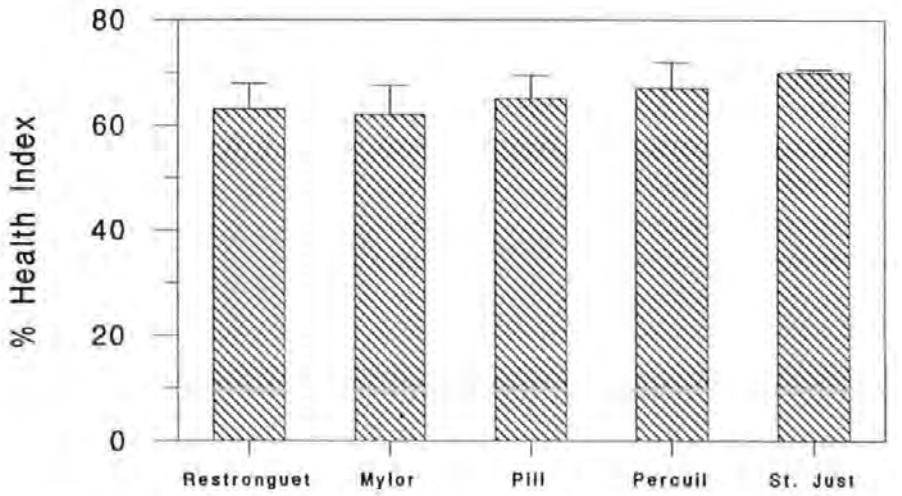


Figure 62: % Health Index for algae sampled from five creeks within the Fal Estuary. Showing SE.

Table 13: Bioaccumulated metals extracted from algae from the Fal Estuary and a number of clean sites near Plymouth (preparation and analysis carried out by John Wedderburn).

Site	Mn conc ppm	Zn conc ppm	Cu conc ppm
Restronguet	377.6	353.3	170.8
Mylor	136.2	187.5	100.2
Pill	102.6	92.8	18.3
St. Just	135.7	56.4	16.6
Percuil	156.0	43.8	18.2
Mothecombe	22.0	6.9	3.7
Thurlestone	40.4	12.1	5.9
Wembury	26.1	11.6	4.6

The data presented in Tables 12 and 13 shows the effect of metals reflected in the physiology of the algae and bioaccumulation data. The % increase in growth varies between sites but did not follow the pattern of bioaccumulation in Table 13, nor the relative levels of exposure due to metals in solution and sediment (Somerfield et al., 1994). In the case of the first four creeks the growth rate was greater than recorded in the study detailed earlier in this Chapter, section *5.3.2.4*, where an average increase of 18.25% over 6 days was recorded. The Health Indices range from 63% $\pm$4.9 for Restronguet Creek to 70% $\pm$0.6 for St. Just, all values obtained being those expected for healthy algae. The chlorophyll *a* content of algae from Restronguet was greater than that extracted from algae collected at St. Just, as was the photosynthetic rate, but the differences are not significant. The metal bioaccumulation data for each site shows that the algae growing in Restronguet Creek accumulate more Cu, Zn and Mn than in the other sites. Algae taken from the Fal

Estuary contained elevated levels of metal compared with algae sampled at Mothecombe, Wembury and Thurlestone. There is however no evidence that these levels of bioaccumulated metals are toxic to *E. intestinalis* growing in the Fal as no physiological effect was seen. Evidence for genetic tolerance to copper has been found in *E. compressa* by Reed and Moffat (1983), who observed uptake of high levels of copper by algae taken from a contaminated and clean site which resulted in toxicity only in the previously unexposed algae.

In addition to the study discussed above, a set of experiments were carried out to establish whether there were differences in the level of tolerance of algae from Restronguet and Wembury to cuprous (Cu^{++}) ions. This was achieved by using two physiological measurements, photosynthetic yield and efficiency (using a Modulated Fluorimeter), and the ion leakage based Health Index. The copper concentrations used (0-500 μg/l) were higher than those usually found in solution in Restronguet Creek, but were chosen to investigate the influence of the extreme situation. In 1973 the concentration of soluble copper in the Carnon River, which flows into Restronguet Creek, was however as high as 550 μg/l, with the concentration in the Estuary of 11 μg/l (Bryan and Hummerstone, 1973).

The photosynthetic yield data is presented in Figure 63 and allows comparison between the algae from the two sources. Algae from Wembury have a higher yield than those from Restronguet at all the exposure concentrations and copper exposure reduced the yield significantly at concentrations greater than 200 μg/l. There was no significant difference between the exposed and control yields in the algae from Restronguet. Photosynthetic efficiency (Fv/Fm) for the Wembury algae showed a similar trend to that of yield (see Figure 64). The control sample had a very healthy Fv/Fm ratio of 0.73, but this declined

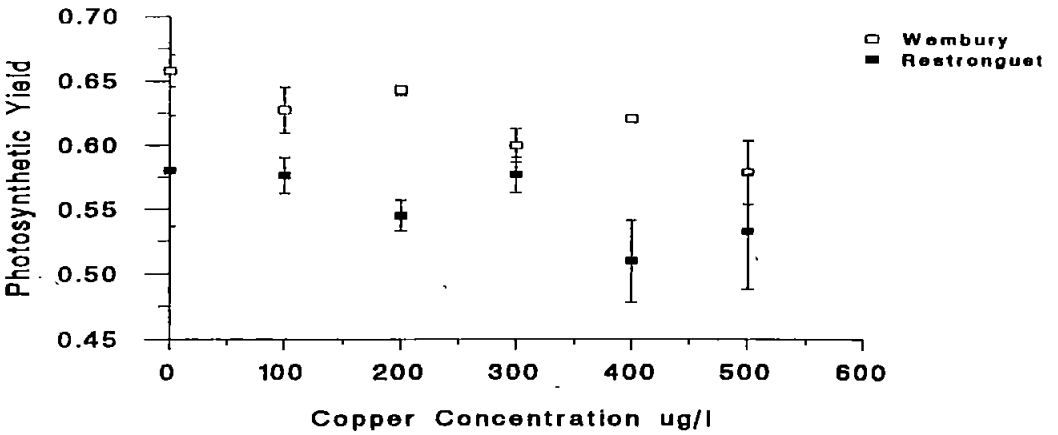


Figure 63: Photosynthetic yield of algae from Wembury and Restronguet Creek (Fal Estuary), following copper exposure. Showing SE.

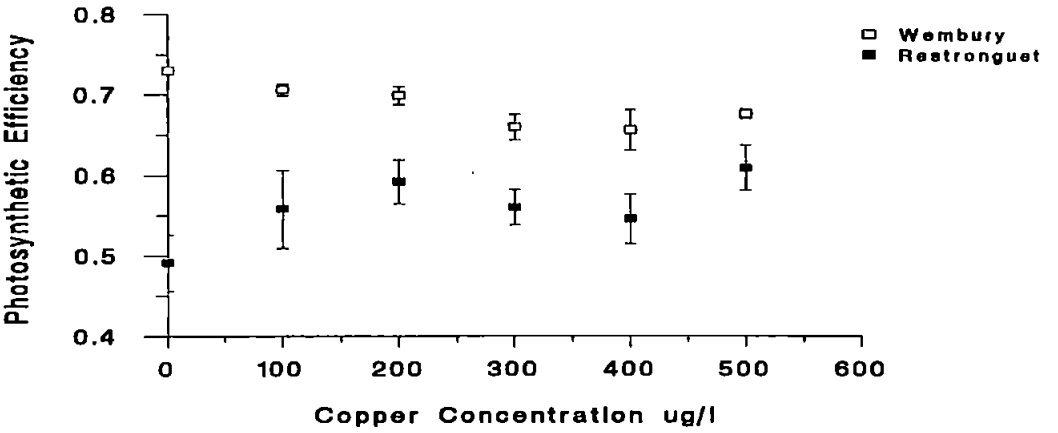


Figure 64: Photosynthetic efficiency of algae from Wembury and Restronguet Creek (Fal Estuary), following copper exposure. Showing SE.

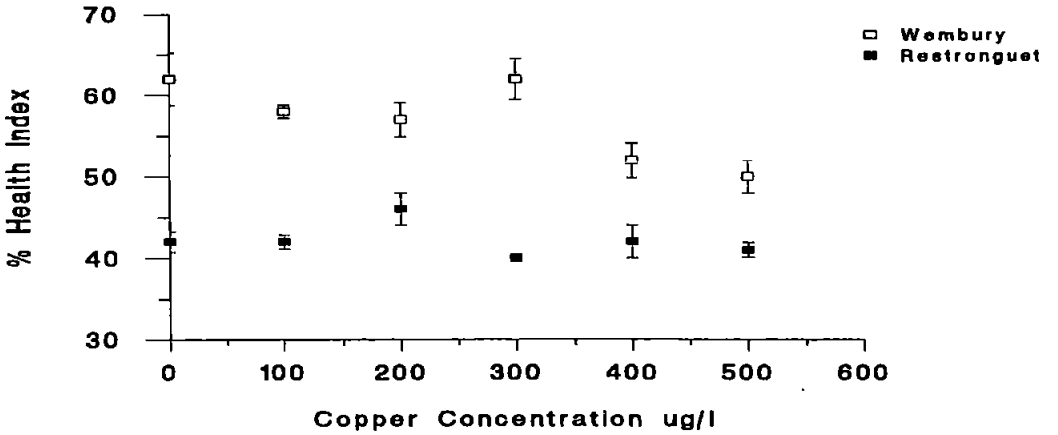


Figure 65: % Health Index of algae from Wembury and Restronguet Creek (Fal Estuary), following copper exposure. Showing SE.

slightly with exposure to copper. The samples taken from Restronguet show a different response to this, having a very low efficiency when incubated in media containing less than 200 μg/l cuprous ions, but this efficiency increased with higher levels of copper exposure. At 200 μg/l photosynthetic efficiency was significantly greater than that for the algae incubated in Instant Ocean with no copper added, showing that the algae from Restronguet exhibited a positive adaptation to high copper exposure, suggesting a higher than normal requirement for this essential micronutrient. The Health Index measured for each sample showed similar responses (Figure 65) to those seen using photosynthetic efficiency. The algae from Restronguet had a low Health Index in comparison to the normal range, which was significantly lower than that achieved for algae taken from Wembury. The copper concentration did not significantly lower the Health Index of the Restronguet algae, although the Health Index was greatest at 200 μg/l. Copper concentration did effect the Health Index of algae from Wembury, causing a decrease with increasing concentration. The algae from Wembury are not exposed to high levels of copper (see bioaccumulation data in Table 13) and therefore have developed no tolerance. The lowest Health Index, achieved with the highest copper concentration, was still however, characteristic of healthy algae.

It is difficult to draw any firm conclusions as to the application of the Health Index to heavy metal pollution effects and clearly further experimentation is needed. The copper exposure experiment does indicate however that the use of a hand held fluorimeter for both laboratory and field operation could be usefully employed in the detection of decline in health due to bioaccumulated metals.

It is still very important to take a unified approach when studying metal exposure, bioaccumulation and effects. Metal bioaccumulation is a very complex study and one which

is affected by many chemical factors, including metal interactions (Munda and Hudnik, 1986) and salinity (Munda, 1984: Bryan et al., 1985). Tidal effects too are very significant in estuaries where pycnoclines are liable to form. Physical factors affect metabolic rate and where uptake is active variations in temperature, turbidity and light will cause variations in bioaccumulation. Changes in seasonal growth rate can result in the concentration or dilution of metals within the thallus and competition for binding sites within an organism can affect the relative quantities of metals which accumulate. Unless all these factors are constant the likelihood of any organism's behaviour accurately reflecting the metal concentrations in the surrounding waters at any given time is remote. However *E. intestinalis* and *Fucus vesiculosus* have been shown to accumulate certain metals, including those tested for here, at a fairly constant rate and in proportion to the water concentrations (Say et al., 1986). They also provide an integrated picture of metal contamination over a long period - one of the criteria required of biomonitors.

The advantages of using biomonitors in the monitoring of metal pollution go largely undisputed as they provide bioconcentrated levels of metals, reducing the complexity of analysis required for water samples which usually contain levels too low for direct detection. Seaweeds have the added advantages that they are sedentary, able to accumulate without being killed by the levels to which they are likely to be exposed, they are generally abundant and unlike animals accumulate largely from solution rather than sediment. *E. intestinalis* is also able to tolerate wide ranges in salinity and other environmental conditions. The limitation of this approach is that it can identify only non-lethal levels of exposure.

Tolerance may be due to an adaptive mechanism, detoxification, an exclusion mechanism (where bioaccumulation data is misleading per se) or lack of toxicity of the accumulated

metal. Whichever the case whether or not the exposure causes stress in the organism it cannot be detected. If the interest in the level of exposure lies in the effect it has on the physiology of the organism it is important to look at that organism and the community in which it lives and note how its physiology might differ given different levels of exposure. From an ecological or ecotoxicological point of view this approach has more relevance than isolated bioaccumulation results. If the interest is purely chemical then the level of accumulated metal gives an indication of the abundance of available metal. A truly unified strategy would incorporate both chemical and physiological analysis, and try to correlate the two data sets. To measure only bioaccumulated metals is invariably inadequate, and only when accompanied by physiological data such as growth (Reed and Moffat, 1983), or by measuring photosynthetic yield or efficiency as here, can it have meaning with respect to their effects.

E. intestinalis is an adequate bioaccumulator of heavy metals, and this study shows that the level of metals to which it is exposed does introduce measurable stress with regard to chlorophyll fluorescence, but not one which can be detected by using the Health Index alone.

CHAPTER 6

FINAL DISCUSSION AND CONCLUSIONS

6.1 Summary of Techniques

6.1.1 Neutral Red

The two techniques initially developed for use with *E. intestinalis* produced useful and informative quantitative structure-activity relationships (QSARs). However the methods varied greatly in ease of execution and the results in reproducibility. The neutral red technique was easily adapted for use with this alga and was successfully utilised as a laboratory based toxicity test for the effects of non-specific narcotics on *E. intestinalis*. Within each test reproducibility was statistically good, each concentration point having small standard errors, and the resultant QSAR showing the technique to be reasonably sensitive. However between experiments the uptake of this vital stain varied greatly, each test bearing no relationship to the rest in terms of the amount of stain taken up and retained within the frond. This highly variable level of uptake was due largely to failure to produce stain solutions of the same concentration for each toxicity test. This had no effect on the outcome of any particular toxicity test and the subsequent calculation of the EC_{50} for that test. This variation prohibits the use of the test in biomonitoring however, because it is axiomatic that all tests must be reproducible regardless of where and when they are carried out, and they must also be repeatable (Calow, 1993). Because uptake is so variable this can not be achieved, uptake having no determinable relationship to membrane disruption. In addition to this criticism the stain has a tendency to act as a mortal stain on exposure to algae which have suffered extensive membrane damage. This confuses the subsequent interpretation of results and again renders the technique inappropriate for use in environmentally based toxicity testing. This was demonstrated in Chapter 5 where it was employed without success to determine the state of health of algae

collected from beaches around Plymouth.

6.1.2 Health Index

In contrast to the neutral red technique the ion leakage Health Index did prove to be both reproducible and reliable in laboratory based toxicity tests, and in application to algae collected from the field. The Health Index also has the advantage of being a very simple and efficient technique. The development of this test for *E. intestinalis* has resulted in a lower 'resolution' between healthy and unhealthy states than the same test does when applied to *Laminaria spp.* This more restricted range imposes no practical disadvantage because the Health Indices achieved are easily interpreted as 'normal' or 'abnormal', the cut-off between these two states varying a little in time and between populations of *E. intestinalis*, but lying on average close to 40%. It is possible mathematically to extrapolate the range of values gained using the Health Index. Such transformation has been used by a number of researchers in the utilisation of the ion leakage technique and was first introduced by Flint et al. (1967). Although this type of manipulation improves the resolution, it is artificial and serves only to increase the range without improving discrimination. What I believe is actually required is a measure of normality and abnormality for the algae at any given time, an end point to which such mathematics does not offer any real advantage.

Essentially this technique's major advantages are that it can be used in both laboratory based toxicity tests and as a tool to gaining information about pollution effects on populations within the environment. This is a property which many toxicity tests do not possess but one that is potentially very significant. The ability to measure toxicity *in situ* is especially relevant as the Environment Agency moves towards direct toxicity measurements and introduces toxicity based consents for effluents. The ability to utilise

the same organism both in the laboratory under ideal conditions and when taken from the environment allows comparisons to be drawn between environmental affects following pollution exposure and those observed under controlled conditions. Production of EC_{50}'s and formation of QSARs also calibrates the technique, creating a relatively simple way of linking the two sets of data from field and laboratory-based experiments. This has the potential to eliminate the need to measure ambient water concentrations. The technique could also be made more convenient by using algae cultured within the laboratory, which is easily achieved for *E. intestinalis*.

This is essentially a very simple and inexpensive technique and is one which can be used routinely. Toxicological investigations involving SAR's and QSARs are therefore easily and swiftly carried out, which is a property required of such tests by industrialists and government. It also adds a diagnostic aspect missing from toxicity tests which are simply quantal, (e.g. measuring mortality), knowledge of a toxicant's action aiding the prediction of that toxicant's fate within the environment. The technique is also unaffected by fluctuations in environmental salinities and is therefore appropriate for use in the biomonitoring of estuarine ecosystems.

This technique is subject to limitations which relate to the range of toxicants whose actions are detectable through this method. The Health Index is primarily dependent upon the state of membrane integrity, a fundamental mode of toxic action of a large number of organic pollutants. In the absence of membrane damage the Health Index will normally range from $\sim 55\%$ to $\sim 70\%$, which is reduced following damage. Toxicants with specific actions not related to cell membranes do not cause such a decline in the Health Index until general cell death occurs. The result is therefore insensitivity to such compounds with respect to this technique. Other types of damage must therefore be determined using other techniques.

It is not possible to use the index categorically to diagnose whether algae are from an ecologically fit population. Maintenance of individual health can result in low, but normal, Health Indices which could be at the expense of successful reproduction. It is therefore vitally important that morphological observations and bioaccumulation studies be carried out during monitoring programs (c.f. Chapter 5, discussion).

6.1.3 Other techniques

6.1.3.1 Growth

Few studies exist to date which apply *effects-based* biomonitoring to algae, for example photosynthesis and growth (see Introduction and Chapter 5). Growth was therefore included in the techniques investigated during this study. Growth has been used in toxicity studies using algae (Fletcher, 1991), but has not been employed in the biomonitoring of algae taken from the field. Two studies included the measurement of growth in *E. intestinalis*, one which was designed to investigate the effects of suspected organic pollution, and one which was aimed at assessing the effects of trace metals on populations taken from different creeks within the Fal Estuary, each experiencing various levels of metal contamination. In the first study the results indicated no significant difference between the populations in terms of growth, but interestingly the rate of survival of the excised pieces taken from the relatively polluted site was considerably lower than those taken from the clean sites. There was no significant difference between the growth rates of the algae taken from the Fal Estuary. This may indicate metal tolerance. There is also no evidence that growth rates measured in the laboratory reflect the intrinsic health of the algae in the environment, and in neither case was growth significantly different between samples.

The use of growth as a biomonitoring technique has neither been dismissed nor affirmed

in this programme of work and clearly more research must be carried out in this area. Limitations of the technique are that the generation of results is slow and the production of a suitable environment where growth will be optimal is both difficult to achieve and essentially artificial. This type of long term study is however necessary if we are to be able to assess the action of pollutants in environmentally realistic situations and concentrations. Growth is also fundamental to an organism's existence and has real ecological significance; its inclusion is therefore essential in any ecotoxicological study. However, the lack of sensitivity and reproducibility must be overcome before this technique can be productive. This might be achieved by developing a germination test such as that used by Fletcher (1989).

6.1.3.2 Photosynthetic techniques

Photosynthesis has been used in many studies as a measure of the physiological condition of plants. Measurements have included both photosynthetic oxygen evolution and carbon dioxide fixation. In this study little success was achieved using measurements of oxygen evolution and dark uptake with *E. intestinalis*. This can largely be attributed to the collection of gases within the hollow frond of the alga thus restricting the movement into the surrounding water allowing detection and greatly affecting the accuracy. An alternative to this technique is the use of a specialised fluorimeter, designed to detect rapid changes in chlorophyll fluorescence induction within photosynthesising plants. At present there are two types of instrument which can achieve this, using slightly different approaches. Both have been used during this study. Parameters can be selected according to the aspect of fluorescence which is being measured, and the employment of one which is most affected as a result of pollution exposure.

At present researchers conflict over which is the better system for measuring

photosynthetic fluorescence, but there is great potential in this technique. The advantage common to both systems is that measurement is fast, and *in vivo* readings can be achieved with minimal sample preparation both in the laboratory and field. Sensitive, non-destructive and non-invasive, this technique has been proven to detect damage caused by a wide variety of stresses. Both instruments used were borrowed from the manufacturers for a short period of time, restricting how much could be achieved. However the simplicity of use meant that results were easily obtained, the PEA meter being the simplest and most 'user friendly' of the two systems. Each system had advantages and disadvantages which will not be discussed here, but both yielded interesting results which compliment the ion leakage Health Index technique effectively. Measuring photosynthetic 'efficiency', and in doing so allowing some measurement of stress to be achieved, the fluorimeters were able to detect differences between treatments which were not highlighted by other techniques. Confirmation of similar trends was also achieved using the two systems e.g. the affects of different copper concentrations on *E. intestinalis*. Here fluorescence analysis not only detected a difference in response but showed that the tolerant algae were healthier in the presence of relatively high concentrations than in the total absence of copper. The Health Index measured at the same time yielded comparable results. Chlorophyll fluorescence reflects PSII activity which is also the site of action of PAH's (see Chapter 4). It would therefore be very interesting to look at the photo-induced effects of anthracene and other PAH's using one of these systems, damage not being associated with membrane disruption and consequently not detected by the Health Index technique.

6.1.3.3 Bioaccumulation techniques

Macroalgae have been used successfully in the biomonitoring of trace metal contamination (Phillips, 1994), the concentrations of elements present being able to provide information about the extent of exposure. There are few records of the use of macroalgae in the

biomonitoring of organic pollutants, the received wisdom being that the low lipid content of algae results in only low accumulation of these predominantly lipophilic compounds. However macroalgae are certainly exposed to such pollution and the steam distillation process carried out to extract small PAH's from *E. intestinalis* was successful and revealed that exposure to these compounds does result in bioaccumulation.

Macroalgae make good biomonitors of trace metal contamination and this is due primarily to their sedentary, ubiquitous and abundant nature, and their bioaccumulation and bioconcentration rates. This has been shown to be particularly true for *E. intestinalis* which was recommended for use in the biomonitoring of trace metals in a Department of the Environment report (Say et al., 1986). The extraction and detection method (Flame Atomic Absorption Spectrophotometry - FAAS) used in this project produced good recovery rates for the elements which were investigated, as shown by the use of the standard material. The analysis of algae collected from the Fal Estuary and the area surrounding Plymouth revealed different metal concentrations precisely as expected. It also confirms a distinct metal gradient within the Fal, which has been previously recorded in a study of micro- and macro-invertebrate communities (Somerfield et al., 1994). Other studies in this area have revealed that algae from the Fal exhibit tolerance to the metals present and it is therefore necessary to obtain a measure of metal exposure, arguing strongly for such bioaccumulation studies. Conversely it is also important to show lack of exposure and lack of tolerance in other populations in order to be able to predict their response should metal contamination occur.

Bioaccumulation studies on organic pollutants are just as important as those on metals. Although algae may not be the perfect bioaccumulators of such contaminants, analysis has shown that they *are* accumulators although the physiological effects are not at present

known. A reduction in the Health Index was observed for the algae collected from Sutton Harbour, as was a slightly lower photosynthetic efficiency and this suggests that even at the relatively low concentrations recovered some effect is seen. It is impossible at this stage to suggest whether this observed reduction in performance is due solely to the presence of organic contaminants or to some other factor, but it does seem likely. Further investigation should therefore be carried out to look into the effects of probable contaminants using a complex 'substance' such as diesel oil or creosote both of which contain a broad range of hydrocarbons. By using a combination of photosynthetic fluorescence techniques, ion leakage and bioaccumulation analysis confirmation of any relationship between the level of contamination and the measured effects should be achieved.

6.2 The Future of Biomonitoring

The future of biomonitoring is assured in so far as it is the only technique available to assess the impact of contaminants released into the environment as a result of human activity, utilising both *effects-based* biomonitoring and bioaccumulation studies. Similarly laboratory based toxicity studies will also remain a necessity to allow for the prediction of chemical toxicity, mode of action and ultimate fate within the environment. Any investigation which did not employ both these approaches would be incomplete. The development of biomonitoring techniques that can be used at all these levels therefore offer the greatest environmental protection, allowing comparison between results obtained in laboratory tests and those achieved using algae taken from or tested within environmental situations. Many *effects-based* biomonitoring techniques can be simultaneously applied, e.g. both the ion leakage Health Index and the photosynthetic fluorescence techniques. With complimentary bioaccumulation studies much can be determined about a chemical compounds effect, mode of action and partitioning into the organism.

At present this relatively new discipline of ecotoxicology is being driven by legislators, both within government and industry. This is mainly because historically it was these bodies which set the targets for chemical levels based upon toxicity tests alone. With the increasing awareness that ecosystems also need protection, research-based ecotoxicological programmes have also been developed, but initially this was seen as being unrelated to toxicity testing. This lack of understanding has therefore meant that ecotoxicological research has followed behind in the wake of the industrial toxicologist and legislators. Today there are attempts to break away from this fragmented approach, and with the introduction of bodies such as the US EPA and the new Environment Agency in the UK cooperation is increasing to produce a new discipline characterised by the diversity of its membership; one which includes both industrial and research chemists, toxicologists, ecologists, physiologists and statisticians in addition to government officials. This level of cooperation must in time make the ultimate aim of the ecotoxicologist, environmental protection, far easier to achieve. Whilst legislative decision makers are a necessity it must be acknowledged by all involved within this field that decisions must be based upon basic knowledge, the vital role fulfilled by the research covered in this project.

The use of macrophytes and macroalgae in ecotoxicology has been extremely sparse. Their main use has been in bioaccumulation studies investigating trace metal contamination. It is common for textbooks on ecotoxicology to discuss and dismiss the use of plants in one or two pages. This study argues forcefully for the revision of this traditional view, by demonstrating that plants, including micro and macroalgae, can be as sensitive to pollutants as animals (Thursby et al., 1991). Ecotoxicologists can no longer afford to overlook the use of plants as biomonitors as they provide an additional and much needed dimension to the discipline. There is mounting social pressure to find alternatives to using animals for all toxicity tests. Issues of legislation, licensing and ethics all point towards the adoption

of plant-based monitoring in the 20th century for the detection and control of environmental pollutants.

Plants have the advantage that many are sedentary and this immobility means that they have no mechanisms by which they can avoid pollutants within their surroundings. Their resources are therefore directed towards detoxification and tolerance. By measuring bioaccumulation and stress an insight into the ecosystem within which they are found is gained. Transplantation and reproduction success are similarly tools which can be used. Their advantages over the use of invertebrates and fish go beyond their sedentary nature, as plants also provide more tissue for bioaccumulation analysis, are simple to locate and identify, are easy to collect and transport and require minimal attention following collection. Because of their physiology it is also possible to develop and execute tests which are both reliable and reproducible as demanded by legislators and desired by those who routinely perform them.

Plants are also likely to be highly responsive organisms for use in direct toxicity testing. This is the likely future of biomonitoring, providing *in situ* measurements of toxicity. Such techniques are becoming increasingly important as changes occur in the approach to toxicity testing. In the past chemical consents for effluent release were set by legislators according to the levels of chemical concentrations believed to result in toxicity. This is all set to change to consents in future being set according to levels of toxicity within a given environment. Any test that can be carried out in the field will then be of primary importance. In addition to this the philosophy that the 'polluter pays' for toxic effluent discharge is dependent upon scientists being able to prove the source of any pollutant incident. To achieve this, sensitive and reliable methods must be available that can be used for direct toxicity testing. The best candidates investigated in this work are the

photosynthetic fluorescence induction techniques, but the ion leakage Health Index would also have an application.

The plants which dominate the marine environment are of course the algae. The aim of this work was specifically to use members of the Ulvaceae, an order of the Chlorophyta. These algae, which include the sheet-like *U. lactuca* and *E. intestinalis* the thallus of which is composed of a long thin hollow tube, are ideally suited to the role of biomonitors. They fulfil the criteria listed above and are ubiquitous, allowing for comparison between sites and populations. *E. intestinalis* was used for the majority of the studies carried out in this project because it is abundant throughout most of the year, and is still present during the other months (January and February) though only in a reduced growth form. However even in this state it is apparently identical physiologically to the plant during maximum growth and reacts identically to toxicants and stress, the Health Index being only slightly lower than in the 'warmer' months. *U. lactuca* is not as tolerant of harsh conditions and in the South West it disappears between October and April. This reduces its use considerably as most monitoring programs need to continue all year round. *E. intestinalis* is also more wide spread than *Ulva*, increasing the possibility of locating it within a given marine benthic ecosystem, whether coastal or estuarine. Although for the purposes of this project it was not necessary to culture the alga, this is easy to achieve and could add to the diversity of use that *E. intestinalis* presents.

6.3 Further Work

As with all studies this research has generated as many questions as it has provided answers. We are able to say that *E. intestinalis* fulfils in principle and in practice the requirements of a biomonitor of various pollutants, both specific and non-specific in nature. The techniques developed that allow for this use include the Health Index,

photosynthetic fluorescence analysis and the use of the alga as a bioaccumulator of trace contaminants. However many of the toxicological studies developed throughout investigation have involved short term exposure, the translation of which into chronic exposure is difficult to achieve. It is therefore necessary that more studies of this type be carried out in the validation of both pollution toxicity and contaminant bioaccumulation.

As a biomonitoring technique applied to *E. intestinalis*, the Health Index has been shown to be effective, but this is a tool which may usefully be applied to the investigation of the effects of a wider range of toxicants, in more varied environmental situations than it was possible to cover here. Further use of this technique should also be carried out in combination with photosynthetic fluorescence analysis, especially in a repeat of the investigation of the effects of photo-induced toxicity and UV-B radiation. Fluorescence measurement should be able to identify whether in the presence of PAH's combined with UV-B radiation a photo-induced toxicity does occur (see also Chapter 4), this type of toxicity acting upon PSII, which is the photosystem from which the majority of chlorophyll fluorescence originates. There is also great potential for using this method in toxicity and QSAR studies and in the investigation of the effects of photosynthesis inhibiting pesticides. Such an application would also provide information as to the best use of the different parameters which the instruments are capable of measuring.

The bioaccumulation studies are particularly interesting, especially those used to extract and identify organic compounds. An extension of this study looking at how specific and complex organic compounds act on the algae could be carried out in combination with uptake studies. The investigation of uptake by different species of algae should also be carried out in an attempt to identify which are the best biomonitors for organic pollutants. If this could be achieved an in-depth study into the use of more sensitive species i.e. those

which are lost at low levels of pollution, could then be attempted using the techniques developed during this project.

The techniques evaluated in this thesis have revealed large areas of neglect where the investigation of toxicant effects on macroalgae is concerned. However, also revealed is the exciting work which is now being carried out to extend our knowledge of toxicant effect within the environment, on members of the plant kingdom. This progressive move beyond the hitherto limited range of toxicity based investigations gives me the confidence to believe that Ecotoxicology is now coming of age, not simply as a named discipline but as a living and evolving science.

EPILOGUE

The reference to other programmes of work in the field of plant based toxicology indicates a well-trodden path of public and scientific concern. Along such a path, the significance of one small additional footprint might not easily be recognised, but I offer this work to the scientific community in the hope that by adding to the sum of human knowledge it may quicken the pace to the achievement of a cleaner and safer environment. To all those who aided, abetted and encouraged me to pursue this path I dedicate these lines from T.S. Eliot (East Coker, Section 5):

> And so each venture
> Is a new beginning, a raid on the inarticulate
> With shabby equipment always deteriorating
> In the general mess of imprecision of feeling,
> Undisciplined squads of emotion. And what there is to conquer
> By strength and submission, has already been discovered
> Once or twice, or several times, by men whom one cannot hope
> To emulate-but there is no competition-
> There is only the fight to recover what has been lost
> And found and lost again and again: and now, under conditions
> That seem unpropitious. But perhaps neither gain nor loss.
> For us, there is only the trying.

The tools have at times been blunt, the material stubborn and uncooperative and the pace irritatingly slow. But the inspiration of other pioneers and the fight to recover what has been lost through centuries of neglect has been deeply fulfilling.

'For us there is only the trying' - but for the environment and it's associated organisms the rewards might yet be tangible.

R.Schild, July 1996

APPENDIX 1

The plots in appendix 1 represent the fitted curves achieved for *E. intestinalis* ion leakage data using the Maximum Likelihood Program. The package was used to calculate the EC_{50} for the following chemical compounds.

1. Methanol,
2. Ethanol,
3. Propan-1-ol,
4. Butan-1-ol
5. Pentan-1-ol
6. Hexan-1-ol,
7. Heptan-1-ol,
8. Octan-1-ol.

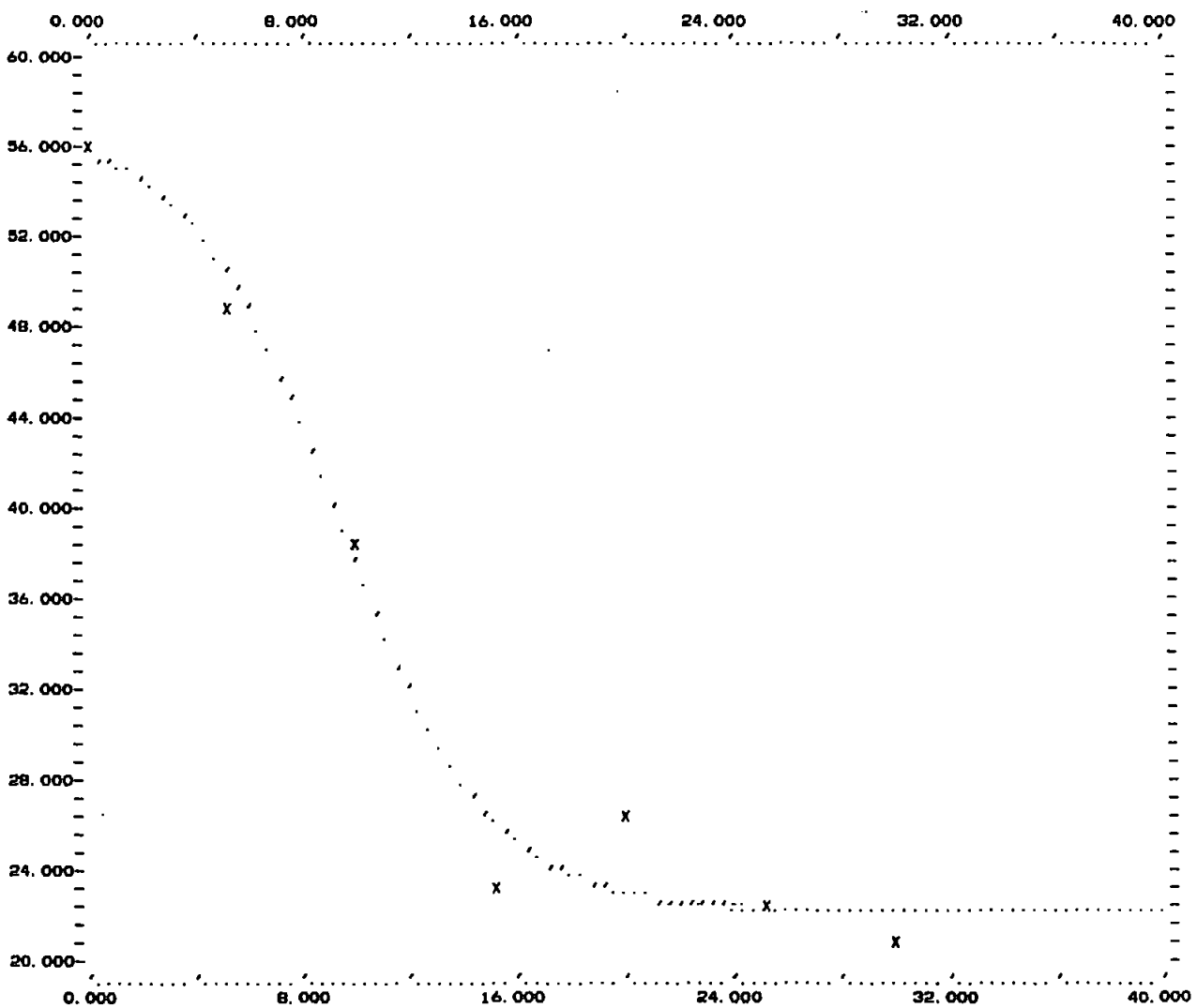
Please note that the concentrations used were 'transposed" in order to run the program, the results were then converted back on completion.

= A + C/(1+EXP(-B(X-M))) LOGISTIC

	PARAMETER	S. E.	CORRELATIONS			
B	-0.34993	0.14998	1.0000			
M	9.38892	0.78689	-0.5757	1.0000		
C	34.80431	4.29378	0.8077	-0.5314	1.0000	
A	22.18454	1.90823	-0.5103	-0.0971	-0.7045	1.0000
A+C	56.98885	3.24541				

S. S. 22.829052 D. F. 3 R. M. S. 7.609684

X	Y	E(Y)	WTD. RES.	W
0.0000	56.0000	55.7333	0.3772	2.0000
5.0000	49.0000	50.8234	-1.3985	0.5882
10.0000	38.0000	37.7332	0.5967	5.0000
15.0000	23.0000	26.4687	-2.9316	0.7143
20.0000	26.0000	23.0136	3.1480	1.1111
25.0000	22.0000	22.3316	-0.2018	0.3704
30.0000	21.0000	22.2102	-1.3530	1.2500



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P 3.08
) 1985  Lawes Agricultural Trust (Rothamsted Experimental Station)

DERIVE CALCULATIONS
1  V3=1.0/V3

= A + C/(1+EXP(-B(X-M)))  LOGISTIC

     PARAMETER
  B        -1.78177
  M         6.52341
  C        31.01507
  A        26.47712

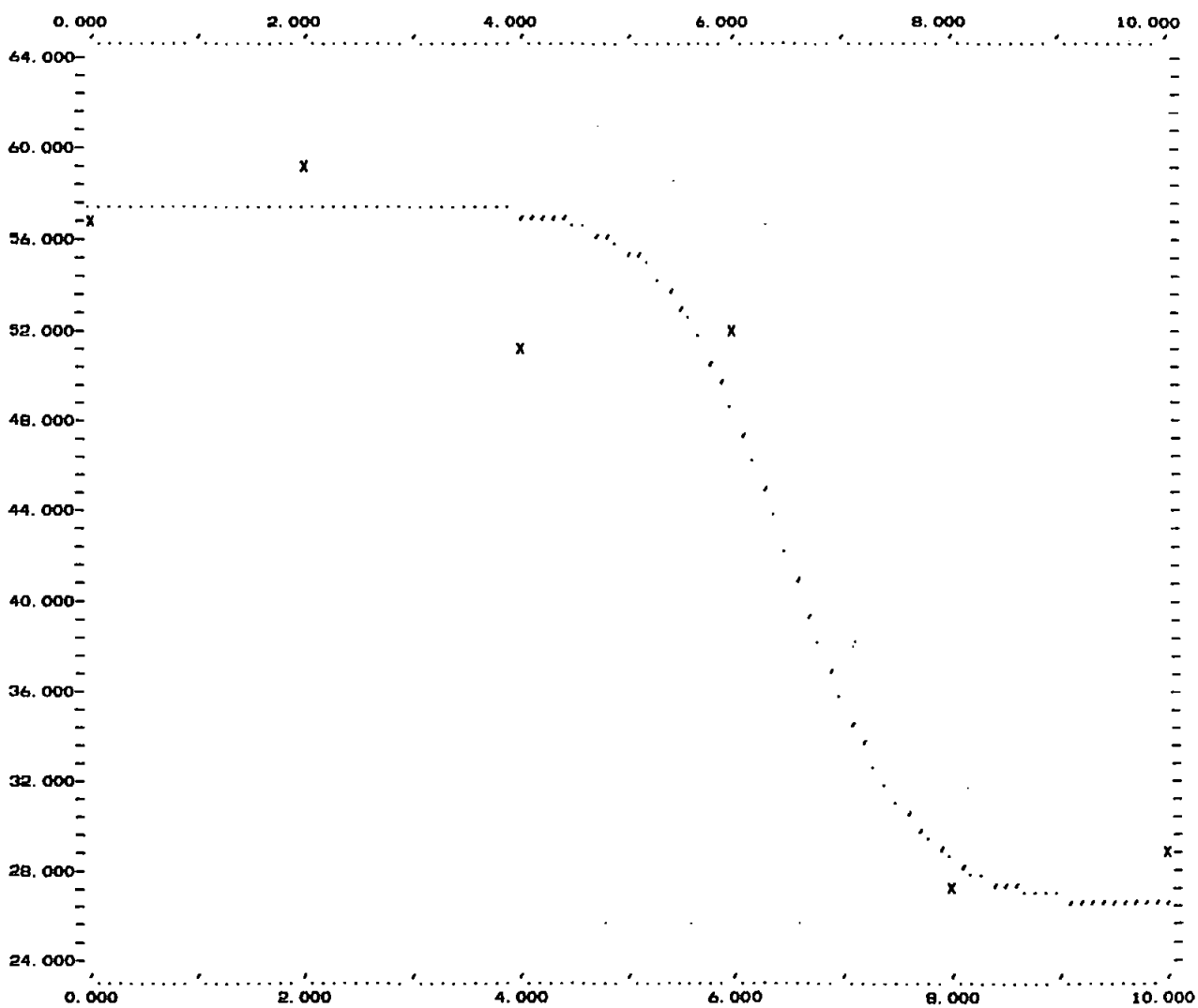
  A+C      57.49219

S.S.      41.829727   D.F.      2   R.M.S.      20.914864

       X          Y        E(Y)   WTD. RES.          W
  0.0000    57.0000    57.4919     -0.4690     0.9091
  2.0000    59.0000    57.4824      2.3995     2.5000
  4.0000    51.0000    57.1501     -5.0215     0.6667
  6.0000    52.0000    48.7336      1.9521     0.3571
  8.0000    27.0000    28.5605     -1.7447     1.2500
 10.0000    29.0000    26.5403      1.9446     0.6250
```

E(Y) fitted value

weighted residual - difference



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P 3.08
) 1985  Lawes Agricultural Trust (Rothamsted Experimental Station)

DERIVE CALCULATIONS
1  V3=1.0/V3

= A + C/(1+EXP(-B(X-M)))  LOGISTIC

    PARAMETER
  B        -1.90185
  M         1.85715
  C        39.83177
  A        13.31871

  A+C      53.15048

S.S.     29.824577   D.F.      2   R.M.S.     14.912289

       X          Y       E(Y)  WTD.RES.          W
  0.0000    52.0000    52.0187   -0.0197     1.1111
  2.0000    31.0000    30.5457    0.3709     0.6667
  4.0000    12.0000    13.9840   -3.6222     3.3333
  6.0000    13.0000    13.3338   -0.6094     3.3333
  8.0000    15.0000    13.3190    3.0690     3.3333
 10.0000    17.0000    13.3187    2.6031     0.5000
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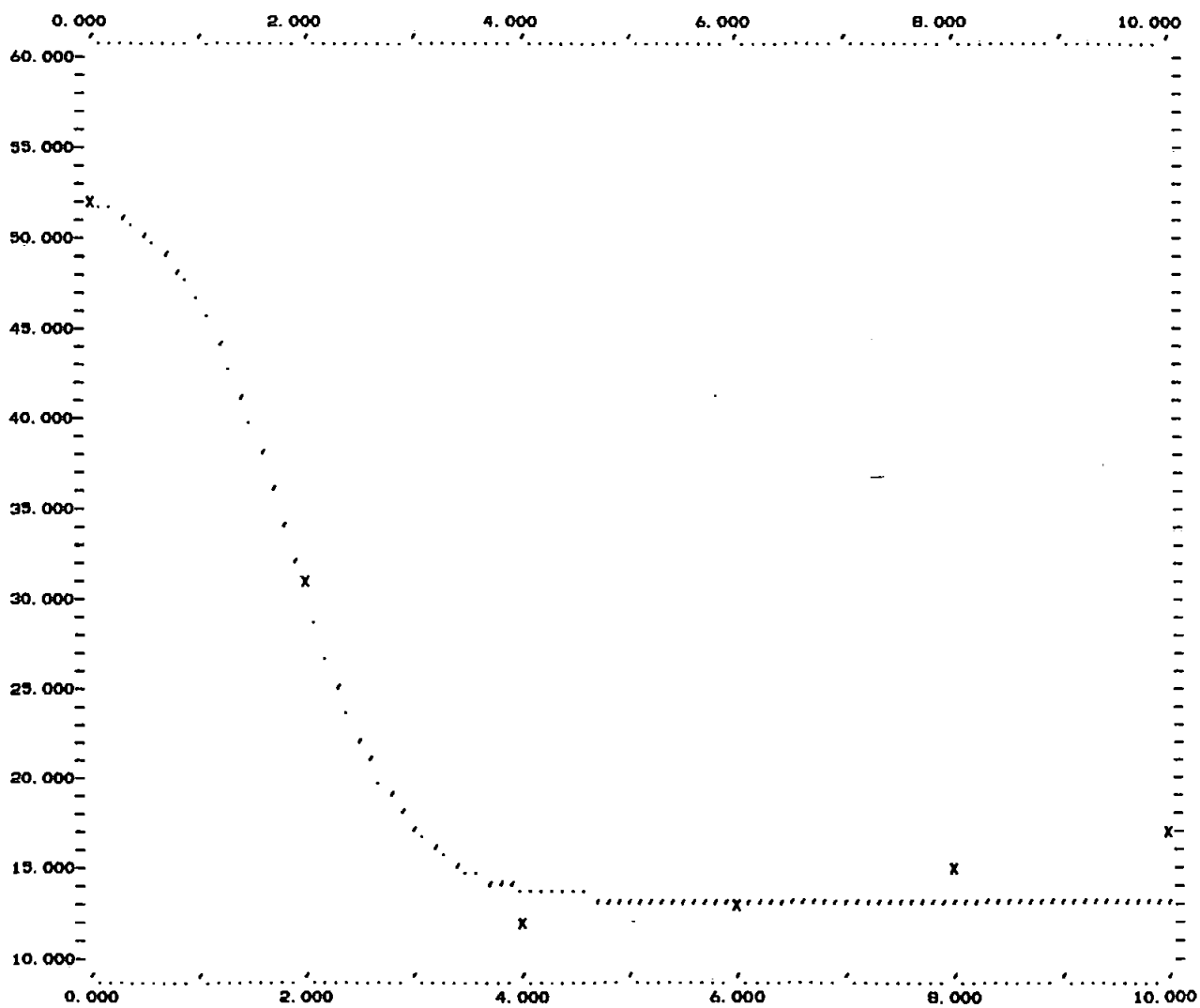
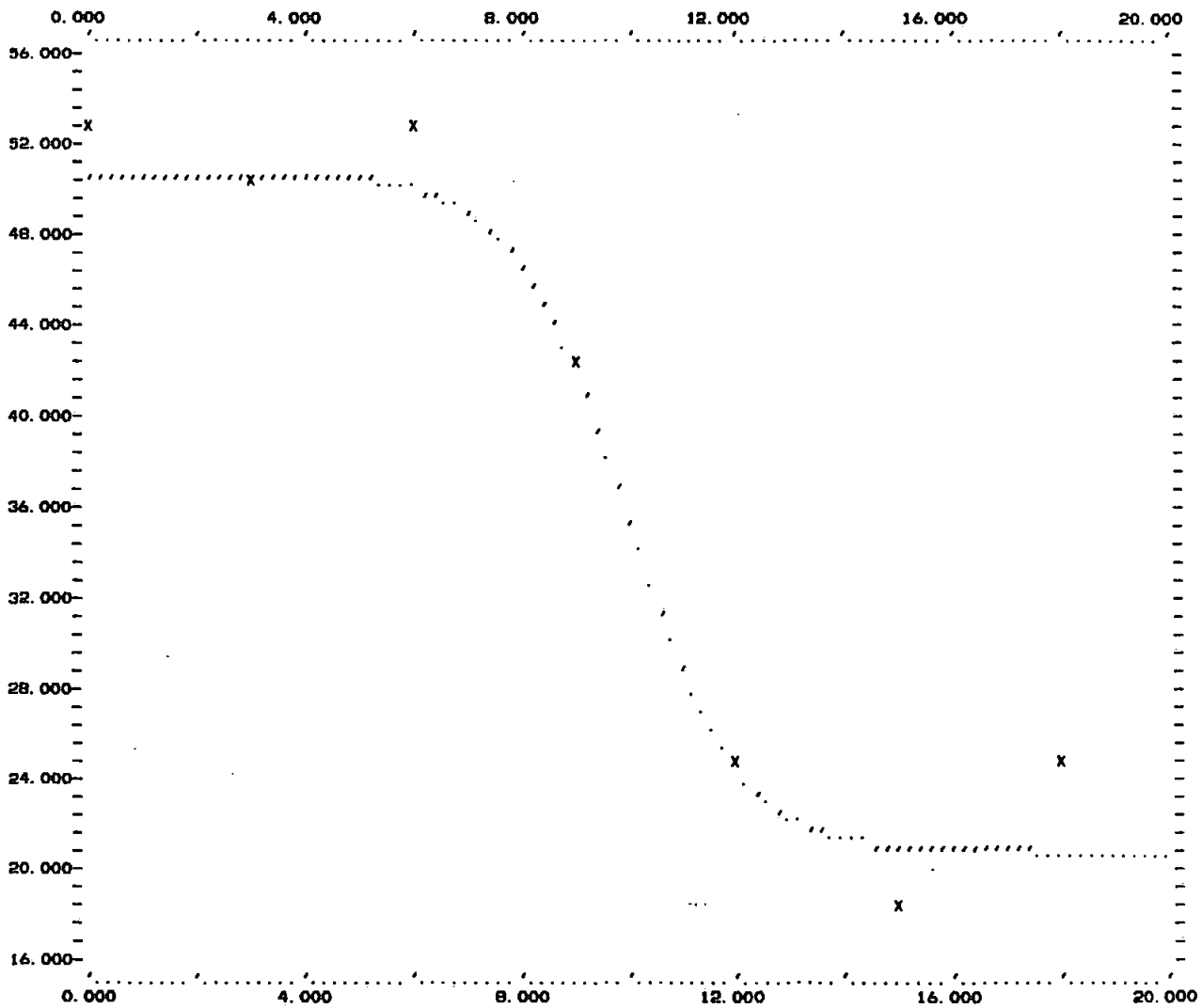


= A + C/(1+EXP(-B(X-M))) LOGISTIC

	PARAMETER	S.E.	CORRELATIONS			
B	-0.94168	0.33918	1.0000			
M	9.94932	0.46511	0.7355	1.0000		
C	29.98706	3.15865	0.6367	0.4722	1.0000	
A	20.77665	2.63686	-0.6368	-0.7215	-0.8653	1.0000
A+C	50.76370	1.58634				

S.S. 46.495720 D.F. 3 R.M.S. 15.498573

X	Y	E(Y)	WTD. RES.	W
0.0000	53.0000	50.7611	2.0438	0.8333
3.0000	50.0000	50.7206	-1.6114	5.0000
6.0000	53.0000	50.0535	2.4902	0.7143
9.0000	42.0000	42.0586	-0.1855	10.0000
12.0000	25.0000	24.9739	0.6737	2.5000
15.0000	18.0000	21.0323	-3.9147	1.6667
18.0000	25.0000	20.7919	4.2081	1.0000



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P 3.08
) 1985  Lawes Agricultural Trust (Rothamsted Experimental Station)

DERIVE CALCULATIONS
1  V3=1.0/V3

= A + C/(1+EXP(-B(X-M)))  LOGISTIC

     PARAMETER          S.E.          CORRELATIONS
  B      -5.61429      3.06134     1.0000
  M       4.41723      0.24255     0.9715  1.0000
  C      28.52777      2.60998     0.7767  0.7809  1.0000
  A      20.97186      2.57633    -0.7785 -0.8025 -0.9876  1.0000

  A+C    49.49964      0.41035

S.S.     10.897247   D.F.      3   R.M.S.      3.632416

       X          Y        E(Y)  WTD.RES.         W
  0.0000    50.0000    49.4996     1.5823   10.0000
  1.0000    49.0000    49.4996    -1.5800   10.0000
  2.0000    52.0000    49.4996     1.9177    0.5882
  3.0000    48.0000    49.4896    -1.4896    1.0000
  4.0000    47.0000    46.9987     0.0041   10.0000
  5.0000    22.0000    22.0146    -0.0146    1.0000
  6.0000    21.0000    20.9758     0.0160    0.5556
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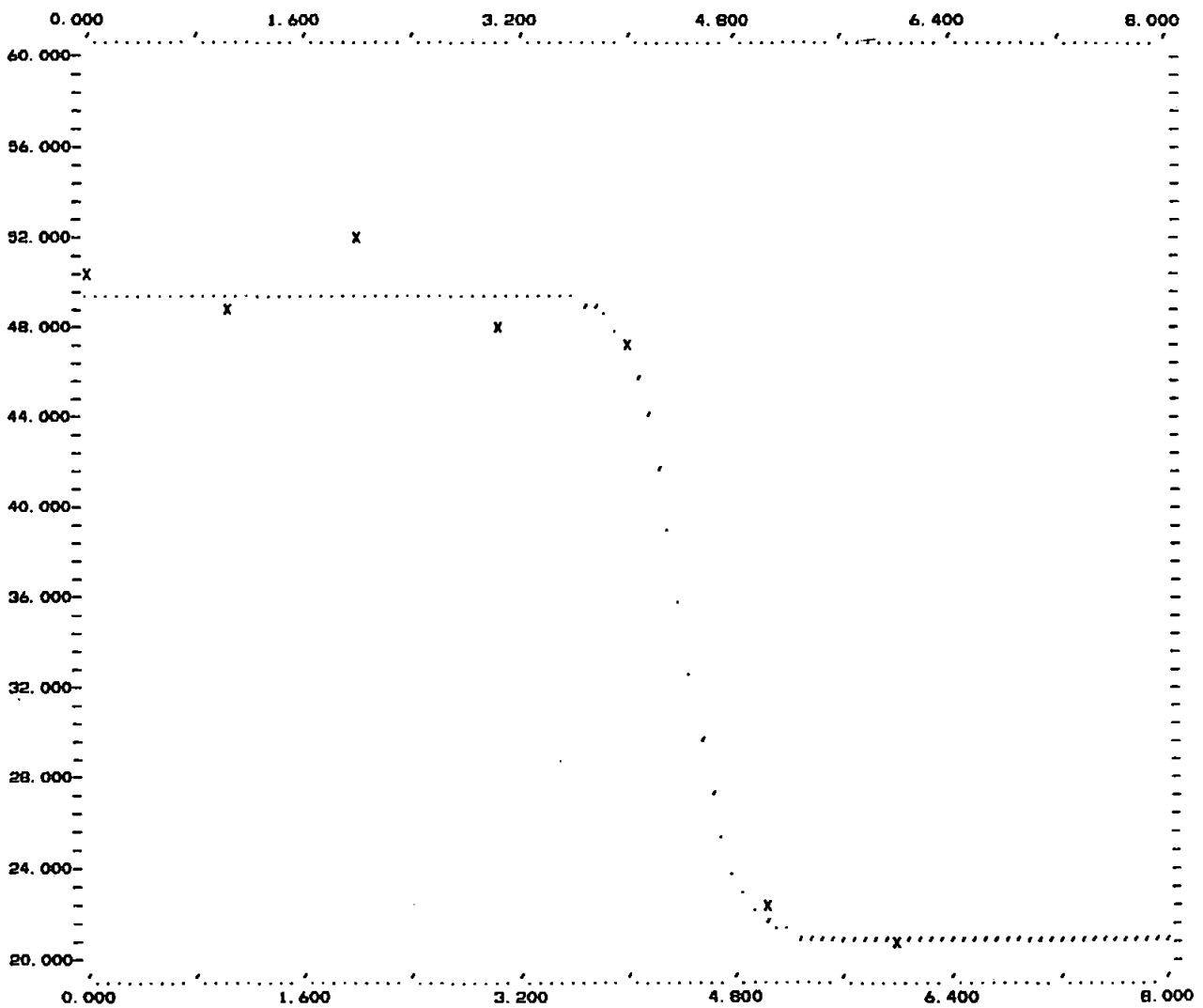

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DERIVE CALCULATIONS
1  V3=1.0/V3

= A + C/(1+EXP(-B(X-M)))  LOGISTIC

     PARAMETER
 B        -4.36373
 M         3.95769
 C        34.82286
 A        16.18437

   A+C     51.00723

S.S.      2.102012   D.F.      2   R.M.S.      1.051006

        X          Y       E(Y)  WTD. RES.          W
   0.0000    51.0000    51.0072    -0.0061     0.7143
   4.0000    32.0000    31.9932     0.0088     1.6667
   8.0000    16.0000    16.1844    -0.5830    10.0000
  12.0000    16.0000    16.1844    -0.2204     1.4286
  16.0000    17.0000    16.1844     1.0530     1.6667
  20.0000    17.0000    16.1844     0.7777     0.9091
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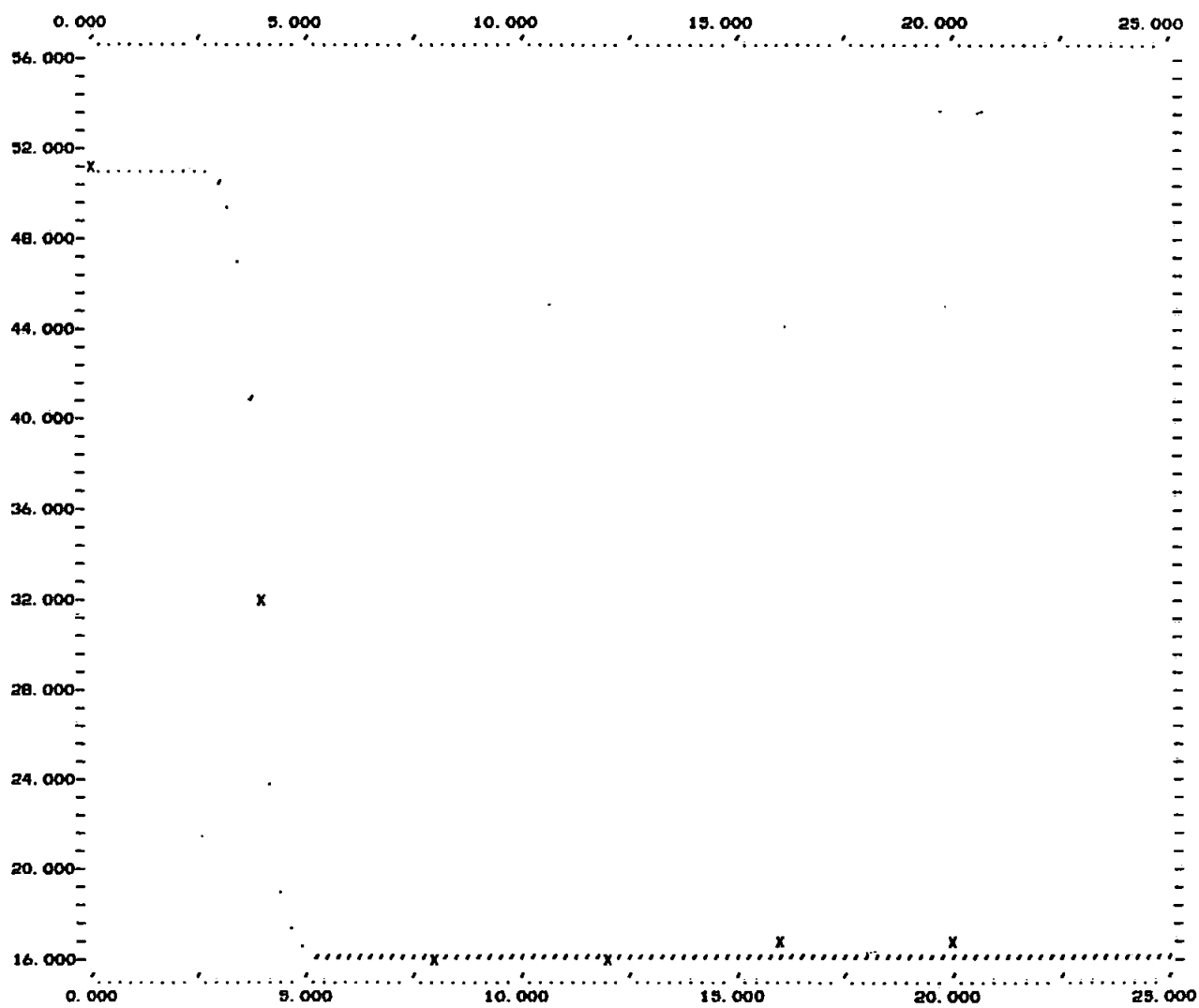
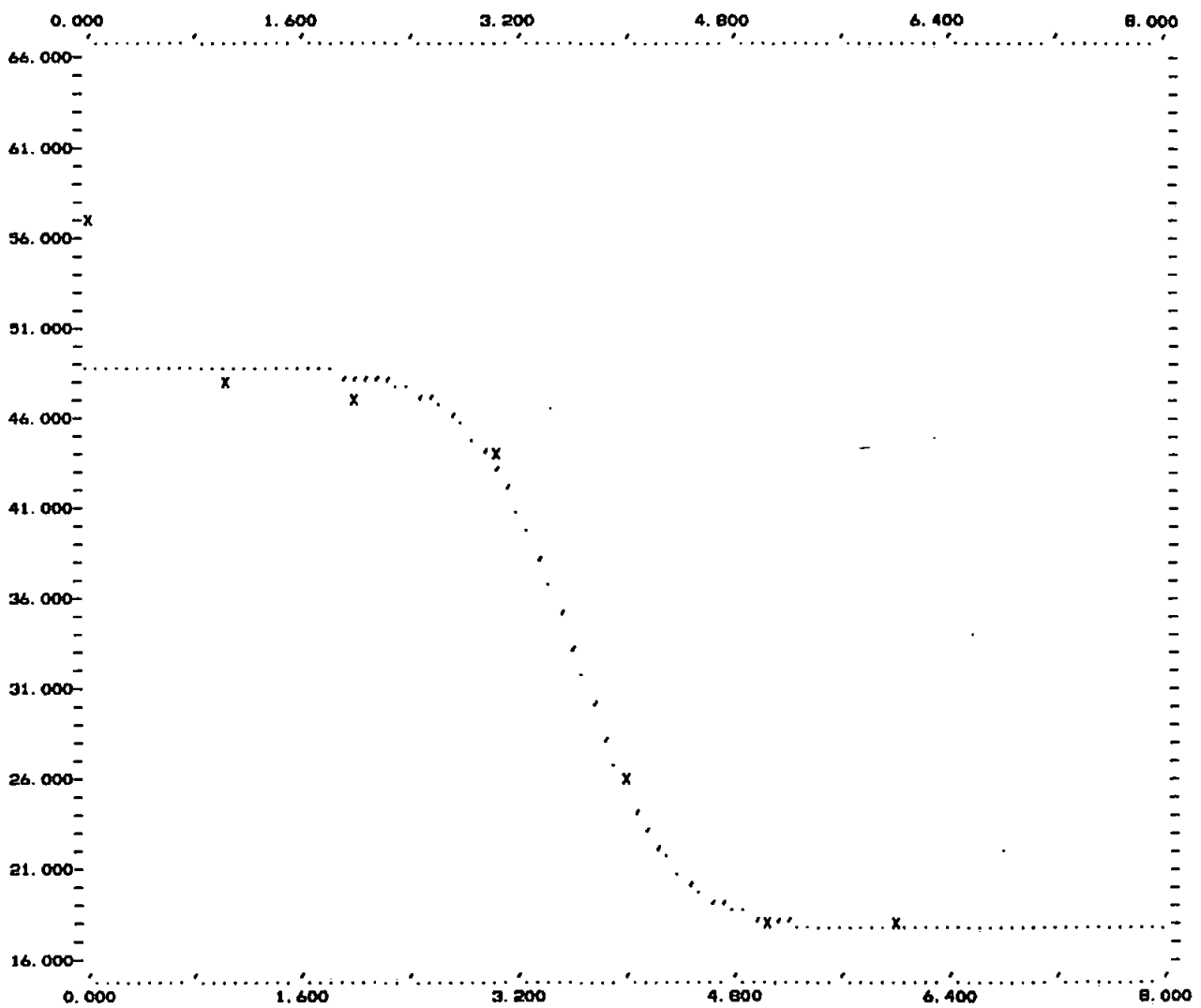


= A + C/(1+EXP(-B(X-M))) LOGISTIC

	PARAMETER		S.E.	CORRELATIONS			
B		-2.68509	1.21982	1.0000			
M		3.60737	0.25637	0.0677	1.0000		
C		31.21864	2.53262	0.6000	-0.0079	1.0000	
A		17.58241	1.52271	-0.9595	-0.3421	-0.7032	1.0000
A+C		48.80105	1.81914				

S.S. 24.117893 D.F. 3 R.M.S. 8.039297

X	Y	E(Y)	WTD. RES.	W
0.0000	57.0000	48.7991	4.5144	0.3030
1.0000	48.0000	48.7726	-0.9235	1.4286
2.0000	47.0000	48.3896	-1.4648	1.1111
3.0000	44.0000	43.6901	0.2248	0.5263
4.0000	26.0000	25.6497	0.1929	0.3030
5.0000	18.0000	18.3072	-0.5609	3.3333
6.0000	18.0000	17.6329	0.5804	2.5000



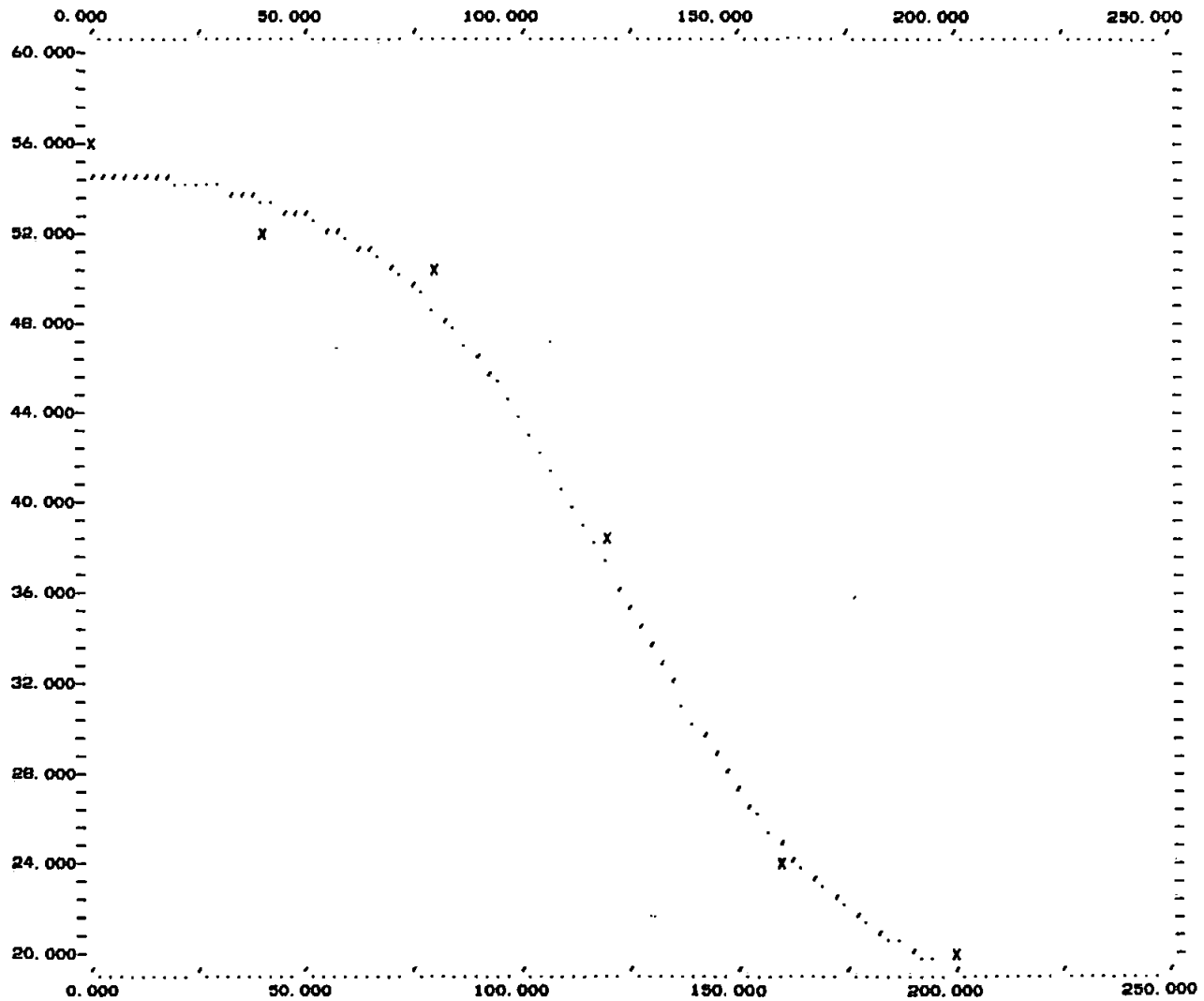
1 WG=1.0/W3

= A + C/(1+EXP(-B(X-M))) LOGISTIC

	PARAMETER		S. E.	CORRELATIONS			
1	B	-0.03728	0.01260	1.0000			
2	M	122.82330	10.54358	0.4013	1.0000		
3	C	37.82542	5.57927	0.8590	0.5902	1.0000	
4	A	17.33425	4.55383	-0.7734	-0.7364	-0.9581	1.0000
	A+C	55.15968	1.78312				

S. S. 5.252602 D. F. 2 R. M. S. 2.626301

X	Y	E(Y)	WTD. RES.	W
0.0000	56.0000	54.7753	1.2247	1.0000
40.0000	52.0000	53.5098	-1.5915	1.1111
80.0000	50.0000	48.7871	0.7123	0.3448
120.0000	38.0000	37.2414	0.4114	0.2941
160.0000	24.0000	24.9013	-0.6219	0.4762
200.0000	20.0000	19.3501	0.3955	0.3704



APPENDIX 2

Key and Legend

Abbreviations: Excitation wavelength = Xλ; Excitation Slit Width = XSW; Emission Slit width = EmSW.

1. Yellow Fuel Oil, 1.48 μg/ml iso-hexane.

   Xλ = 253 nm; XSW = 5; EmSW = 0.

2. Environmental Sample 1., 1st distillation into iso-hexane.

   Xλ = 253 nm; XSW = 5; EmSW = 0.

   Mothecombe - Red

   Restronguet - Yellow

   Sutton Harbour - Orange

   Thurlestone - Purple

   Wembury - Blue

3. Environmental Sample 1., 2nd distillation into iso-hexane.

   Xλ = 253 nm; XSW = 5; EmSW = 0.

   Mothecombe - Red

   Restronguet - Yellow

   Sutton Harbour - Orange

   Thurlestone - Purple

   Wembury - Blue

4. Environmental Sample 2., 1st distillation into iso-hexane.

   Xλ = 253 nm; XSW = 5; EmSW = 0.

   Mothecombe - Red

   Restronguet - Yellow

   Sutton Harbour - Orange

   Thurlestone - Purple

   Wembury - Blue

5. Environmental Sample 2., 2nd distillation into iso-hexane.

   Xλ = 253 nm; XSW = 5; EmSW = 0.

   Mothecombe - Red

   Restronguet - Yellow

   Sutton Harbour - Orange

   Thurlestone - Purple

   Wembury - Blue

6. Yellow Fuel Oil, 1.48 μg/ml iso-hexane.

   Xλ = 280 nm; XSW = 5; EmSW = 0.

7. 2,3-Dimethylnaphthalene, 3.56 μg/ml iso-hexane.

   Xλ = 280 nm; XSW = 5; EmSW = 0.

8. Environmental Sample 1., 1st distillation into iso-hexane.

$X\lambda$ = 280 nm; XSW = 5; EmSW = 0.

Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Thurlestone - Purple

Wembury - Blue

9. Environmental Sample 1., 2nd distillation into iso-hexane.

$X\lambda$ = 280 nm; XSW = 5; EmSW = 0.

Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Thurlestone - Purple

Wembury - Blue

10. Environmental Sample 2., 1st distillation into iso-hexane.

$X\lambda$ = 280 nm; XSW = 5; EmSW = 0.

Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Thurlestone - Purple

Wembury - Blue

11. Environmental Sample 2., 2nd distillation into iso-hexane.

Xλ = 280 nm; XSW = 5; EmSW = 0.

Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Thurlestone - Purple

Wembury - Blue

12. Yellow Fuel Oil, 1.48 μg/ml iso-hexane.

Xλ = 310 nm; XSW = 5; EmSW = 0.

13. 1-Methylphenanthrene , 146 μg/ml iso-hexane.

Xλ = 310 nm; XSW = 5; EmSW = 0.

14. Environmental Sample 1., 1st distillation into iso-hexane.

Xλ = 310 nm; XSW = 5; EmSW = 0.

Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Wembury - Purple

15. Environmental Sample 1., 2nd distillation into iso-hexane.

Xλ = 310 nm; XSW = 5; EmSW = 0.

Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Thurlestone - Purple

Wembury - Blue

16. Environmental Sample 2., 1st distillation into iso-hexane.

Xλ = 310 nm; XSW = 5; EmSW = 0.

Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Thurlestone - Purple

Wembury - Blue

17. Environmental Sample 2., 2nd distillation into iso-hexane.

Xλ = 310 nm; XSW = 5; EmSW = 0.

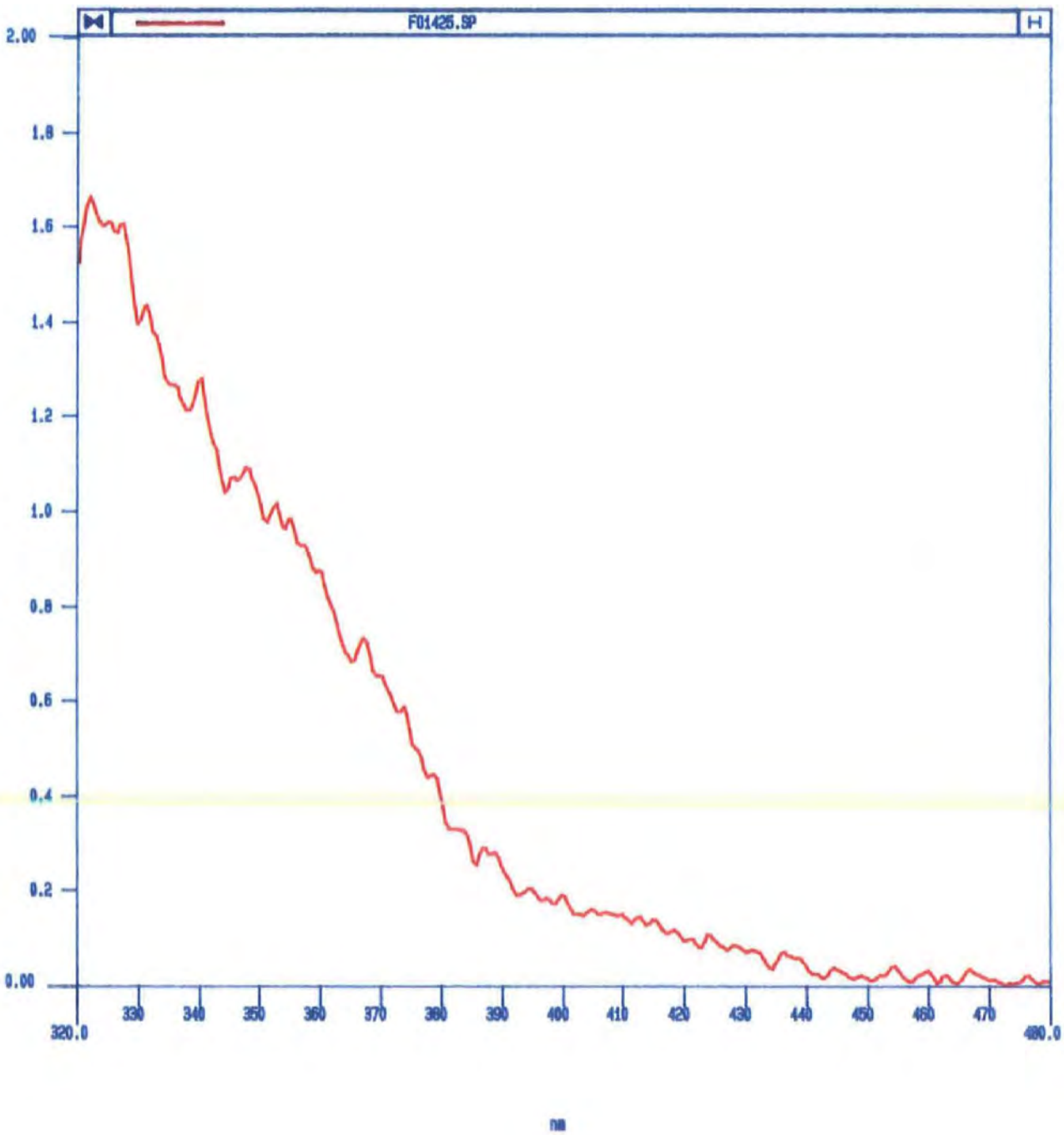
Mothecombe - Red

Restronguet - Yellow

Sutton Harbour - Orange

Thurlestone - Purple

Wembury - Blue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360
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420
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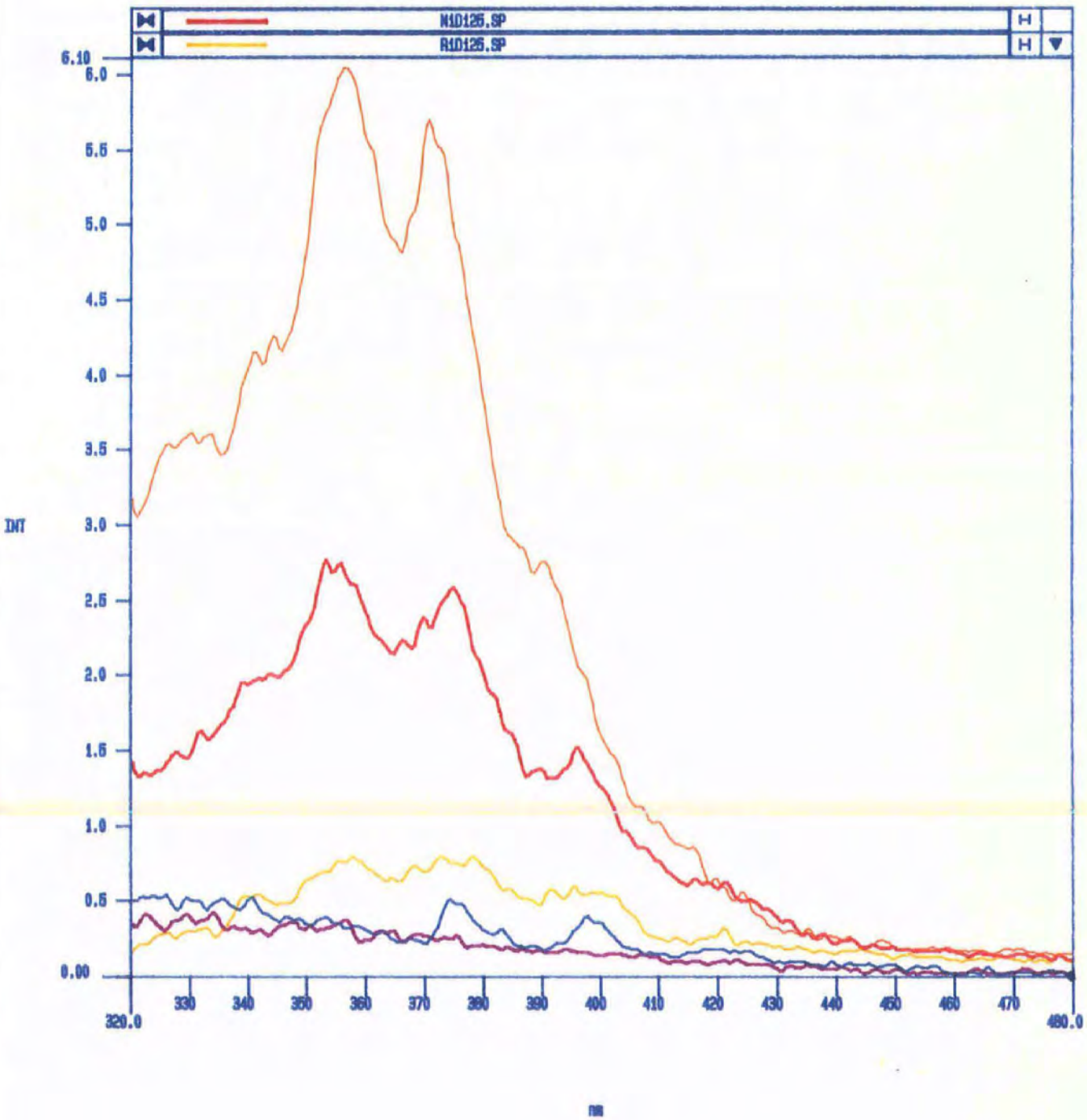
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R1D126.SP
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1.0
0.5
0.00
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420
430
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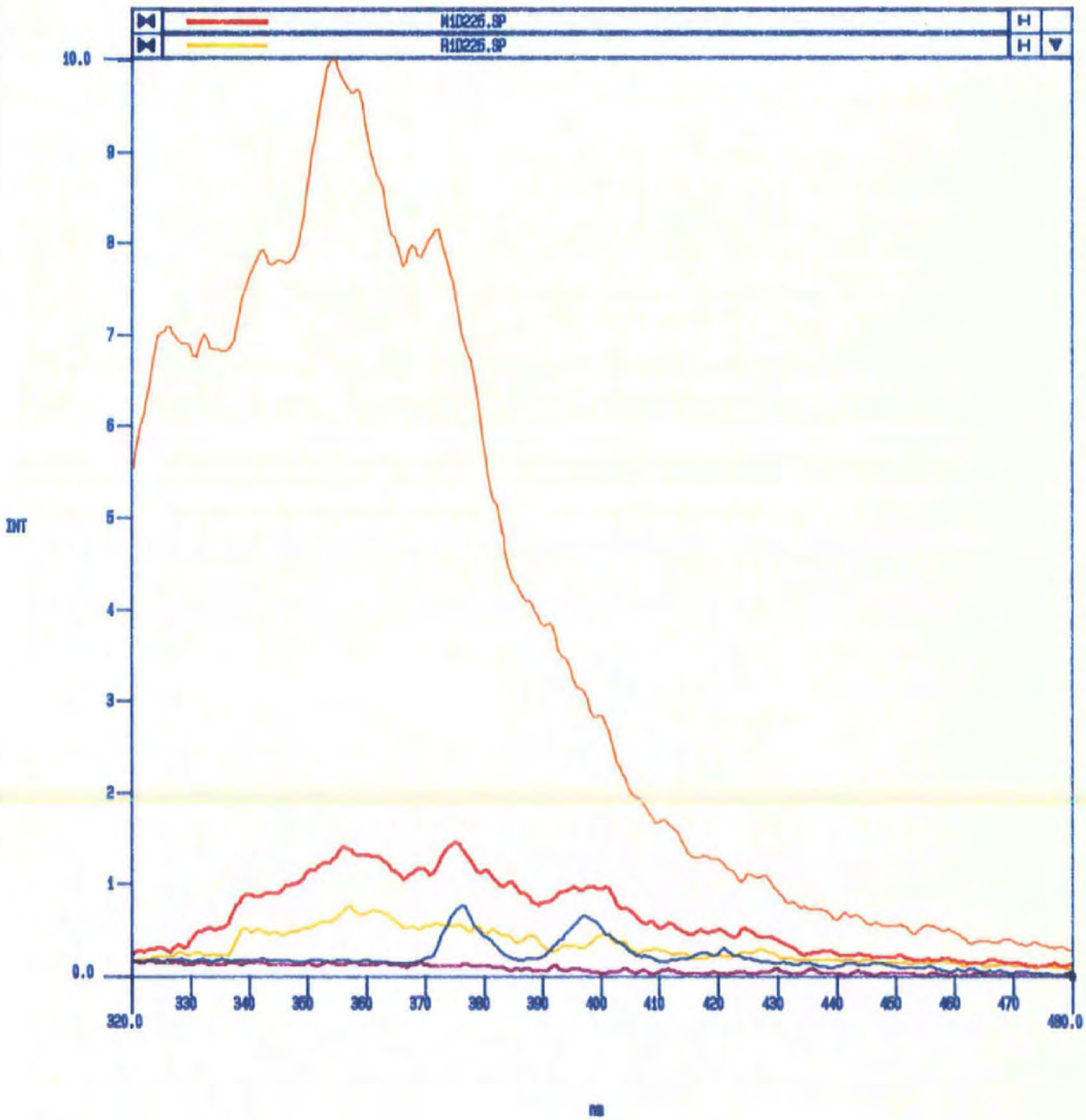
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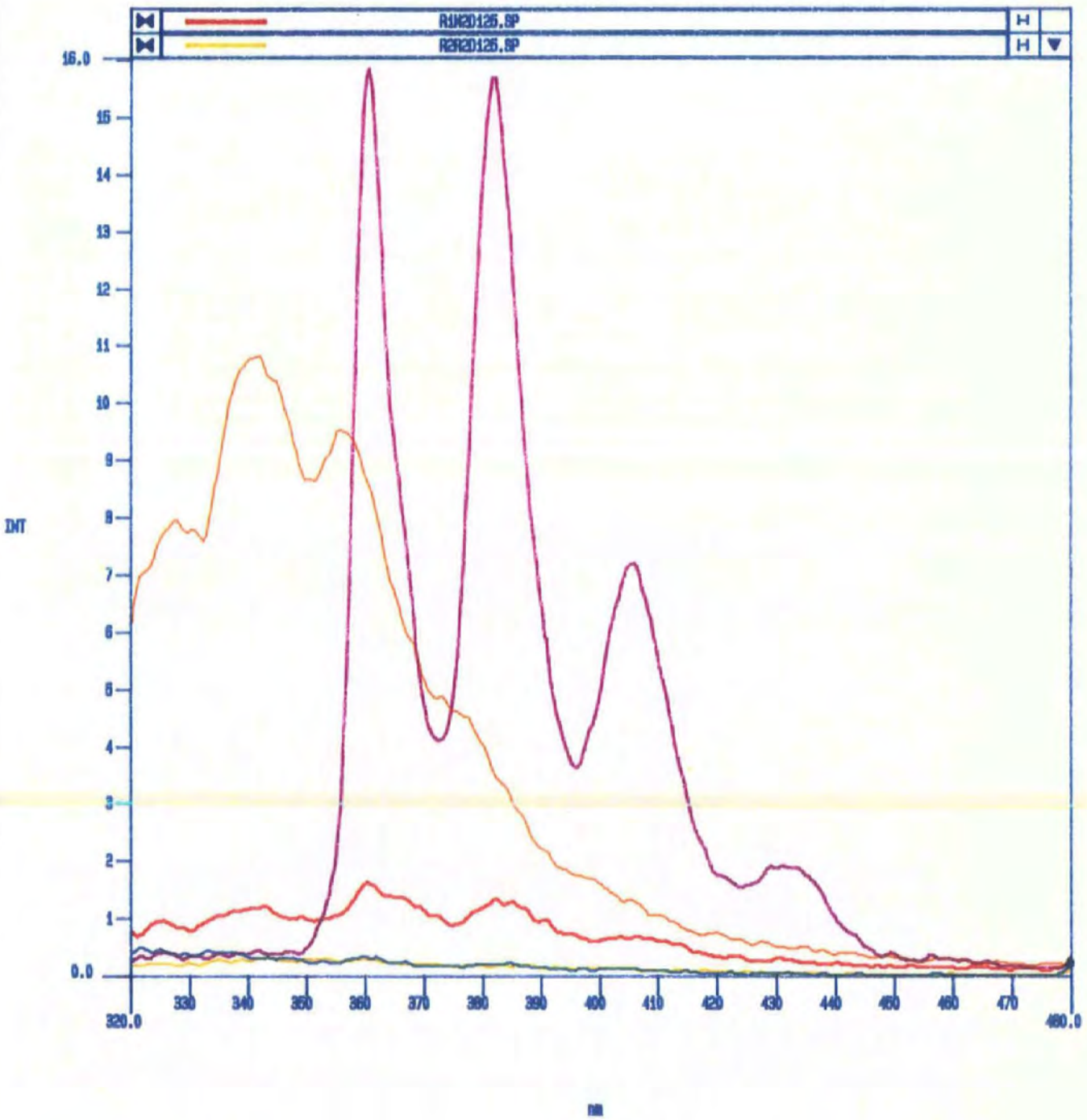
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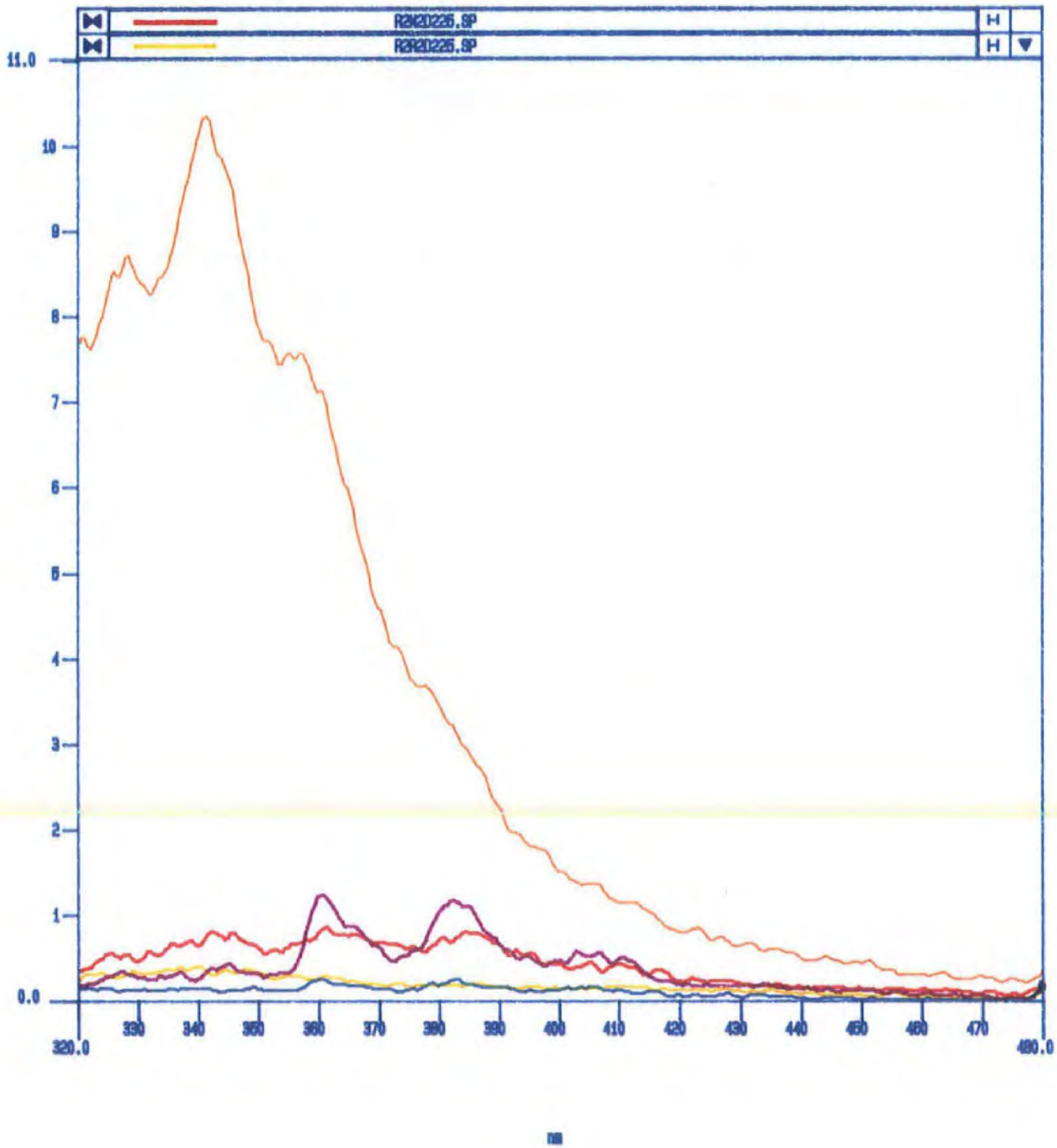
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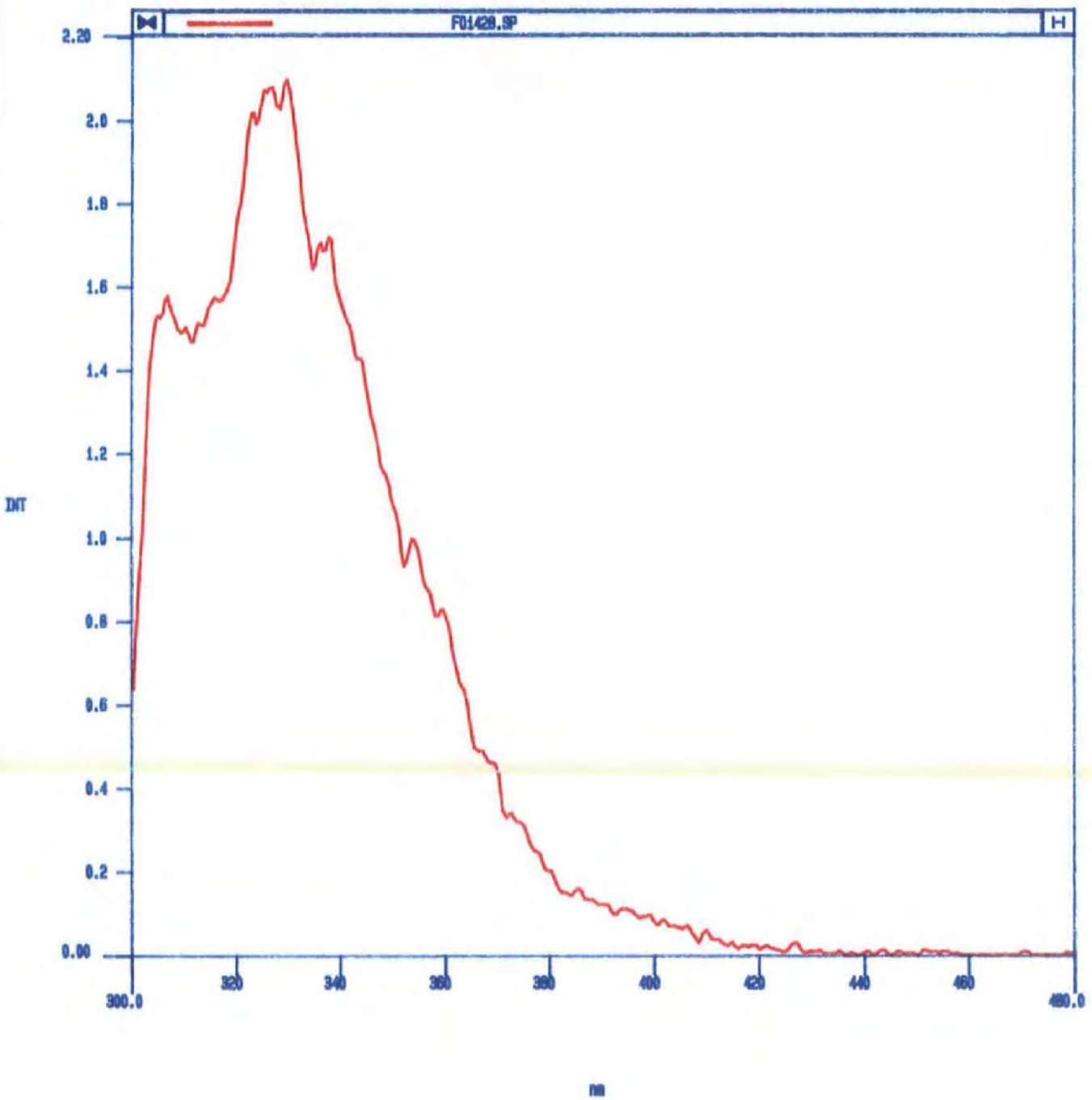
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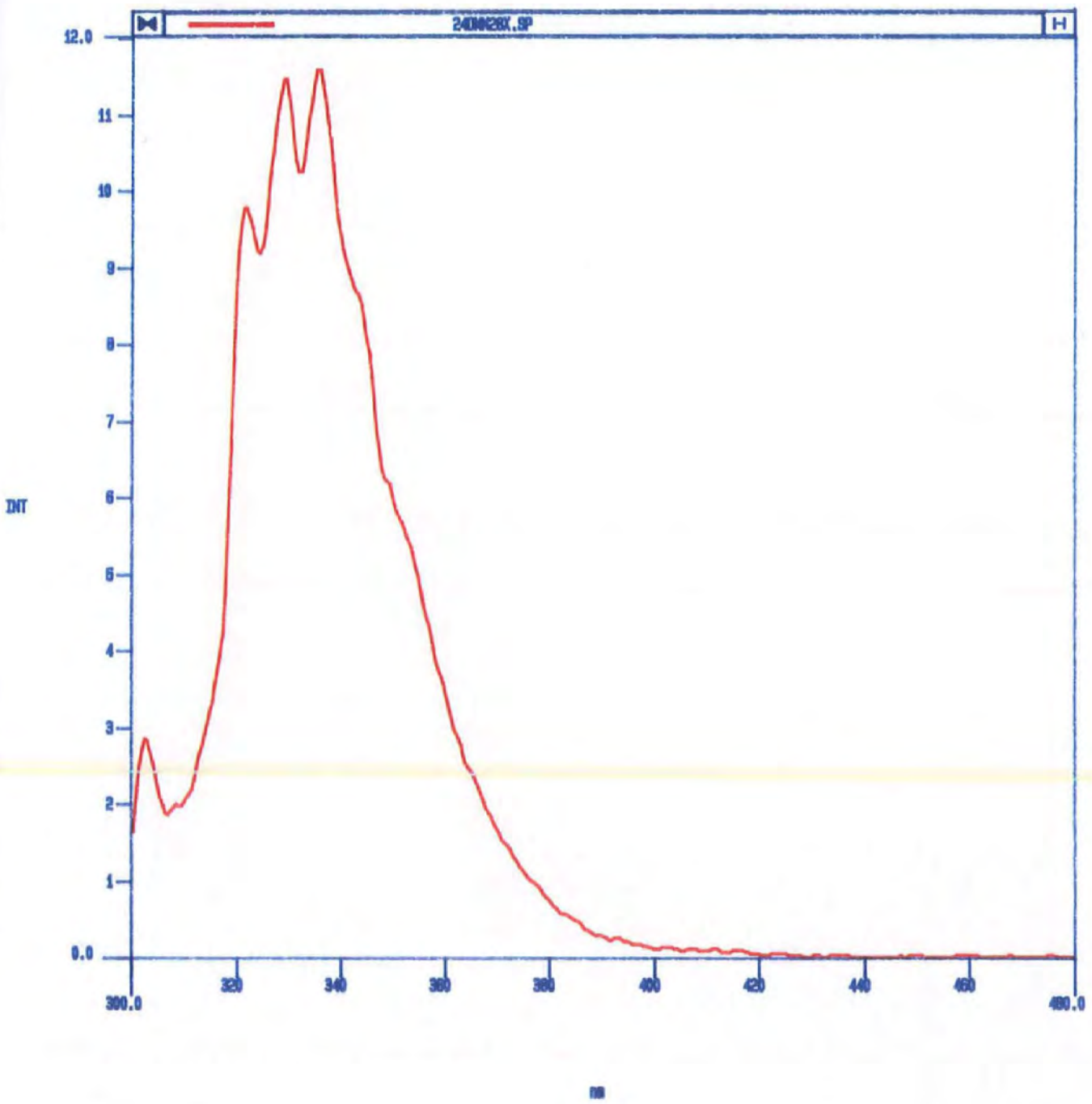
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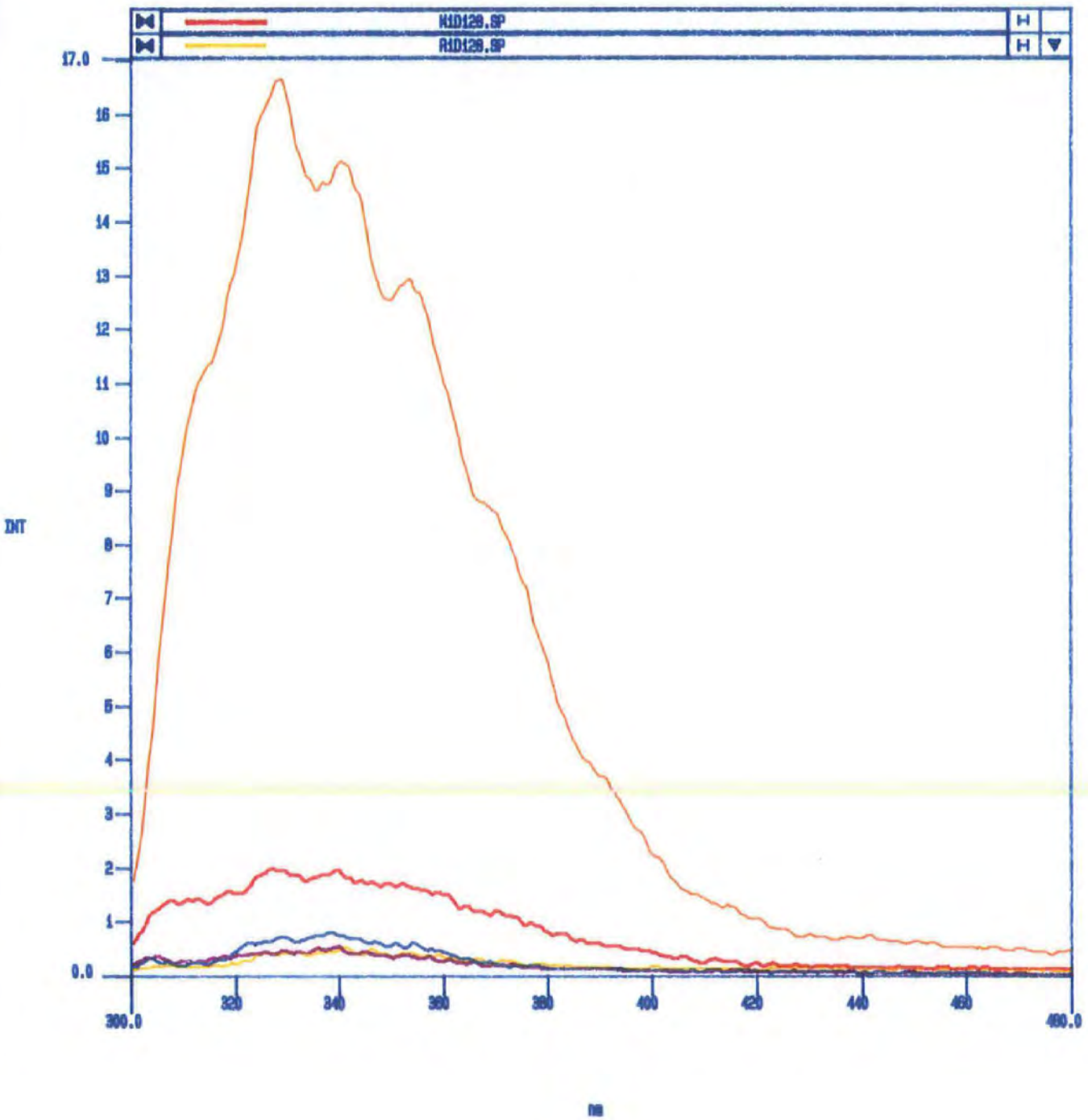
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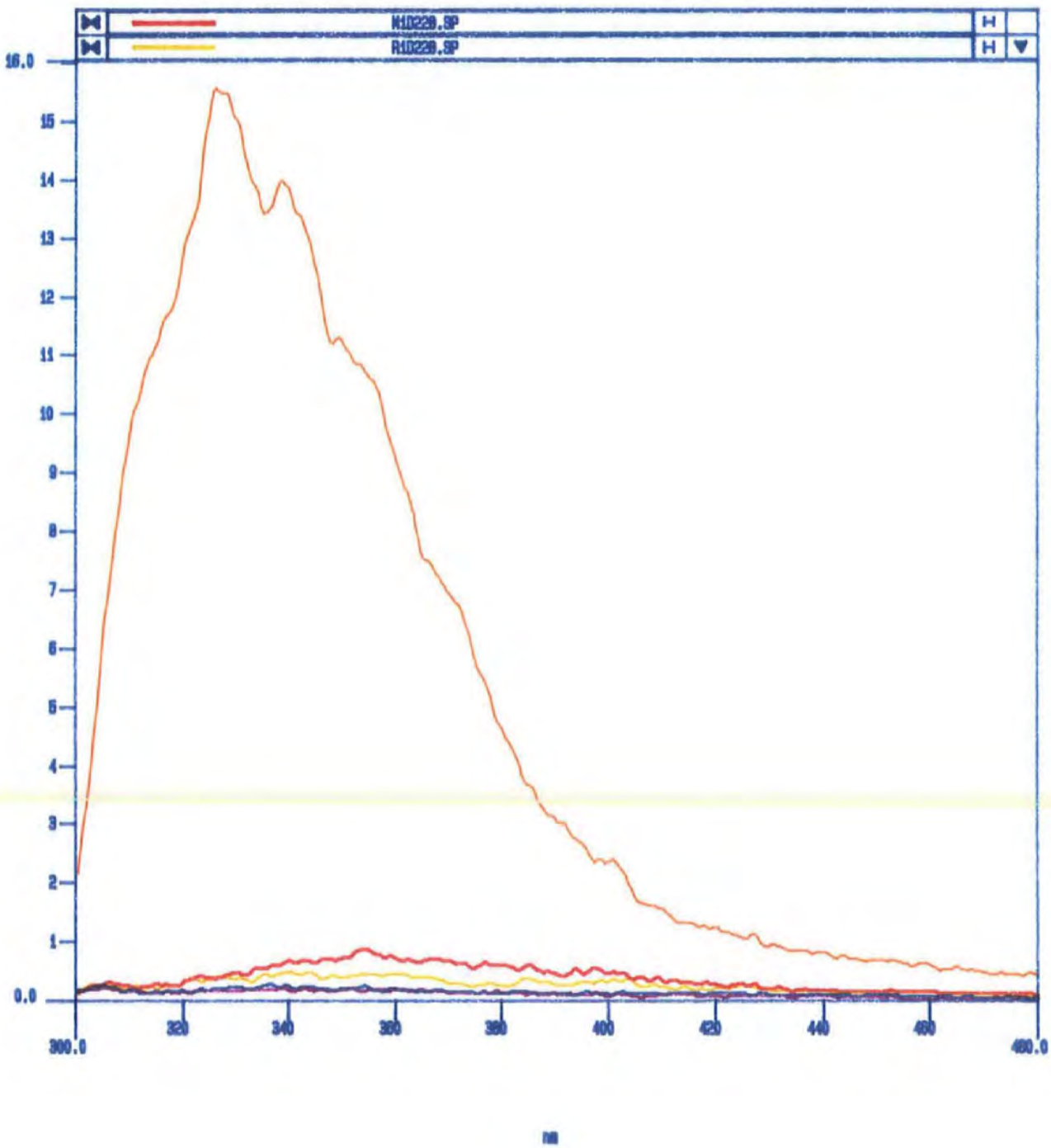
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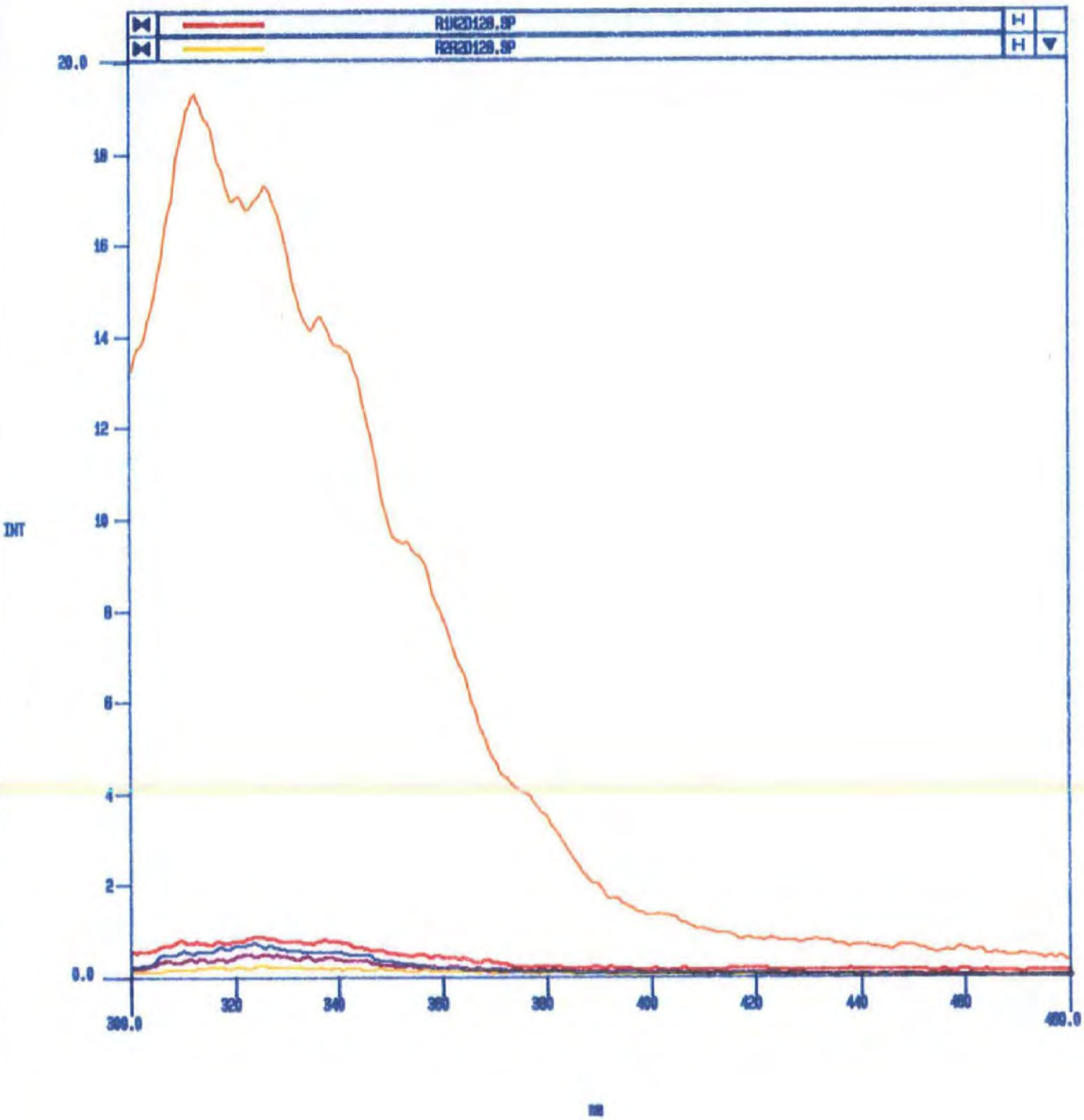
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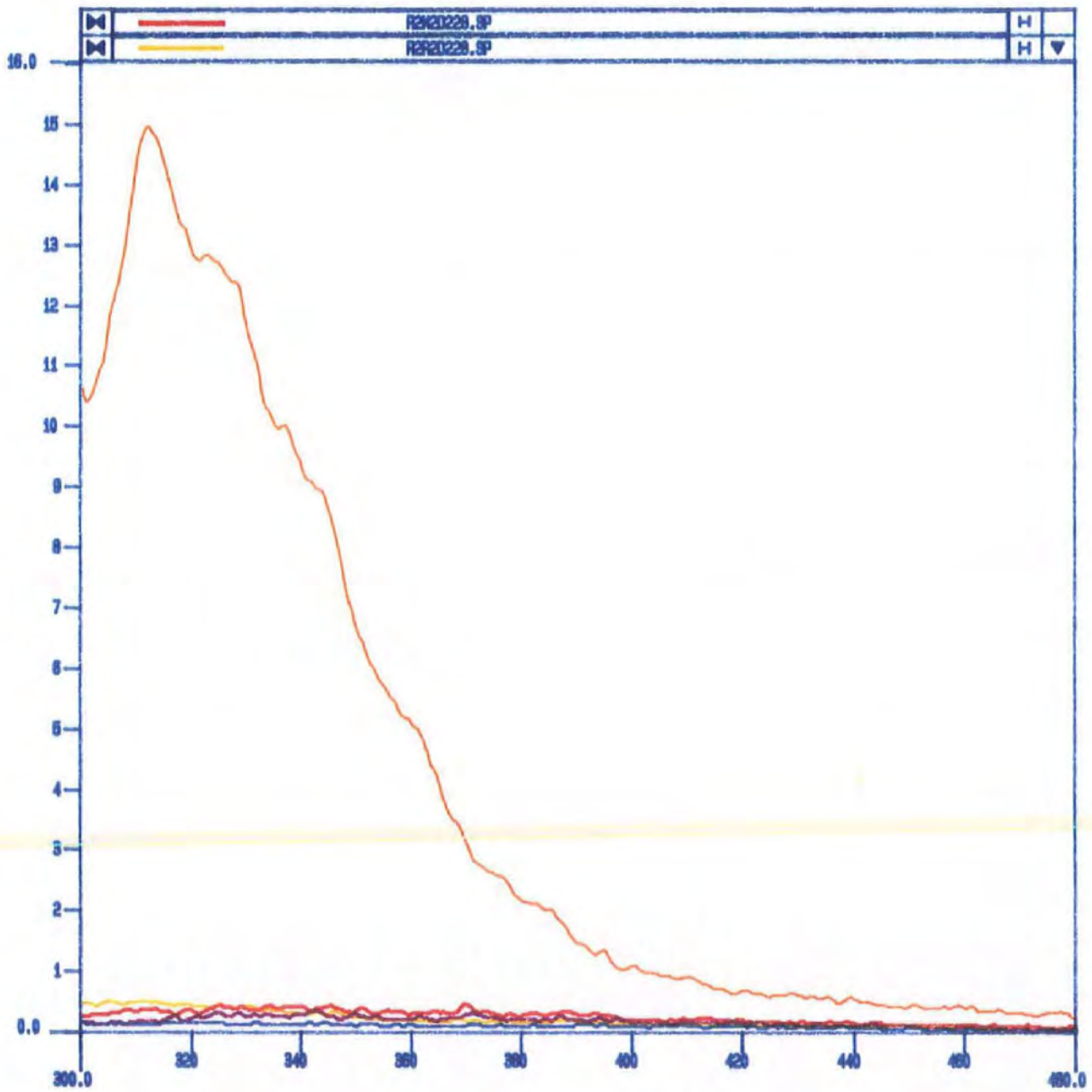
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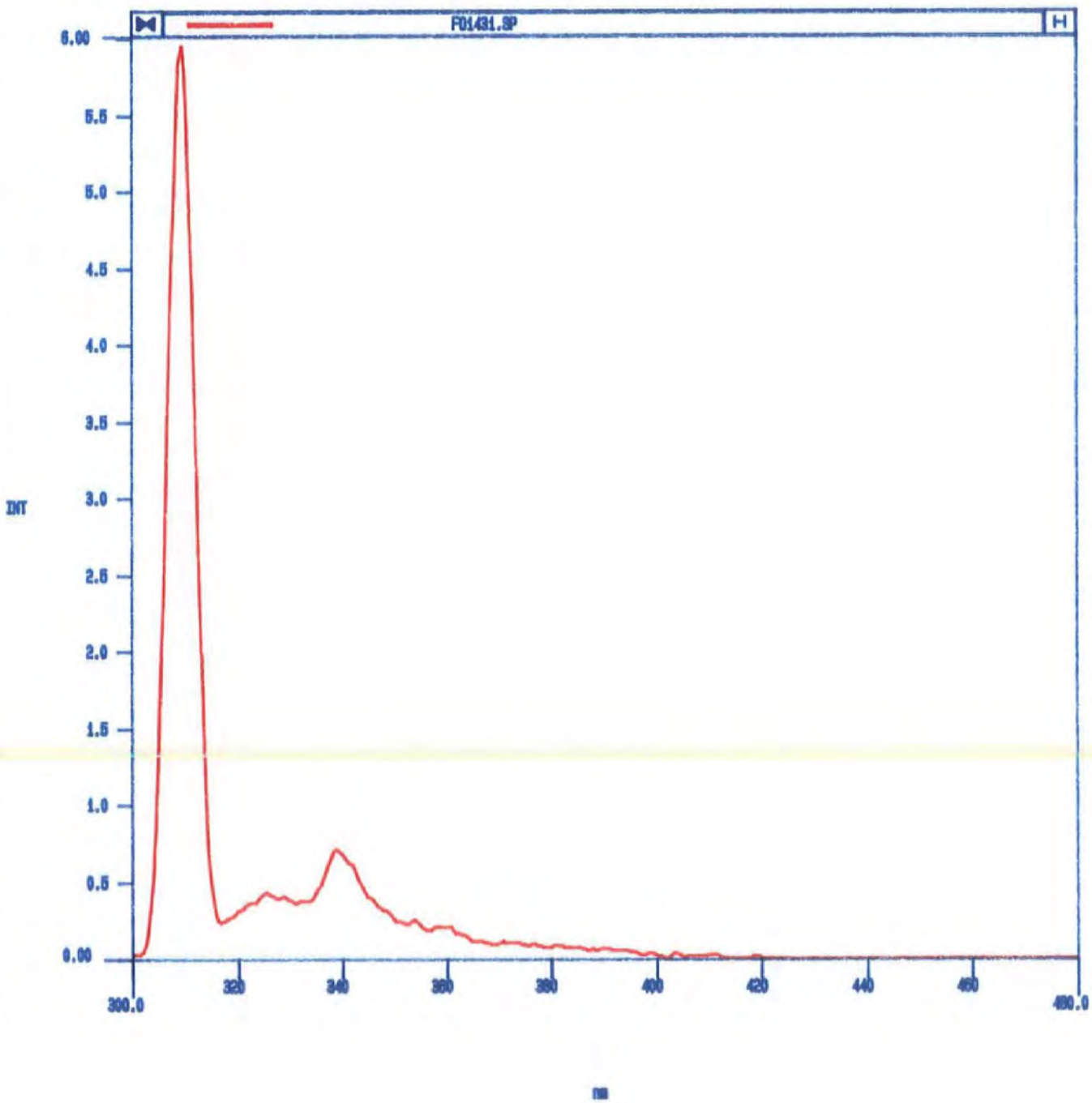
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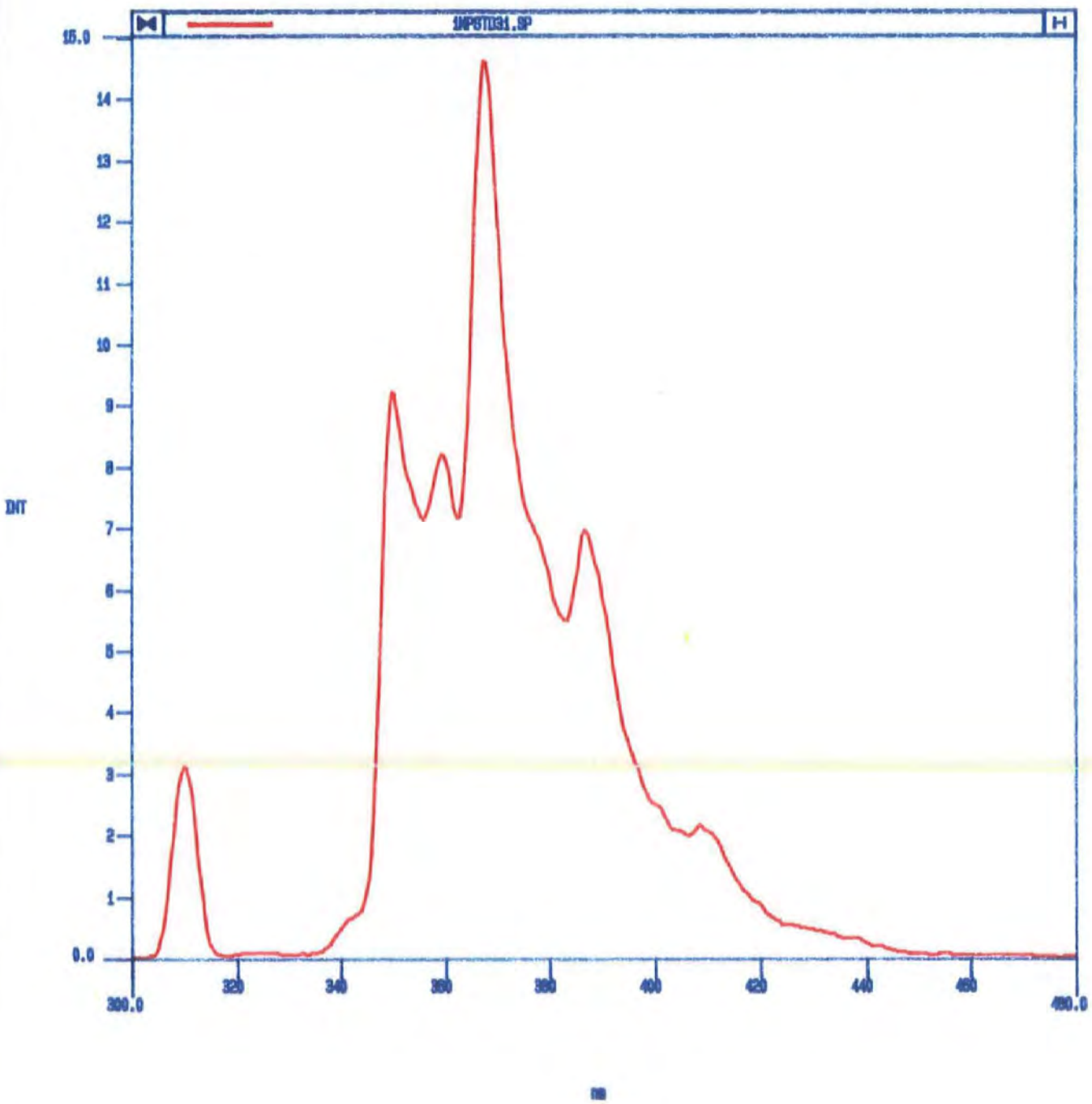
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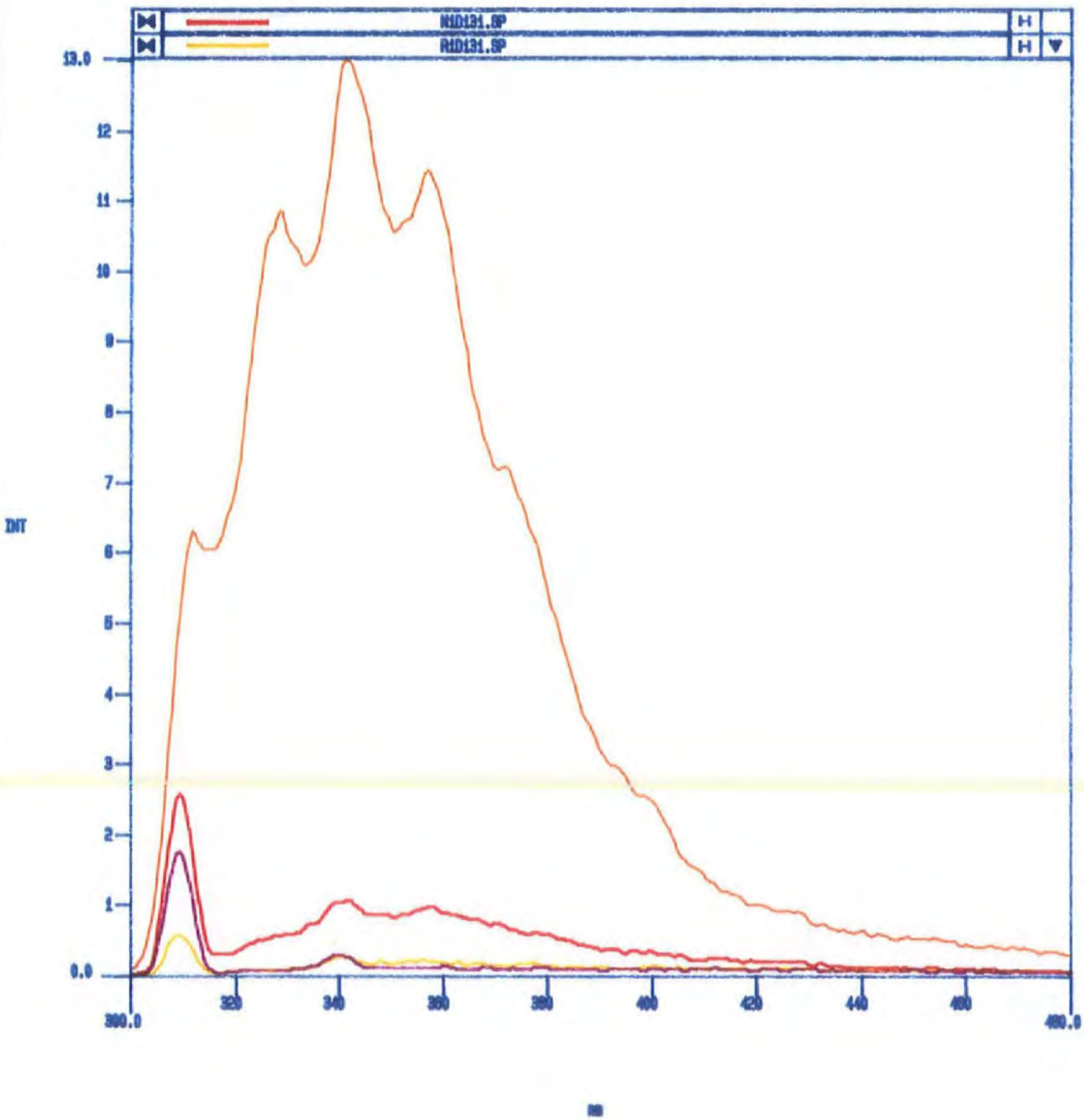
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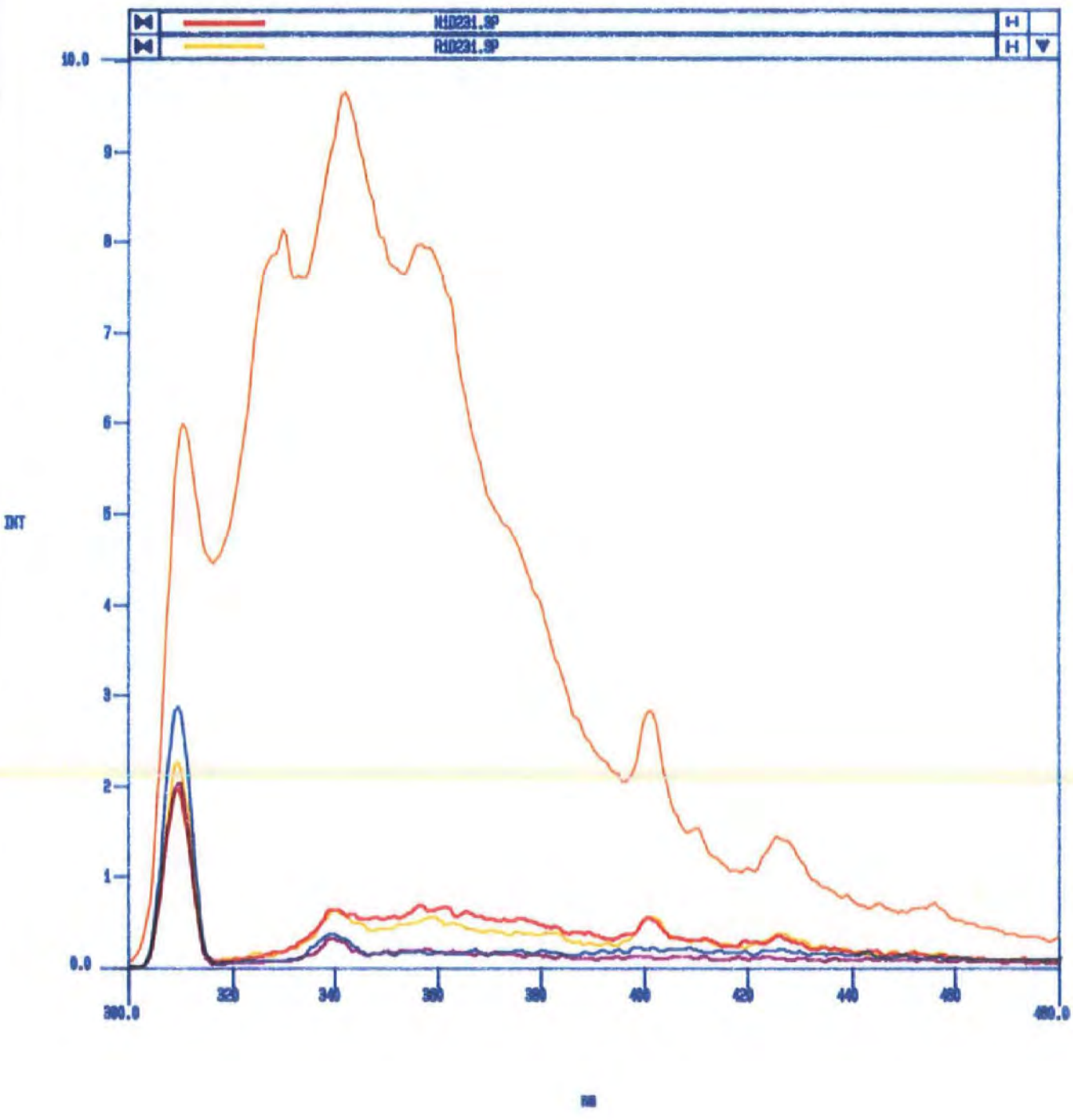
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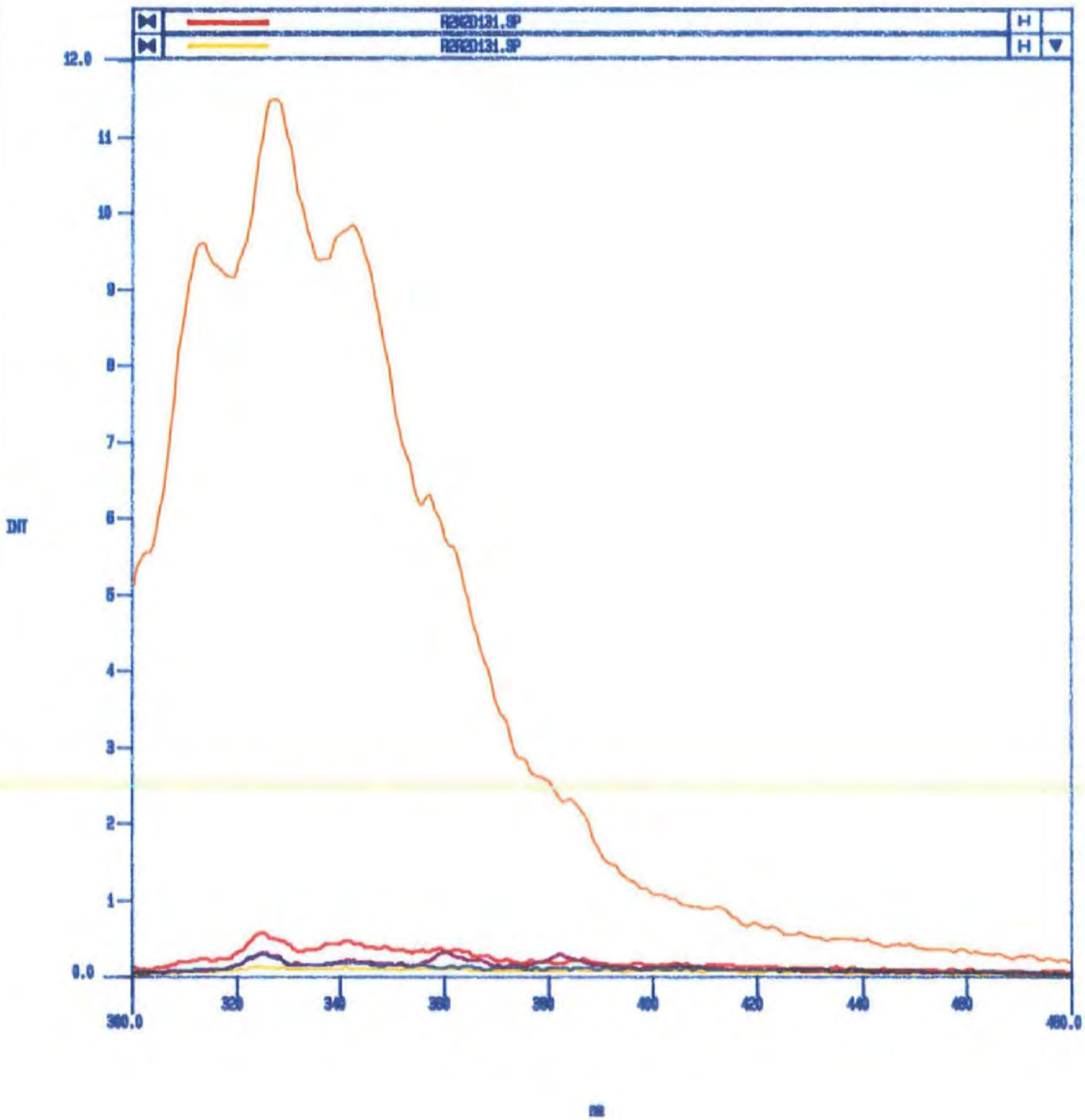
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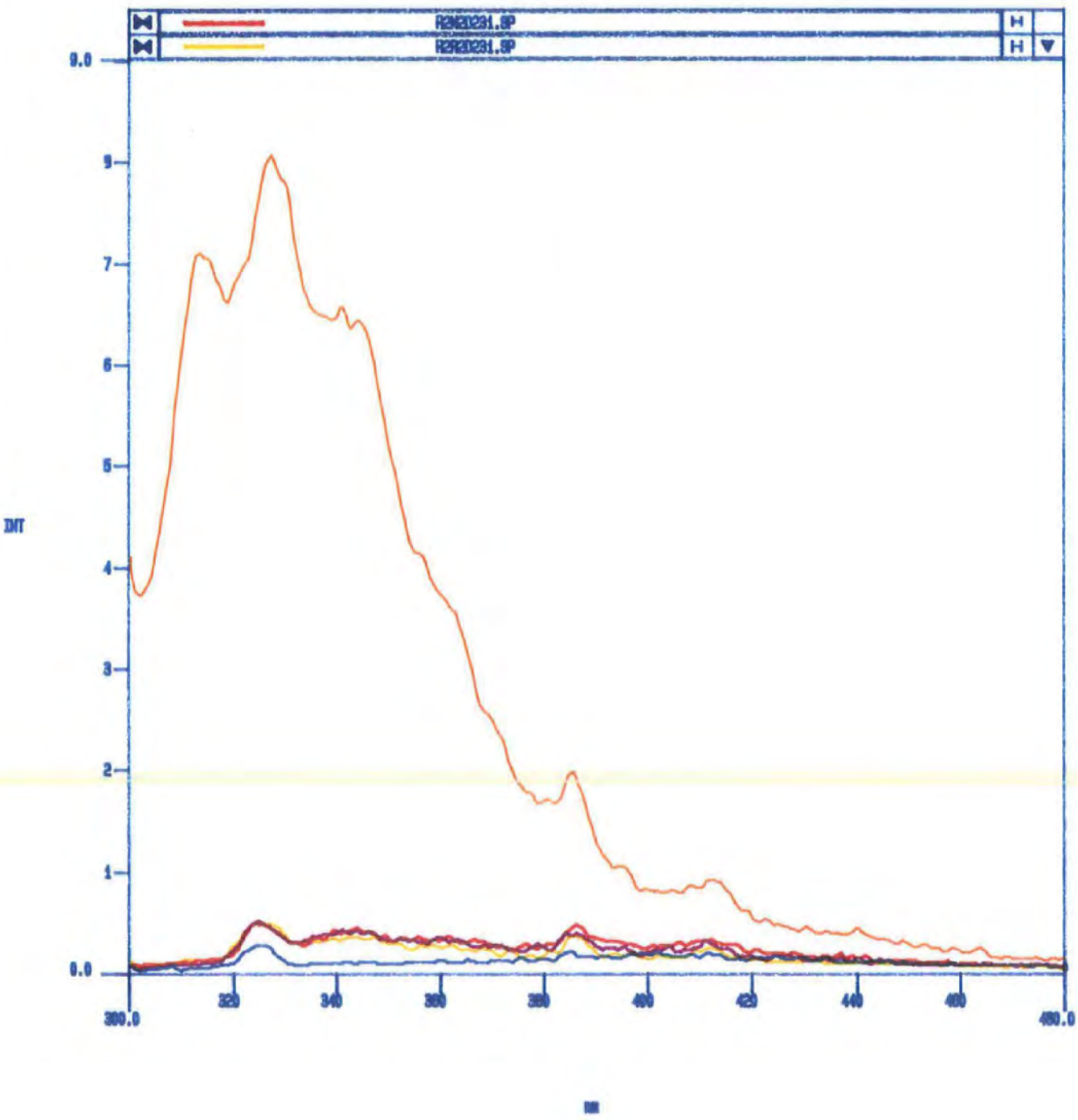
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