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RESPONSES OF THE DECAPOD CRUSTACEAN, *CARCINUS MAENAS*, FOLLOWING EXPOSURE TO ENVIRONMENTAL CONTAMINANTS: DEVELOPMENT AND APPLICATION OF MULTIPLE BIOMARKERS

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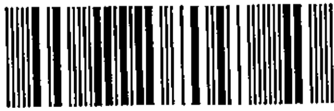
PhD 2004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RESPONSES OF THE DECAPOD CRUSTACEAN, *CARCINUS MAENAS*, FOLLOWING EXPOSURE TO ENVIRONMENTAL CONTAMINANTS. DEVELOPMENT AND APPLICATION OF MULTIPLE BIOMARKERS.

by

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A thesis submitted to the University of Plymouth

in partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences, Faculty of Science

and

Plymouth Environmental Research Centre

In collaboration with

Akvamiljo Aquatic Environment Research Centre,

Randaberg, Norway.

January 2004

Responses of the decapod crustacean, *Carcinus maenas*, following exposure to environmental contaminants - Development and application of multiple biomarkers.

Giles Marcus Watson

ABSTRACT

Marine, coastal and estuarine ecosystems continue to be contaminated by anthropogenic chemicals. Improving our mechanistic understanding of the toxicity of environmental pollutants to aquatic organisms is therefore an important priority. This work is focussed on detecting and measuring the molecular, cellular and physiological responses of the shore crab *Carcinus maenas* following exposure to environmental chemicals, and uses biomarkers to do so.

To better understand the response of *C.maenas* to polycyclic aromatic hydrocarbons (PAHs) a novel biomarker of exposure to this class of compounds was developed. Direct fluorimetric techniques revealed that the shore crab *C.maenas* eliminated metabolites of pyrene in the urine following waterborne exposure to this PAH in the laboratory. The levels of excreted metabolites were exposure concentration and time dependent. Levels of pyrene metabolites were also detected in the haemolymph and their levels were exposure concentration dependent. HPLC/F analysis of urine samples revealed metabolites were conjugates of 1-OH pyrene. Levels in urine determined by fluorimetry correlated well with HPLC/F and ELISA methods of analysis. Levels in haemolymph did not correlate well with the ELISA, due to sample matrix interference with the assay.

Detection of urinary PAH metabolites following laboratory exposure is evidence that the parent PAH has been taken up, metabolised by the enzymatic machinery of the organism and eliminated. Whilst this is an important finding and illustrates the response of *C.maenas* when faced with PAH exposure, confirmation of this phenomenon in the field is also required. Field trials revealed that comparable metabolites were eliminated in the urine following exposure to pyrogenic PAH discharges from a Norwegian aluminium smelter. The assay also provided evidence of exposure to petrogenic PAH in crabs from petroleum contaminated field sites and crabs exposed to crude oil. Both laboratory and field studies illustrated the potential for this exposure biomarker to be used for environmental monitoring.

The responses of *C.maenas* following exposure to combinations of PAH, metal and pesticide were measured using biomarkers of exposure and effect. Increasing the number of contaminants did not affect the responses of certain biomarkers (metallothionein, micronucleus, antioxidant activity), which were not induced or remained largely unchanged. These biomarkers also showed no significant differences between exposed and control groups. Specific biomarkers of exposure (urinary PAH assay) and effect (carboxylesterase activity) responded in a concentration dependent manner when exposed to PAH and pesticide, but the level of their response was not altered upon addition of further contaminants at each concentration. The lack of induction or alterations in biomarker responses unfortunately provides little additional information on the complex mechanisms of toxicity in organisms following exposure to these contaminants. Similarly, investigations into the effects of environmental chemicals on endocrine mediated processes in *C.maenas* did not provide conclusive information on mechanisms of endocrine toxicity. Exposure to 20-hydroxyecdysone and its insecticidal analogue tebufenozide had no gross effects on the processes investigated in *C.maenas* (moulting, vitellogensis and locomotor activity) and consequently, no suitable endpoints were established as markers of endocrine disruption in this species. The quantitative Vg ELISA proved to be applicable for monitoring potential endocrine disruption in female *C.maenas*, but only after the temporal progression of vitellogenesis is understood.

The present work has investigated and measured the responses of *C.maenas* following exposure to exogenous chemicals. This has resulted in a better understanding of PAH disposition, a field applicable PAH exposure biomarker and evidence for robust responses following exposure to multiple contaminants. It has also highlighted the need for greater understanding of endocrine mediated processes before endocrine disruption can be confirmed as a realistic mechanism of toxicity in this species.

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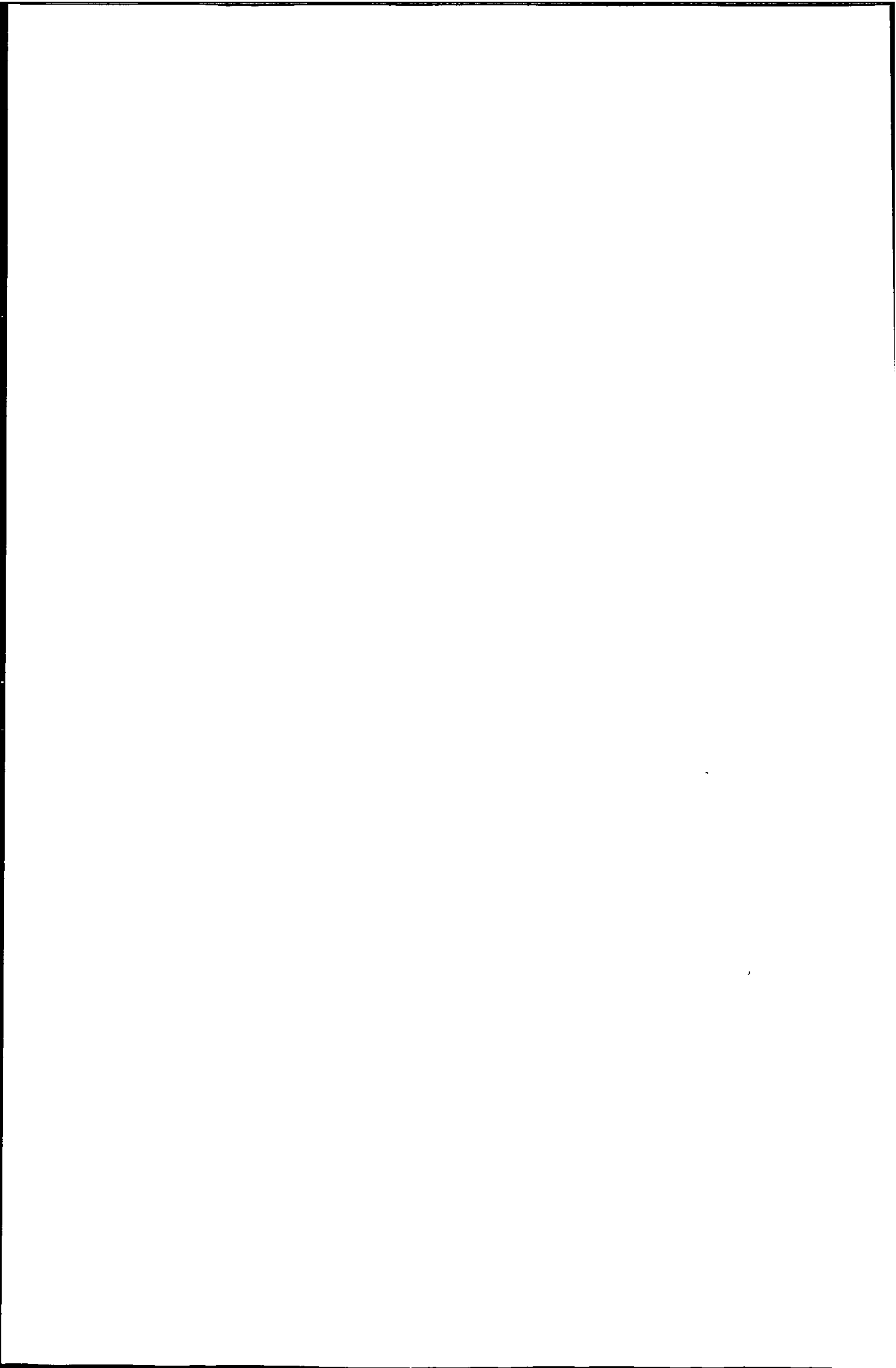

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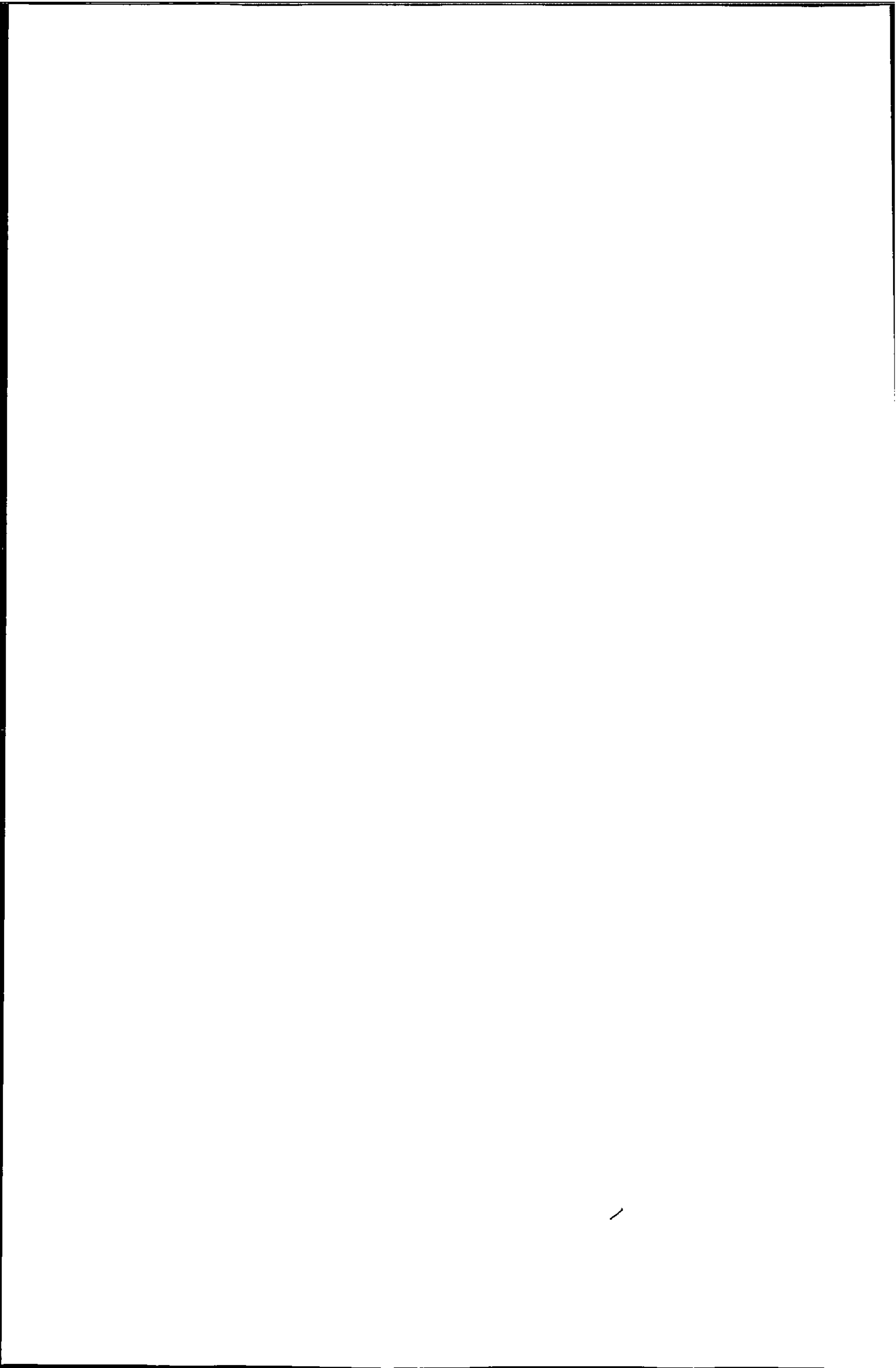

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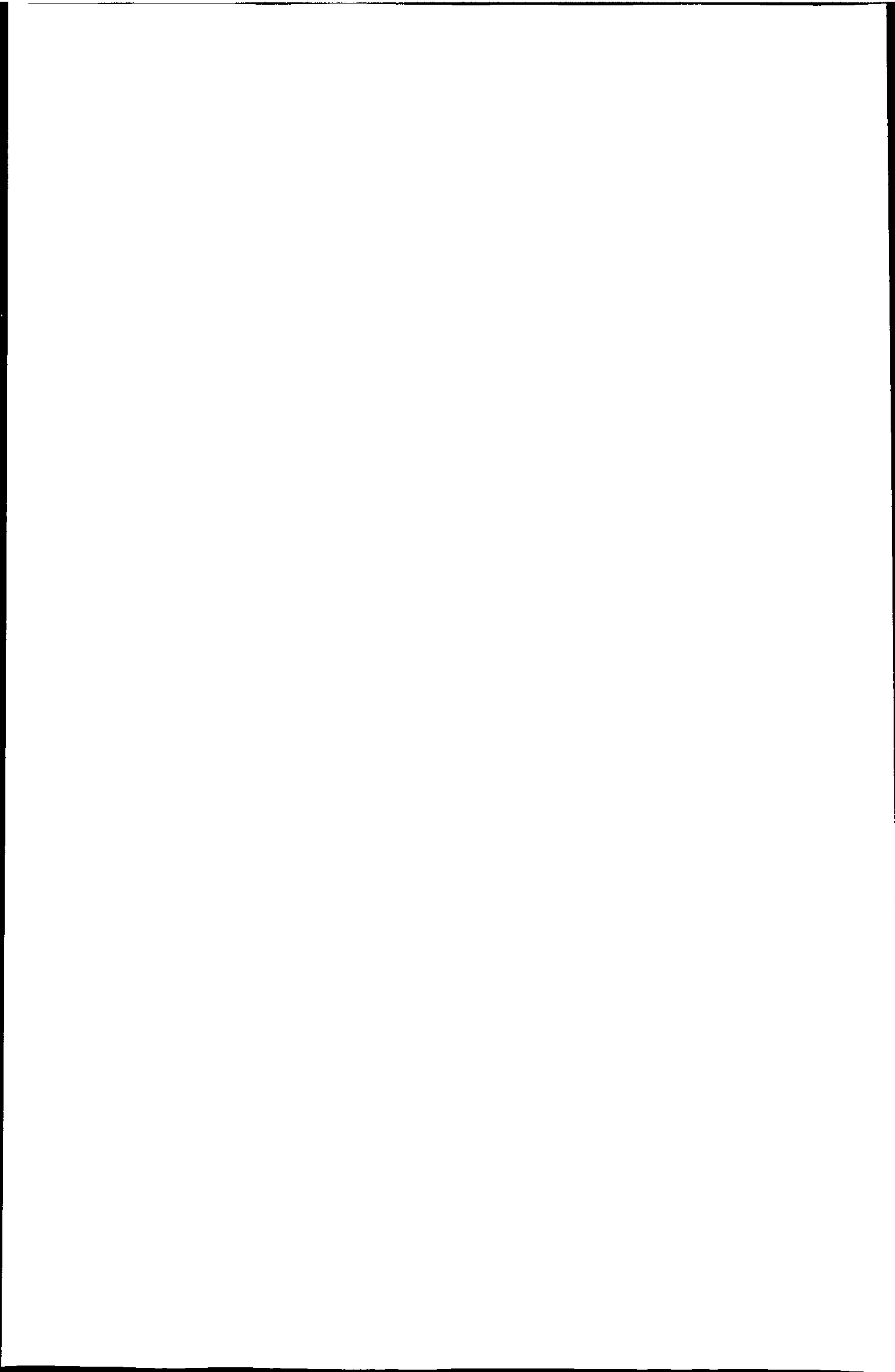


ACKNOWLEDGEMENTS

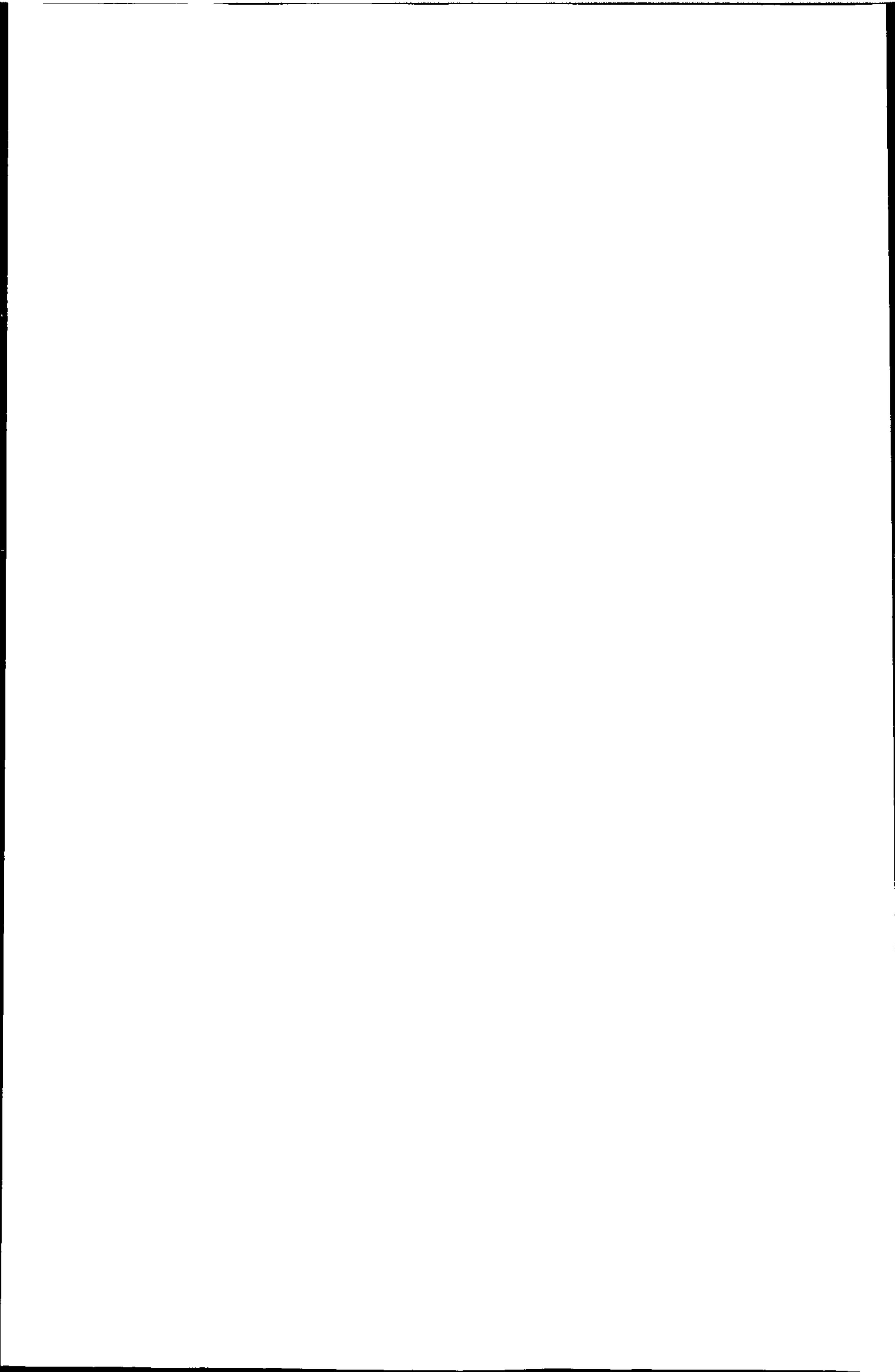
This research was made possible by the award of a Research Studentship from the Norwegian Science Council.

I would like to thank my supervisors Dr. Tamara Galloway and Prof. Mike Depledge for their guidance, inspiration and motivation and for reading my drafts and papers. I would also like to thank Dr. Shaw Bamber for his practical expertise and day-to-day support during my thesis work. I am grateful to Dr. Odd-Ketil Andersen and Anne Bjornstad from Akvamiljo for their help and the opportunity to collaborate with their laboratory and on field studies in Norway. I would also like to thank Dr. Gilberto Fillmann for his collaborative efforts and Prof. Jim Readman for his invaluable advice on PAHs and analytical chemistry. Also, I am grateful to Dr. Dave Lowe for his help and support on BEEP sampling trips and workshops.

I am thankful to all my fellow researchers in lab 404 who created such an enjoyable and supportive work atmosphere and particularly to Jo and Phil for their friendship and gig partnership. I would also like to thank all my non-local friends, who have provided me with some much needed mental relief from my PhD stress, and to Tom Foster, whose telephonic rants never failed to cheer me up and put my "problems" into perspective.

I would also like to thank the North and South coasts of Cornwall and Devon for their variety of beaches, reefs and points, which, whilst not being world class, have provided me with a welcome respite from the rigours of scientific research and kept me stoked and invigorated for 4 years.

Last, but most definitely not least, I am extremely grateful to my parents and family for all their support, both financial and personal, during the course of my PhD.



AUTHOR'S DECLARATION

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

This study was financed with the aid of a studentship from the Norwegian Science Council as part of the Strategic Institute Program (SIP) - Hormone Disruption in Marine Invertebrates.

A programme of advanced study was undertaken, which included postgraduate courses on COSSH assessment and teaching/demonstrating skills.

Collaborative studies were carried out with Akvamiljo, Aquatic Environment Research Centre, Norway, including involvement in and work for the EU's BEEP (Biological Effects of Environmental Pollution in coastal marine ecosystems) project.

Work included in this thesis was also submitted in fulfillment of a contract entitled "Evaluation of the risks posed by exogenous chemicals on the mechanisms of endocrine control in crustaceans", for the DEFRA (Department of Environment, Food and Rural Affairs) research program *Endocrine Disruption in Invertebrates and Top Predators.*

Relevant scientific seminars and conferences were regularly attended at which work was often presented and several papers were prepared for publication.

PUBLICATIONS:

G.M. Watson, O-K. Andersen, T.S. Galloway and M. H. Depledge (2004). Rapid assessment of polycyclic aromatic hydrocarbon (PAH) exposure in decapod crustaceans by fluorimetric analysis of urine and haemolymph. *Aquatic Toxicology,* **67**, pp127-142.

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PRESENTATIONS AND CONFERENCES ATTENDED:

Pollutant Responses in Marine Organisms (PRIMO) 12 Conference, Florida, USA, 2003. **G.M.Watson**, O-K. Andersen, T.S. Galloway and M.H. Depledge. Detecting a field gradient of PAH exposure in decapod crustacea using a novel urinary biomarker. (POSTER PRESENTATION).

UK - Japan Research Cooperation workshop, CSL, York, April 2003. Evaluation of the risks posed by exogenous chemicals on the mechanisms of endocrine control in crustaceans. (ORAL PRESENTATION).

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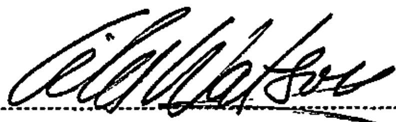
Signed 

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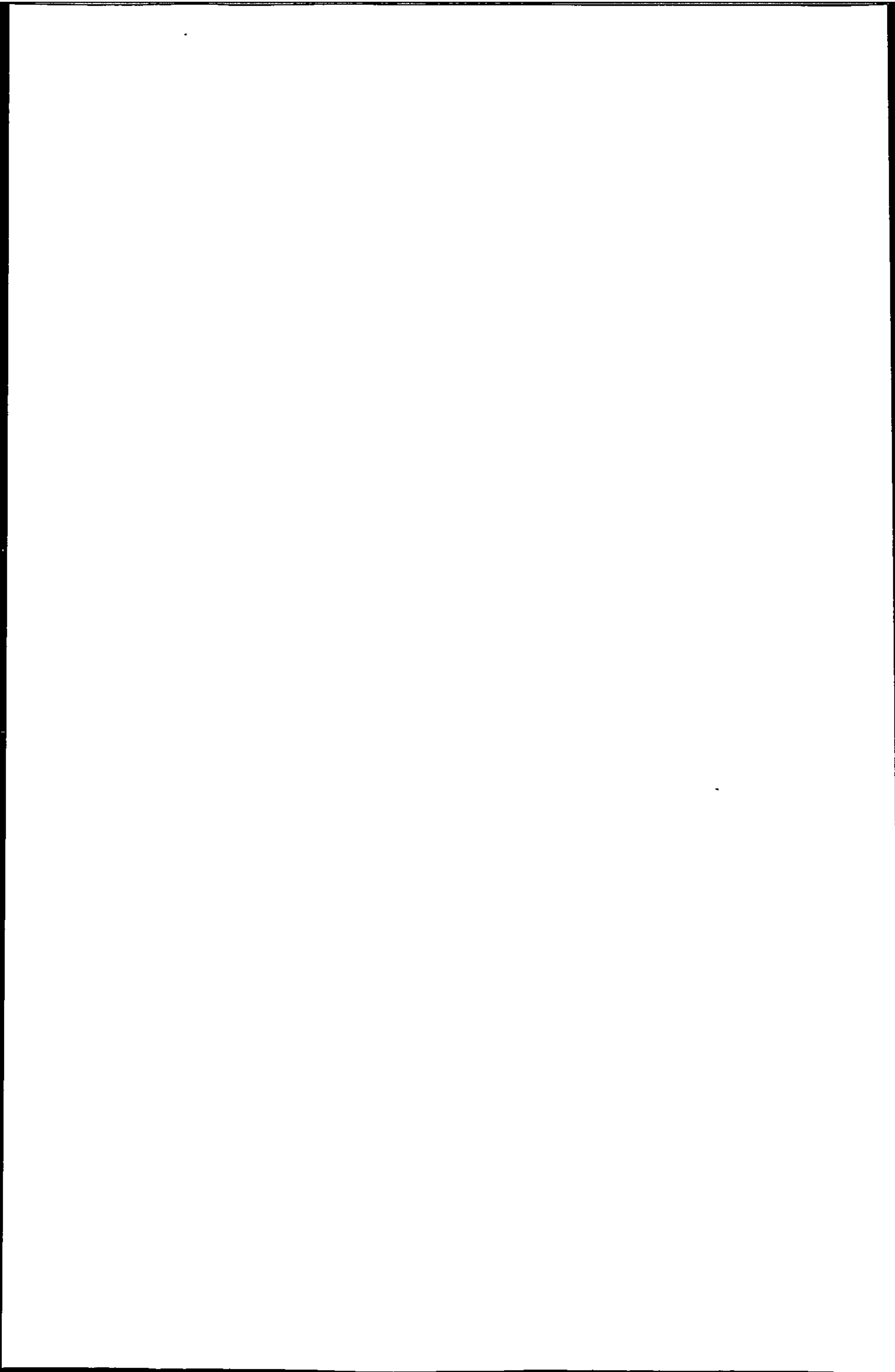

Chapter 1: Introduction: Understanding the responses of the decapod crustacean, *Carcinus maenas*, following exposure to environmental contaminants – Development and application of multiple biomarkers.

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1.1 Thesis outline

The world's marine, coastal and estuarine ecosystems continue to be contaminated by anthropogenic chemicals. Consequently, there is a requirement to improve our mechanistic understanding of the toxicity of environmental pollutants and to devise better methods of assessing the exposure of organisms that inhabit these ecosystems. The work in this thesis is focussed on understanding and measuring the molecular, cellular and physiological responses of a decapod crustacean, the shore crab *Carcinus maenas*, following exposure to environmental chemicals. Biomarkers at various levels of biological organisation, both novel and documented, were employed to achieve this. The main body of this work describes the development and field-testing (in the UK and Norway) of novel biomarkers to determine exposure to and the effects of environmental pollutants in *C.maenas*. To gain an understanding of how this organism responds when exposed to contaminant mixtures, such as those encountered in the environment, investigations into its responses following exposure to multiple contaminants were carried out using suites of biomarkers.

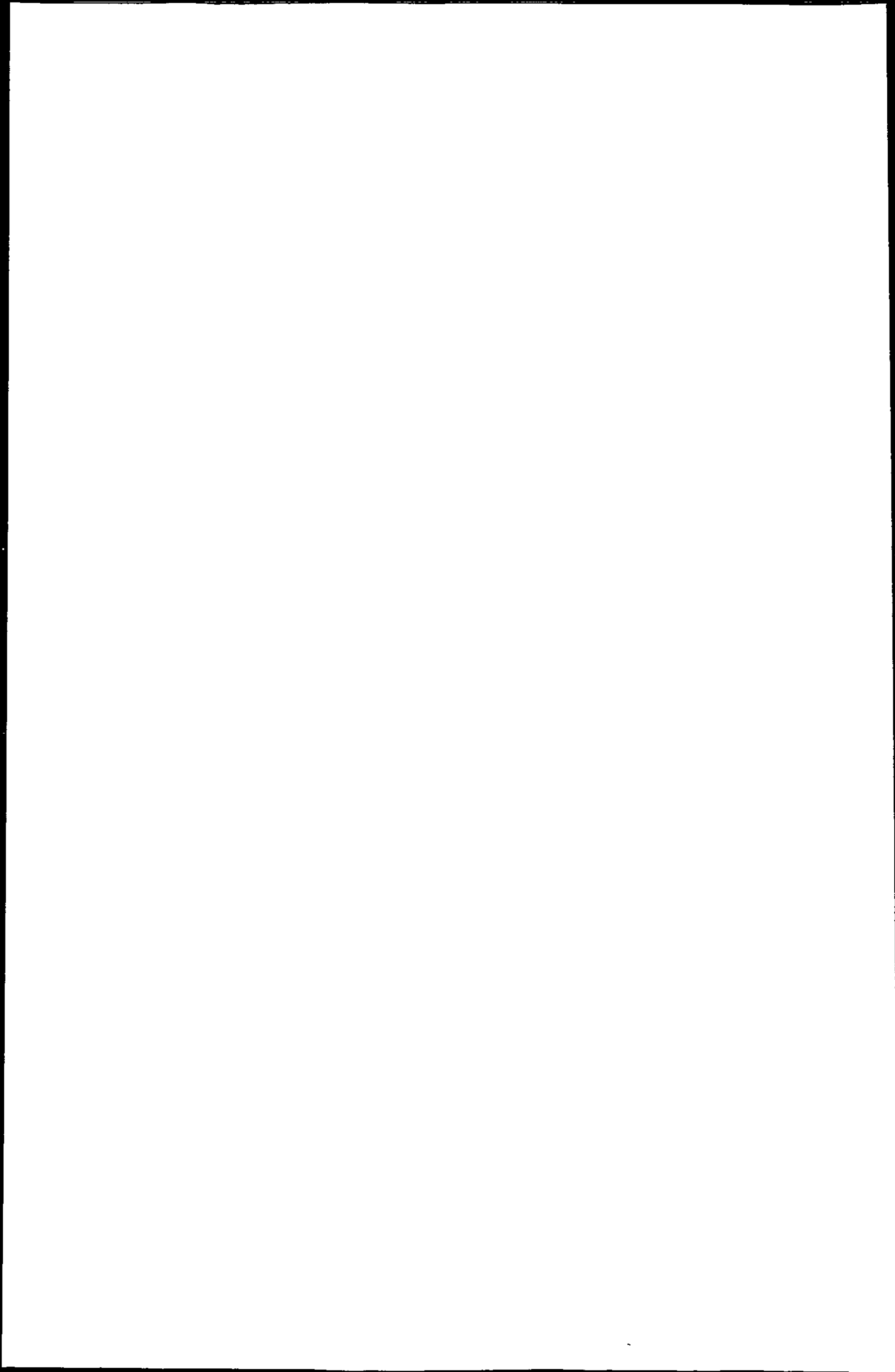
Chapter 2 describes a PAH exposure biomarker in *C.maenas*, using urine and haemolymph as the sample media. Analysis was perfomed using direct fluorimetry, an approach adapted from studies on fluorescent bile metabolites in PAH-exposed fish. The technique was developed and refined using laboratory exposures to a model PAH (pyrene). The chapter describes the detection of metabolite equivalents in the two sample matrices and the dose and time-dependent variation in their levels. Implications for potential effects, field applications and the possible identity of the metabolites detected are discussed.

Chapter 3 is concerned with the analytical and diagnostic performance of the urinary biomarker. Tests for matrix and inner filter effects were performed and

preliminary field trials were carried out. Comparisons were made between fluorimetry and alternative methods of analysis. Both field and lab-exposed samples were analysed by HPLC/F and immunoassay techniques to validate results derived from fluorescence analyses. HPLC/F also provided information on the identity of urinary metabolites. Additionally, a crude oil exposure was carried out as part of an interlab monitoring programme which provides evidence of the assay's applicability to oil spill situations.

Chapter 4 presents the results from a field study, carried out at a series of well-characterised sites in Norway, impacted with high levels of polycyclic aromatic hydrocarbons. The urinary PAH biomarker was applied along a gradient of PAH exposure from a contamination point source (an aluminium smelter). Additional biomarkers of sublethal effect at the biochemical and physiological level in *C.maenas* (measurement of heart rate and haemolymph carboxylesterase enzyme activity) were applied selectively alongside the PAH biomarker to further evaluate the impact and extent of the smelter discharges. Sediment data and body burdens of PAH in mussels and edible crab are also presented, providing data on contamination of these environmental compartments to compare with the biomarker results.

Chapter 5 describes a series of experiments designed to elucidate the responses of *C.maenas* following exposure to combinations of contaminants and to investigate any interactions occurring between biomarkers. The PAH biomarker was employed alongside a battery of other biomarker assays to determine what happens upon exposure to mixtures of trace metals, PAH and pesticides. Does the organism's response differ (i.e. are biomarker results changed) when it is exposed to several as opposed to single contaminants? PRIMER analysis of all biomarker data attempted to identify any differences between their responses in the different treatments.



Chapter 6 deals with a mechanism of toxicity of environmental chemicals that has received considerable attention in recent years; that of interaction with the endocrine systems of aquatic organisms. Experiments were designed to determine the effects of exogenous chemicals on endocrine-mediated processes in *C.maenas* and reveal possible endpoint biomarkers of endocrine disruption in this species. Exposure to two compounds, previously reported to interact with endocrine systems of arthropods (the ecdysteroid moulting hormone 20-hydroxyecdysone and the insecticidal ecdysone agonist tebufenozide) were carried out in an attempt to disrupt three selected endocrine mediated processes - moulting, vitellogenesis and locomotor activity. The rationale behind these experiments was to investigate whether exposure to certain exogenous chemicals causes disruption to endocrine processes in *C.maenas*. If so, can these changes be measured and used as biomarkers of endocrine disruption?

Chapter 7 is a summary and discussion of the overall findings from this research, how they fulfil the original aims and objectives and the direction future research should take in this area.

1.2 Aims and objectives

The primary aim of this thesis is to develop a better understanding of the effects of environmental contamination on the European shore crab, *Carcinus maenas*. One way of doing this is by employing biomarkers of exposure and effect. This thesis has utilised such an approach, investigating molecular mechanisms of toxicity using biomarkers and improved methods of detection for environmental pollutants in the biological fluids of this organism. The development and field evaluation of novel biomarkers for specific environmental contaminants in *C.maenas* has been central component of this research. *C.maenas* is a numerous and widespread representative of a group of important invertebrates, the decapod crustacea, and therefore information on the disposition and mechanisms of toxicity of environmental contaminants in this species is highly desirable.

Mechanisms of toxicity following exposure to single contaminants have received considerable attention in *C.maenas*, although the use of biomarkers for measuring organismal response following exposure to mixtures of contaminants is limited. Biomarkers themselves are also often developed and used under conditions of single contaminant exposure only. It follows therefore that a deeper understanding of the responses mounted by *C.maenas* following contaminant challenge can be attained only by investigating numerous mechanisms of toxicity following exposure to more environmentally realistic combinations of chemicals. This thesis aims to address this particular issue and uses numerous biomarkers to do so. Additionally, the use of multiple biomarkers in multi-contaminant systems allows us to investigate whether any changes occur in the response of individual biomarkers and whether biomarkers can be relied upon to provide meaningful results when numerous chemicals are causing toxicity. In the process, any interactions between biomarker responses can be identified, resulting in a more thorough understanding of the responses of individual biomarkers observed in the

field, where organisms are exposed to numerous contaminants and often to complex mixtures.

1.2.1 Specific aims

The experiments described in this thesis use biomarkers to measure the exposure and effects of sub-lethal concentrations of chemical contaminants upon *C.maenas*. It is hypothesised that the biological responses of *C.maenas* following exposure to environmental contaminants are indicative of their toxicity and the organism's attempts to compartmentalise, detoxify and excrete them. The specific aims of this work are as follows.

To better understand the disposition of polycyclic aromatic hydrocarbons (PAHs) in the biological fluids of *C.maenas*, following waterborne exposure in the laboratory.

To determine the extent, nature and time course of PAH metabolism in this species, and identify any metabolic products.

To develop an inexpensive, rapid and non-destructive technique that enables *C.maenas* to be used as an indicator of PAH exposure.

To validate/correlate any findings using other analytical techniques.

To apply the technique to PAH contamination situations in the field in order to test its suitability for environmental PAH monitoring.

To investigate, at the molecular and cellular level, the toxic responses of *C.maenas* to combinations of contaminants, using multiple biomarkers of exposure and effect.

To evaluate the effects and mechanisms of toxicity of selected chemicals on endocrine-mediated processes in *C.maenas*, with a view to developing a reliable biomarker for endocrine disruption in this species.

1.3 Pollution of estuarine, coastal and marine ecosystems

70% of the world's population (approximately 3 billion people) live within 60km of a coastline, and this percentage is increasing (Gray, 1991), with the rate of population growth in coastal zones approximately twice that of the global rate (Dewailly *et al.*, 2002). The coastal environment comprises a large proportion of the world's natural resources (Costanza *et al.*, 1997), and although only representing 10% of the total oceanic area, accounts for more than half of the oceans biological productivity. Over 2 billion people rely on seafood as a major source of protein in their diet (Dewailly *et al.*, 2002), yet despite supporting this large proportion of the world's population, the vulnerable estuarine, coastal and marine environments of the world are some of the most abused zones of our planet. They have been and continue to be dramatically impacted by human activities, with estuaries in particular subject to intense pressure (Forbes and Forbes, 1994). Much of the waste generated by human activities, both industrial and domestic, ends up in these environments of greatest economic and biological significance (Moore, 2002). As a result, coastal waters and sediments, particularly in industrialised and urban areas, may contain a multitude of contaminants. For example, the water column and sediments of Puget Sound, USA, contains thousands of different chemical compounds as a result of anthropogenic activities (Malins and Ostrander, 1991). Such chemical contamination has a deleterious impact on the quality of waters and sediments and poses a potential toxic risk to exposed aquatic organisms and to the ecosystems they underpin.

Before describing the nature of the most common compounds affecting coastal marine environments, it is pertinent to make a distinction between the terms

"contamination" and "pollution". Contamination has been defined as an anthropogenic "increase of a substance (the contaminant) in water sediment or organisms above the natural background level for that area and for the organisms" (Clark, 1992). "Pollution" is reflective of the effects such contaminants might have. Clark (1992) defines marine pollution as "the introduction by man, directly or indirectly, of substances or energy to the marine environment resulting in deleterious effects such as: hazards to human health; hindrance of marine activities, including fishing; impairment of the quality for the use of seawater; and reduction of amenities". Walker *et al.*, (2001) define a pollutant more simplistically as an "environmental chemical which exceeds background levels and has the potential to cause harm" where "harm" is defined as "biochemical or physiological changes which adversely affect individual organisms' birth, growth or mortality rates". Similarly, Moriarty (1983) defines pollutants as "substances that occur in the environment at least in part as a result of man's activities, and which have a deleterious effect on living organisms" and contaminants as "substances released by man's activities". Walker *et al.*, (2001) argue that whether a contaminant is a pollutant or not depends on its level, the organism in question and whether the organism is harmed. These authors conclude that the two terms can be used synonymously, since it is difficult to determine unequivocally that a contaminant has no potential to cause harm in any situation.

It has been estimated that approximately 100,000 chemical substances are at present in common use, and about 2000 new ones come onto the market every year (WHO, 2003). Clearly, the quantity, number and variety of different chemicals produced by man are continually increasing (Livingstone, 1993) and all such chemicals have the potential to reach aquatic environments. Inputs to estuarine and coastal ecosystems are numerous and include direct outfalls and discharges (municipal waste, industrial waste, sewage), riverine discharges, shipping (e.g. oil spills), offshore inputs (dredging spoil, sewage sludge) and atmospheric inputs (Clark, 1992). It has been estimated that 300 billion cubic metres of wastewater are discharged into the world's oceans and seas each

year (UNEP, 2003), and that 5 million tonnes of oil (petroleum hydrocarbons) reach the marine environment annually (Fossi *et al.*, 2000). In addition, most terrestrial, freshwater and air pollutants ultimately reach the ocean (Depledge, 1992) so marine environments can be considered the final repository for most anthropogenic wastes (UNEP, 2003).

The major classes of pollutants as described by Walker *et al.* (2001) include metals (particularly the inorganic heavy metals), hydrocarbons (petroleum products, crude oils), polycyclic aromatic hydrocarbons (PAHs), polycholorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated biphenyls (PBBs), organochlorine, organophosphate, carbamate and pyrethroid insecticides, phenoxy herbicides, rodenticides, detergents, chlorophenols, organometallic compounds (methyl mercury, tributyltin) and radioactive isotopes. Other contaminants include nutrients (N, P), nitrogenous compounds (nitrate, nitrite, ammonia), oxygen demanding compounds (putrescible materials in sewage effluent), organochlorine alkenes (Newman, 1998), environmental oestrogens in sewage effluent (Pinder *et al.*, 1999), pharmaceutical compounds (Christensen, 1998, Lanzky and Halling-Sørensen, 1997) and phthalates (Matthiessen and Law, 2002).

1.4 Modern challenges in Ecotoxicology

The field of ecotoxicology was developed to combine classical toxicology with ecology (and environmental chemistry) (Baird *et al.*, 1996). Numerous definitions have been proposed, ranging from Truhaut's original of "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context" (Truhaut, 1977) to modern refinements such as "the study of the adverse effects of materials on ecological systems" (Cairns, 1998), "the science of contaminants in the biosphere and their effects on constituents of the biosphere, including humans" (Newman,

1998) and the slightly less ambitious "study of harmful effects of chemicals upon ecosystems" (Walker *et al.*, 2001). Depledge (1994) suggests that the key problem facing ecotoxicological scientists is to "recognize the damaging effects of chemical pollutants on natural biota". In the last quarter century, ecotoxicologists have attempted to determine whether the myriad chemical contaminants entering the environment are likely to affect ecosystems adversely (Depledge, 1994). However, much early work in the field was not strictly faithful to the above definitions and was more concerned with detection and determination of "xenobiotic" chemicals in samples of animals and plants (Walker *et al.*, 2001). Xenobiotics are defined as foreign compounds, playing no part in the normal biochemistry of the organism. The biological significance of xenobiotic residues at the level of the organism (let alone of populations or communities) was often not addressed. As the science progressed, measures of biological effect were incorporated into ecotoxicological studies (Lam and Gray, 2003) but their relationship to higher levels of biological organization have continued to prove challenging (Moore, 2002, Malins and Ostrander, 1991).

It has long been argued that ecotoxicology is more "toxicology" than "ecology" (Baird *et al.*, 1996, Chapman, 2002). Much of the work within ecotoxicology to date has been more akin to the experimental tradition of toxicology and the behaviour/fate of chemicals in the environment. A large number of studies have also used laboratory-based approaches with limited ecological relevance, making the contrast between the proposed aims of ecotoxicology and the reality even more striking. Some authors believe that ecology has yet to make a significant impression on most "ecotoxicological" studies and insist that cooperation between the two traditional disciplines must be fostered to take the subject forward (Baird *et al.*, 1996). Toxicological studies often extrapolate potential ecosystem effects from the effects of contaminants in single species tests (the "reductionist" approach), but this may suffer from a lack of ecological relevance. The immediate effects of pollutants are at the organismal or sub-organismal level, but the

ecological significance is related to the indirect impact on the population of species (Moriarty, 1983). While a pollutant might kill half of the individuals in a population, the ecological significance may be minimal. In contrast, a pollutant that kills no individuals but retards development or reproduction may have considerable and wide ranging ecological consequences (Moriarty, 1983). The complexity of higher levels of biological organisation has precluded direct measures of effect on natural ecosystems and so research has focussed on lower levels of complexity with the hope of deducing ecosystem function from the sum of its parts (Forbes and Forbes, 1994). Clearly, understanding effects at the level of the organism and below (biochemical, cellular, histological, physiological and behavioural) is an extremely valuable endeavour, and allows linkage to specific causative agents with greater ease than perhaps community/population effects. However, the greatest challenge is to find ways of detecting the effects of xenobiotic chemicals on higher levels of organisation (Moore, 2002) (figure 1.1). To quote Forbes and Forbes (1994), the ultimate goal of ecotoxicologists is "determining the effects of pollutants on the structure and function of intact ecosystems, communities or assemblages".

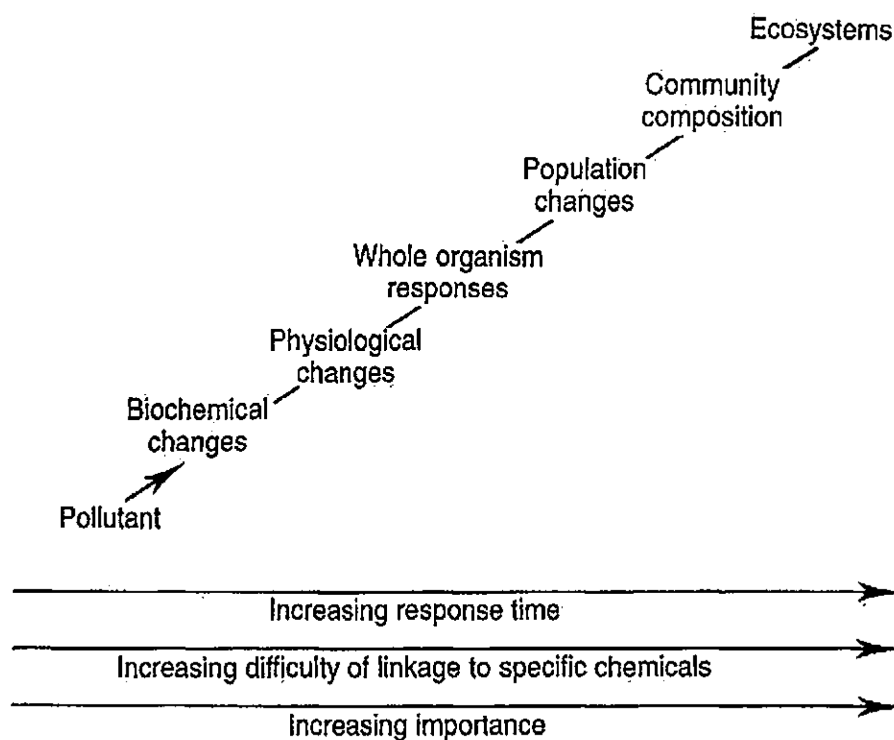
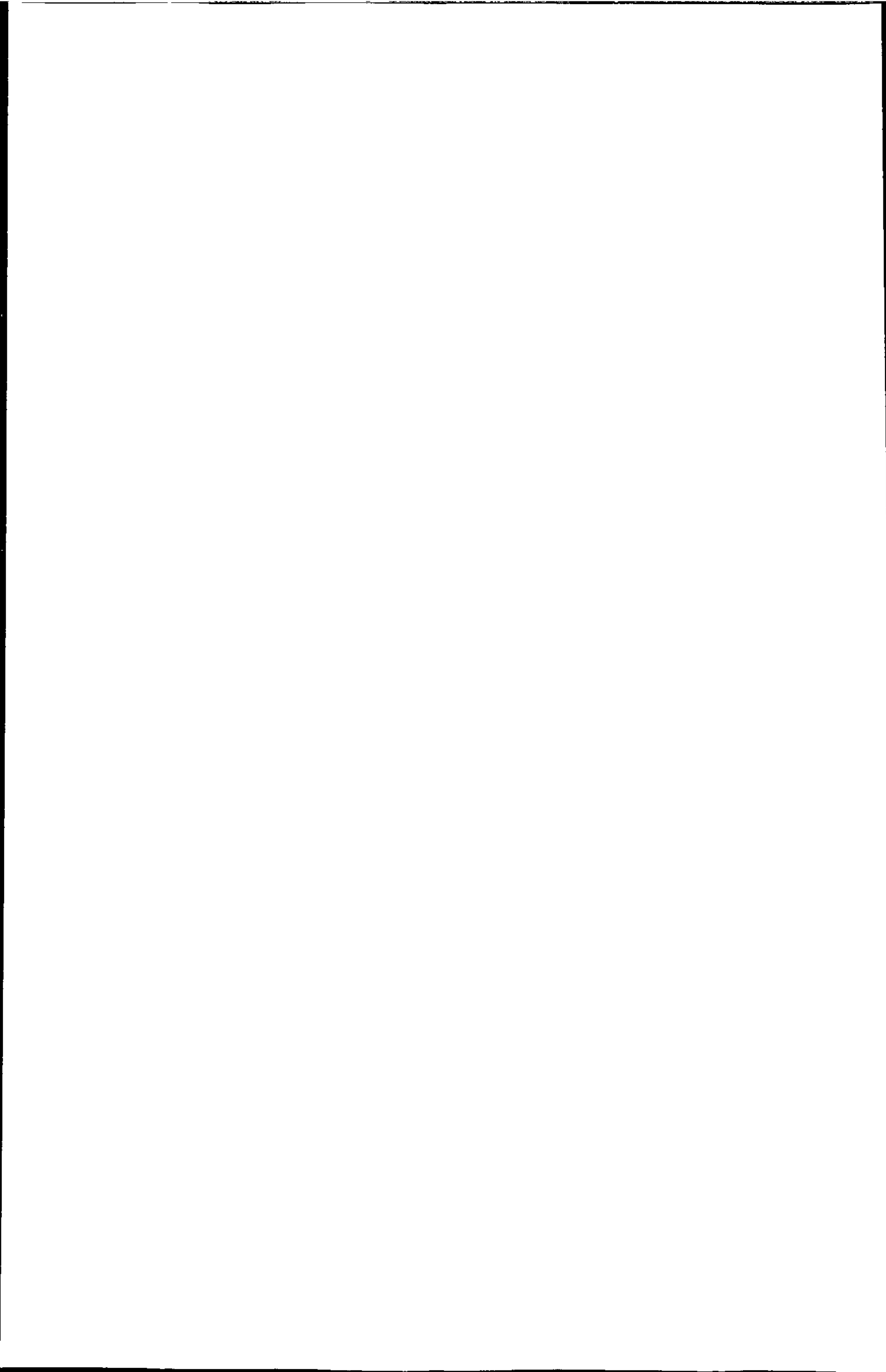


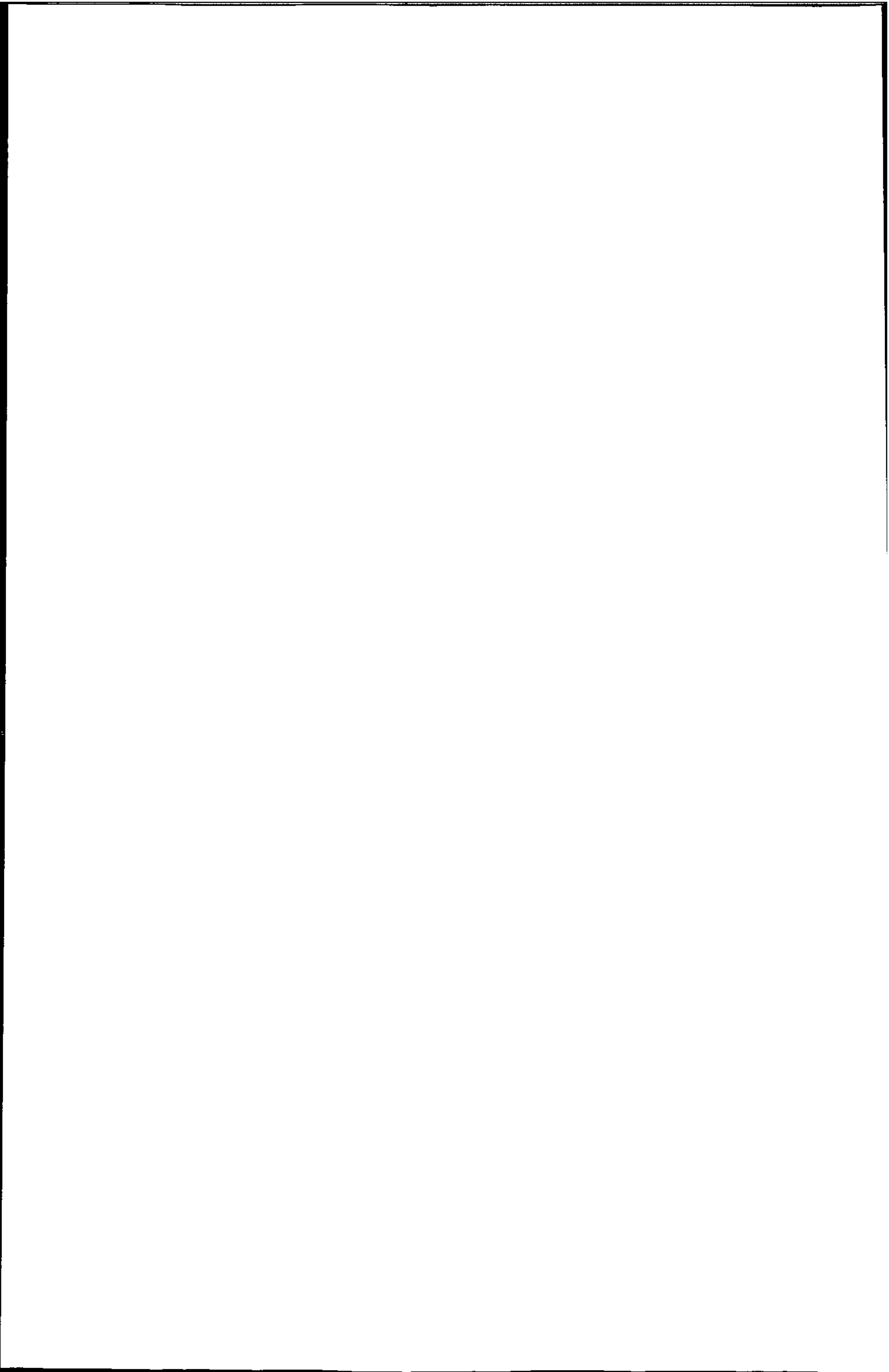
Figure 1.1 Relationship of linkages between responses at different levels of biological organisation (from Walker *et al.*, 2001).



1.5 Biomarkers and biomonitoring

The continued anthropogenic contamination of estuarine, coastal and marine environments worldwide has driven the development of methods to detect the impacts of such contamination on these fragile aquatic ecosystems. Environmental risk assessments traditionally rely on studies on the physico-chemical characteristics of compounds (e.g. QSAR approach) and laboratory toxicity tests (e.g. LC_{50}, LD_{50}) (Moore, 2002). Such procedures are practical and relatively easy to perform but are restricted to certain species and tell us little about bioavailability in the environment, sub-lethal toxicity and other adverse effects on organisms and ecosystems (Moore, 2002). Ecotoxicological studies have identified sub-lethal effects of pollutants at single levels of biological organisation, but whilst useful, these are still insufficient to evaluate the potential effects at the level of communities and whole ecosystems (Cairns, 1997). One of the approaches developed in an attempt to address this problem is the application of biomarkers at various levels of biological organisation. This is presented as a way to detect deleterious impacts on biochemical, physiological and behavioural processes of exposed individuals before populations, communities and ecosystems are adversely affected (Depledge, 1994).

Various definitions of biomarkers have been proposed. A broad definition is "a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect" (Depledge and Fossi, 1994). Depledge (1994) provides a more thorough definition in "biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples, or at the level of whole organisms that provide evidence of exposure to and or/effects of, one or more chemical pollutants". (N.B. Although the above definitions refer to chemical pollutants, other environmental stressors such as radiation, thermal pollution and noise may also influence biological responses).



Biomarkers have been used in pollution research in one capacity or another for more than 35 years (Handy *et al.*, 2003). The biomarker approach was proposed initially as a means to assess the health of pollutant exposed organisms and chart their responses to increasing pollutant stress (Depledge, 1992, Depledge and Fossi, 1994). Biomarkers can be utilised to determine where on a health-disease continuum an organism lies, since it is assumed that a progressive deterioration in health, eventually fatal, results from exposure of healthy individuals to increasing pollutant loads (Depledge and Fossi, 1994) (figure 1.2). This is a known as the Multiple Response Paradigm (Depledge, 1992).

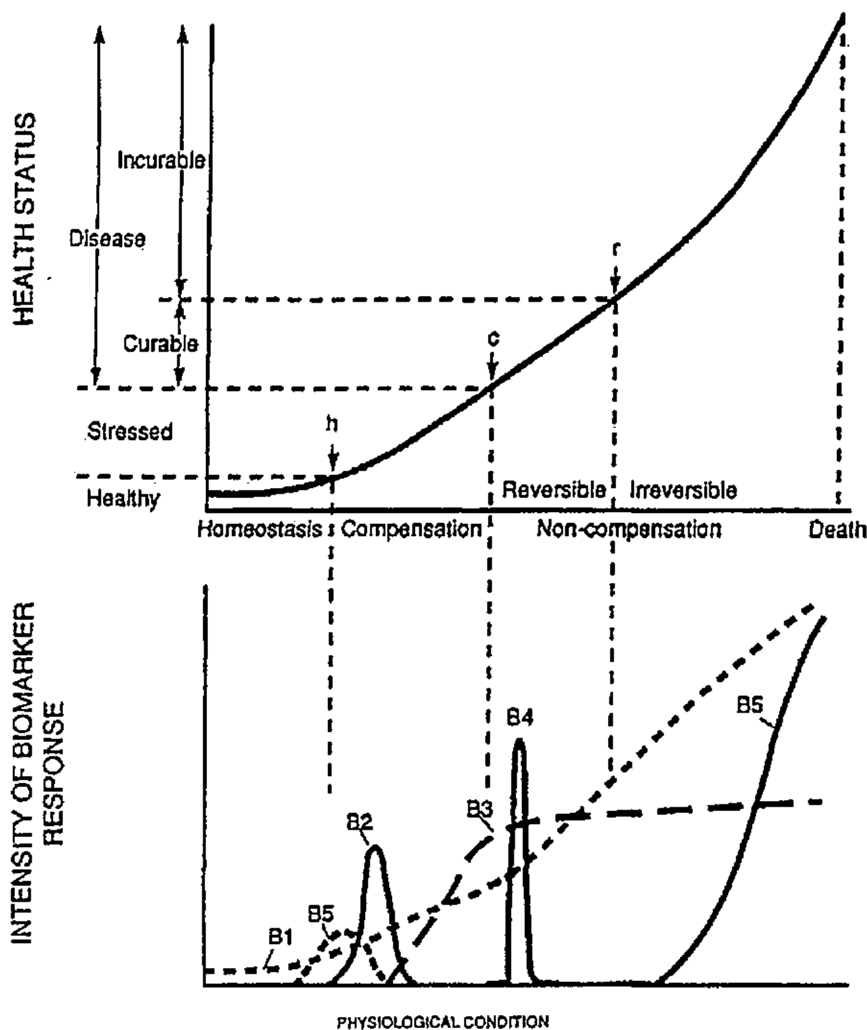


Figure 1.2 The health-disease continuum: The relationship between health status, biomarker response and physiological condition. (h= the point at which departure from normal homeostatic response is initiated, c= the limit for prevention of overt disease by compensatory responses, r= the point beyond which repair mechanisms are unable to reverse pathological damage) (from Depledge, 1994).

As an organism is exposed to increasing concentrations of a pollutant, it will mount biochemical and physiological responses in order to tolerate, detoxify or excrete that pollutant stress. Such compensatory responses are not as apparent as overt disease, but signify departures from health. Within this compensatory zone, the organism's ability to respond to further environmental challenges is compromised, which may adversely affect its chances for survival. Should the organism be unable to tolerate, detoxify or excrete the pollutant load, pathological response will result in disease then death (Depledge, 1994). Should the quality of the organism's environment improve and pollutant stress be reduced or removed, recovery is still possible as repair mechanisms may be able to restore compensatory responses. The organism may therefore be able to return from a diseased to a healthy state (Depledge and Fossi, 1994). Biomarkers provide a way of determining where on this health status curve an organism lies and identify any sub-lethal departures from health before overt disease is established.

It is also clear that the application of multiple biomarkers offers greater possibilities for the detection of effects than do single biomarkers. Departures from homeostasis at various levels of biological organisation can be detected by simultaneous use of biomarkers at each of those levels. Suite of biomarkers can potentially offer a comprehensive early warning of deleterious effects to marine organisms and their dependent ecosystems and this has been proposed as a way to assess ecosystem integrity (Depledge and Fossi, 1994) (figure 1.3). This provides an opportunity for remediation to reverse any pollutant-induced departures from health before permanent damage is inflicted.

Ideally, biomarkers should also be capable of indicating any alterations to "Darwinian fitness". Any biomarker response that can be related to impairment of growth, reproductive output or energy utilisation is invaluable, since this has significant implications for the survivorship of the individual. However, such causal links are difficult to establish and more difficult still is linking biomarker responses at the level of the

individual and below, with adverse affects at the population, community and ecosystem levels. These are the greatest challenges presently facing ecotoxicologists (Moore, 2002).

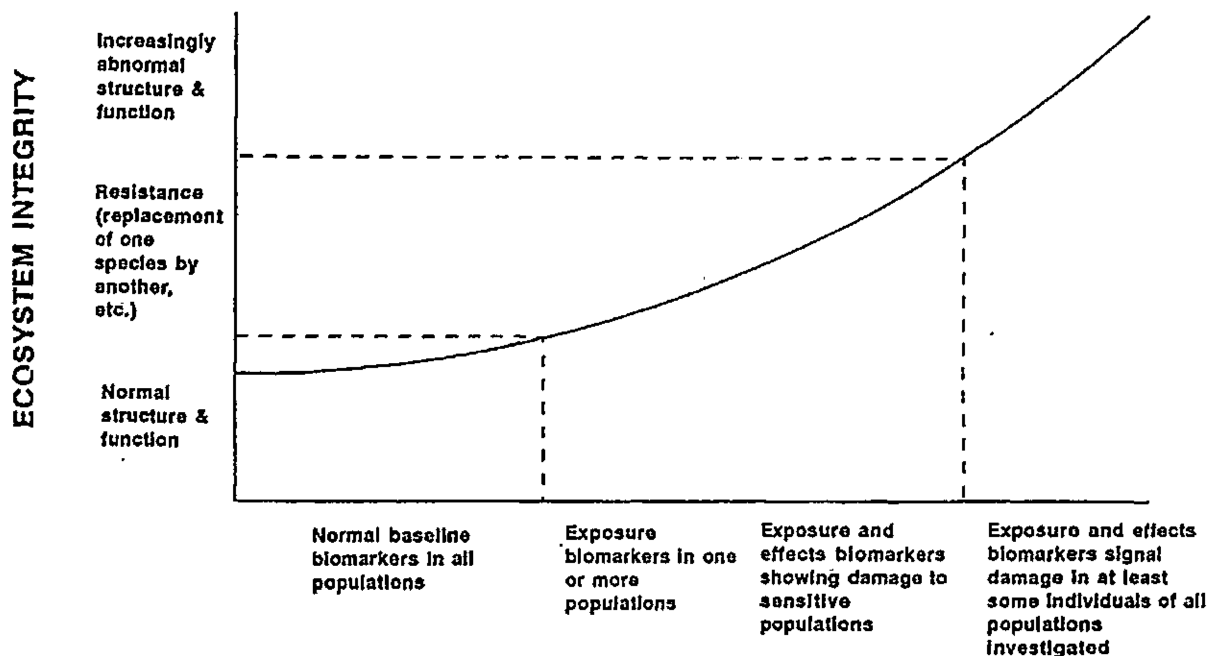
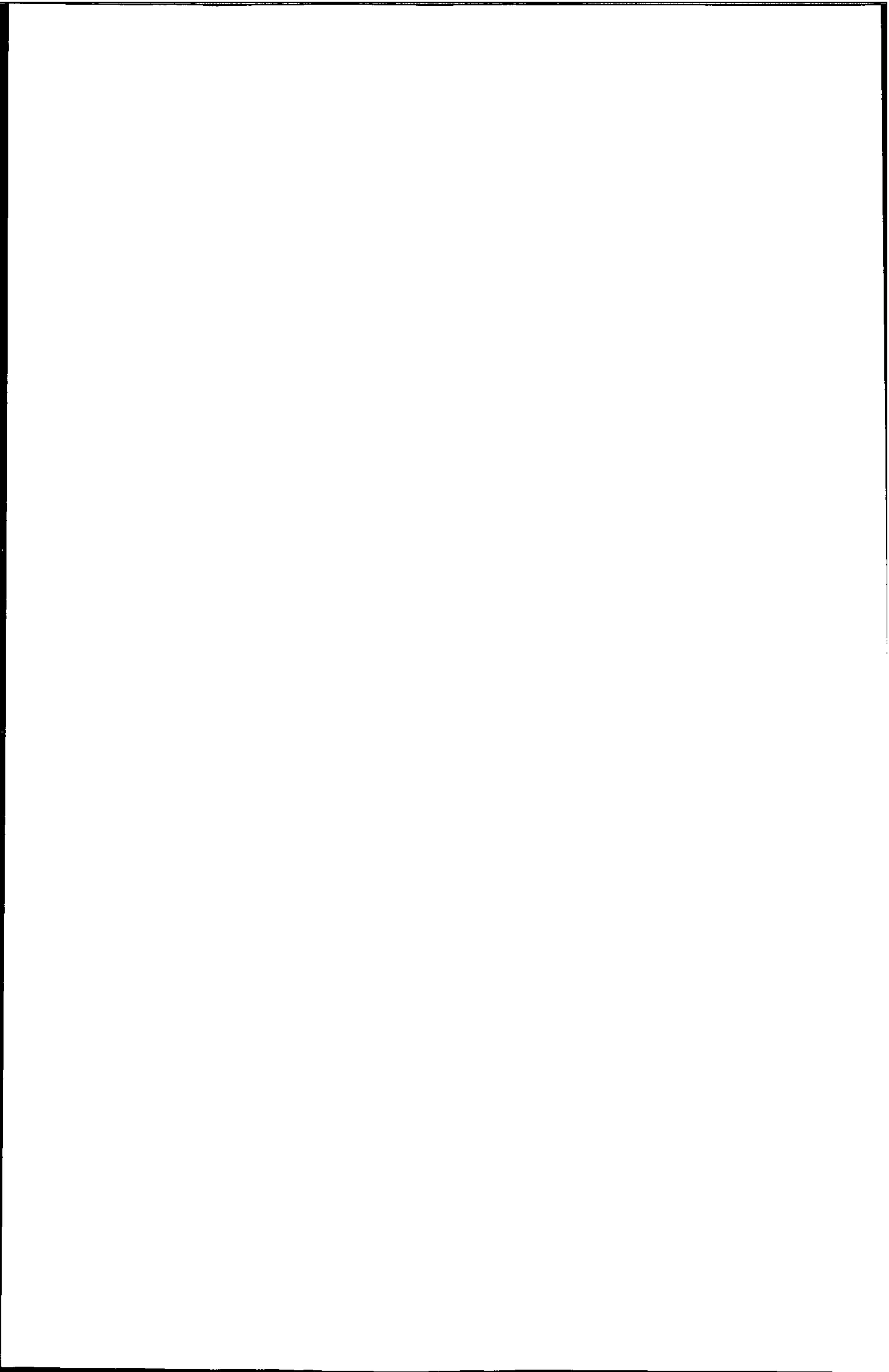


Figure 1.3 The use of biomarkers to assess ecosystem integrity (from Depledge and Fossi, 1994).

Biomarkers are broadly of 4 different classes, (assigned by Depledge, 1994 and Depledge and Fossi (1994)). Class 1- Biomarkers of exposure; class 2- Biomarkers of effect; class 3- Biomarkers of exposure/effect; class 4- Biomarkers of latent effect.

A biomarker of exposure indicates an organism (or population or community) has been exposed to general or specific pollutant stress. Examples include the detection of metal-binding metallothionein proteins induced by exposure to certain trace metals. Whilst these biomarkers signify that exposure has taken place, they tell us little about the potential deleterious consequences for the individual or its population (Depledge and Fossi, 1994).

A biomarker of effect signals an organism (or population or community) has been affected (normally adversely) by exposure to one or more pollutants, although often this does not provide information on the precise nature or identity of the pollutant stress (Depledge, 1994). Biomarkers of effect include altered heart rate in invertebrates upon



exposure to PAHs and trace metals (Fossi *et al.,* 2000, Bamber and Depledge, 1997a, Lundebye and Depledge, 1998) and reduced haemocyte lysosomal stability in bivalves (Lowe *et al.,* 1995, Cheung *et al.*, 1998). An exposure/effect biomarker indicates that an organism (or population or community) has been exposed to single or multiple pollutants and that this exposure is linked to an adverse effect (Depledge, 1994). Inhibition of acetylcholinesterase activity in marine bivalves and crustaceans, following exposure to organophosphate and carbamate pesticides, is an example of an exposure/effect biomarker (Lundebye *et al.*, 1997, Galloway *et al.*, 2002a). Inhibition is specific to exposure to these classes of pesticides and reduced cholinesterase activity has deleterious implications for neural function in the exposed organism.

Finally, there are the class 4 biomarkers, or biomarkers of "latent effect", which indicate that previous exposure to pollutant or physiological stress, from which the organism may be fully recovered, may limit the ability to survive or adapt to future environmental challenges (Depledge, 1994). Scope for Growth in marine bivalves (Widdows *et al.,* 2002, Widdows and Johnson, 1988) is such an example. Whilst individuals with relatively reduced Scope for Growth (e.g. reduced energy stores) may survive well in favourable environmental conditions in the absence of pollutant stress, they may be more vulnerable to pollutant stress following a contamination incident.

Further biomarker distinctions have been made by Depledge (1994), with the introduction of the terms "functional" and "non-functional" biomarkers, to discriminate between biomarkers that are integral components of the compensatory response or detoxification mechanism initiated by pollutant exposure and those that are not. Functional biomarkers include induction of metallothionein, which sequesters trace metals that might otherwise interact with cellular targets and initiate toxic effects. Non-functional biomarkers, e.g. serum acetylcholinesterase inhibition, signal contaminant exposure and/or effects but do not play a role in the prevention of toxic effects.

Combining exposure biomarkers with biomarkers of effect and biological damage provides data on the distribution of deleterious impacts, allowing environmental managers to make informed decisions regarding ecosystems at risk and take appropriate remediation measures. The biomarker approach is now an integral part of what has become known as the RAMP (Rapid Assessment of Marine Pollution) program. This is a program designed specifically to develop and apply simplistic, non-destructive, rapid and inexpensive assays for determining pollutant exposure and impacts on natural ecosystems (Depledge, 2000, Wells *et al.*, 2001, Galloway *et al.*, 2002b). Such assays are particularly important in developing nations whose coastlines are under immense anthropogenic pressure and in urgent need of pollution monitoring. The very nature of RAMP techniques means developing countries without the resources and skills to carry out complex and laborious water/sediment based analyses can still obtain meaningful data on the pollution status of their marine environments.

1.5.1 Invertebrates and biomonitoring

The present thesis is concerned specifically with biomarkers of exposure and effect in invertebrates - a group of organisms whose suitability for biomonitoring studies has been highlighted in recent years (Depledge and Fossi, 1994).

Invertebrates make up 95% of all recognised animal species (Barnes, 1968) and are major components of all ecosystems, occupying numerous trophic levels. The diversity and abundance of invertebrates far exceeds that of vertebrates and they occupy important positions in every food web. They are major food sources for both carnivorous invertebrates and vertebrate species and are fundamental in the transfer of energy to higher trophic levels. The loss of such important constituents of an ecosystem might jeopardize the viability of a diverse array of organisms (deFur *et al.*, 1999). Additionally, invertebrate populations are often numerous, so sampling for biomonitoring purposes does not

significantly affect population dynamics (Depledge and Fossi, 1994). In aquatic ecotoxicological studies and in contrast to fish, invertebrates are relatively restricted in their spatial ranges and are arguably therefore more useful for evaluating localised contaminant impacts (Fossi *et al.*, 2000). Our increasing knowledge of the biochemistry and physiology of invertebrates also allows biomarker responses to be interpreted with respect to the risks posed to their health (Stegeman *et al.*, 1992, Depledge and Fossi, 1994).

In the present thesis, the specific invertebrate used for study is the decapod brachyuran crustacean *Carcinus maenas*. It is an extremely eurythermal (6-18°C) and euryhaline (4-33ppt) species (Crothers, 1967), distributed widely along the coastal areas of NW Europe from Morocco to southern Iceland (Crothers, 1968). Due to its widespread distribution and predominantly estuarine habit, it may be exposed to a broad range of anthropogenic contaminants, yet it remains common and abundant. This suggests the mechanisms that allow this species to tolerate widely fluctuating environmental conditions (salinity, temperature, aerial exposure) afford it a degree of tolerance to contaminant exposure (Hebel *et al.*, 1997). This hardy species is well suited to laboratory studies and as a result is used extensively in ecotoxicological research (Fillmann *et al.*, 2002, Legras *et al.*, 2000, Astley *et al.*, 1999, Weddeburn *et al.*, 1998, Bamber and Depledge, 1997a, Hebel *et al.*, 1997, Pedersen and Lundebye, 1996, Depledge *et al.*, 1995). Its basic biology and life history are well understood (Crothers, 1967, 1968) and its biochemical, physiological and behavioural systems are perhaps the most extensively studied of the European decapod Crustacea. This wealth of background information provides a fundamental basis for interpreting biomarker responses.

1.5.2 Novel biomarker development

Biomarkers are used increasingly in ecotoxicological research to understand how organisms respond when exposed to selected environmental contaminants. New biomarkers increase our knowledge of organismal responses following contaminant stress and provide valuable additional information for monitoring purposes. This permits a more comprehensive assessment of potential impacts on ecosystems at different levels of biological organisation.

The present thesis investigates, using specifically developed novel biomarkers, responses of *C.maenas* following exposure to chemicals which have the potential to cause metabolic and cellular toxicity and to disrupt endocrine processes.

Biomarker development is focussed on measuring exposure to polycyclic aromatic hydrocarbons (PAHs) and disturbances to endocrine mediated processes (see below). Field applicable and reliable biomarkers of exposure/effect for PAH and endocrine disruption do not exist presently for decapods, highlighting the importance of this work.

1.6 Polycyclic Aromatic Hydrocarbons (PAHs)

One group of contaminants of great environmental concern is the polycyclic aromatic hydrocarbons (PAHs). These are highly lipophilic chemicals (log K_{ow}= 3-8) (Readman *et al.,* 2002), ubiquitous in soils, sediments, air and water (Lin *et al.,* 1994) and are almost exclusively anthropogenic in origin (Onuska, 1989, McElroy *et al.,* 1989). The aquatic environment receives PAHs from inputs of petroleum and its products, from sewage effluents, runoff and atmospheric deposition from the incomplete combustion of organic matter (Readman *et al.,* 2002, Law *et al.,* 1994). Industrial activities such as metal smelting (Naes *et al.,* 1995, Beyer *et al.,* 1996) and the electrolytic production of aluminium (Beyer *et al.,* 1998) also produce PAHs that contaminate aquatic environments.

In addition to these aerial and aqueous modes of transport, the coastal marine environment also receives substantial amounts of PAHs from products such as creosote, coal tar and coal tar pitch which are used as preservative and antifouling agents (Uthe and Musial, 1986). The aqueous solubility of PAHs is low, and their hydrophobic nature means they readily associate with particulates and deposit in sediments (Readman *et al.*, 2002).

PAHs can broadly be divided into two groups depending on their source (see figure 1.4). Petroleum derived or petrogenic PAH are constituents of crude oils and are formed by low temperature alteration of organic matter, such as in the formation of fossil fuels (Readman *et al.*, 2002). They are characterised by a 2 or 3 ring structure and a large proportion of alkylated homologues (Readman *et al.*, 2002). Examples include naphthalenes, phenanthrenes and anthracene (Onuska, 1989). Combustion derived or pyrogenic/pyrolytic PAH are formed as a result of high temperature combustion of organic matter and high temperature industrial processes (metal smelters, electrolytic production of aluminium) and are characterised by non-alkylated 4, 5 and 6 ring structures (Readman *et al.*, 2002). Some pyrogenic PAH are fluoranthene, pyrene, benzo(a)anthracene, chrysene and benzo(a)pyrene (Onuska, 1989). Some PAH do occur naturally, such as perylene (synthesised by bacteria, algae and fungi), but inputs to aquatic systems are low in comparison to the anthropogenic sources described above (Readman *et al.*, 2002, UNEP, 1992).

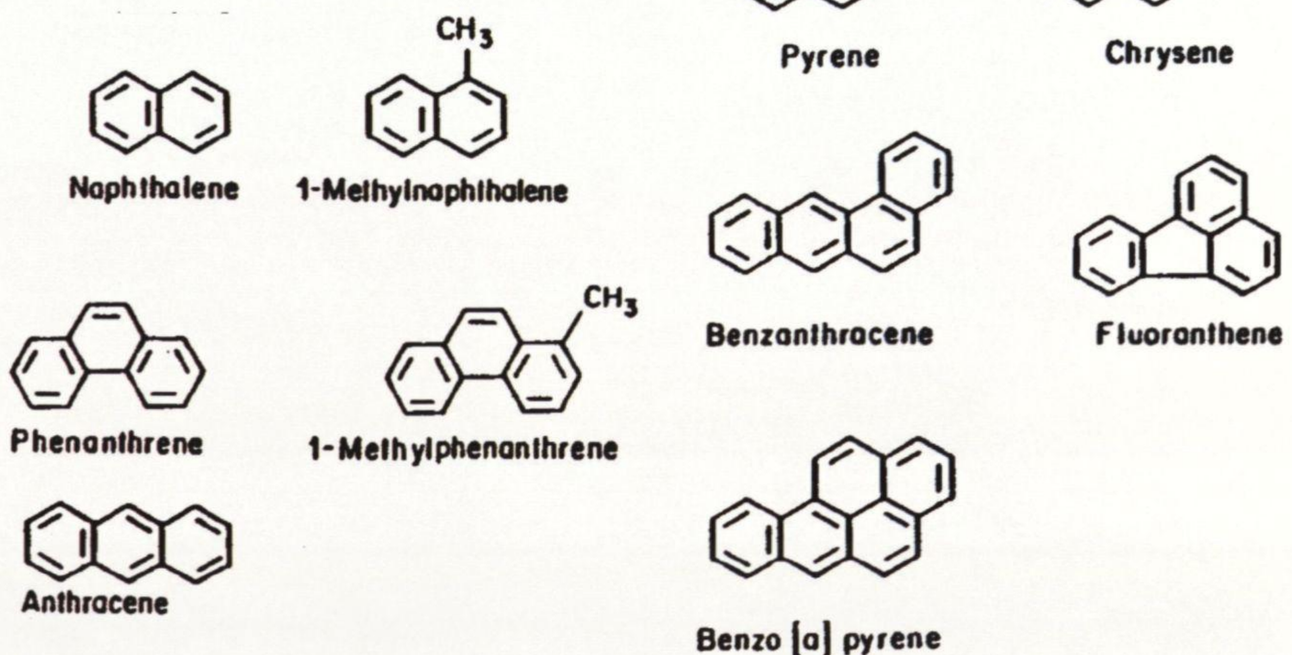
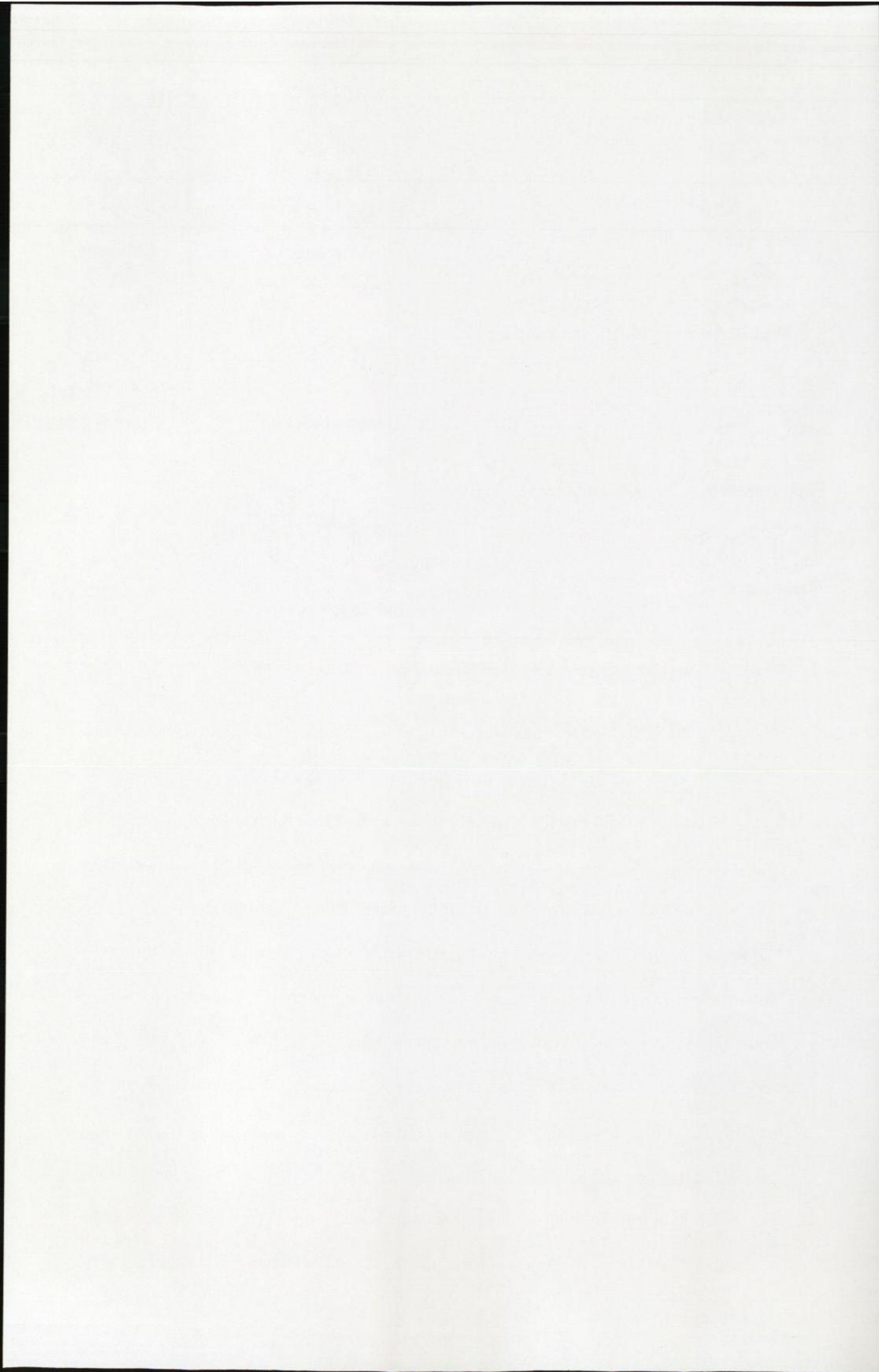


Figure 1.4 Examples of petrogenic and pyrogenic PAH (from McElroy *et al.*, 1989).

The lower molecular weight, lesser-ringed forms are associated with acute toxicity (Woodhead *et al.,* 1999). Major health concerns focus on the carcinogenic properties of higher molecular weight PAH, which have been implicated in the incidence of vertebrate cancer (IARC, 1983). For example, PAH contamination of sediments has been linked to liver neoplasia and other abnormalities in associated benthic fish (Krahn *et al.,* 1986, Malins *et al.,* 1988). PAH can only exert their latent carcinogenic effects following biotransformation to active mutagenic, carcinogenic and teratogenic metabolites which then bind to and disrupt important cellular macromolecules, such as DNA and RNA, the basis for tumour formation (Wild and Jones, 1995). The most potent carcinogens are the benzofluorathenes, benzo(a)pyrene, benzo(a)anthracene, dibenzo(a,h)anthracene and indeno(1,2,3-cd)pyrene (IARC, 1983). It is therefore important to understand the disposition (absorption, biotransformation, distribution and excretion) of potentially carcinogenic PAH in invertebrates, in particular those used as human food (James, 1989a).



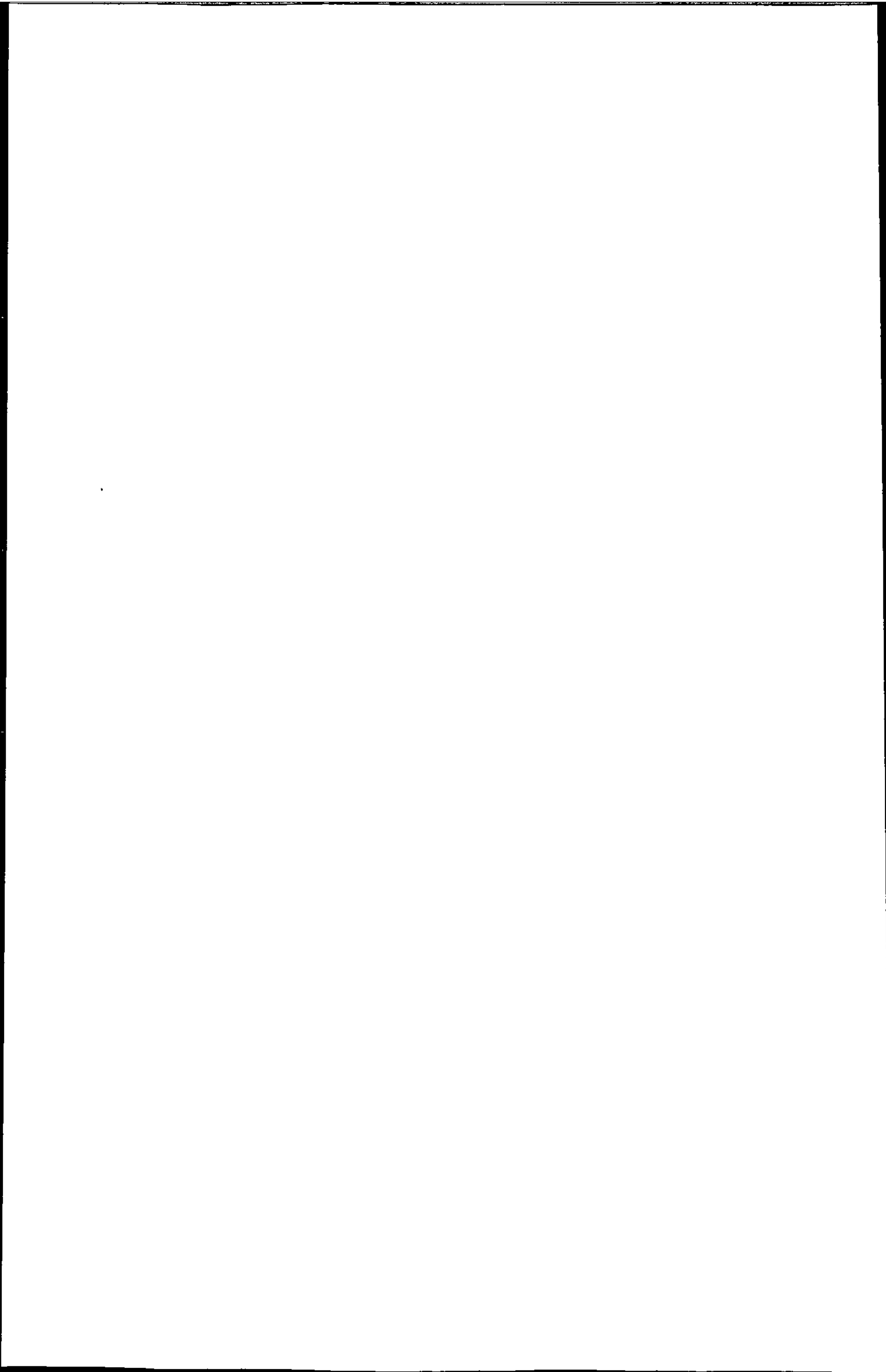
1.6.1 Biomarker of PAH exposure

PAHs are ubiquitous environmental contaminants found without exception in areas affected by industrial activities (see above). This combined with their often persistent nature, their high concentrations in sediments and concerns over their carcinogenic and mutagenic potential, has resulted in extensive study into their disposition and fate in the environment (Woodhead *et al.*, 1999, Wild and Jones, 1995, James, 1989a).

PAH residues in sediments and water are widely used as indication of environmental contamination by PAH, but analysis of these environmental media is often problematic (Law and Biscaya, 1994). PAH contaminants are sparingly soluble (Onuska, 1989) and their concentrations in water are very low (Lin *et al.*, 1994) and consequently are difficult to measure, since large volumes are needed for analysis. A large part of the higher molecular weight PAH will be adsorbed onto organic or inorganic particulates, causing problems for interpretation and in the separation of dissolved and particulate phases if they are to be analysed separately (Law and Biscaya, 1994). This hydrophobicity of PAH also means they preferentially partition into sediments (Readman *et al.*, 2002, Lin *et al.*, 1994). Analysis of sediments is laborious and time consuming, involving numerous clean up and extraction steps. In addition to the problems associated with these traditional water/sediment based measurements, the results often tell us little about the bioavailability of contaminants and the potential to cause adverse effects in aquatic organisms (Malins, 1989, Livingstone, 1993, Lam and Gray, 2003). Therefore, to assess satisfactorily the exposure of aquatic organisms, it is more pertinent to detect PAH parent compounds and their metabolites in the tissues and biological fluids of these organisms. Suitably reliable PAH exposure biomarkers can then be used as potential surrogate measures of bioavailable PAH in environmental media (Handy *et al.*, 2003). It must be noted here however, that chemical residue data are still very valuable, as they unequivocally demonstrate the presence of particular contaminants in the ecosystem in question

(McCarthy and Shugart, 1990). Linking biological and chemical information allows cause-effect relationships to be established (Gray, 1992). Ideally, therefore, biomonitoring programs should employ biomarkers alongside traditional measures of contaminants in water and sediments (Handy *et al.*, 2003, Sanchez-Hernandez *et al.*, 1998) in order to obtain a complete picture of the degree of environmental degradation and the risks posed to organisms. Biomarkers can alert environmental managers to potential problems and suggest the types of chemicals responsible for the effects seen in indicator organisms. Traditional chemical analyses can then be focussed cost effectively to provide confirmation of contaminants (Sanchez-Hernandez *et al.*, 1998).

Organismal responses integrate the relative concentration and bioavailability of contaminants temporally and over the organism's spatial range (McCarthy and Shugart, 1990), thus providing a measure that is more relevant to evaluating the risks posed to populations, communities and ecosystems. With reference to PAH, tissues such as liver, hepatopancreas, kidney, muscle and gills, and fluids such as blood/haemolymph, bile and urine can be analysed to determine the levels and types of PAHs an organism has been exposed to. However, differences in the ability of aquatic species to metabolise xenobiotics such as PAHs influences their suitability as biological indicators of PAH contamination. In organisms capable of metabolising PAH, measuring metabolites instead of simply quantifying parent compounds avoids underestimation of true uptake (McElroy *et al.,* 1989). Measurement of parent PAH residues is more suited to species such as bivalve molluscs, whose limited scope for biotransformation means they tend to accumulate these compounds in their tissues (Baumard *et al.,* 1999, Hellou *et al.,* 2002, Hufnagle *et al.,* 1999, James, 1989a, McElroy *et al.,* 1989). Measurement of PAH metabolites is better suited to fish, which metabolise PAH rapidly and extensively (Stegeman and Lech, 1991) and excrete it in the bile. Analysis of PAHs in the liver and muscle of fish from polluted sites reveals only very low levels (Varanasi *et al.,* 1985, Stegeman and Lech, 1991), reflecting their ability to transform and clear these compounds



from their tissues. Detection of PAH metabolites in fish bile is used as a measure of environmental PAH contamination (Krahn *et al.*, 1984).

Crustacean species have a lesser capacity to metabolise and excrete PAH than fish, but do so more extensively than bivalves (Lee *et al.*, 1976, James, 1989a, Stegeman and Lech, 1991, Miles and Roster, 1999, M. Krahn pers. comm.). Studies have shown that hepatopancreatic and other organ's microsomes contain cytochrome p450 (Quatrocchi and Lee, 1984, James, 1989b, Li and James, 1993, James and Boyle, 1998), capable of metabolising PAH. PAH metabolites can therefore be detected in their excreta (James *et al.*, 1991), whilst parents are still measurable in their tissues (Mothershead *et al.*, 1991), albeit at much lower levels than in bivalves (Miles and Roster, 1999). Since PAHs must be biotransformed to active metabolites in order to exert their toxic, mutagenic and carcinogenic effects (James, 1989a, Stroomberg *et al.*, 1996) it is important to study metabolite levels for the purposes of environmental risk assessment as well as pollution monitoring.

The development of accurate and cost effective techniques for the measurement of PAHs and their metabolites (and other contaminants) in the various matrices of aquatic biota is becoming increasingly relevant, as it provides us with useful information regarding contaminant exposure in field populations. This information is then used for assessing the pollution status of the ecosystem in which these organisms live, and can also be used to assess the potential risk to humans who consume PAH-contaminated animals. Essentially, this modern approach aims to determine the distribution of exposure to organisms as opposed to simply the levels and distribution of contaminants in sediments and water. This approach is one of the foundations of the biomarker concept.

In comparison to fish, fewer PAH studies have been carried out on invertebrates. However, PAH residues have been reported in crustaceans captured at polluted sites

(Mothershead *et al.,* 1991, Uthe and Musial, 1986) and studies have looked at disposition and excretion following exposure (via a number of different routes) in the laboratory (Corner *et al.,* 1973, Lee *et al.,* 1976, Lauren and Rice, 1985, Moese and O'Conner, 1985, James *et al.,* 1991, Palmork and Solbakken 1980, Li and James, 1993, James *et al.,* 1995 and Watson, 1998). The early findings of Corner *et al.,* (1973) are particularly relevant as they show that a marine crab species (the spiny spider crab, *Maia squinado*) is capable of biotransforming naphthalene and excreting it's hydroxylated and conjugated metabolites in the urine. While this study was primarily concerned with the disposition and metabolism of naphthalene, several of the authors above propose the respective species as potential bio-indicators and discuss their findings in the context of *in situ* pollution monitoring applications.

More recently, Eickhoff *et al.,* (2003a) developed and field-tested a novel assay for determining PAH exposure in Dungeness crabs (*Cancer magister*), detecting 1-OH pyrene equivalents in haemolymph samples. The present thesis describes work of a similar nature in *C.maenas* and is an attempt to provide information on the metabolic mechanisms employed by this species following exposure to PAHs. Allied to this is the development of a RAMP-style exposure assay to PAH. This is a continuation of work using HPLC/F to detect phenanthrene metabolites in the urine of phenanthrene exposed *C.maenas* (Watson, 1998). This work, adapted from studies pioneered by Krahn *et al.,* (1984) looking at PAH metabolites in the bile of exposed fish, provided compelling evidence for the use of *C.maenas* as a potential biomarker species for PAH monitoring. Emphasis in this thesis has been placed on the disposition of pyrogenic PAH in *C.maenas*, following laboratory and environmental exposure.

1.7 Field application of newly developed biomarkers

Biomarkers are of limited value for biomonitoring unless they can be applied to environmentally realistic situations. It is therefore of vital importance to field trial any biomarker which shows promise in laboratory studies. Under controlled laboratory conditions it is relatively straightforward to regulate chemical exposures and standardise biomarker assays in order to establish cause-effect and dose-response relationships (Astley *et al.*, 1999). Biomarkers responses in the field are more difficult to interpret, and vary widely due to the dynamism of natural systems and phenotypic and ontogenetic differences in susceptibility of organisms to anthropogenic stress (Astley *et al.*, 1999). In order to determine if a biomarker/bio-indicator species is suitable for *in situ* biomonitoring of pollutants it must first be applied to contamination situations in the field and be able to fulfil what Hopkins (1993) has called the "5R's".

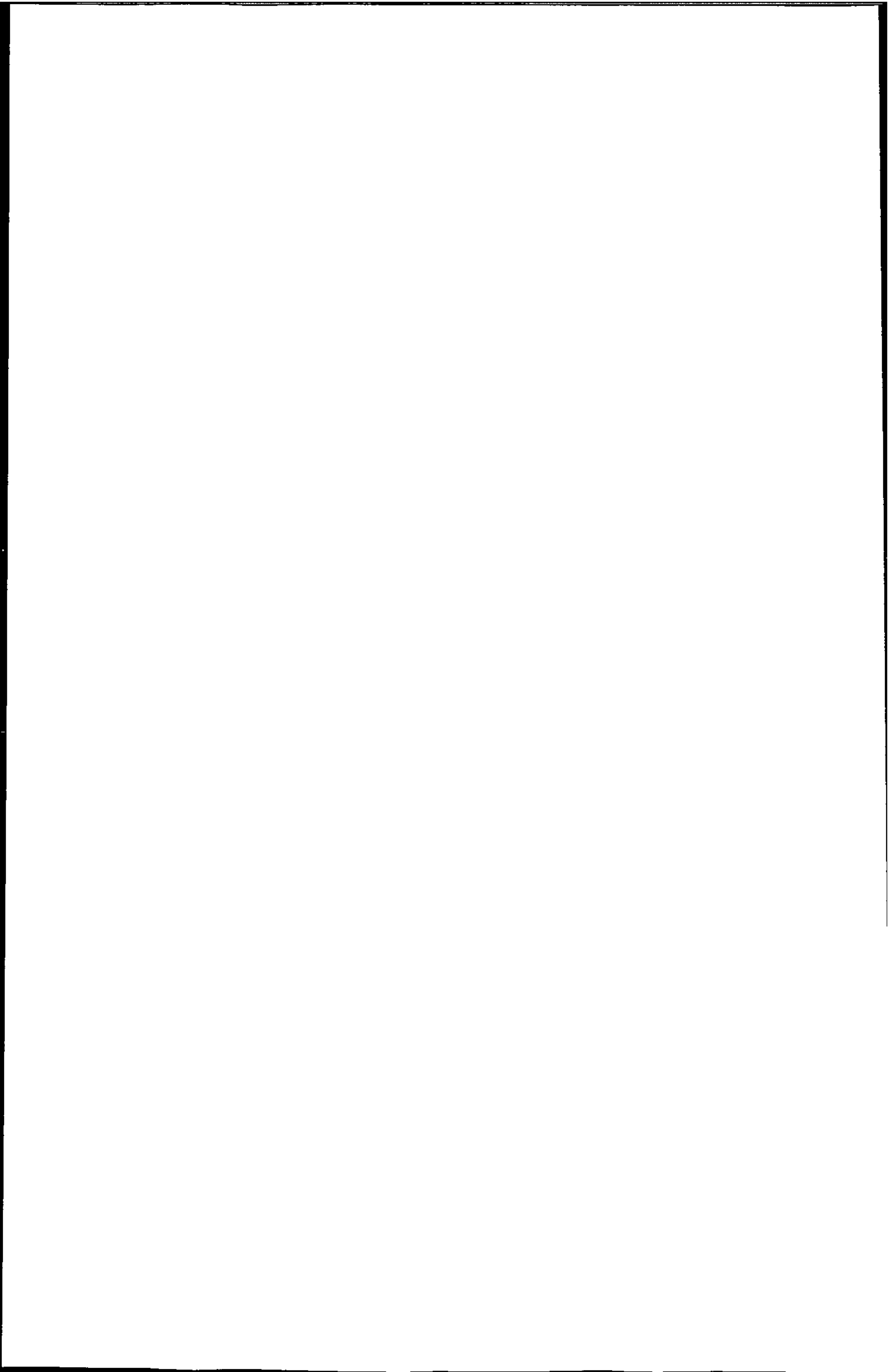
Relevant- the biomarker should be measurable in an ecologically important species, which plays a role in the functioning of the ecosystem.

Reliable- the biomarker species should be widely-distributed, common and easily collected to allow comparison between sites.

Robust- the biomarker species should be able to tolerate low levels of contamination and be hardy enough to withstand sampling/caging.

Responsive- the biomarker should exhibit measurable responses to pollutant exposure and/or effects.

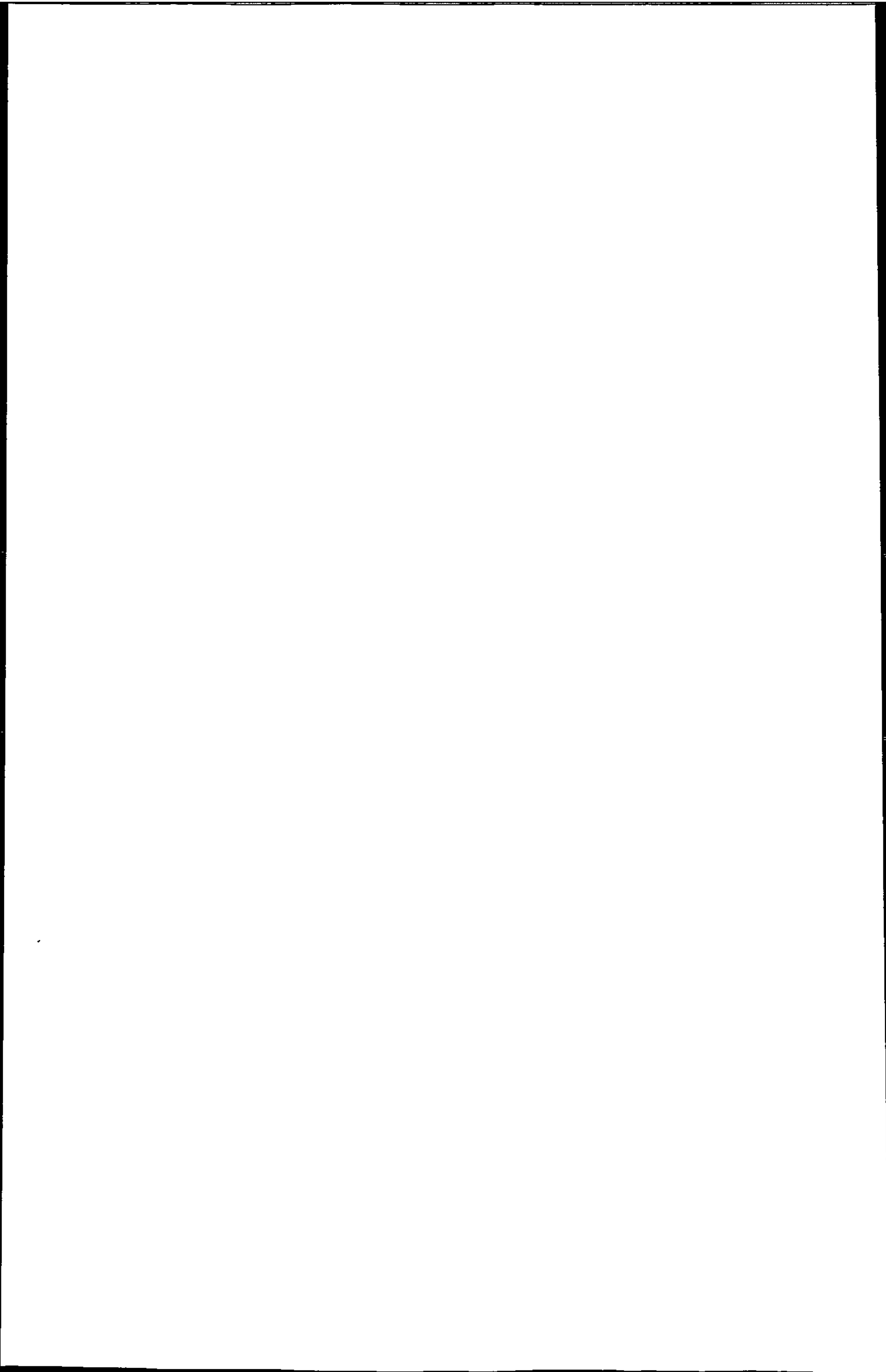
Reproducible- the magnitude of the response should be similar following exposure to the same levels of pollutants at different sites.



The biomarker must also be able to discriminate between reference and impacted sites and where possible identify gradients of pollution from point sources. Testing of novel biomarkers and validation of common biomarkers has been a primary objective of the recent European BEEP project (Biological Effects of Environmental Pollution in coastal marine ecosystems) (Garrigues *et al.*, 2002). One specific aim of the project is to "validate the use of selected biomarkers in specific sites for both routine assessment of chemical contamination and for the improvement of national and international monitoring programmes". The present thesis shares this aim and devotes a considerable portion of the present work to this end.

1.8 Understanding the integrated toxic response to multiple contaminants through the use of multiple biomarkers

In the natural aquatic environment, organisms are frequently exposed to complex mixtures of contaminants and exposure to single contaminants in isolation rarely occurs (Moore, 2002, Walker *et al.*, 2001). Particularly in heavily industrialised estuaries or harbours, organisms may be faced with complex mixtures of PCBs, PCDDs, PCDFs, PAHs, metals, pesticides and numerous other contaminants (Walker, 1998) under fluctuating environmental conditions. It is not uncommon for coastal waters to receive literally thousands of different compounds (Malins and Ostrander, 1991). The uptake of these complex mixtures is poorly understood and it is not known how contaminants in such mixtures influence each other's uptake, biotransformation and toxicity (Moore, 2002). In stark contrast, laboratory-based studies rarely reflect the nature of field contamination. Whilst undoubtedly valuable, many biomarker studies have relied too heavily on single contaminant exposures. Most ecotoxicological investigations involve exposing organisms to chemicals singly, under controlled laboratory conditions. Studies on effect mechanisms have therefore tended to focus on the predominant mechanisms of toxicity, for example, metabolic toxicity, immunotoxicity, genotoxicity, endocrine



disruption. A present priority requirement for ecotoxicologists is now to assess the risks presented by combinations of chemicals (Walker, 1998) and to disentangle the complex nature of interactions between biomarker responses observed in natural populations. The patterns of toxicity displayed in nature are often cumulative, complex and difficult to attribute to specific causative agents. Understanding how biomarker responses change in relation to others provides insight into how organisms prioritise their responses when faced with xenobiotic challenge.

Complex mixtures also present the ecotoxicologist with the problem of synergism or potentiation of toxicity (Walker *et al.*, 2001). This is defined as occurring when the toxicity of a combination of chemicals exceeds the sum of the toxicities of its individual components (Walker, 1998). This should be clearly distinct from additive effects, where the toxicity of a mixture is approximately equal to the sum of the toxicity of its individual components (Walker *et al.*, 2001). It is generally accepted that effects of different chemicals tend to impact additively on ecological systems when they are applied in mixtures (Calow, 1996). However, mixtures of PAHs have shown synergistic effects on invertebrates, and illustrate the risks associated with extrapolation of toxicity data on single substances to mixtures (Verrhiest *et al.*, 2001). Evaluating the toxicity of complex mixtures is complicated further by antagonism, where the toxicity of a mixture is less than the sum of the toxicity of its parts (Newman, 1998).

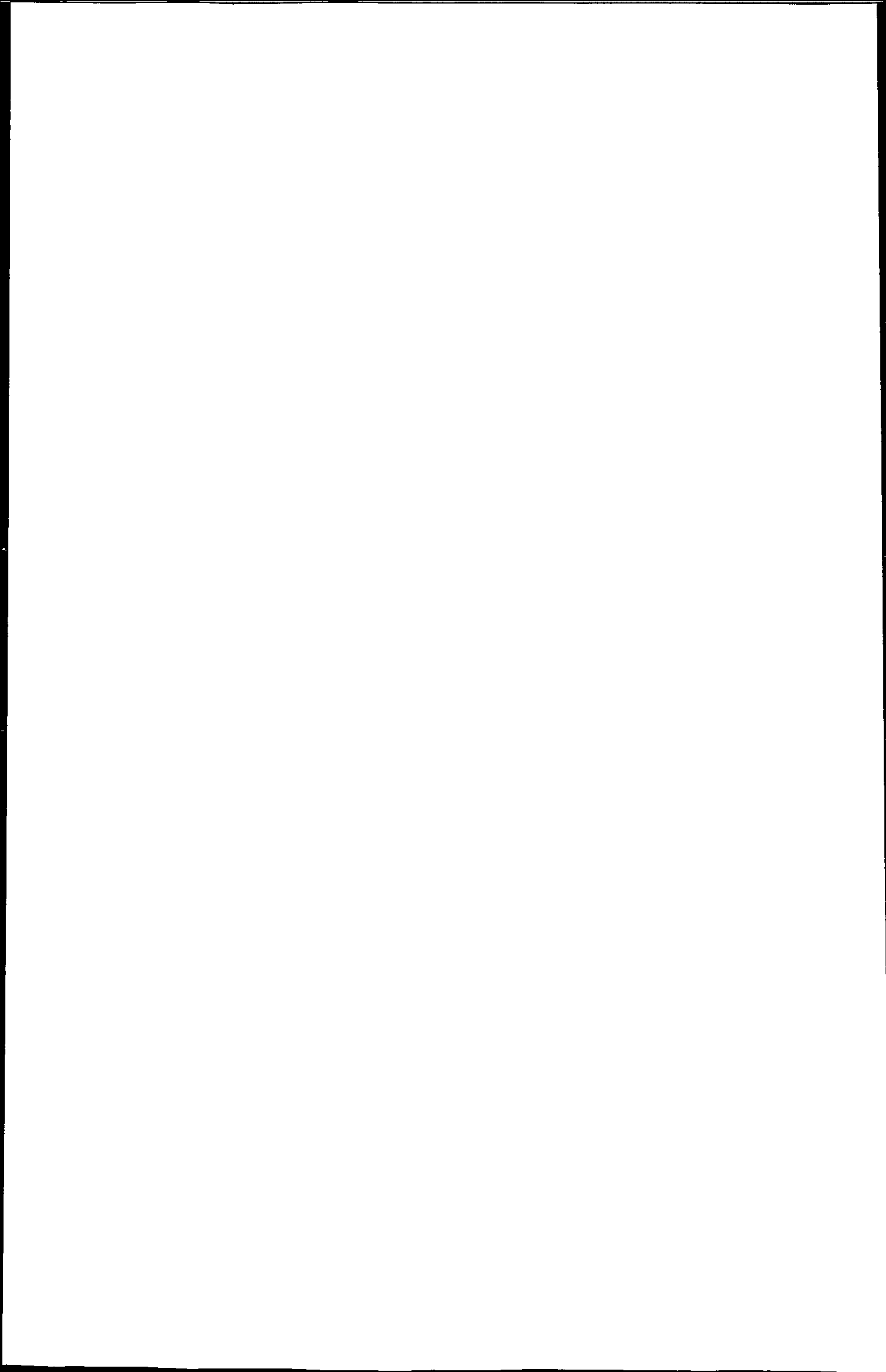
Potentiation is often encountered by ecotoxicologists investigating acute toxicity (Johnston, 1995) but this thesis is more concerned with using biomarkers to observe the sublethal responses of the organism (*C. maenas*) following exposure to mixtures of chemicals. To address this it is necessary to apply suites of biomarkers to a multi-contaminant system. The biomarkers chosen can be used to detect the integrated impact of contaminants and any interrelationships between biomarkers can be investigated. Responses that are well understood (e.g. induction of metal binding proteins, inhibition of

neuronal enzyme function, induction of oxidative stress defence mechanisms, genotoxic damage) can be investigated alongside those for which novel biomarkers have been developed (exposure to PAH, disruption to endocrine proceses). For example, biomarkers can be applied to measure the response of crabs exposed to both a trace metal and a PAH. Which, if any of these responses will be altered for the maintenance of the other? Will levels of metal binding protein fall to allow metabolic energy to be shunted to the metabolism and excretion of the PAHs, or will PAH metabolism be less of a priority than chelating the offending metal? Do crabs sustain greater genotoxic damage when additional contaminants are added to the exposure system?

The use of a suite of biomarkers also allows us to evaluate the validity of individual biomarkers in a multi-contaminant system. For example, does PAH exposure invalidate the response of a biomarker for exposure to trace metals? If so, could this mean that a crab's response to metal exposure is no longer clear when other contaminants are present?

1.9 Disruption of endocrine mediated processes as a mechanism of toxicity - risks posed by exogenous chemicals.

Information presented so far has introduced the concept of responses of aquatic organisms following exposure to environmental contaminants. Responses such as the binding of toxic levels of non-essential metals to specialised proteins or the metabolism and elimination of potentially mutagenic PAHs. Interactions of foreign chemicals with biochemical, cellular and physiological processes within exposed organisms can also result in effects that are detrimental to their health. Consequently, considerable effort has been put into the study of these mechanisms of toxicity. In recent years, the deleterious interaction of environmental chemicals with endocrine systems of aquatic organisms (endocrine disruption) has been a mechanism of toxicity of great concern, as it has far



reaching implications for their reproductive success. For example, PAHs and PCBs, in addition to their well-documented toxic and carcinogenic effects, have been shown to interact with hormone receptors associated with moulting in crustaceans (Oberdorster *et al.*, 1999).

The International SETAC Workshop on Endocrine Disruption in Invertebrates, December 1998, defined an endocrine disrupter as "a substance which causes adverse effects in an intact organism, or its progeny, subsequent to changes in endocrine function" (deFur *et al.*, 1999) from an established definition by Holmes *et al.*, (1997). A growing body of evidence from laboratory and field studies indicate that environmental endocrine disrupting chemicals may be affecting wildlife adversely (deFur *et al.*, 1999). The phenomenon is well-documented in vertebrate species, including man (Sharpe and Skakkebaek, 1993, Colborn *et al.*, 1996). The effect of vertebrate type hormones on aquatic organisms has received considerable investigation, with reports of sex changes in riverine fish (Sumpter, 1995) and abnormalities in the reproductive organs of alligators (Guillette *et al.*, 1994).

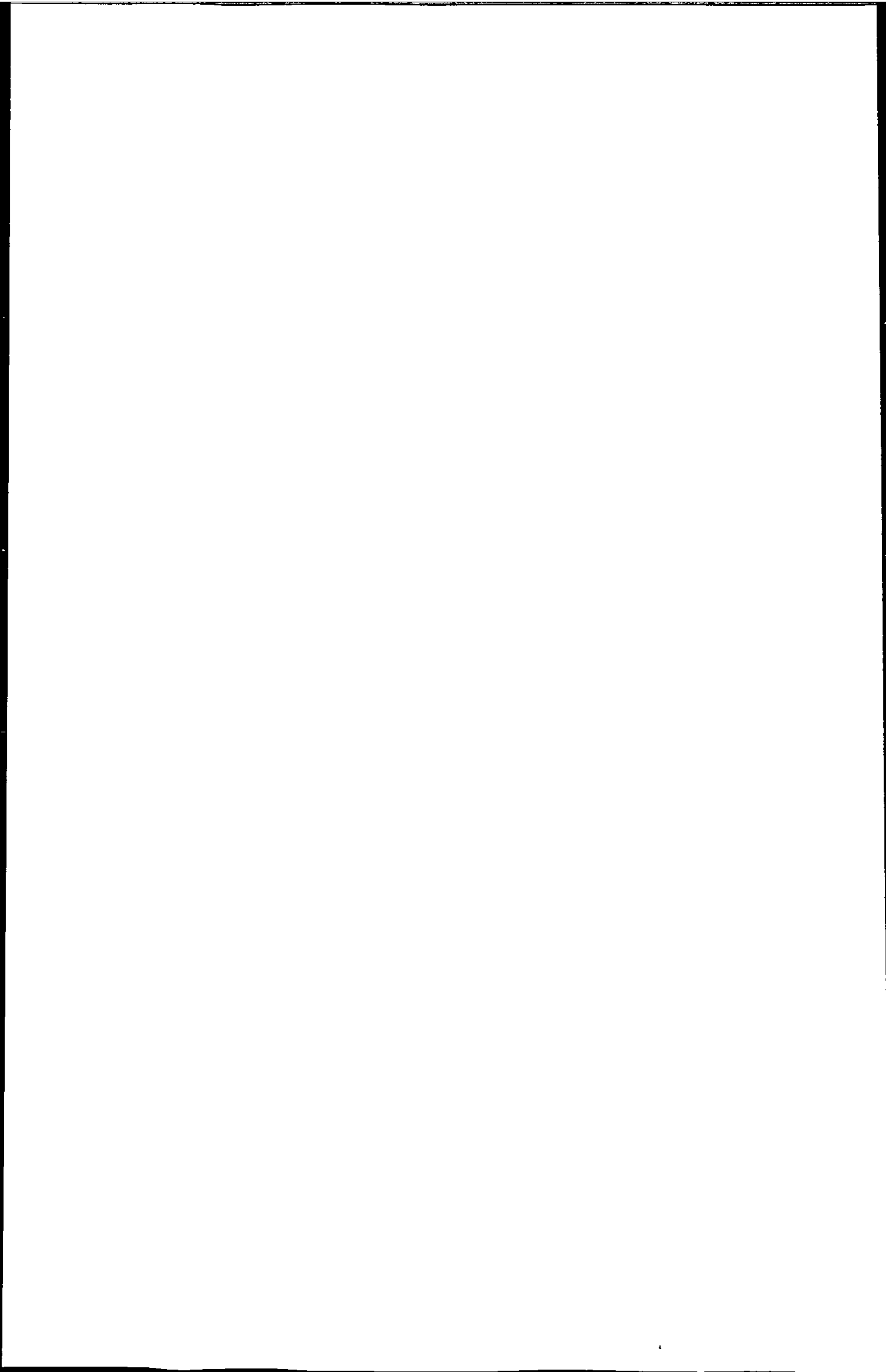
Much less is known regarding the potential for chemicals to disrupt endocrine processes in invertebrate species, and a high level of uncertainty remains in our understanding of endocrine disruption in this group of organisms (see Depledge and Billinghurst, 1999 and deFur *et al.*, 1999 for reviews). As mentioned previously, invertebrates account for 95% of all described animal species and are vital components of marine ecosystems (deFur *et al.*, 1999). It is pertinent therefore to study the potential effects of anthropogenic contaminants on their hormonally-regulated functions. Investigations into endocrine disruption in invertebrates have thus far focussed primarily on the potential influence of vertebrate type hormones and their analogues. There is little direct evidence that natural and anthropogenic compounds, capable of modifying endocrine control in vertebrates, have similar effects in invertebrates such as crustaceans

(Pinder *et al.,* 1999, Matthiessen *et al.*, 2002). Despite the gaps in our knowledge regarding invertebrate endocrine systems, preliminary findings suggest that the pathways and chemical messengers used by invertebrates differ from those of vertebrate species. Thus, exogenous sources of androgens and oestrogen and its mimics, do not appear to have significant effects on endocrine controlled physiological or reproductive processes investigated in decapod species.

Little is known of the potential for exogenous contaminants to mimic or antagonise the action of specific invertebrate hormones. Recent research has therefore shifted its focus from vertebrate-type hormones to the potential for exogenous invertebrate type hormones and their anthropogenic analogues to adversely effect hormonally regulated processes in model invertebrates.

1.9.1 Biomarker of endocrine disruption

To the present, there has been limited success in establishing a reliable endpoint biomarker for endocrine disruption in adult decapods such as *Carcinus* (deFur *et al.*, 1999). In contrast to riverine fish (McMaster, 2001, Sumpter, 1995), there is also very limited information in the literature concerning the occurrence of this kind of toxicity in wild crustacean populations, particularly decapods (Pinder *et al.,* 1999). Indeed, evidence for endocrine disruption in the field is distinctly lacking for invertebrates in general, with the notable exception of TBT-induced imposex in gastropod molluscs (Matthiessen and Gibbs, 1998, Bryan *et al.*, 1986). Decapod examples seem restricted to reports of intersex in lobster (*Homarus americanus*) (Sangalang and Jones, 1997) and imposex in freshwater crab (*Geothelphusa dehaani*) (Takahashi *et al.,* 2000) populations. However, specific causative agents were not identified in either study. High prevalence of intersex has been reported in harpacticoid copepods near a sewage outfall in the Firth of Forth, Scotland, but again no causal relationship between sewage and levels of intersex was determined

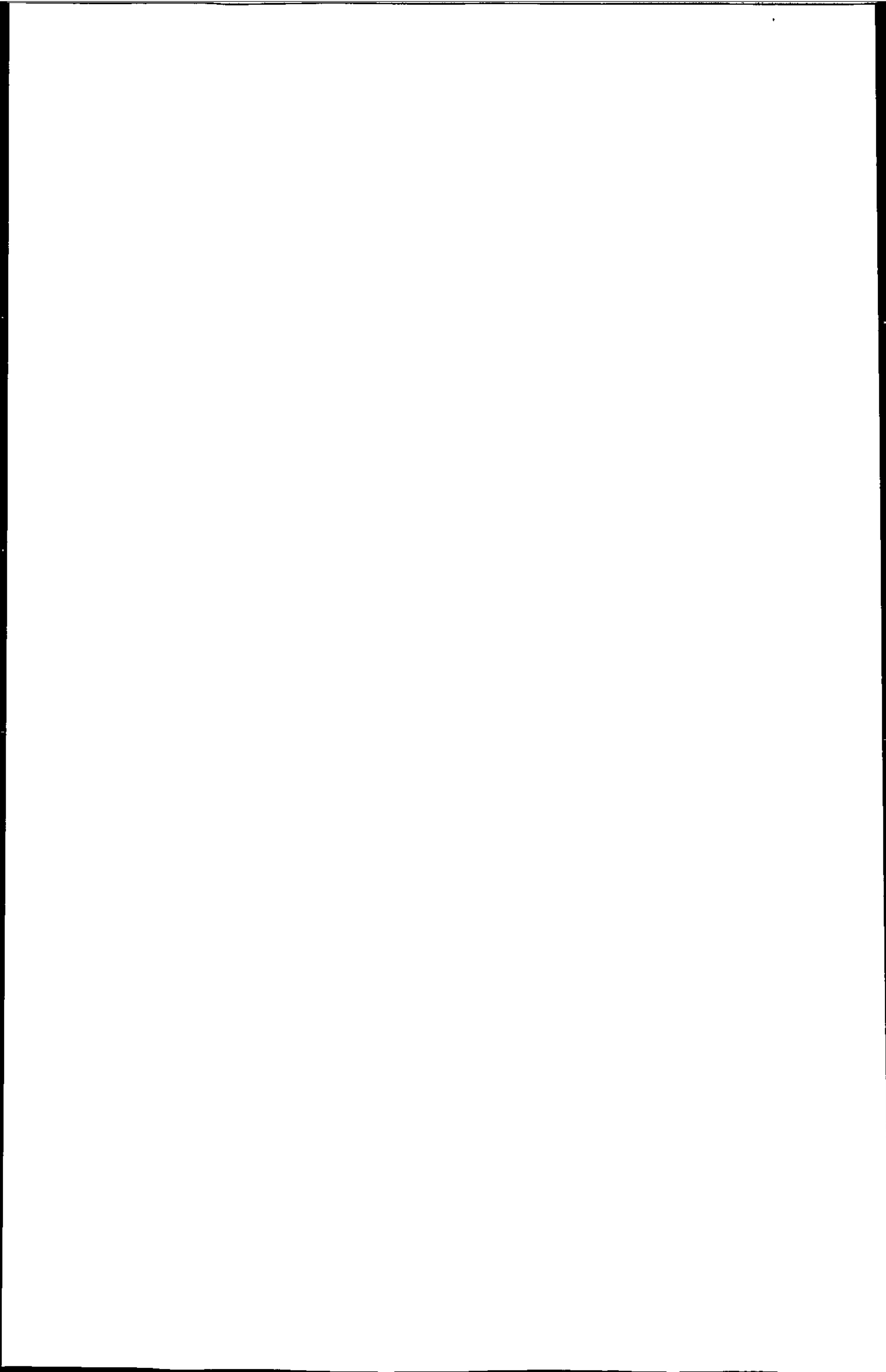


(Moore and Stevenson, 1991, 1994). Investigations carried out as part of the national EDMAR (Endocrine Disruption in the Marine Environment) programme did not show any alterations in the hormonally-controlled processes of vitellogenesis, heart rate or osmoregulation in shore crabs (*C.maenas*) following exposure to various natural and synthetic vertebrate sex steroids (oestrogen and its mimics and androgens) (Matthiessen *et al.*, 2002).

Various contaminants have been shown to disrupt endocrine-mediated processes in crustaceans *in vivo,* although studies largely have involved the use of *Daphnids* (deFur *et al.*, 1999, Zou and Fingerman, 1997a,b). Some studies have utilised decapod Crustacea looking at the effects of contaminants such as trace metals, PAHs and TBT as well as those specifically designed to do so for the chemical control of arthropod pests (e.g. moult hormone agonist pesticides) (see Pinder *et al.,* 1999 for review). *In vitro* studies have shown that PAHs and PCBs can enhance the effect of ecdysteroids (Oberdorster *et al.*, 1999). It would appear therefore that endocrine systems of crustaceans are susceptible to disruption by compounds previously not considered endocrine disruptors, perhaps due to the focus on vertebrate type sex steroids. The final part of the thesis is aimed therefore at determining if exposure to chemicals capable of interacting with the endocrine systems of crustaceans (hormones and analogs) can disrupt processes under their control. This will provide a more thorough understanding of their mechanisms of toxicity, should deleterious effects be induced. The main objective is to identify a "disruptable" endocrine mediated process with quantifiable endpoint(s) with the potential to be used as a biomarker of exposure to endocrine disrupting chemicals. Fingerman *et al.* (1998) approached a recent review with similar objectives, making suggestions of hormonally–regulated functions that could be used as biomarkers of environmental pollution. These included gonadal index, blood glucose levels and limb generation. Taylor and Harrison (1999) emphasise the importance of developing appropriate endocrine disruption biomarkers in invertebrates and in particular, tests with ecologically-relevant endpoints for detecting reproductive

consequences. One of the conclusions of the International SETAC Workshop on Endocrine Disruption in Invertebrates was that there was an urgent need for "validated biomarkers and other indicators of ED exposure and/or effects in invertebrates" (deFur *et al.*, 1999). The present thesis aims to tackle this problem using *C.maenas*.

Several variables have the potential to be adapted for such purposes. Reproduction (vitellogenesis), moulting and locomotor activity are three endocrine mediated processes with easily observable endpoints. The temporal progression of vitellogenesis can be followed using a quantitative ELISA for vitellogenin in crab haemolymph samples. Fluctuations/perturbations in the normal vitellogenic cycle of female crabs caused by endocrine disruptors might therefore be identified. The nature of the agent responsible would be difficult to determine in the field, but since vitellogenesis is an endocrine mediated process, any gross deviations from its normal pattern could constitute endocrine disruption. Altered moult frequency, interval and growth following moult could be used to indicate disruption to the ecdysteroid pathways controlling moulting, and have previously been suggested as endpoints for evaluating endocrine disruption (deFur *et al.*, 1999). Rhythmic locomotor activity is a parameter that can be quantified using an actograph system, and any gross changes to this highly predictable behaviour might be considered endocrine disruption.



Chapter 2: Assessing the responses of *C.maenas* to polycyclic aromatic hydrocarbons – development of a non-destructive biomarker of PAH exposure.

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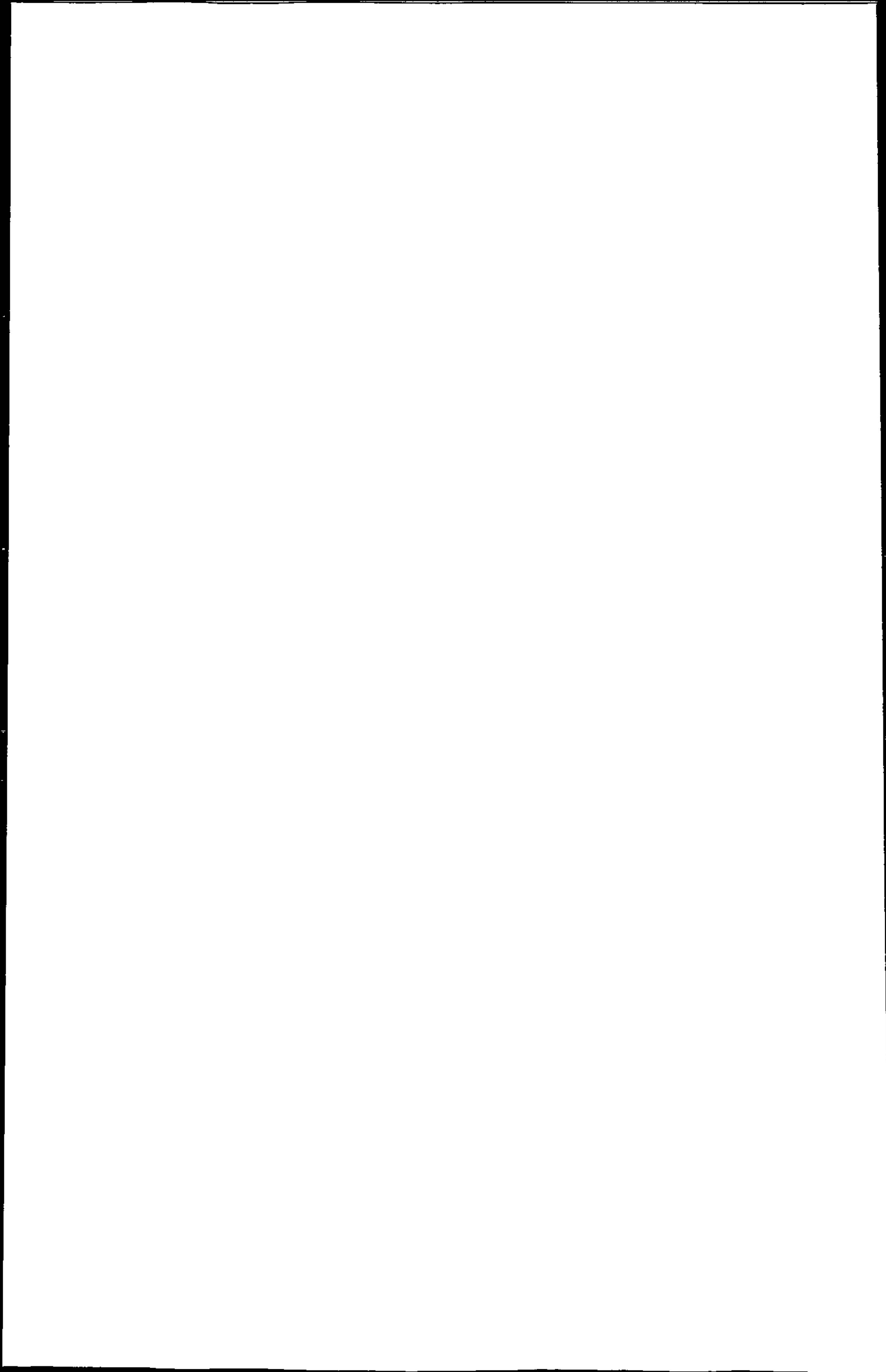
2.1 Introduction

Marine waters and sediments, particularly those subject to anthropogenic inputs, contain a multitude of xenobiotic contaminants, which invariably include numerous polycyclic aromatic hydrocarbons (PAHs). Traditional analysis of PAH in sediment and water provides us with measures of PAH in the environment in absolute terms, but tells us little about the bioavailabilty of this PAHs and their potential to cause adverse effects in exposed organisms. Biomarkers of exposure to PAH are therefore useful for biomonitoring purposes as they provide a measure of the available PAH and allow more accurate predictions of adverse effects (see introduction).

When exposed to waterborne pyrene in the laboratory, crustaceans take up the compound over their gills (James *et al.,* 1995). Subsequent metabolism in the hepatopancreas is mediated by cytochrome p450 enzymes (James and Boyle, 1998), which attach various functional groups that enhances the solubility and hence the excretability of this hydrophobic contaminant. Hydroxyls and dihydrodiols (phase I metabolism) and their sulphate and glucoside/glucuronide derivatives (phase II) are some of the more common metabolites formed from various parent PAHs (James and Boyle, 1998), and their presence is evidence of exposure to PAH. This chapter presents a series of exposure experiments where *C.maenas* is exposed to waterborne pyrene and sampled for urine and haemolymph. Urine is an important excretory route for xenobiotics and their metabolites in higher invertebrates such as crustaceans (Holiday and Miller, 1980, 1984, Solbakken and Palmork, 1981, James *et al.,* 1991, Corner *et al.,* 1973) and in vertebrate species including fish (Solbakken *et al.,* 1980). Urinary PAH metabolite levels are also used routinely to monitor occupational exposure to PAH in humans (Angerer *et al.,* 1997, Grimmer *et al.,* 1997), with 1-OH pyrene being the most widely-utilised marker metabolite (Jongeneelen *et al.,* 1987, Vu-Duc and Lafontaine, 1996).



In crustaceans, excretory transport of endogenous organic metabolites, xenobiotics and xenobiotic metabolites is mediated by the antennal glands (Holiday and Miller, 1984). In crabs, each of the paired antennal glands forms urine by ultrafiltration of the haemolymph at the coelomosac (see figure 2.1). This ultrafiltrate is then modified as it flows through the labyrinthine/tubular parts of the gland and is stored in the terminal bladder before being voided (Holiday and Miller, 1980). Since parent compounds can diffuse across membranes back into the system but metabolites are not reabsorbed, renal excretion in metabolite form is a more efficient way of eliminating any xenobiotic burden (Lauren and Rice, 1985). Urine and haemolymph can be sampled repeatedly and non-destructively from *C.maenas* and need little or no clean up or preparation before analysis, aside from dilution in the appropriate solvent. Urine and haemolymph samples can then be analysed for pyrene and its metabolites using direct fluorescent spectrometry.

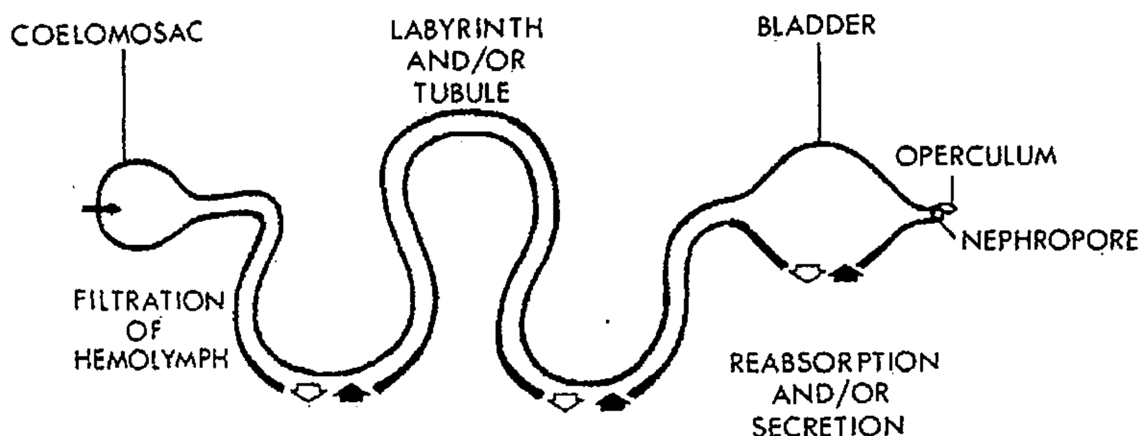


Figure 2.1 A generalised crustacean antennal gland, the site of urine ultrafiltration (from Holliday and Miller, 1980).

2.1.1 Fluorescence spectrophotometry as an analytical tool

Direct fluorescent spectrophotometry/spectrometry or fluorimetry, is a relatively rapid and inexpensive technique for the analysis of PAHs (Aas *et al.*, 1998). The strong characteristic fluorescent properties of PAHs, derived from delocalised electrons associated with their aromatic rings, make them ideally suited to detection by fluorescent means. PAH molecules absorb UV light and emit it at longer wavelengths. The optimal excitation and emission wavelength of different PAHs vary according to their size, structure and substituent groups, with the optimal excitation wavelength generally increasing with the number of rings present. This variability can be exploited by analytical techniques to discriminate between different groups of individual PAHs in environmental and biological samples. Direct fluorescence spectrometry has proved to be a useful tool for environmental PAH biomonitoring in recent years. Most studies have concentrated on the analysis of fish bile with this technique (Ariese *et al.*, 1993, Lin *et al.*, 1994, 1996, Beyer *et al.*, 1996, 1998, Aas *et al.*, 1998, Aas and Klungsoyr, 1998, Camus *et al.*, 1998), but it has been used for analysis of PAH metabolites in invertebrates (Stroomberg *et al.*, 1996, Fillmann *et al.*, 2002, Eickhoff *et al.*, 2003a).

Direct fluorescence spectroscopy is generally of two basic types. Both fixed wavelength fluorescence (FF) and synchronous fluorescent (SFS) methods retain a good level of discrimination and PAH specificity (Lin *et al.*, 1996). In FF, a standard/sample (dissolved in a suitable solvent) is excited at a specific wavelength and the fluorescent intensity at a specific emission wavelength is measured or the emission monochromator is scanned to obtain an emission spectrum). First developed by Vo Dinh (1978, 1981), SFS involves scanning both excitation and emission monochromators simultaneously, with a constant wavelength difference ($\Delta\lambda$) between them. This results in a greatly simplified spectrum with observed fluorescence bands narrower and more clearly resolved than with FF but without the loss of spectral information (Sharma and Schulman, 1999). In complex

mixtures, fluorescent overlap from different compounds is reduced and in some cases specific peaks can be resolved and quantified without the need for chromatographic separation. SFS has met with considerable success in several studies (e.g. Ariese *et al.,* 1993, Beyer *et al.,* 1996, Lin *et al.,* 1994, Stroomberg *et al.,* 1996). Validation studies have shown that results are comparable with more powerful techniques such as HPLC/F (Ariese *et al.,* 1993, Lin *et al.,* 1994, 1996).

In accordance with the method reported by Aas *et al.,* (1998) for analysis of fish bile, dilution of the urine and haemolymph samples may be needed to avoid inner filter (light absorption) effects and to allow the analysis of small sample volumes. While Aas *et al.,* (1998) diluted bile by a factor of 1000 or more, the optimum dilution for urine is likely to be lower (<1:100), due to its less viscous nature. Measurements of protein and biliverdin, required to standardise results when analysing bile, can be omitted from the methodology, as urine is ultrafiltrated in the antennal gland and free from such high molecular weight compounds. This ultrafiltration also means that the urine is relatively free of compounds which might contribute fluorescent interference during analysis and does not require extraction or clean-up before analysis. PAH metabolite concentrations are lower in haemolymph and so dilution factors are reduced.

Detection of pyrene metabolites in the urine and haemolymph can provide compelling evidence of pyrene exposure, a principle that can then be applied in the field to assess PAH exposure to wild populations, providing a meaningful measure of environmental contamination and potential risk to organisms. It is hoped that the detection of urinary/haemolymph metabolites of PAHs (such as pyrene) in crustaceans can be used as a biomarker for general PAH contamination in aquatic environments. This work tests the suitability of *C.maenas* as a sentinel species for PAH contamination and the sensitivity of fluorescence spectrometry as an analytical technique.

2.2 Materials and Methods

2.2.1 Collection of experimental animals and laboratory conditions

Male, intermoult, shore crabs, *Carcinus maenas* (carapace width 50-72mm) were collected on incoming tides from Jenkins Quay on the Avon estuary at Bantham, South Devon, England (OS ref: SX 667438 GB) on three occasions between the months of April and July, 2000. On return to the laboratory, they were maintained in holding tanks containing filtered (10µm carbon filtered), well aerated 34ppt, 15+/-1°C seawater, under a 12h light: 12h dark regime for one week to permit acclimation. Crabs were fed twice weekly with irradiated cockles. Water was changed within 12h of every feeding session.

2.2.2 Chemicals

Pyrene (98%, cat. no: 18, 551-5) and 1-OH pyrene (98%, cat. no: 36, 151-8) were purchased from Sigma-Aldrich (The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, England). Ethanol (GPR™, 96% v/v) and acetone (GPR™) were obtained from BDH Laboratory Supplies (Poole, BH15 1TD, England).

2.2.3 Exposure- response experiment

Crabs were transferred to glass aquaria containing 10L of filtered (10µm carbon filtered), well-aerated seawater (34ppt, 15+/-1°C), under a 12h: 12h light: dark regime. After measurement of carapace width, the animals were assigned to one of 14 groups of 4 in separate aquaria, with groups of 8 crabs exposed being exposed to one of seven treatments. Individuals were labelled according to their treatment by super-gluing a printed label onto the dorsal surface of the carapace. For exposed crabs, pyrene was added to the water in an acetone carrier (at a ratio of 1:1, w:v, pyrene/acetone) to increase solubility. Pyrene exposure was at five nominal concentrations (200, 100, 50, 25 and 10µg l^{-1}).

Solvent controls were exposed to acetone only and 8 crabs were held in seawater alone (Controls). Animals were not fed during the exposure period. The crabs remained in the aquaria for the duration of the exposure, whereupon they were transferred to clean seawater to depurate for up to 3 weeks.

2.2.4 Time-response experiment

In a separate experiment, groups of 8 crabs were exposed, under the conditions described above, to waterborne pyrene at the nominal concentrations of 400 or 200μg l^{-1}. An acetone control group and a seawater control group, each comprising 8 individuals, were also included as described above. Individuals were exposed and kept in their respective aquaria for a period of 10 days. After this time, they were removed and held in clean aerated seawater.

2.2.5 Urine and haemolymph sampling

In the exposure- response experiment, urine and haemolymph samples were taken from each crab after 48h exposure using the technique described by Bamber and Naylor (1997). Briefly, crabs were removed from their aquaria and placed in a bucket containing clean seawater. After being drained of residual seawater, they were restrained with the ventral surface uppermost on a plastic board using rubber bands (see figure 2.2). The third maxillipeds were moved aside and kept apart by inserting absorbant paper between the base of the appendage and top of the sternum. The epistome was dried (to prevent seawater contaminating the urine) and the crab placed under a dissecting microscope (x10). The operculum of each antennal gland bladder was lifted using a hooked seeker, causing urine to flow from the bladder, through the opercula, where it was collected using a 200μl Gilson© pipette fitted with a flexible flat tip. Samples (20-400μl per crab) were then transferred to siliconised microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C until analysis.

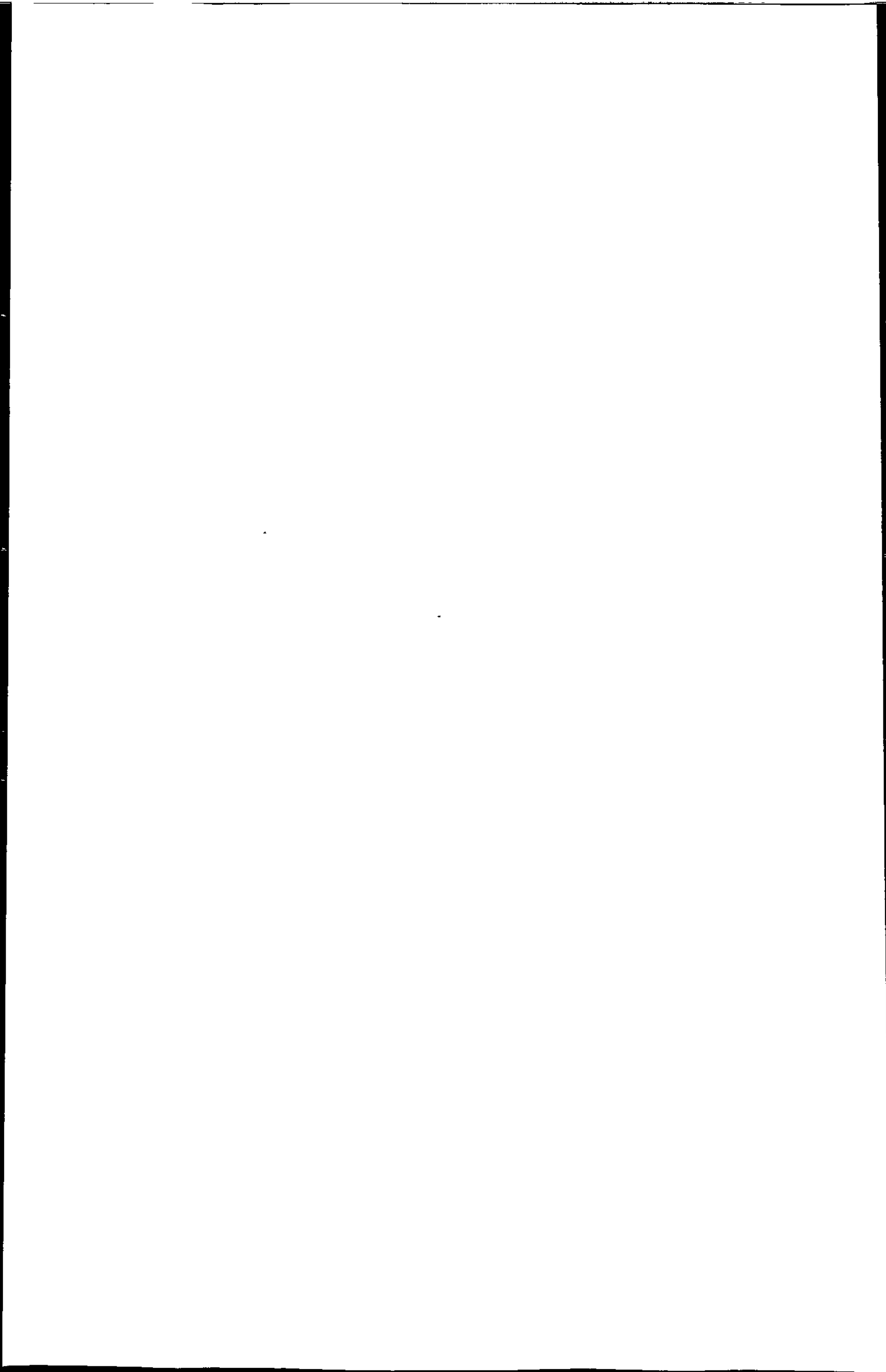


Figure 2.2 Urine sampling *Carcinus maenas*.

With the crab still restrained, the absorbant paper holding the maxillipeds was removed and a haemolymph sample was taken from a suitable arthrodial membrane at the base of a walking leg (see figure 2.3). The membrane was first gently punctured using a fine bore needle and then a fine capillary, with plunger, was used to draw out 200µl of haemolymph. Samples were then expelled into siliconised microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C until analysis. Test animals were returned to their respective aquaria immediately after sampling.

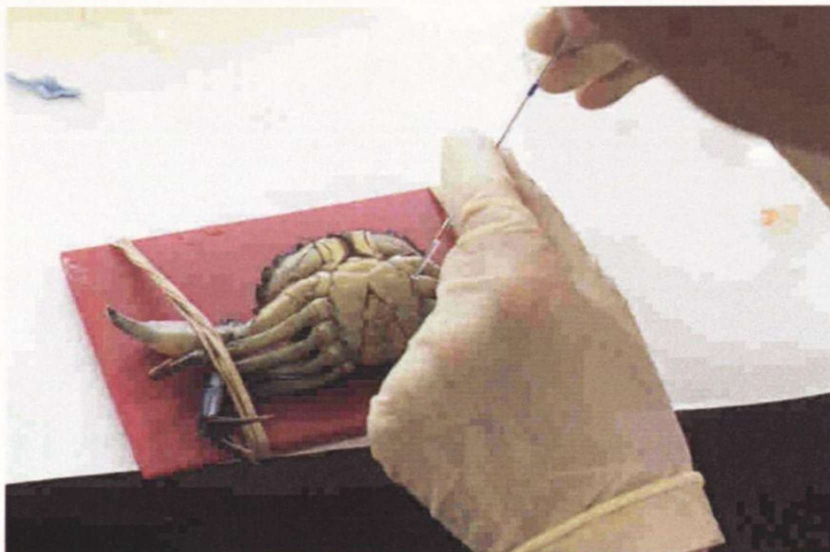
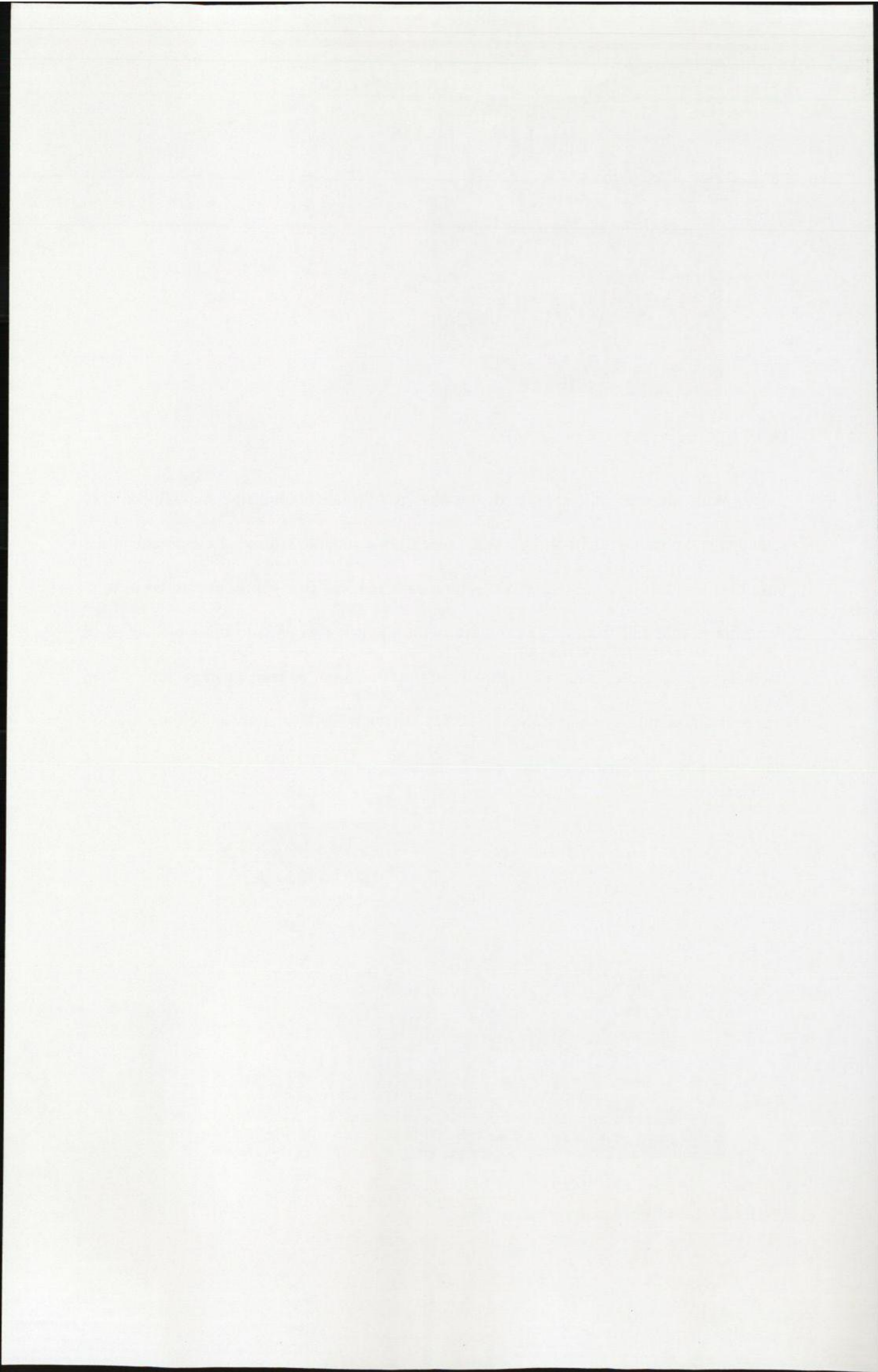


Figure 2.3 Haemolymph sampling *Carcinus maenas*.



Urine and haemolymph samples were taken in this way wherever possible, but occasionally urine could not be obtained since the urinary bladders of some crabs were empty. It was necessary therefore, to repeat the above exposure and sampling procedure three times, to obtain sufficient replicates (n=>10) of urine samples. For the time-response experiment, urine samples were taken from all crabs (as described above) after 12h, 24h, 48h, 96h, 8d and 10d.

2.2.6 Analysis of standards and urine samples

Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer, coupled to a software package operated by Windows 3.1. Ethanol (50%) was the solvent used for samples, standards and blanks. Measurements were made out in a 3.5ml quartz fluorescence cuvette. All FF and SFS measurements were made with excitation and emission slit widths of 2.5nm. Resulting FF and SFS spectra were smoothed and fluorescence intensity measured at a fixed excitation and emission wavelength pair for solvent blanks, standards and samples alike (see below).

2.2.7 Determination of wavelength pairs

Various authors have reported differing wavelength pairs for the detection of pyrenes in fish bile, including 341/383nm (Aas *et al.,* 1998, Camus *et al.,* 1998, Beyer *et al.,* 1998), 345/395nm (Ariese *et al.,* 1993, Escartin and Porte, 1999a,b) and 349/391nm (Sundt and Goskoyr, 1998). For synchronous fluorescence analyses of fish bile, wavelength differences of 34nm (Lin *et al.,* 1994) 37nm (Ariese *et al.,* 1993, Lin *et al.,* 1994) and 42nm (Aas and Klungsoyr, 1998, Aas *et al.,* 2000a,b) have been used.

The optimum wavelength pairs for the detection of pyrenes (the parent compound and its metabolites) in the present study were selected after first analysing a subset of samples and several pyrene and 1-OH pyrene standards using a range of different excitation and emission wavelengths.

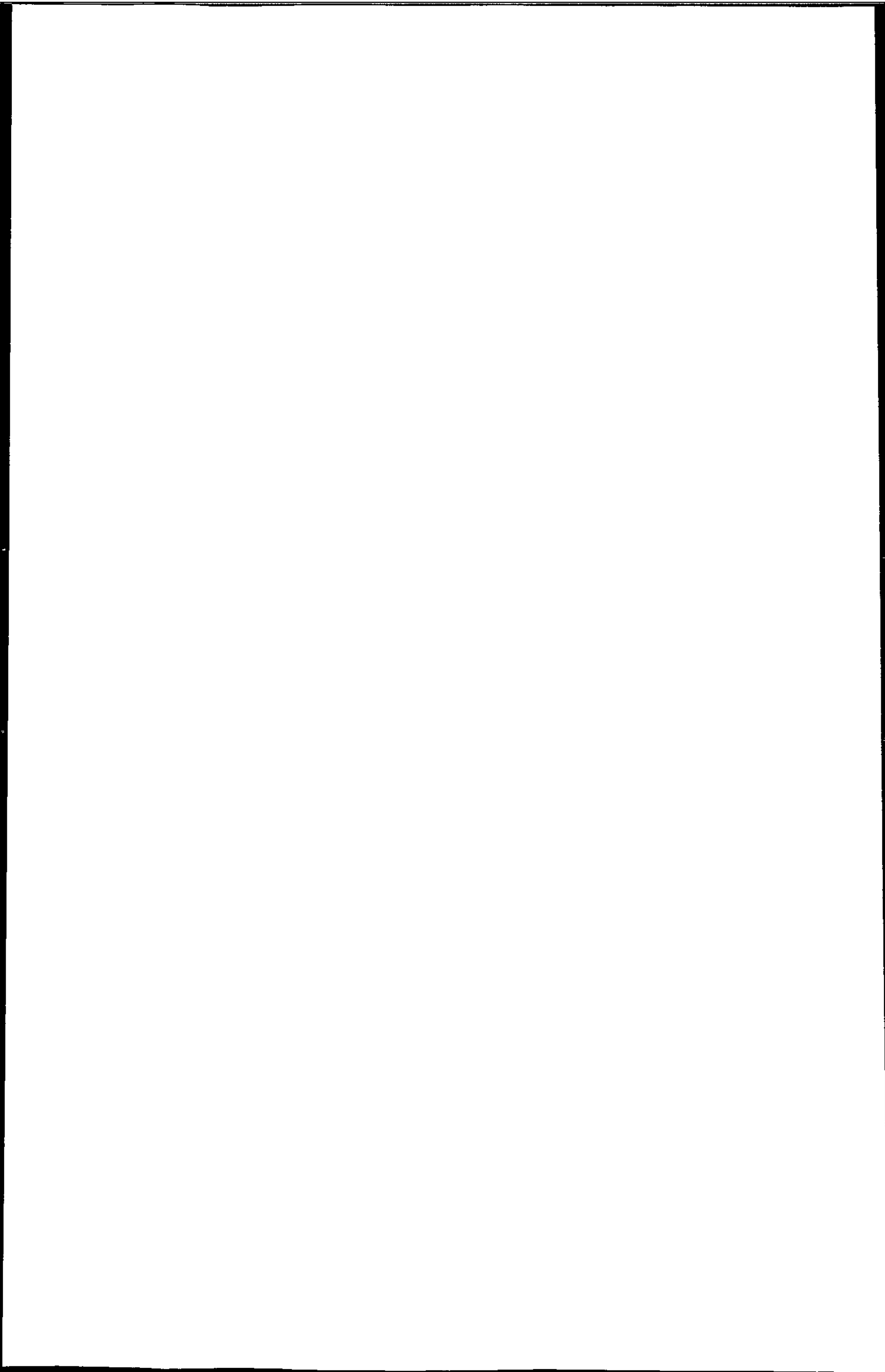
2.2.8 Preliminary analysis of standards and samples

Various concentrations (200, 100, 75, 50, 25, 10, 5 μg l^{-1}) of pyrene and 1-OH pyrene were prepared in 50% ethanol and analysed to determine the optimal wavelength pair for these compounds. The standards were analysed using fixed fluorescence (FF) at a range of excitation wavelengths (including 341,345 and 349nm, according to the papers reported above), with emission spectra taken from 330-500nm. For synchronous fluorescence (SFS) analysis, Δλ of 34, 37 and 42nm were tested, with excitation/emission spectra taken from 240-500nm.

Several representative urine and haemolymph samples from each treatment were also analysed. A 20μl/50μl aliquot of urine/haemolymph was dissolved in 1980μl/950μl of 50% ethanol in the cuvette, to yield a 1:100/1:20 dilution, respectively. Analyses were performed using the above wavelength regimes for detection of pyrenes.

FF at Ex345nm produced the largest and most clearly resolved peaks for standards and samples. For synchronous fluorescence spectrometry (SFS) analysis, 37nm was the optimal Δλ. The predominant urinary peaks more closely approximated those produced by the 1-OH pyrene standards than the parent standards (see figures 2.4 and 2.5). Therefore, analysis of samples and 1-OH pyrene standards was undertaken with a fixed excitation wavelength of 345nm and synchronous analysis was performed with a Δλ of 37nm.

For operational purposes, the fluorescence value assigned to individual standards and samples was the intensity measured at a fixed wavelength pair rather than at the maximum peak height of the dominant peak on the emission spectra. In all cases this value was within 5% of the peak maxima. The fixed wavelength pair was determined by calculating the mean emission wavelength of the dominant peak on both standard and sample spectra. For analyses, this was Ex345/Em387nm (standards) and Ex345/Em382nm



(samples). For SFS analyses, wavelengths were Em387nm (standards) and Em381.4nm (samples).

2.2.9 Quantification of sample peaks

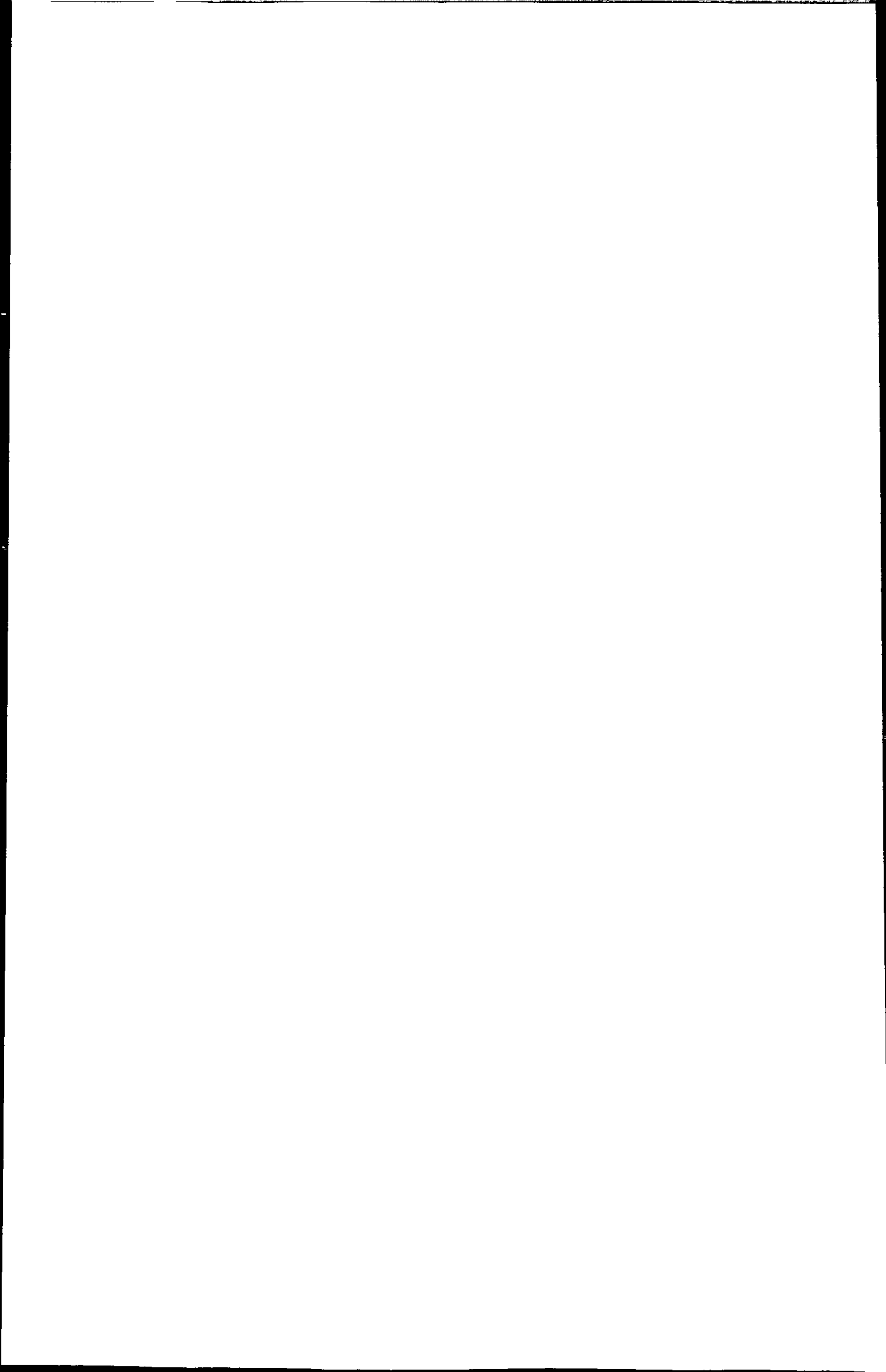
To take into account instrument drift over the lifetime of the experiment and to allow all samples to be compared despite fluctuations in instrument response, sample peaks were quantified with respect to a series of 1-OH pyrene standards (200, 100, 75, 50, 25, 10, 5μg l^{-1} for urine samples and 10, 5, 2, 1, 0.5μg l^{-1} for haemolymph samples), analysed at the fixed wavelength pair of Ex345/Em387nm (FF) and Em387nm (SFS). The use of 1-OH pyrene was favoured over the parent compound as it more closely approximates the peaks seen in the samples. 1-OH pyrene does not serve to quantify sample peaks in terms of "μg l^{-1} of 1-OH pyrene" as the peaks seen in samples are blue shifted by 5nm and are unlikely to be the pure standard. Results are therefore reported in terms of μg l^{-1} of 1-OH pyrene "equivalents".

2.2.10 Urine samples

Urine samples taken from exposed and control crabs during all three exposure experiments were analysed for 1-OH pyrene equivalents as described above (1:100 dilution). Fluorescence intensity was measured at the assigned wavelength pair of Ex345/Em382nm (FF) and Em381.4nm (SFS).

2.2.11 Haemolymph samples

Haemolymph samples from exposed and control crabs were analysed as described above (1:20 dilution). Fluorescence intensity was measured at the assigned wavelength pair of Ex345/Em382nm (FF) and Em381.4nm (SFS).

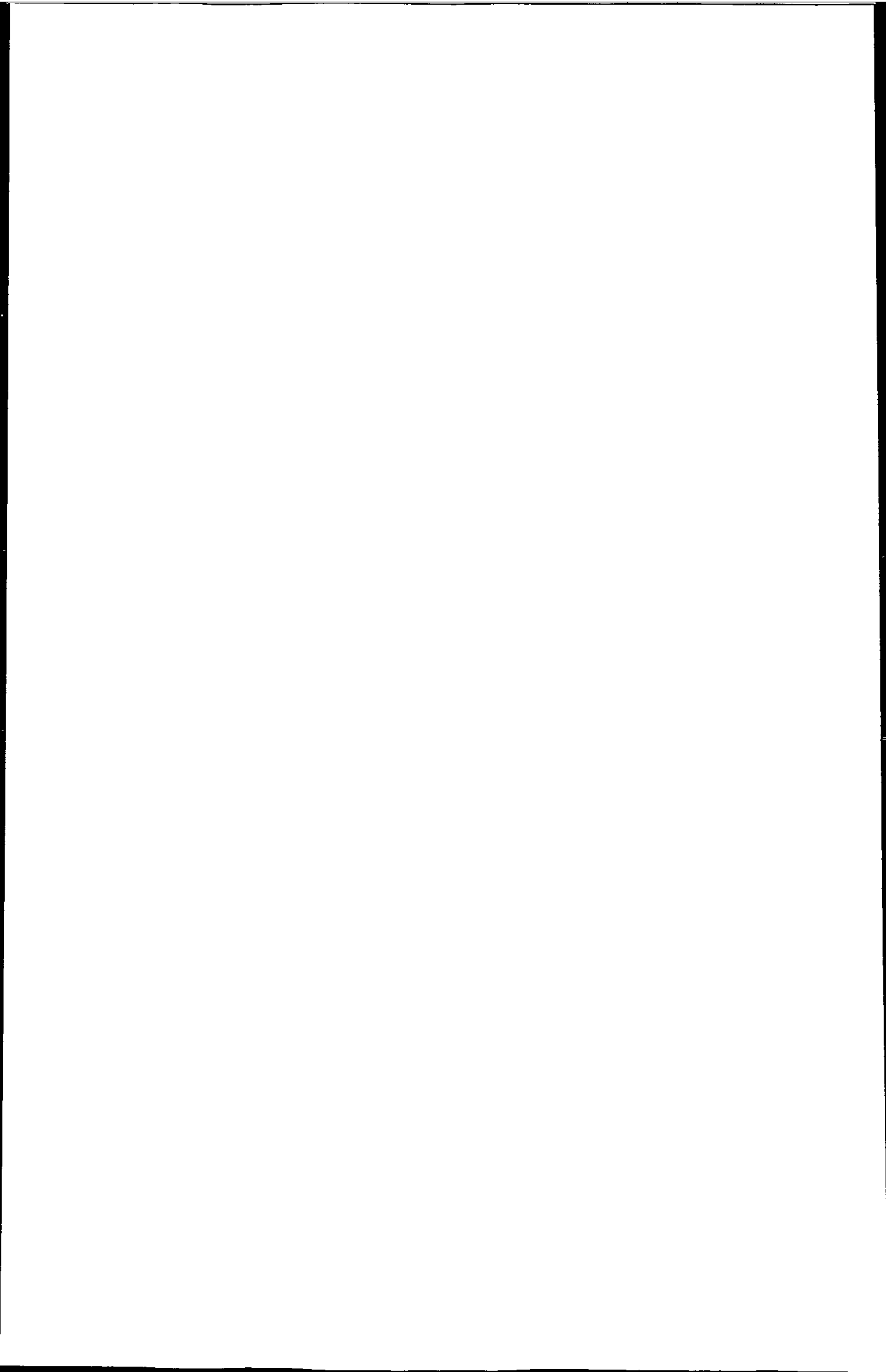


Before analysis of either standards or samples, a blank of 50% ethanol was scanned by FF and SFS. This provided a value for the background fluorescence contribution of the solvent, for comparison with samples and standards

2.2.12 Inner filter effects and dilution of urine samples

An important factor that must be accounted for when using direct fluorescence spectroscopy is the "inner filter effect" (UNEP/IOC/IAEA, 1992). The intensity of any emitted fluorescence is dependent on several factors - the fluorescent characteristics of the compounds within the sample, their respective concentrations, the dimension of the sample in the light path of the spectrophotometer and the intensity of the excitation source. Provided the total absorbance of any sample is low, a linear relationship exists between the intensity of light emitted by a compound and its concentration. If the sample absorbs more than 5% of the incident light, fluorescence intensity will be reduced by any compound in the sample which absorbs either the excitation or emission light. This is known as "inner filter effect" (UNEP/IOC/IAEA, 1992). Energy may also be absorbed by other compounds present in the sample, rather than being emitted as fluorescence, a phenomenon known as "quenching" (UNEP/IOC/IAEA, 1992). To determine whether inner filter effects are interfering with the fluorescent compounds under investigation, it is necessary to carry out specific tests, one of which being sample dilution.

Practicalities inherent in the method meant dilution was required for analysis of samples. Dilution generated the larger volumes required for the cuvette-based fluorescence analyses and reduced fluorescence levels sufficiently to allow quantification within a convenient range of standard concentrations. Small sample volumes and the desire to conserve them for additional validation experiments also favoured dilution. Information on the fluorescence behaviour of the urinary metabolites upon dilution was, therefore, important in order to ascertain whether absolute values for undiluted urine could be



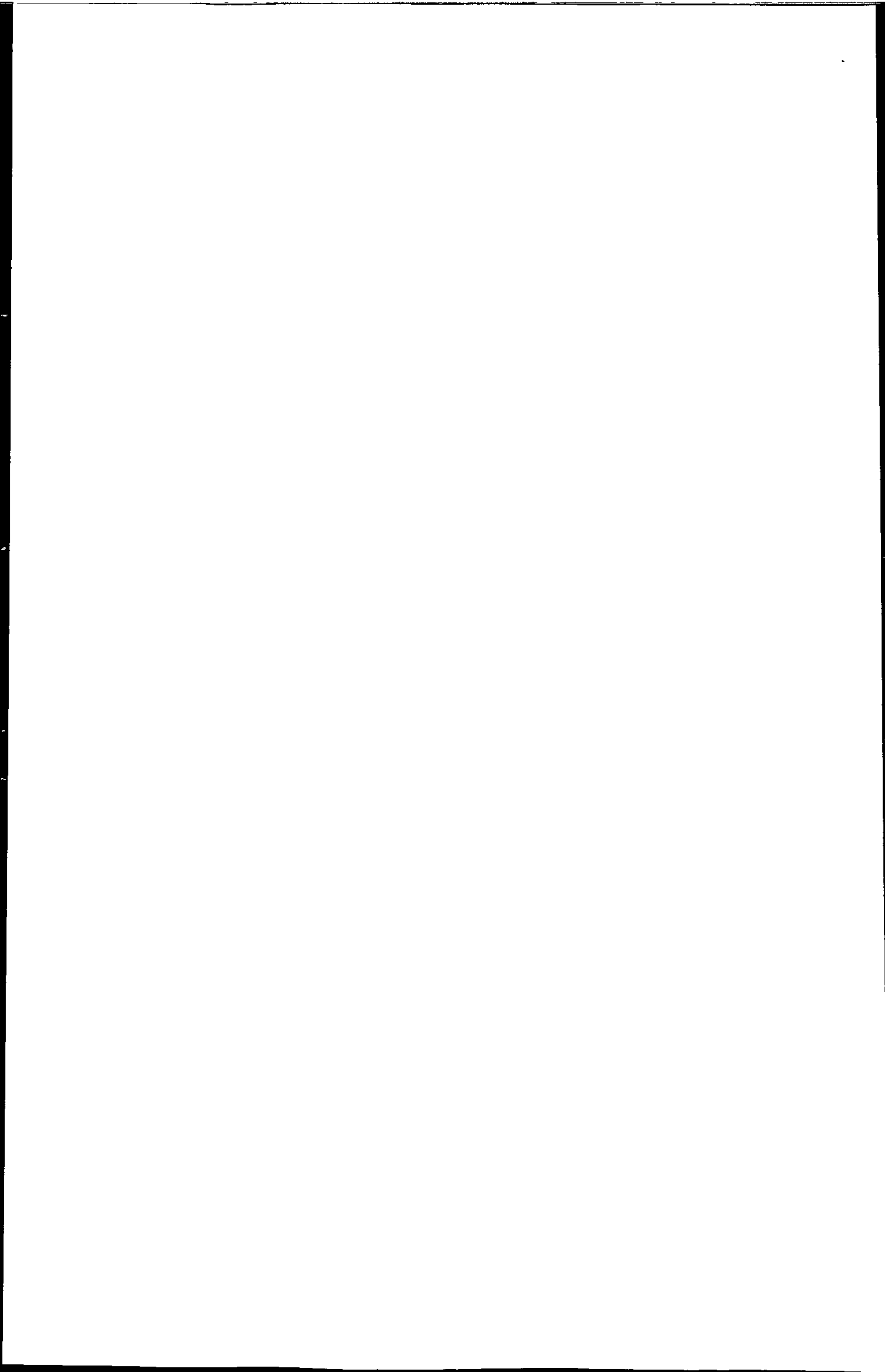
extrapolated from values obtained from diluted samples. Additionally, dilution of urine samples must be performed for determination of any inner filter effects.

Representative urine samples from each treatment were serially diluted in 50% ethanol (1:100, 1:50, 1:25, 1:10, 1:5) and analysed as above (FF Ex345/Em382nm). Fluorescence intensity results were plotted against the relative concentration of the sample. A linear relationship indicates there is no significant inner filter effect, permitting any measurements to be used for quantification (UNEP/IOC/IAEA, 1992). A straight line also signifies that fluorescence from the compounds of interest increases in a linear fashion with their concentration, allowing absolute values for pure samples to be extrapolated directly from diluted ones.

Standard addition is another technique designed to test for inner filter effects. A sample of known fluorescence intensity is diluted with an equal volume of standard of the same fluorescence intensity. The intensity of the mixture should then be equal to half the intensity of the standard plus half the intensity of the sample. Should the intensity of the mixture be more than 20% lower than predicted, then significant inner filter effects exist and any sample intensities must be multiplied by this correction factor for quantification (UNEP/IOC/IAEA, 1992). However, in the present study, standard addition was not a valid test for inner filter effects, since the dominant urinary metabolite and the closest available standard emitted fluorescence at different wavelengths.

2.2.13 Statistical analysis

Data were analysed using the software package Statgraphics Plus 5.1. Variance checks were performed on all data (urine and haemolymph) for homogeneity of variance between groups and were found to be not normally distributed (a statistically significant difference amongst the SDs, $p=<0.05$). This large variability in the data (more than a 3 to 1 difference between the smallest SD and the largest) invalidates most statistical tests,



including analysis of variance (ANOVA), which assumes that the SD at all treatments is equal. All urinary data were therefore log-transformed, after which they were found to be normally distributed. This permitted comparison of means at the 95% confidence level by one-way ANOVA (with treatment as the independent variable), using Fisher's least significant difference (LSD) procedure. Log transformation of the haemolymph data did not render it normally distributed, and therefore the Kruskal-Wallis test was applied to compare medians at the 95% confidence level.

In order to illustrate the variability of the results, all original, untransformed data are presented using notched box and whisker plots. The rectangular part of the plot ("box") extends from the lower quartile to the upper quartile, covering the middle 50% of data values (interquartile range). The centre lines within each box show the position of the medians, whilst the plus signs show the position of the mean. The "whiskers" extend from the upper and lower quartiles to maximum and minimum values lying within 1.5 times the interquartile range. Outliers are shown as individual points. Also included are notches covering a distance either side of the median line, representing the 95% confidence interval for the median. Where notches for any pair of medians overlap, there is no statistically significant difference between medians at the 95% confidence level. Where no overlap occurs, the difference is significant at the 95% confidence level. Data that was normally distributed following log-transformation i.e. urinary data, is also presented as means plots (± SD).

2.3 Results

2.3.1 Pyrene standards

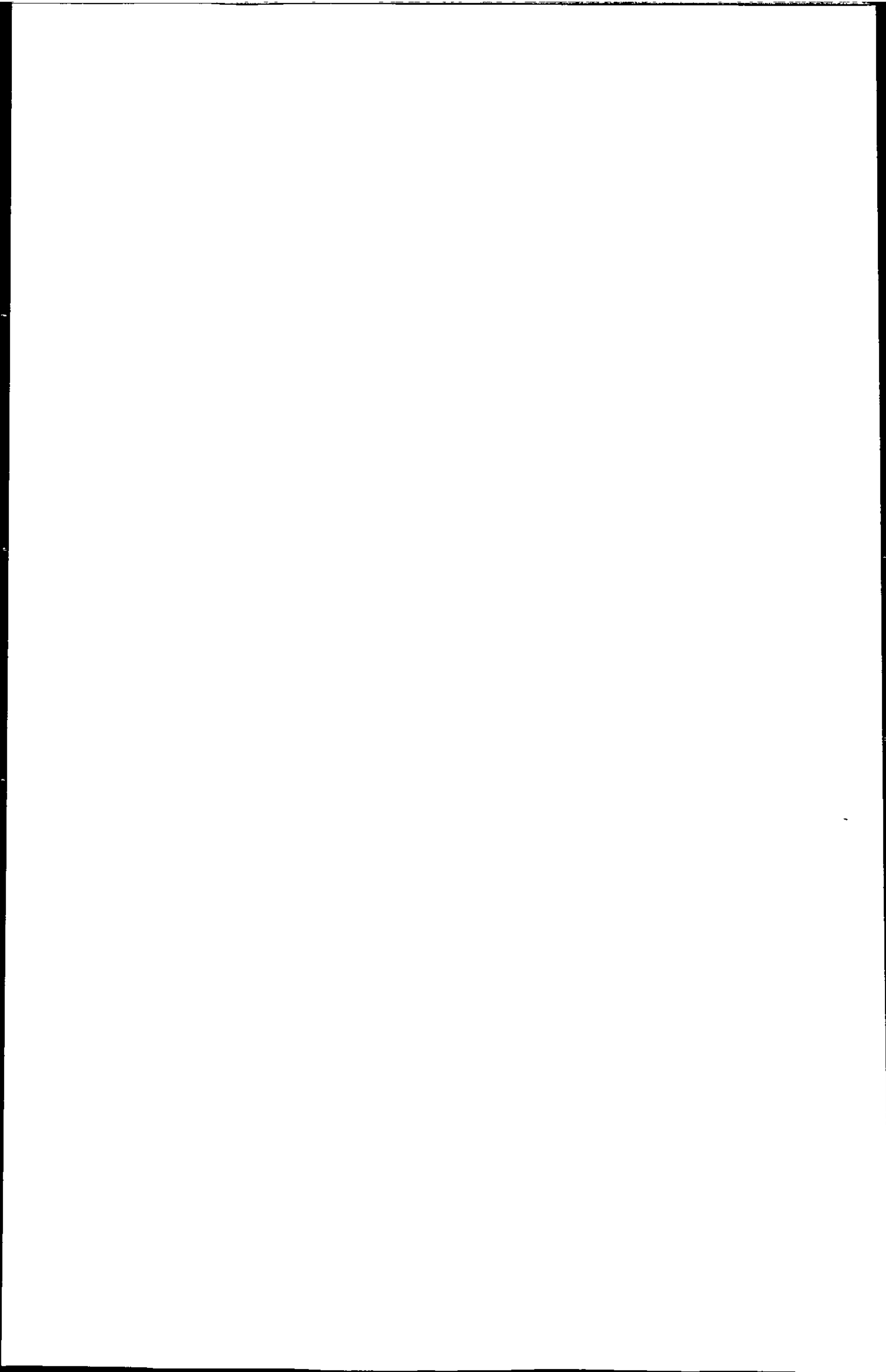
FF with an excitation wavelength of 243nm produced a predominant peak at Em372-373nm, with an additional smaller peak at Em392nm. Excitation with 345nm produced no peaks. SFS with a $\Delta\lambda$ of 37nm yielded a sharp peak at Em372nm (figure 2.4).

2.3.2 1-OH pyrene standards

FF yielded three peaks, with the largest and most clearly resolved at Em386-7nm (figure 2.4). SFS ($\Delta\lambda$37nm) of the same standards yielded a large sharp peak at the wavelength pair of Ex349-350/Em386-387nm (figure 2.5).

2.3.3 Urine samples

FF analysis of urine samples from exposed crabs (from both dose response and time response experiments) produced three clear peaks on the emission spectrum, with the largest of these being at approximately 381-383nm (figure 2.4). These peaks were conspicuously absent from control samples. SFS reduced these peaks to a single emission band, with a large sharp peak around Em381-383nm in exposed samples (figure 2.5), with controls lacking this peak. The position of the dominant emission peaks seen in FF and SFS is shifted approximately 5nm from that of the largest peak seen in the hydroxylated standards (~Em387nm). This has important implications for quantification and metabolite identification, which will be discussed below. The position of the SFS peak also strongly suggests that the fluorescence signal is produced almost exclusively by pyrenes other than the parent compound.



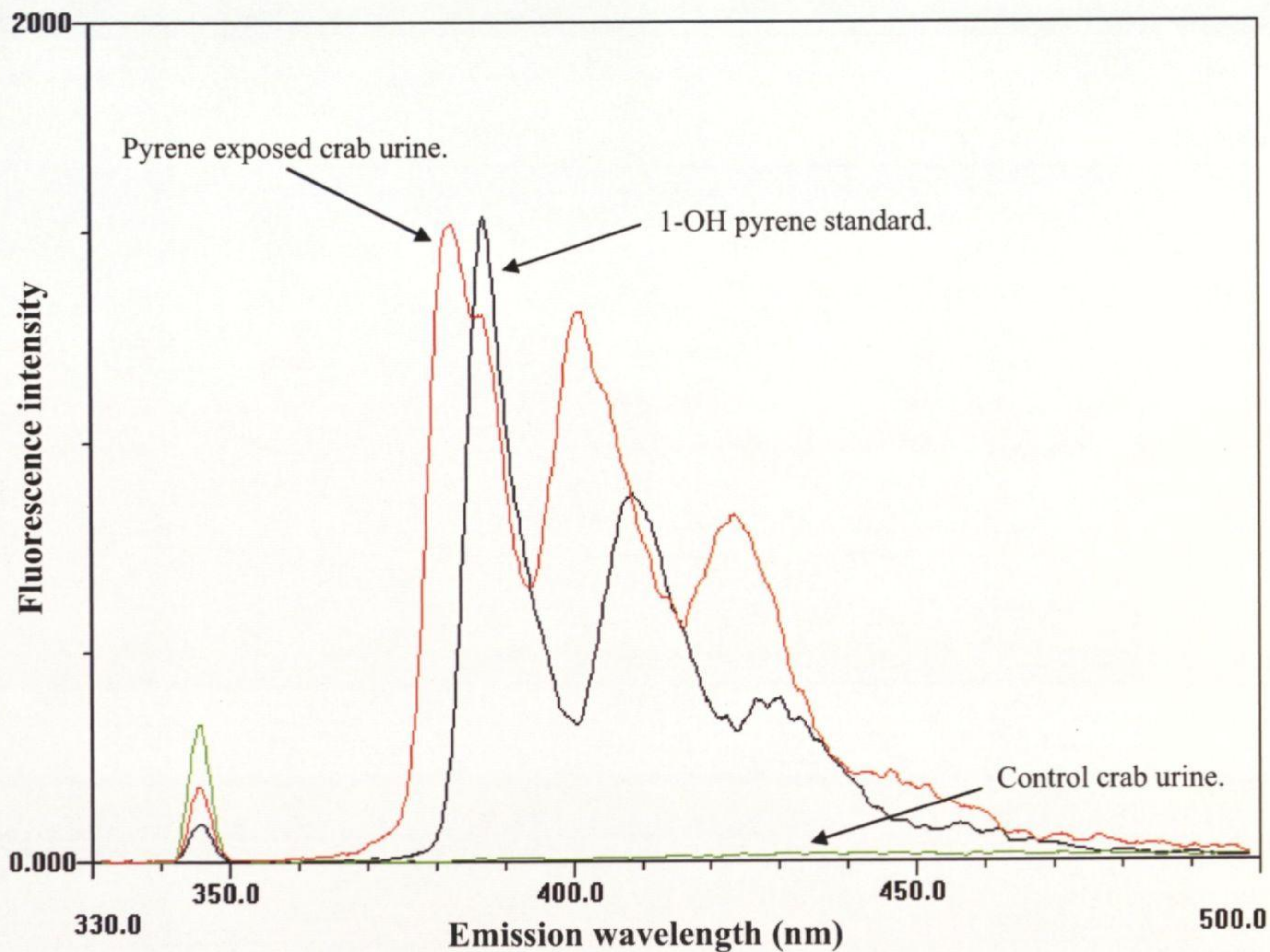
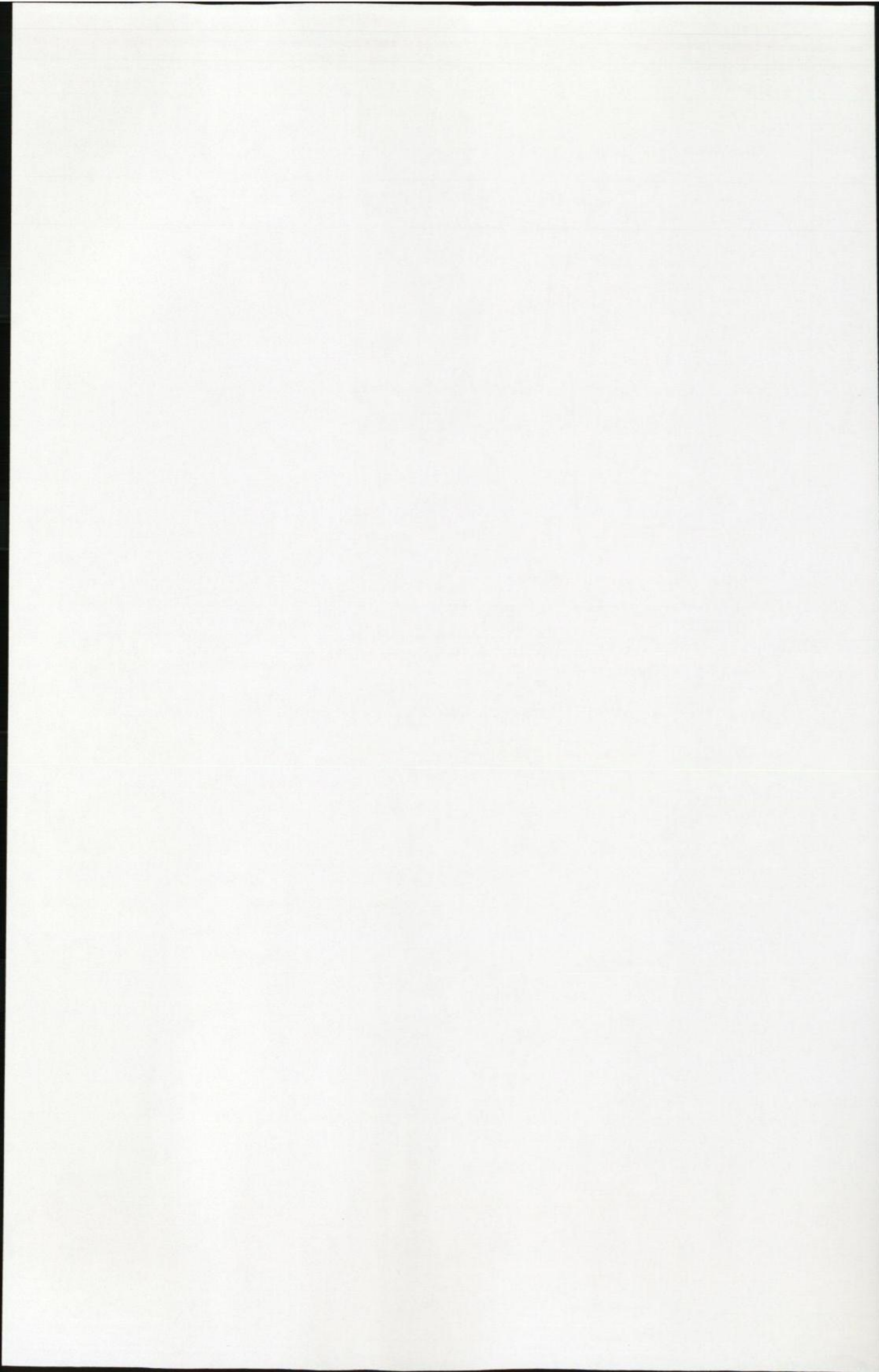


Figure 2.4 Fluorescence (FF, Ex345nm) spectra from 200μgl^{-1} pyrene exposed crab urine, control crab urine and 100 μgl^{-1} 1-OH pyrene standard. Urine samples diluted 1:100.



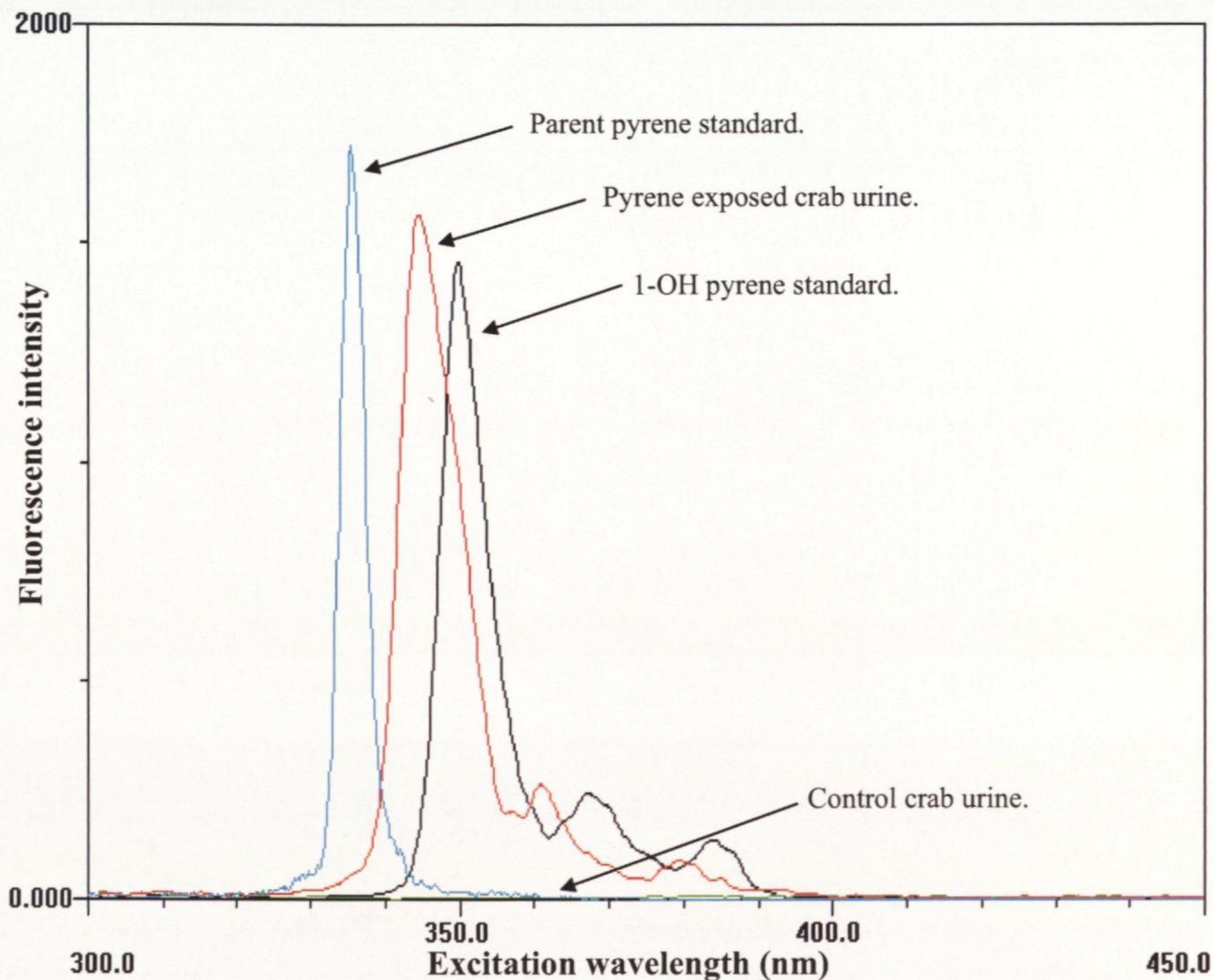
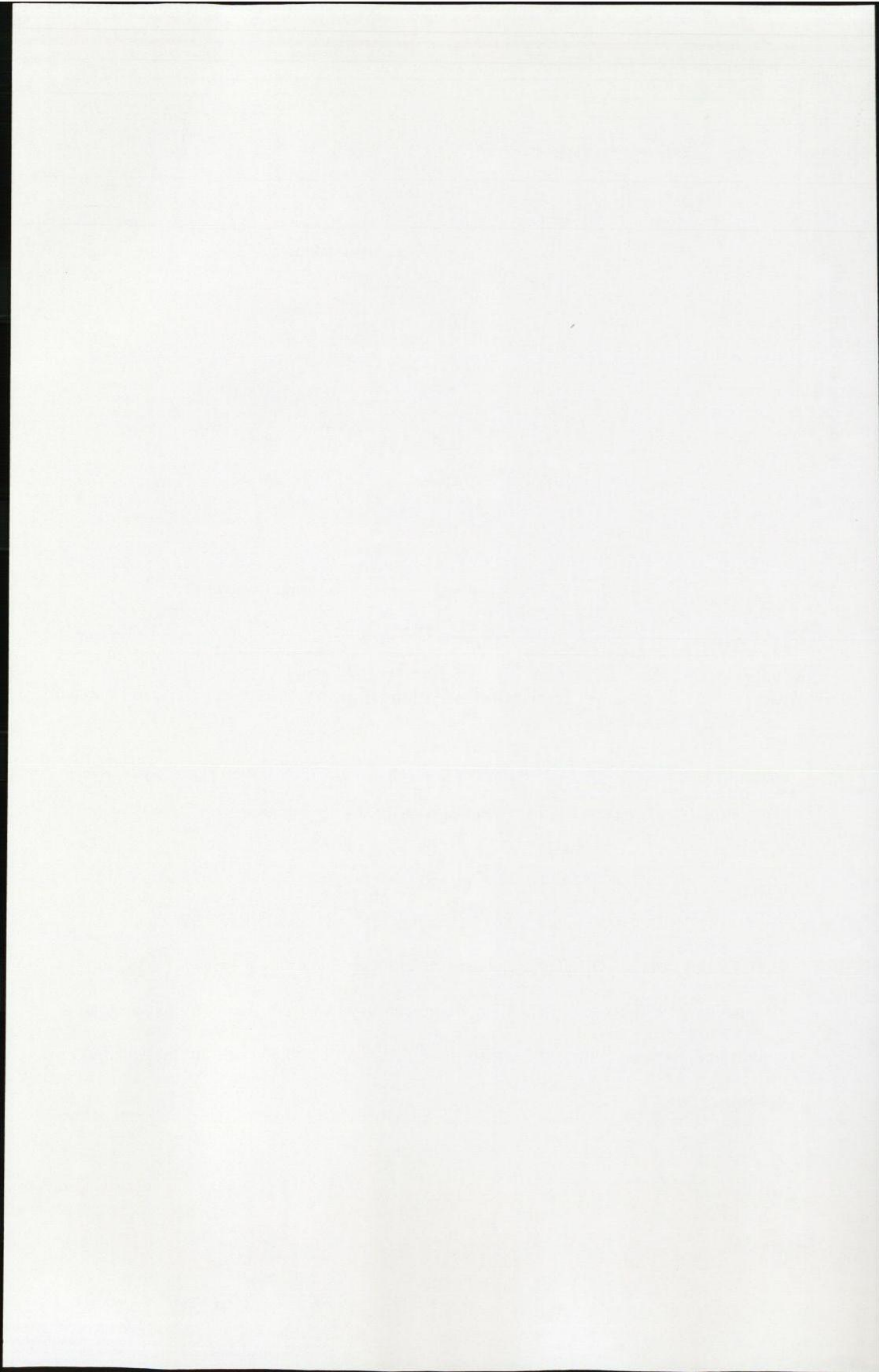


Figure 2.5 Fluorescence (SFS, Δλ 37nm) spectra from 200 μgl^{-1} pyrene exposed crab urine, control crab urine, 100 μgl^{-1} 1-OH pyrene and 100 μgl^{-1} pyrene standards. Urine samples diluted 1:100.

Results are reported in terms of $\mu g\ l^{-1}$ of 1-OH pyrene equivalents, determined by interpolation of sample fluoresence intensities (at FF Ex345/Em382nm and SFS Em381.4nm) from a 1-OH pyrene standard curve (using intensities at FF/SFS Em387nm). No significant differences ($p>0.05$) in fluorescence signal were seen between acetone control and seawater control groups, so these data were combined and are presented as controls.



Inner filter effects

A linear relationship (Figure 2.6) between fluorescence intensity and sample concentration (dilution factor) indicated there was no significant inner filter effect, permitting measurements to be used for quantification (UNEP/IOC/IAEA, 1992). This result also signified that fluorescence from the compounds of interest increased in a linear fashion with their concentration, allowing values for undiluted samples to be extrapolated from diluted values.

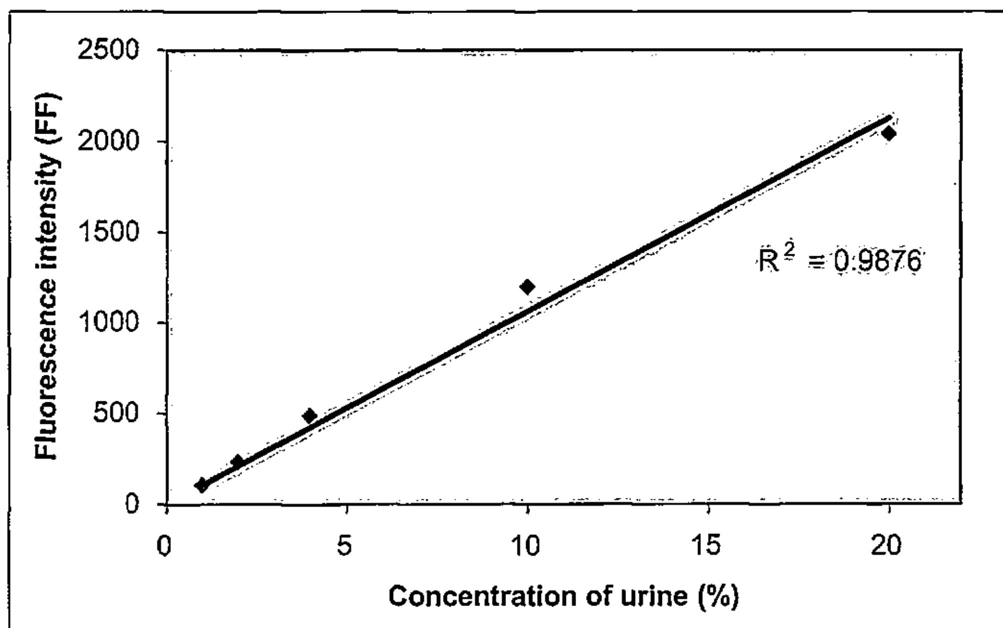


Figure 2.6 Inner filter effects - Linear relationship between urine concentration (1-20% or alternatively, diluted 1:100 - 1:5) and fluorescence intensity of equivalents @FF $\lambda_{Ex/Em}$=345/382nm).

Exposure- response experiment

Untransformed urinary data are presented in Table 2.1 and Figures 2.7 and 2.8. Levels of urinary "1-OH pyrene equivalents" increased in a concentration dependent manner with waterborne pyrene concentration. Regression analysis of these data revealed that a "moderately strong" relationship exists (FF r^2 =68.23%, correlation coefficient =0.826, SFS r^2 =66.12%, cc =0.813). Mean values determined by SFS were marginally

higher than those determined by FF. There was a statistically significant difference ($p=0.0$, KW) between the medians at each treatment, whether determined by FF or SFS, with the exception of the medians from the 100 and 200µg l^{-1} treatment groups. The data from both methods showed considerable individual variability, with this increasing with exposure and being most marked in the 100 and 200µg l^{-1} treatment groups. Regression analysis of treatment means resulted in much higher r^2 values (FF = 0.9953, SFS = 0.9972) (figure 2.9).

Table 2.1 Mean concentrations of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C.maenas*, determined by FF and SFS. Data are means ± SD. Numbers in parentheses indicate the number of samples analysed.

Treatment (µg l^{-1} pyrene)	µg l^{-1} 1-OH pyrene equivalents (FF @ Ex345/Em382nm)	µg l^{-1} 1-OH pyrene equivalents (SFS @ Em381.4nm)
200	89.77 ±47.40 (15)	100.44 ±55.69 (15)
100	40.58 ±20.70 (19)	43.75 ±23.49 (19)
50	21.50 ±11.66 (16)	24.27 ±13.15 (16)
25	8.10 ±3.21 (15)	8.93 ±4.01(15)
10	2.59 ±1.00 (12)	2.92 ±1.22 (12)
Controls	0.38 ±0.21 (20)	0.44 ±0.26 (20)

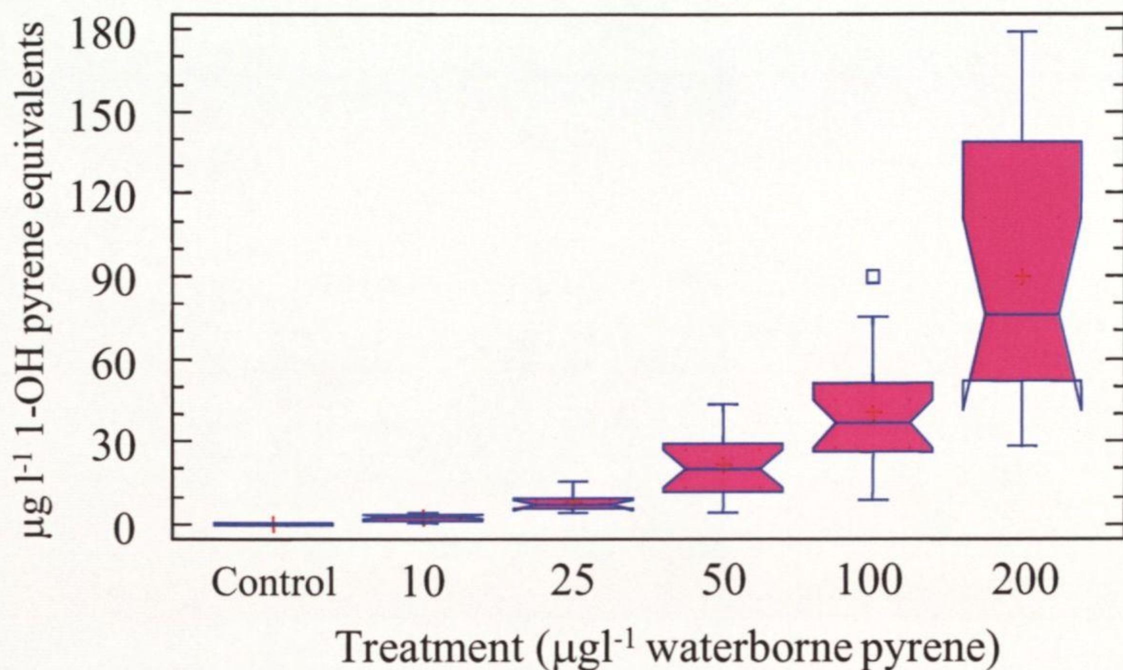


Figure 2.7 Notched box and whisker plot of concentration ($\mu g l^{-1}$) of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C.maenas*, determined by FF.

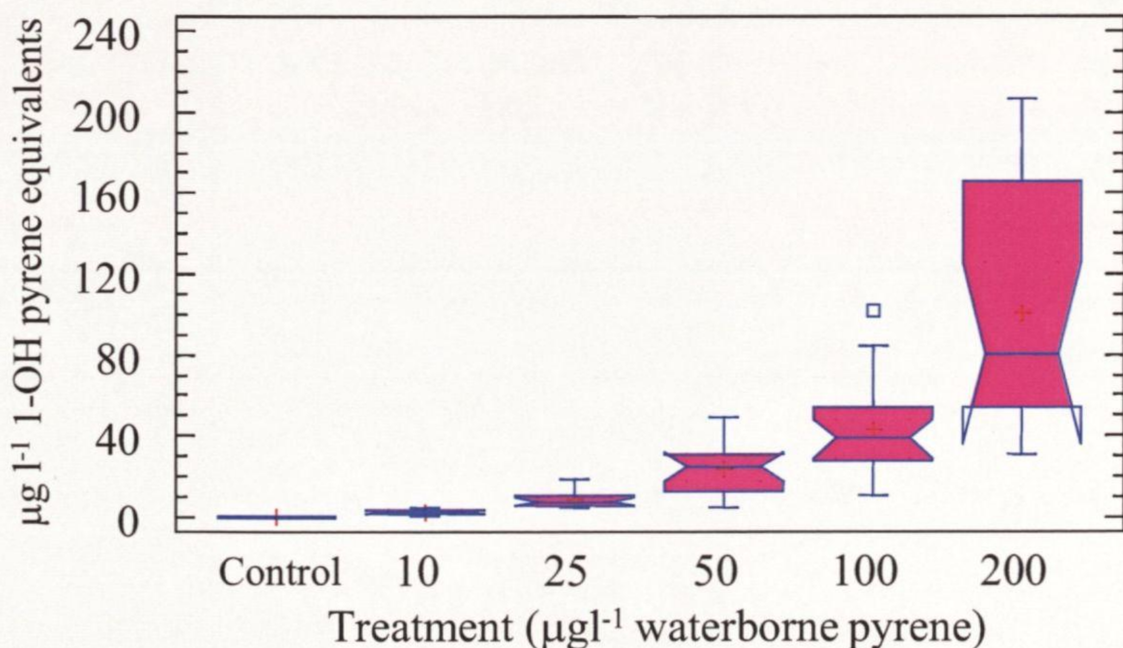
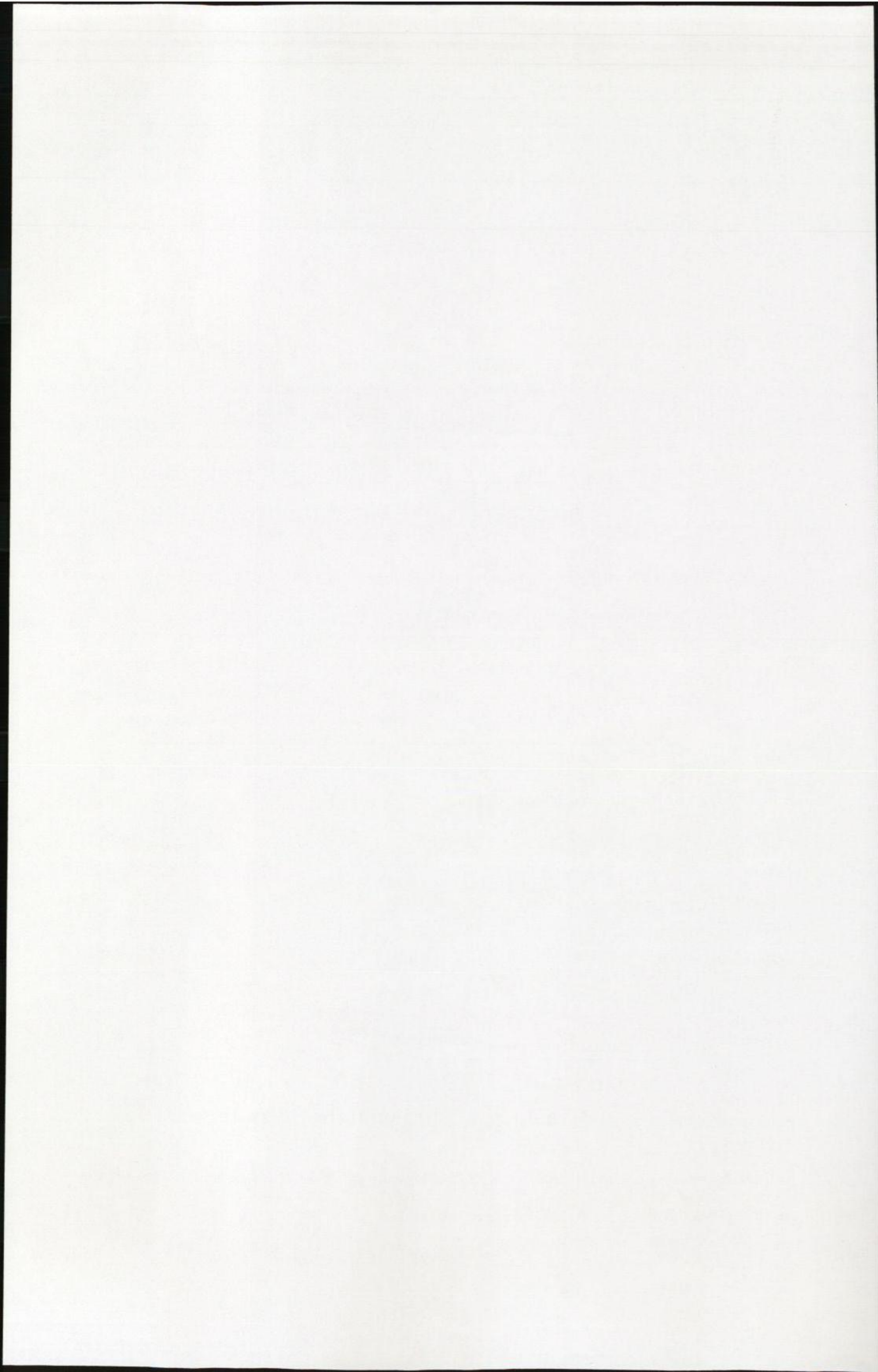


Figure 2.8 Notched box and whisker plot of concentration ($\mu g l^{-1}$) of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C.maenas*, determined by SFS.



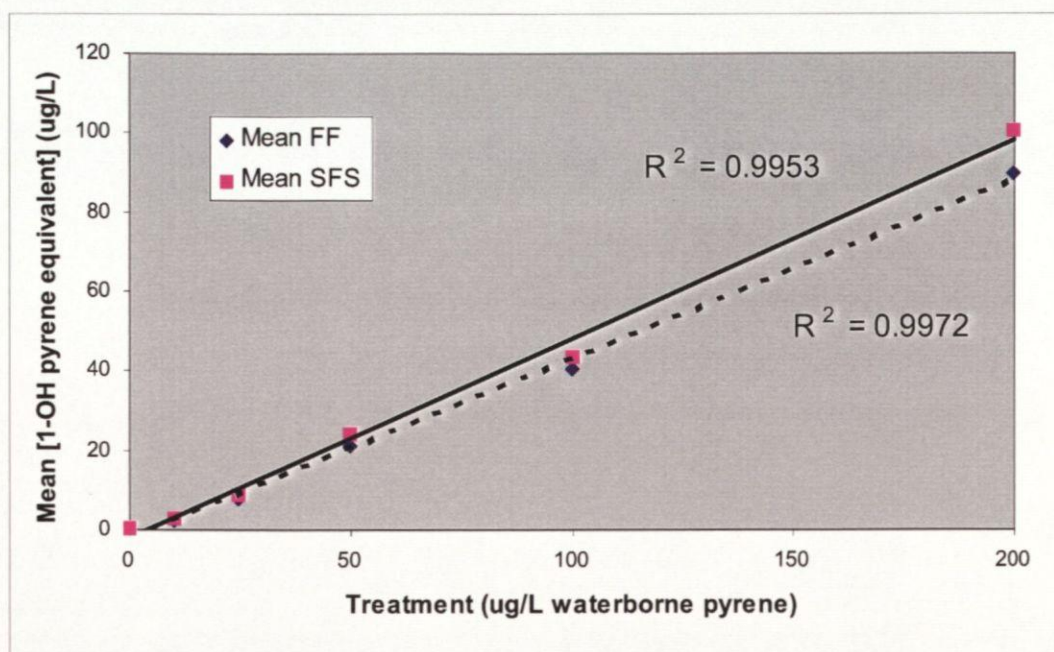


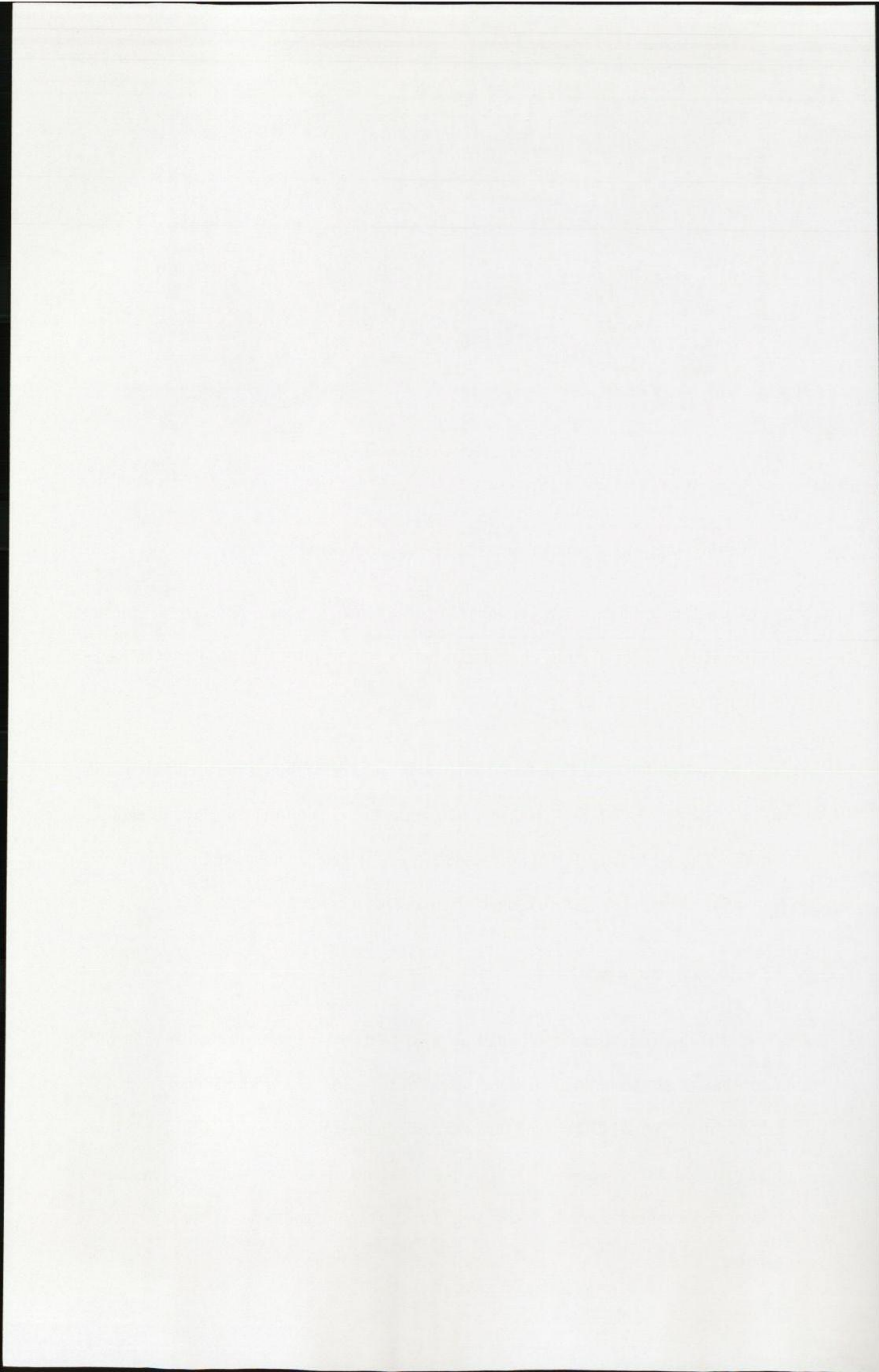
Figure 2.9 Regression plot of mean urinary 1-OH pyrene equivalent levels (FF + SFS).

Analysis of variance of log-transformed FF and SFS data revealed statistically significant differences in mean fluorescence between all treatments at the 95% confidence level (p=0.0001, ANOVA).

Baseline fluorescence at Ex345/Em382nm and Em381.4nm is reported for controls (as these samples lack a peak at the wavelengths), and is very low compared to exposed samples. The control level of fluorescence at these wavelengths was comparable to that seen in the solvent blank, which is negligible (data not presented).

2.3.4 Haemolymph samples

Results are reported in terms of $\mu g\ l^{-1}$ of 1-OH pyrene equivalents, determined by interpolation of sample fluoresence intensities (at FF Ex345/Em382nm and SFS Em381.4nm) from a 1-OH pyrene standard curve (using intensities at FF/SFS Em387nm). No significant differences (p>0.05, KW) in fluorescence signal were seen between acetone control and seawater control groups, so these data were combined and are presented as controls.



FF and SFS analysis of haemolymph samples produced peaks in the same positions as those seen in the urine samples (figures 2.10 and 2.11) but with greatly reduced intensities (table 2.2).

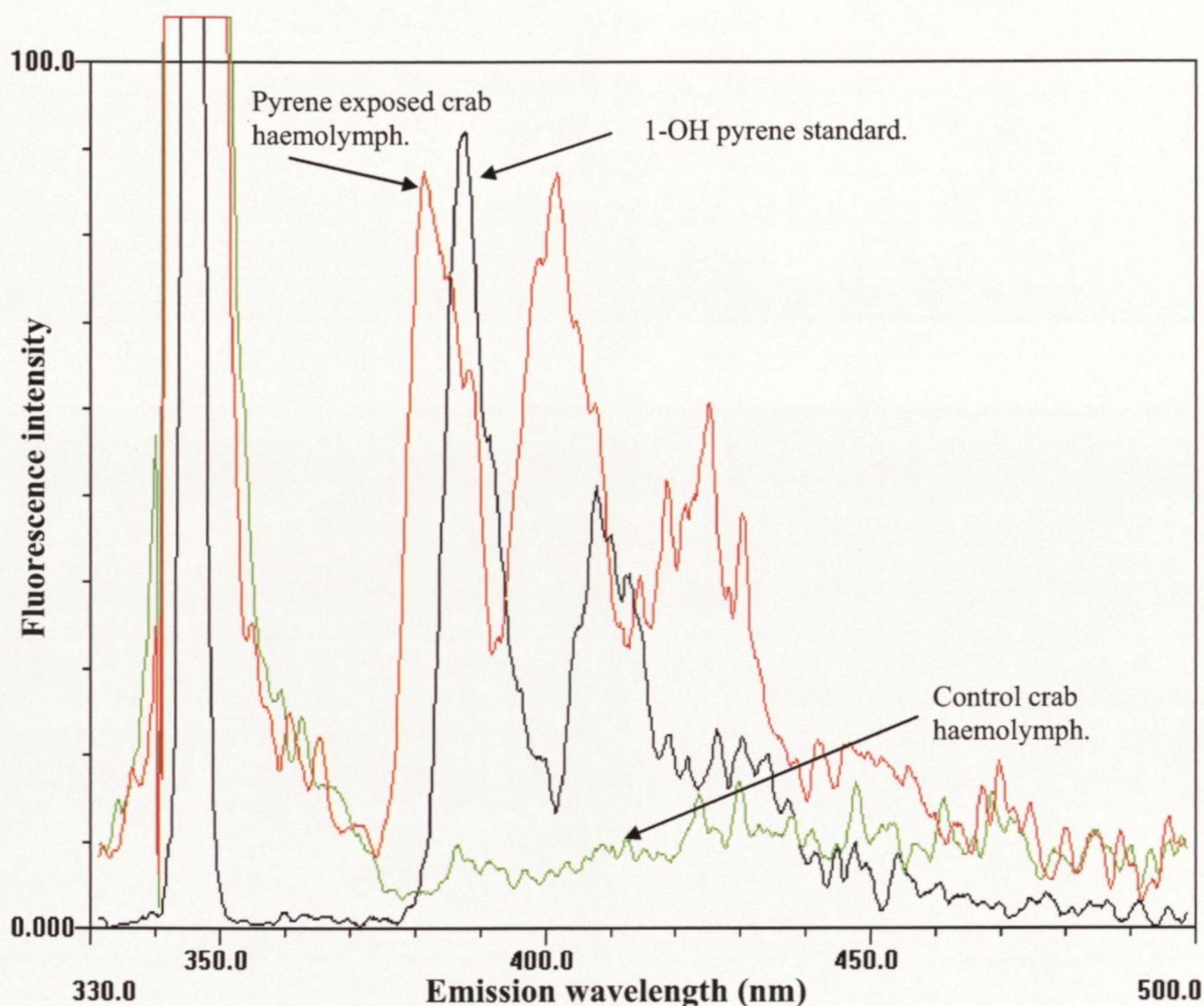
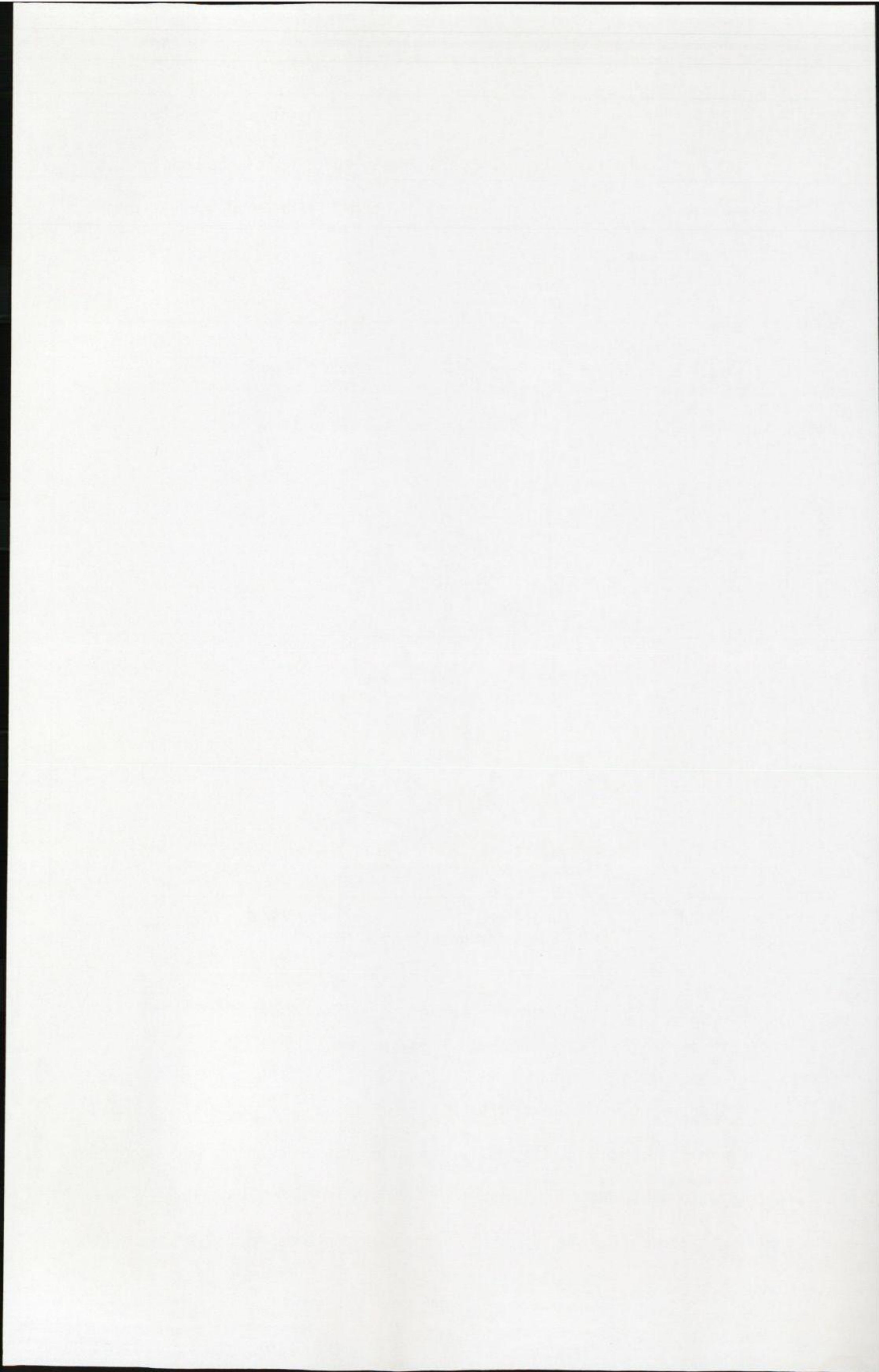


Figure 2.10 Fluorescence (FF, Ex345nm) spectra from 200 μgl^{-1} pyrene exposed crab haemolymph, control crab haemolymph and 5μgl^{-1} 1-OH pyrene standard. Haemolymph samples diluted 1:20.

Untransformed data are presented in figures 2.12 and 2.13 and table 2.2. Levels of 1-OH pyrene equivalents increased in a concentration dependent manner with waterborne pyrene concentration. Regression analysis of these data revealed that a moderately strong relationship exists (FF r^2=49.79%, cc=0.706 and SFS r^2=48.64%, cc=0.697). Mean values



determined by SFS were marginally higher than those determined by FF. Regression analysis of treatment means resulted in much higher r^2 values (FF = 0.8352, SFS = 0.8413), suggesting a stronger relationship between the variables.

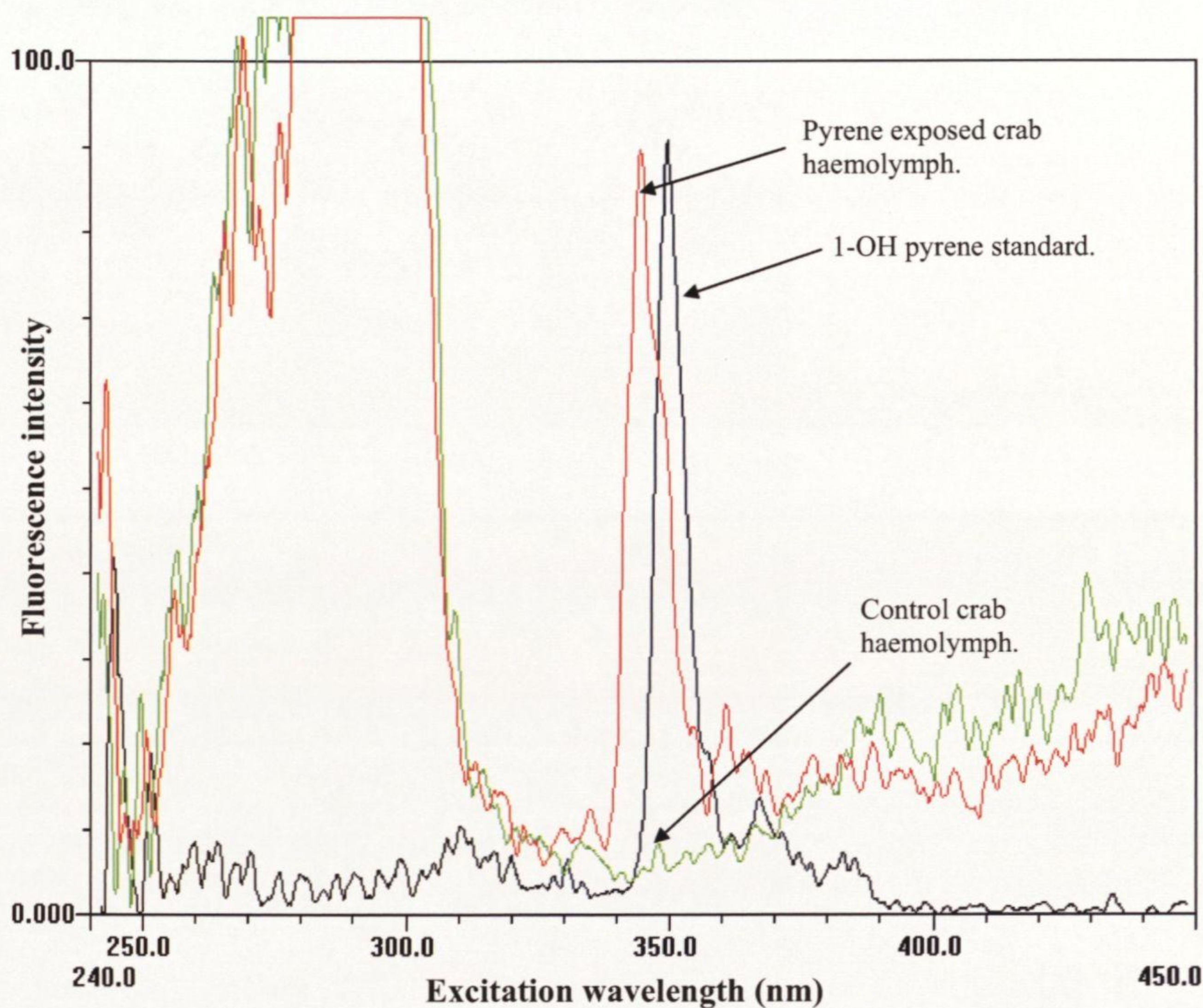
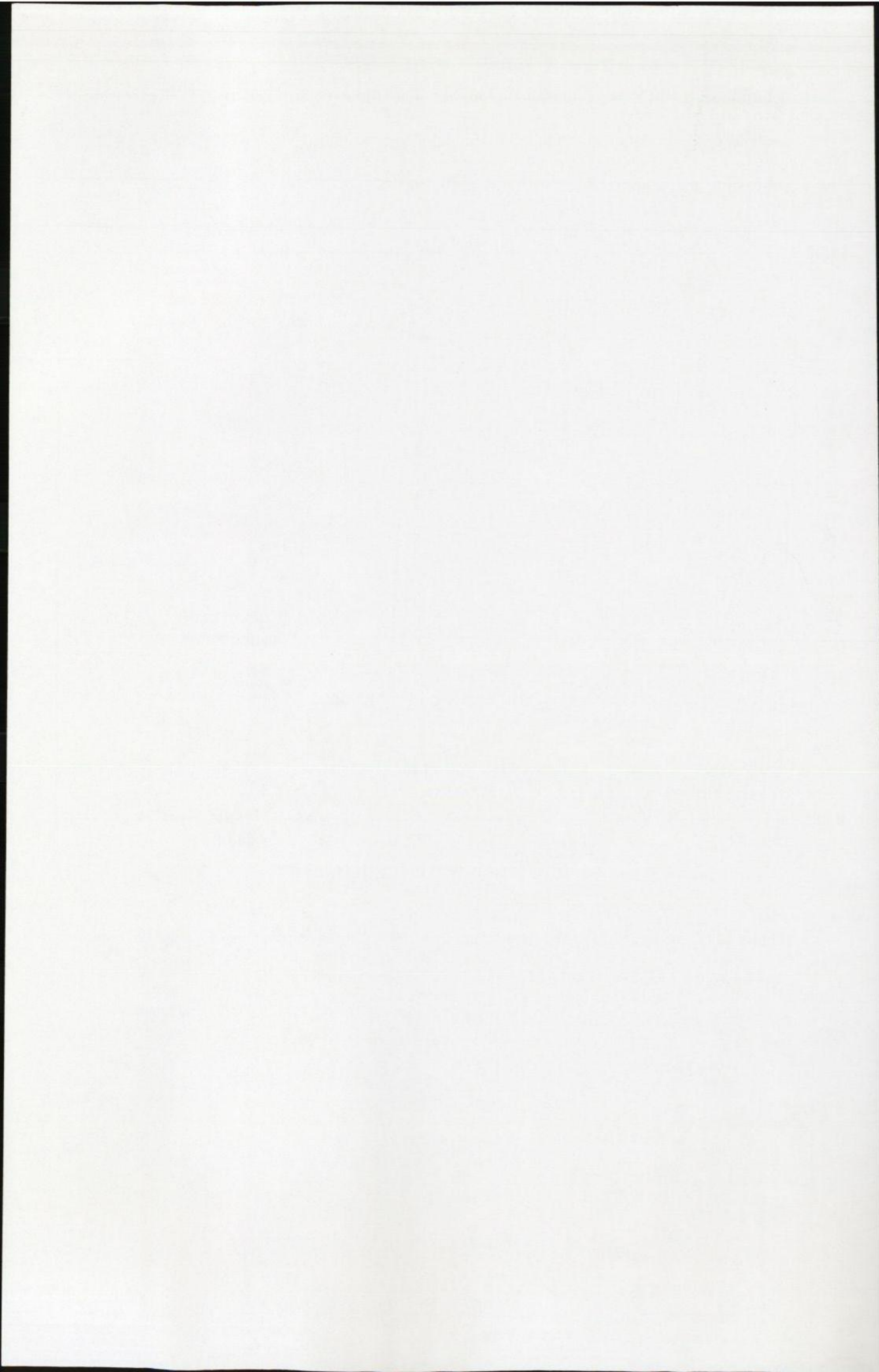


Figure 2.11 Fluorescence (SFS, $\Delta\lambda$ 37nm) spectra from 200 μgl^{-1} pyrene exposed crab haemolymph, control crab haemolymph and 5μgl^{-1} 1-OH pyrene standard. Haemolymph samples diluted 1:20.



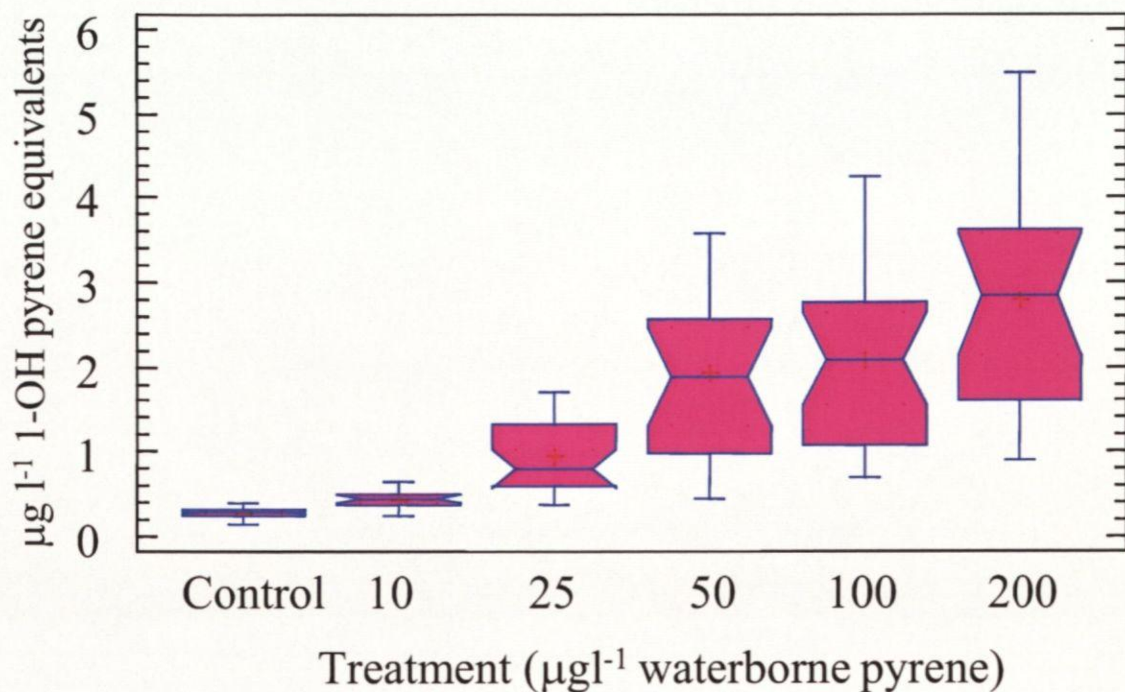


Figure 2.12 Notched box and whisker plot of concentration (μgl^{-1}) of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C.maenas* (FF).

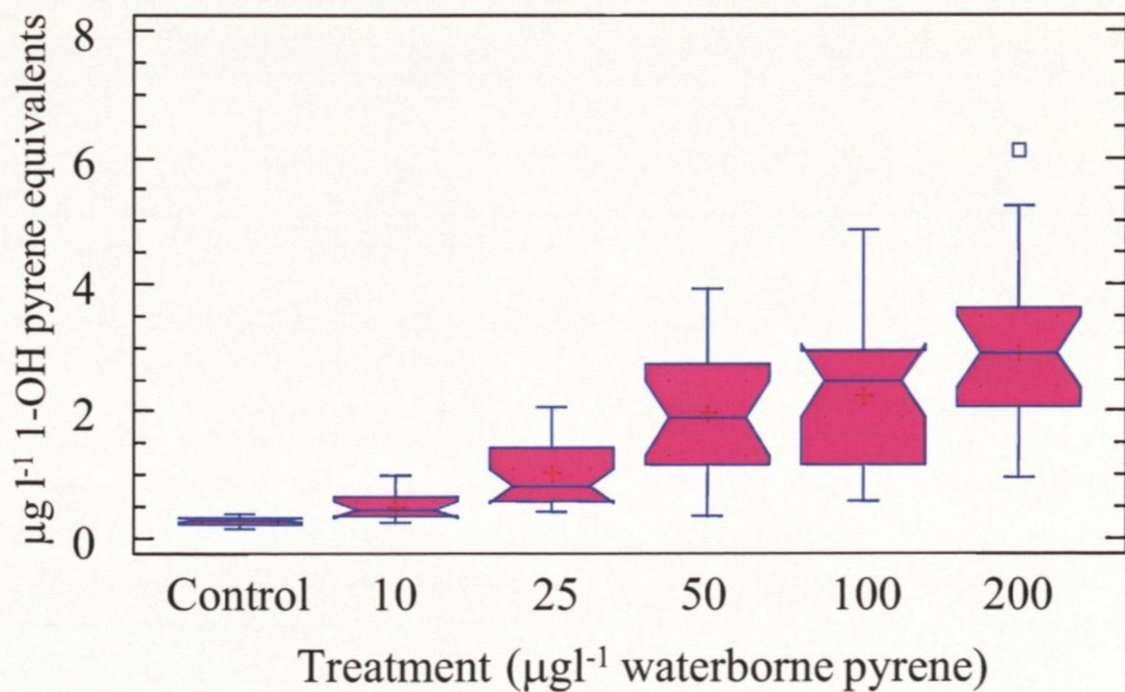


Figure 2.13 Notched box and whisker plot of concentration (μgl^{-1}) of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C.maenas* (SFS).

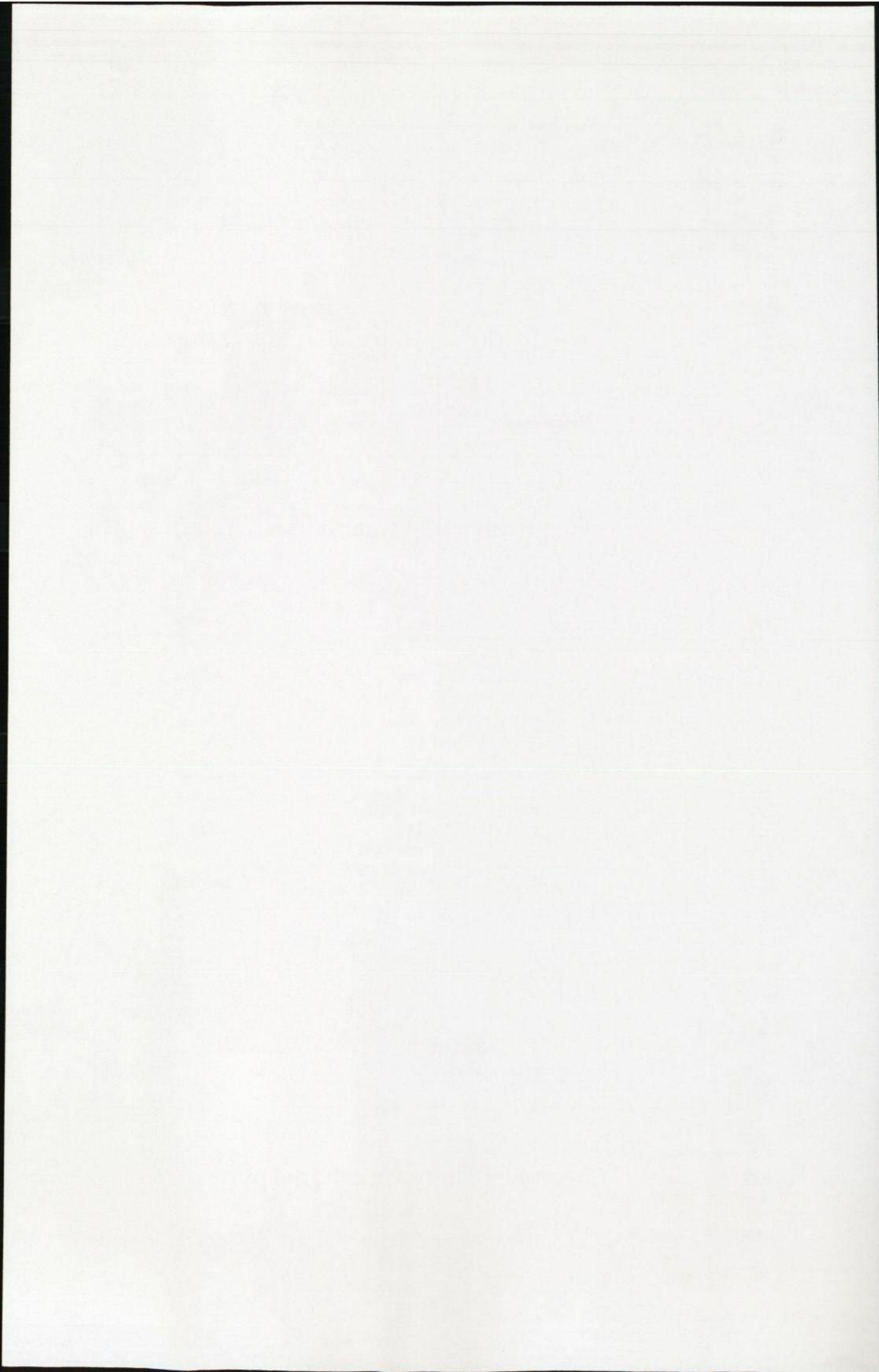


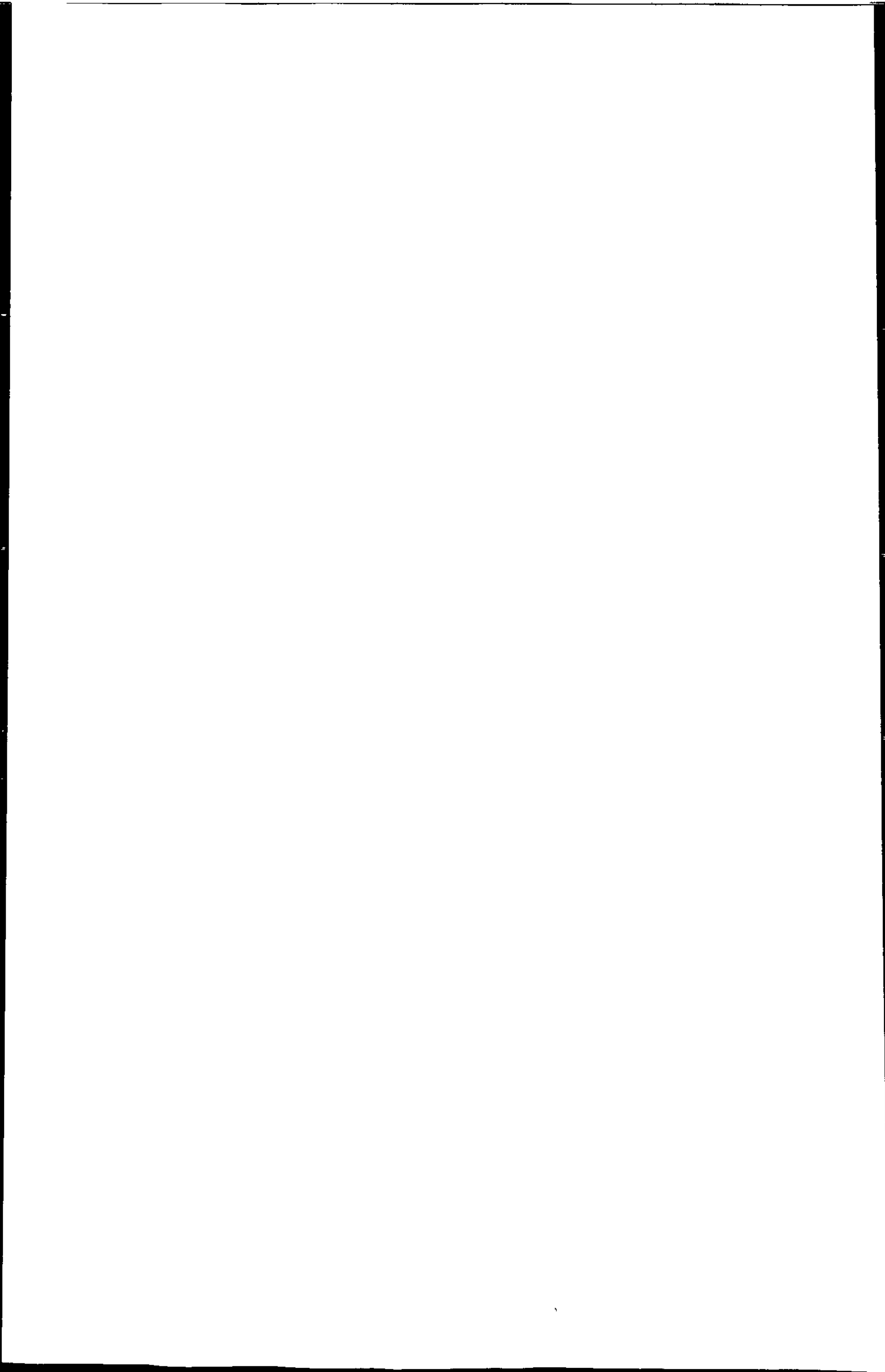
Table 2.2 Mean concentrations of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C.maenas*, determined by FF and SFS. Data are means ± SD. Numbers in parentheses indicate the number of samples analysed.

Treatment ($\mu g\ l^{-1}$ pyrene)	$\mu g\ l^{-1}$ 1-OH pyrene equivalents (FF @ Ex345/Em382nm)	$\mu g\ l^{-1}$ 1-OH pyrene equivalents (SFS @ Em381.4nm)
200	2.79 ±1.25 (19)	2.93 ±1.32 (19)
100	2.08 ±1.02 (23)	2.24 ±1.15 (23)
50	1.93 ±0.99 (17)	2.00 ±1.01 (17)
25	0.97 ±0.43 (24)	1.95 ±0.52 (24)
10	0.44 ± 0.11 (13)	0.51 ±0.21 (13)
Controls	0.29 ±0.06 (26)	0.29 ±0.06 (26)

The FF and SFS data showed large variability, which increased with exposure concentration and was most marked in the 50, 100 and 200$\mu g\ l^{-1}$ groups. This variability was greater than that seen in the urine samples from the same crabs. Despite logging the FF and SFS data, the standard deviations at each treatment differed significantly at the 95% confidence level, excluding ANOVA. A Kruskal-Wallis analysis of FF and SFS data showed that statistically significant differences ($p=0.00$) exist between the medians from the control, 10 and 25$\mu g\ l^{-1}$ groups. These three groups also differed significantly from the 50, 100 and 200$\mu g\ l^{-1}$ treatment groups. However, there were no statistically significant differences between the 50, 100 and 200$\mu g\ l^{-1}$ data sets ($p>0.05$).

2.3.5 Time response experiment

The time response data determined by both FF and SFS (figure 2.14) showed a clear pattern of uptake and elimination of pyrene in *C.maenas*. Levels of 1-OH pyrene equivalents in the urine of 200$\mu g\ l^{-1}$ and 400$\mu g\ l^{-1}$ exposed crabs increased to reach a maximum after 2 days and 3-4 days respectively, and dropped steadily over the remainder of the exposure period. The maximum mean level seen in the 400$\mu g\ l^{-1}$ exposed crab urine



was approximately twice the level in the 200μg l^{-1} exposed crabs. Pyrene fluorescence in controls was not detectable above the background baseline for the entire experimental period. After 10 days, 1-OH pyrene equivalent fluorescence in exposed crabs decreased to levels similar to those after 12hrs exposure, but was still significantly different to controls ($p<0.05$, KW).

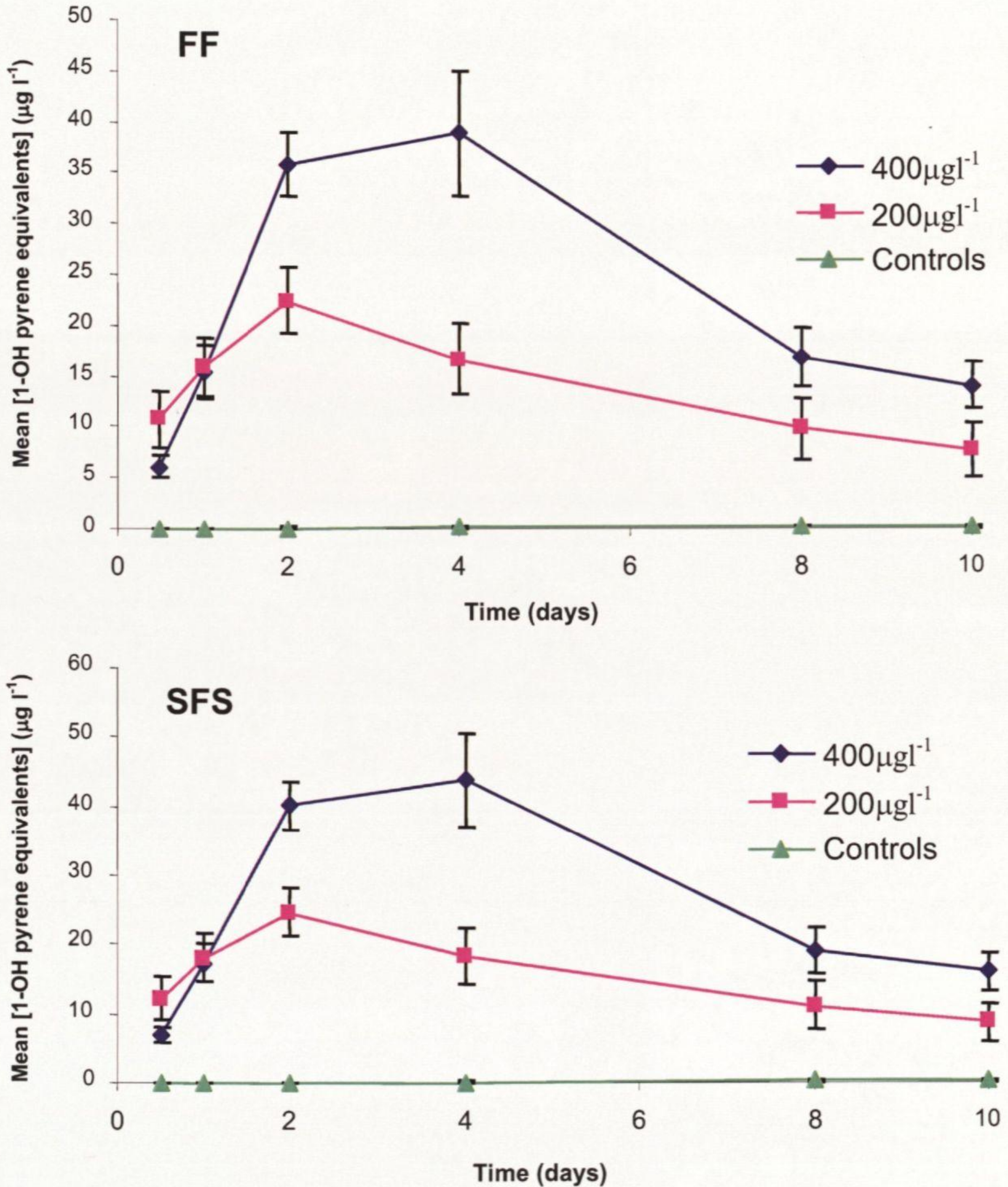
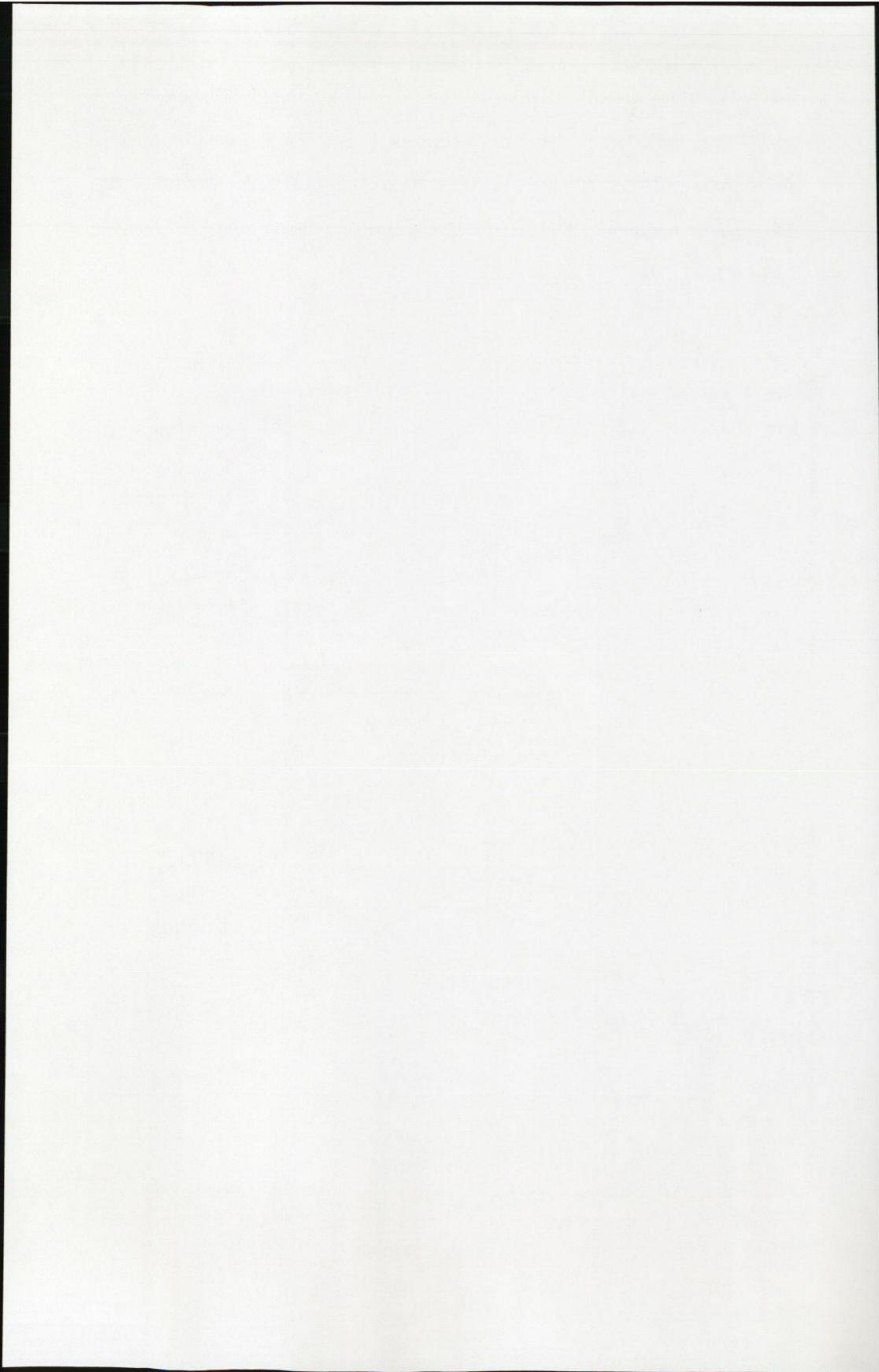


Figure 2.14 Temporal response of 1-OH pyrene equivalent levels in the urine (diluted 1:100) of pyrene exposed *C.maenas*, determined by FF and SFS. Values are means ±SE. (400μgl^{-1} n=7, 200μgl^{-1} n=6-7, Controls n=3-8)



2.4 Discussion

The results above provide compelling evidence of exposure (uptake, metabolism and excretion) in the pyrene-exposed crabs, as large 1-OH pyrene equivalent peaks occur in their urine and to a lesser extent, in their haemolymph. This is in stark contrast to the controls, whose urine and haemolymph are conspicuously free of peaks at Em381-383nm. This rules out the possibility of endogenous compounds contributing sharp fluorescence peaks at these wavelengths. Fluorescence spectrophotometry appears to be a valid method for establishing proof of exposure and has potential for use in ecosystem contamination monitoring. Urinary levels are exposure concentration dependent, a result which may help in prioritising contaminated sites according to PAH exposure. Indeed, in a similar study on fish bile, Aas *et al.,* (2000a) note that a prerequisite for application of the FF technique for monitoring purposes is the establishment of a reasonable dose response relationship. Regression analyses of raw data indicate a "moderately strong" relationship between exposure concentration and levels of pyrene equivalents, meaning this requirement has been fulfilled. Regression analysis of mean levels at each treatment revealss an even stronger relationship between the variables.

A field exposure level equivalent to the 10μg l^{-1} exposure concentration in the present study should be considered the minimum detectable by this method, since pyrene equivalent peaks become obscured by the baseline fluorescence below this level. FF has shown clear dose response relationships in studies on the bile of oil-exposed turbot, *Scophthalmus maximus* (Camus *et al.*, 1998) and Atlantic cod, *Gadus morhua* (Aas *et al.*, 2000b), Atlantic cod exposed intraperitoneally to pyrene (Aas *et al.*, 2000a) and flounder injected with benzo(a)pyrene (Beyer *et al.*, 1997). Levels of pyrene equivalents in the haemolymph are also exposure concentration dependent, although this relationship is not as strong as in urine. The haemolymph fluorescence intensities are, on average, much lower than those in urine and the variability of the data much greater, with the three highest

treatment groups showing no significant differences as a result ($p>0.05$, KW). Urine it seems is a much more suitable biological medium to analyse, where available.

The time response data show urinary levels of 1-OH pyrene equivalents reach a maximum after 48-96h in exposed crabs and steadily decline for a further 6 days to levels similar to those at 12hrs. This level is still significantly different ($p<0.05$, KW) to the controls. This is consistent with the fact that cytochrome p450 biotransformation systems are generally induced in a matter of days (Stegeman *et al.*, 1990). Very similar kinetics are seen in the levels of "BaP-type" fluorescence in the bile of exposed Atlantic cod (Aas *et al.*, 2000a), with a peak level of fluorescence signal observed after 3 days followed by a gradual decrease until day 28. Camus *et al.*, (1998) also observe a temporal component in the levels of pyrene metabolites in the bile of oil-exposed turbot. The maximum signal was measured between 24 and 96hrs of exposure and then levels steadily decreased over 9 days when the fish were transferred to clean seawater. After this recovery period, bile fluorescence had returned to basal levels.

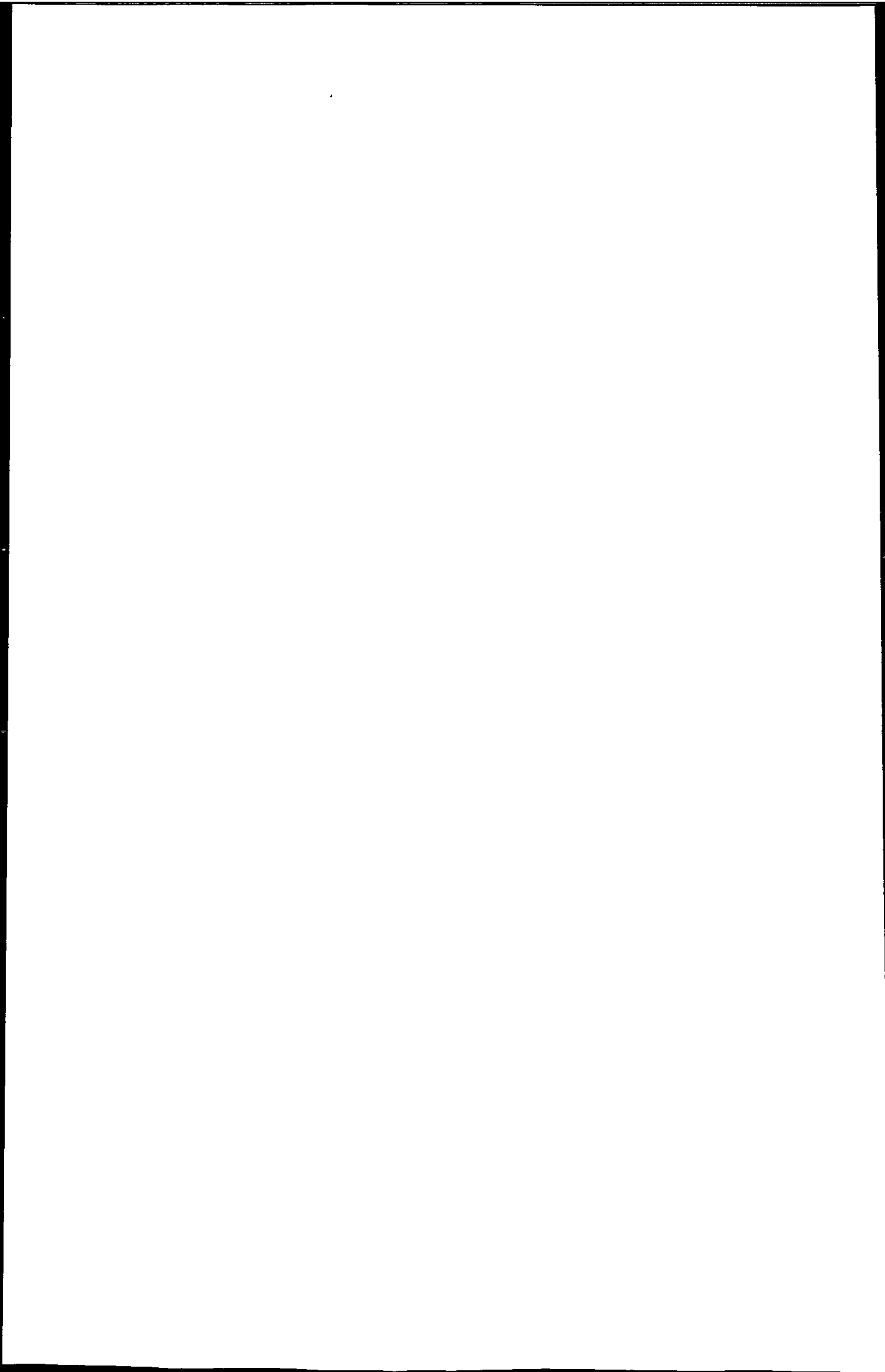
Time-dependent clearance of contaminant load is not restricted to fish species, however, and has been reported in various crustacean species exposed to or dosed with PAHs (James *et al.*, 1995, James *et al.*, 1989, Lauren and Rice, 1985, Solbakken *et al.*, 1980, Lee *et al.*, 1976). In one of the few fluorimetric studies on Crustacea, Sundt and Goksoyr (1998) injected the edible crab (*Cancer pagurus*) with benzo(a)pyrene and showed that levels of B(a)P-type fluorescence in the hepatopancreas cytosol reach a maximum 10 days after injection and fall steadily for another 20 days. However, levels at day 30 were still considerably higher than controls.

Rapid depuration of PAHs in fish results in concentrations of biliary PAH equivalents that reflect current levels of exposure. This can be used to detect rapidly changing exposure levels and is especially useful for monitoring acute pollution incidents such as oil spills (Johnston and Baumann, 1989). Despite slower rates of PAH metabolism

and depuration in crustaceans (Stegeman and Lech, 1991), urinary levels of PAH equivalents may also prove useful for episodic pollution monitoring, given their time-dependent nature.

Several authors with access to suitable standards have identified metabolites of various PAHs in the biological fluids of aquatic species. While most have concentrated on identifying metabolites in fish bile, several studies have investigated PAH metabolism in crustaceans and their results suggest possibilities for the identity of the equivalents in the present work.

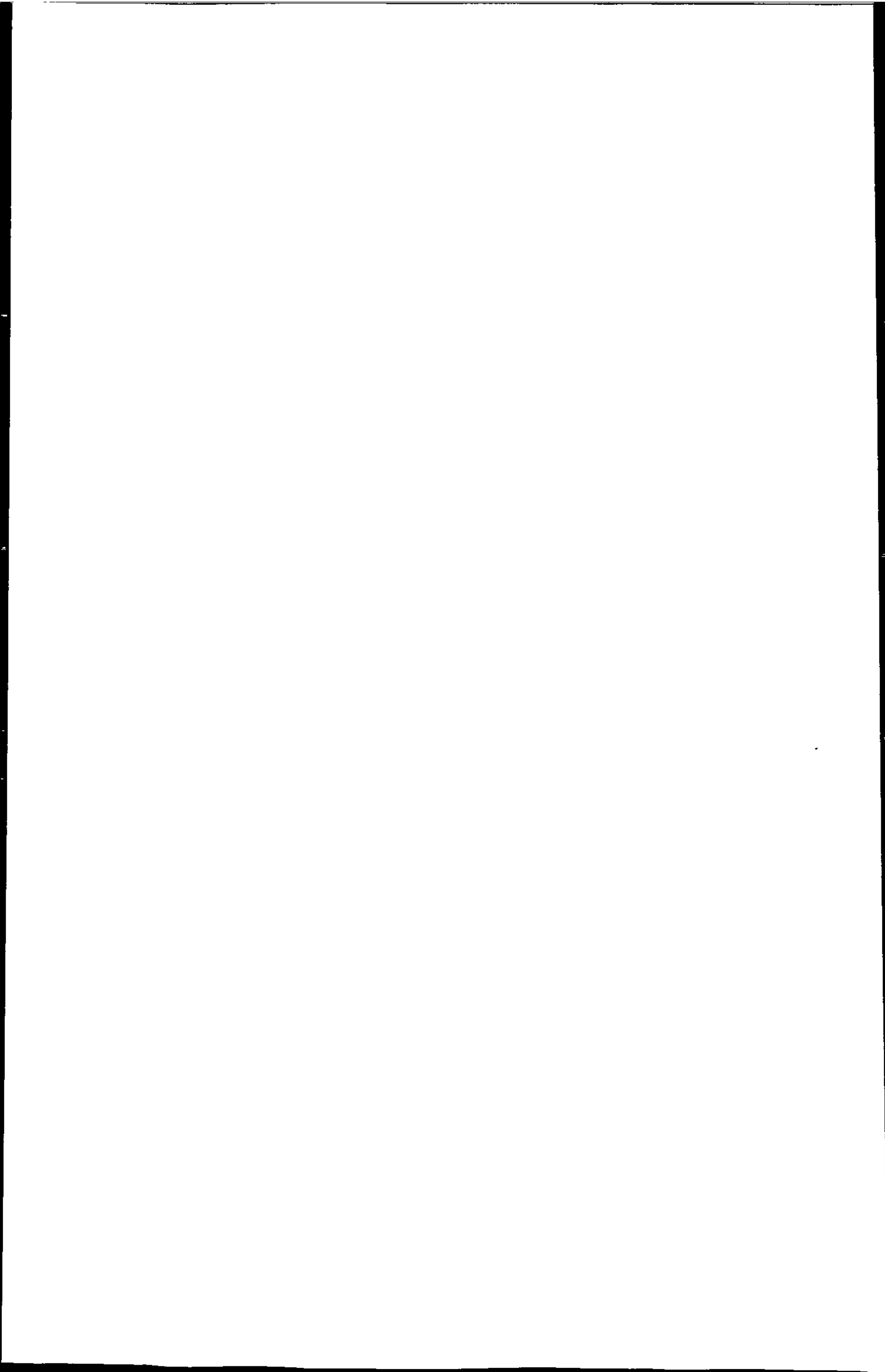
The 1,2- and 9,10-dihydrodiols, and 1-,2-,3-,4- and 9-monohydroxy derivatives of phenanthrene were identified in the urine and bile of the coalfish (*Pollachius virens*) (Solbakken *et al.,* 1980). Law *et al.,* (1994) exposed rainbow trout to pyrene and found that 1-hydroxypyrene was the major oxidation metabolite in the urine and the bile, with small amounts of the 1,6 and 1,8-dihydroxypyrenes also being detected. No parent pyrene was found. *Cancer pagurus* has been shown to metabolise benzo(a)pyrene to hydroxylated and conjugated metabolites (Sundt and Goskoyr, 1998) and James *et al.,* (1992) report that the predominant benzo(a)pyrene metabolites produced by the spiny lobster *Panulirus argus* are the 9,10-,4,5- and 7,8- dihydrodiols. In an early study by Lee *et al.,* (1976), radiolabelled pariffinic and aromatic hydrocarbons, including benzo(a)pyrene, fluorene, napthalene and methylnapthalene, were administered to the water and food of the blue crab, *Callinectes sapidus.* After 6 days, monohydroxy, and dihydroxy metabolites and their conjugates were found in the hepatopancreas. Parent and polar metabolites were also found in the gills, haemolymph, stomach muscle and gonads. Analysis of the green gland, gonads and intestine of Norway lobster, *Nephrops norvegicus,* force fed ^{14}C phenanthrene detected the 9,10- and 1,2-dihydrodiol as well as 1-,2- and 9-hydroxys (Solbakken and Palmork, 1981). In blue crab (*C.sapidus)* fed mussels exposed to ^{14}C phenanthrene, elimination was mainly via dihydrodiol metabolites, in particular the 9,10-type (Moese and O'Conner,



1985). 1, 2-dihydrodiol naphthalene was found in the urine of the spiny spider crab (*Maia squinado*) force-fed naphthalene (Corner *et al.,* 1973).

The phenolic (monohydroxy) and dihydrodiols are clearly the dominant class of metabolites generated by Phase I biotransformation, mediated by cytochrome p450 enzymes in the microsomes of the liver of fish and the hepatopancreas of crustaceans (James and Boyle, 1998). Phase II conjugation reactions, which attach readily soluble organic anions, such as glycosides, sulphates and amino acids to Phase I metabolites (James, 1987), produce metabolites that are rapidly excreted by fish and higher invertebrates. Conjugation is therefore a desirable process and the extent to which it occurs is extremely important in determining excretion rates of pollutants (James, 1987). Examples can be found in most studies that report the occurrence of phase I metabolites. Metabolites conjugated with sulphate and glucuronic acid are seen in the bile and urine of rainbow trout (*Oncorhynchus mykiss*) exposed to pyrene (Law *et al.,* 1994). Sulphate and glucuronide conjugates of hydroxylated phenanthrene metabolites have also been found in the bile and urine of the coalfish (*P.virens*) (Solbakken *et al.,* 1980).

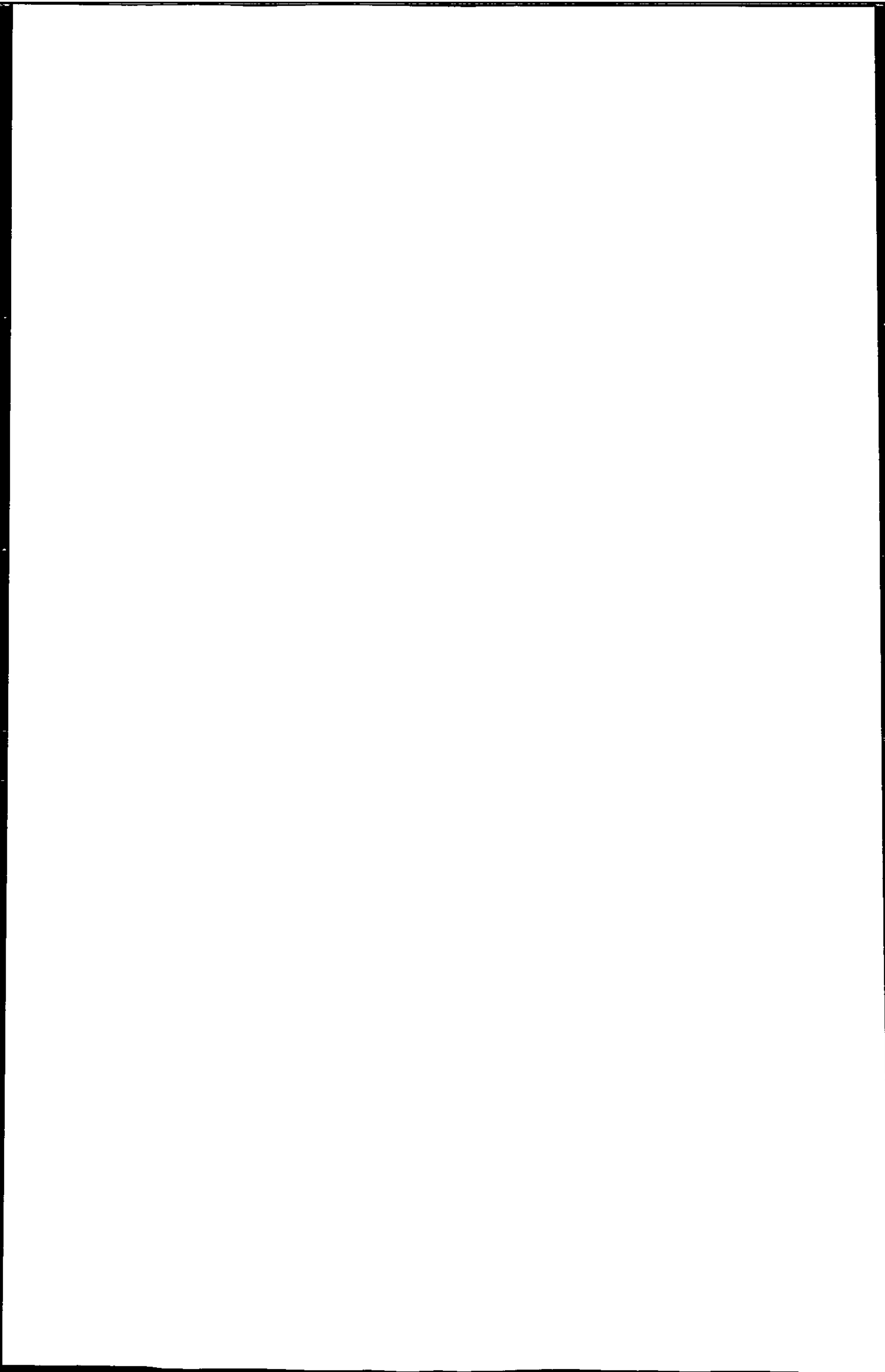
Despite occurring at slower rates and to a lesser extent than in fish species, conjugation of PAH metabolites does occur in a wide variety of crustaceans. Sundt and Goskoyr (1998) report conjugation of B(a)P metabolites in *Cancer pagurus*, whilst Li and James (1993) report sulphation and glucosidation of 3-OH B(a)P in *H.americanus*. The Norway lobster (*Nephrops norvegicus*) is capable of producing conjugated dihydrodiol metabolites of phenanthrene (Solbakken and Palmork, 1981) and the spot shrimp (*Pandulus platyceros*) forms sulphate conjugates of hydroxynaphthalenes (Sanborn and Malins, 1980). McElroy and Colarusso (1988) determined that *C.maenas* rapidly metabolises B(a)P to primary and conjugated metabolites. In one of the first studies of its kind, Corner *et al.,* (1973) showed that the spiny spider crab *M.squinado* converted



naphthalene to a glucoside derivative of 1,2-dihydrodiol naphthalene, 1-naphthyl sulphate and 1-naphthyl glucoside and excreted these compounds in the urine.

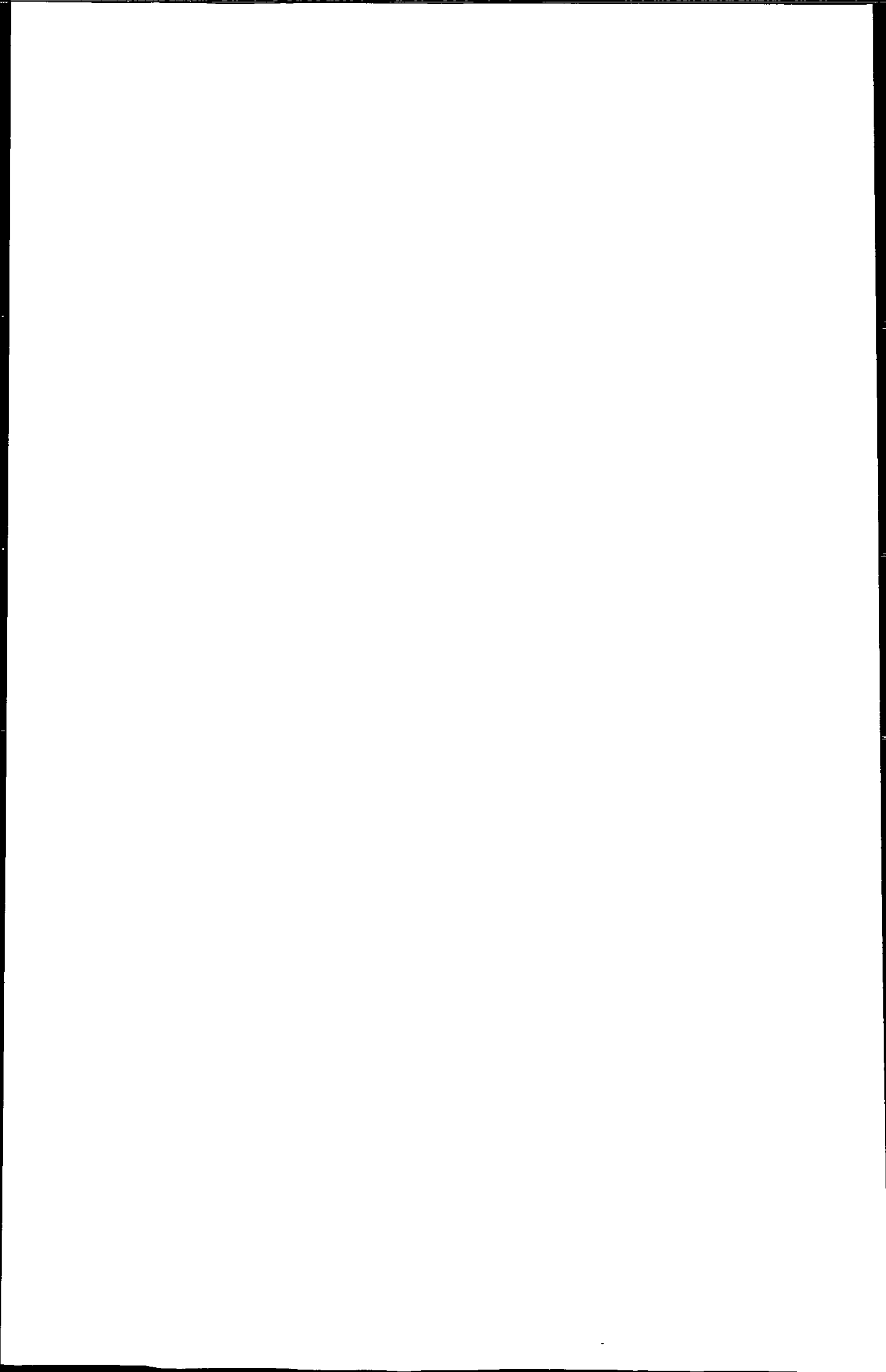
Other authors report that conjugation of 1-OH pyrene is almost certain to be occurring (Aas, E., personal communication) and that glucosides or sulphates are the most likely candidates (James, M.O., personal communication). This is further supported by Ariese *et al.,* (1993) who report that the emission spectrum of conjugated 1-OH pyrene (in their case, pyrene-1-glucuronide) is blue shifted by 5nm and more intense. This would account for the 5nm peak shift seen in the exposed urine samples when compared to the pure hydroxylated standard. To test this, equivalents suspected of contributing fluorescence could be identified and quantified using a set of conjugated standards. However, these standards are not available commercially (Aas *et al.*, 1998) as sulphate and glucuronide conjugates are unstable and cannot be readily supplied (QMX Laboratories, pers. comm.). The subtle wavelength shift of the fluorescent peaks and the lack of conjugated standards present a problem, since the hydroxylated standard cannot be used for quantification of conjugates, but simply "1-OH pyrene equivalents". One solution is to deconjugate the sample before analysis. Several studies on fish bile (Solbakken *et al.,* 1980, Krahn *et al.,* 1984, Ariese *et al.,* 1993, Law *et al.,* 1994, Escartin and Porte, 1999a,b) have included enzymic steps (utilising β-glucuronidase/arylsulphatase) to hydrolyse samples. This produces the free metabolites, which are then extracted and quantitated against the readily available and more stable hydroxylated standards. However, some authors advise against hydrolysis for simple screening purposes as it leads to a reduction in the fluorescence signal produced, complicates the analysis and decreases sample storage stability (Aas *et al.,* 2000a).

Another solution is to apply a correction factor, determined by hydrolysis of synthesised pyrene-1-glucuronide, to sample data, which takes into consideration the increased intensity of the conjugates formed *in vivo,* allowing quantitation with the free



metabolite. Such an approach is used by Ariese *et al.*, (1993), who use a correction factor of 2.2 to quantify the intensities of fluorescent peaks in the bile of exposed fish. Alternatively, fluorescence signals may be quantified by expressing levels in terms of "equivalents" of PAHs (Aas *et al.*, 2000a), which is the approach adopted for the present work.

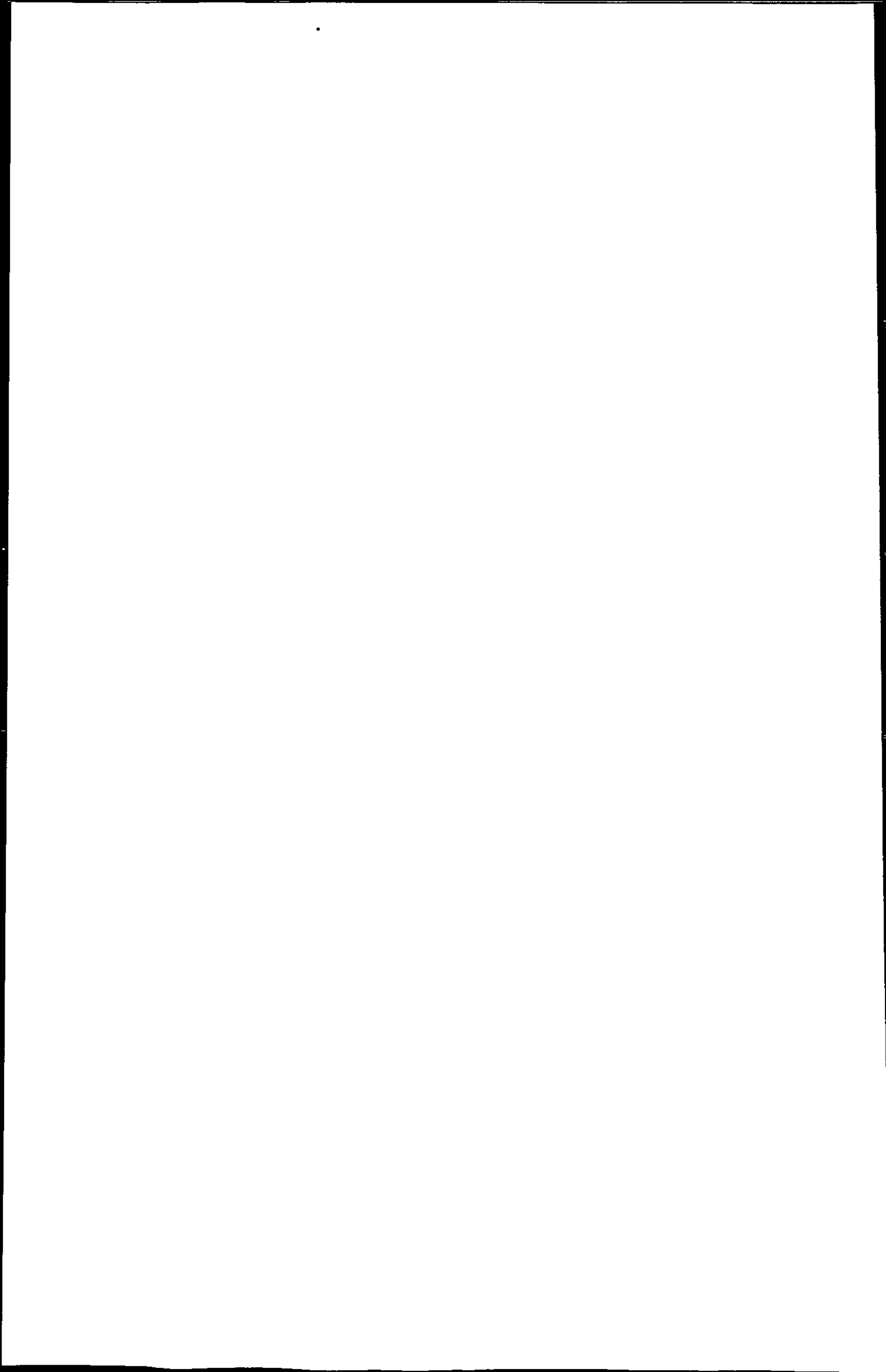
In light of the above, it is highly probable that *C.maenas* is capable of forming and excreting conjugated PAH metabolites, with 1-hydroxypyrene being the most likely intermediate. Analysis of the samples using a more powerful analytical technique is the only way to unequivocally identify the equivalents found in this study. HPLC/F is the most appropriate technique when dealing with conjugated metabolites and was carried out separately, with results reported in the next chapter and also in Fillmann *et al.*, (2004). It should be noted, however, that while identification of specific metabolites is useful, it is not a necessary requirement for PAH exposure monitoring. Direct fluorimetric (FF and SFS) techniques are perfectly adequate for discriminating between organisms exposed to PAHs and those that are not exposed, as demonstrated by the present study and many others using fish bile. However, certain limitations are associated with these techniques that must be considered when interpreting the results of such studies. As both FF and SFS lack any kind of separation technique it is very possible that the fluorescent signals observed at specific wavelength pairs or $\Delta\lambda$s are made up of contributions from a range of PAHs and/or fluorescent aromatic compounds (FACs). The results reported in the next chapter and in Fillmann *et al.*, (2004) highlight this. Also, direct fluorimetry cannot accurately discriminate between compounds with overlapping excitation and emission spectra (Lin *et al.*, 1994). Whilst this phenomenon is not problematic for single contaminant exposures (such as the present study), it is potentially important in samples from field-exposed organisms. In field populations, organisms can be exposed to a multitude of contaminants, including numerous PAHs. Several PAHs or metabolites may



contribute fluorescence at a specified wavelength pair, even if these wavelengths are not optimal for the individual compound.

For example, chrysene and its metabolites contribute fluorescence at the wavelength pairs for pyrene and naphthalene (Aas *et al.*, 2000a) and Camus *et al.*, (1998) report that phenanthrene will contribute fluorescence at pyrene wavelengths. Lin *et al.*, (1996) state that fluorene, phenanthrene and anthracene contribute fluorescence at the naphthalene wavelength pair and perylene fluoresces at the wavelength pair 380/430, optimised for the detection of B(a)P and its metabolites. Fluorescence may also be contributed at a particular wavelength pair by alkylated derivatives of the parent and its metabolites and also by N-, S- and O- containing compounds with the same ring structure (Krahn *et al.*, 1984). Contribution from alkylated forms is particularly important in the case of petrochemical pollution.

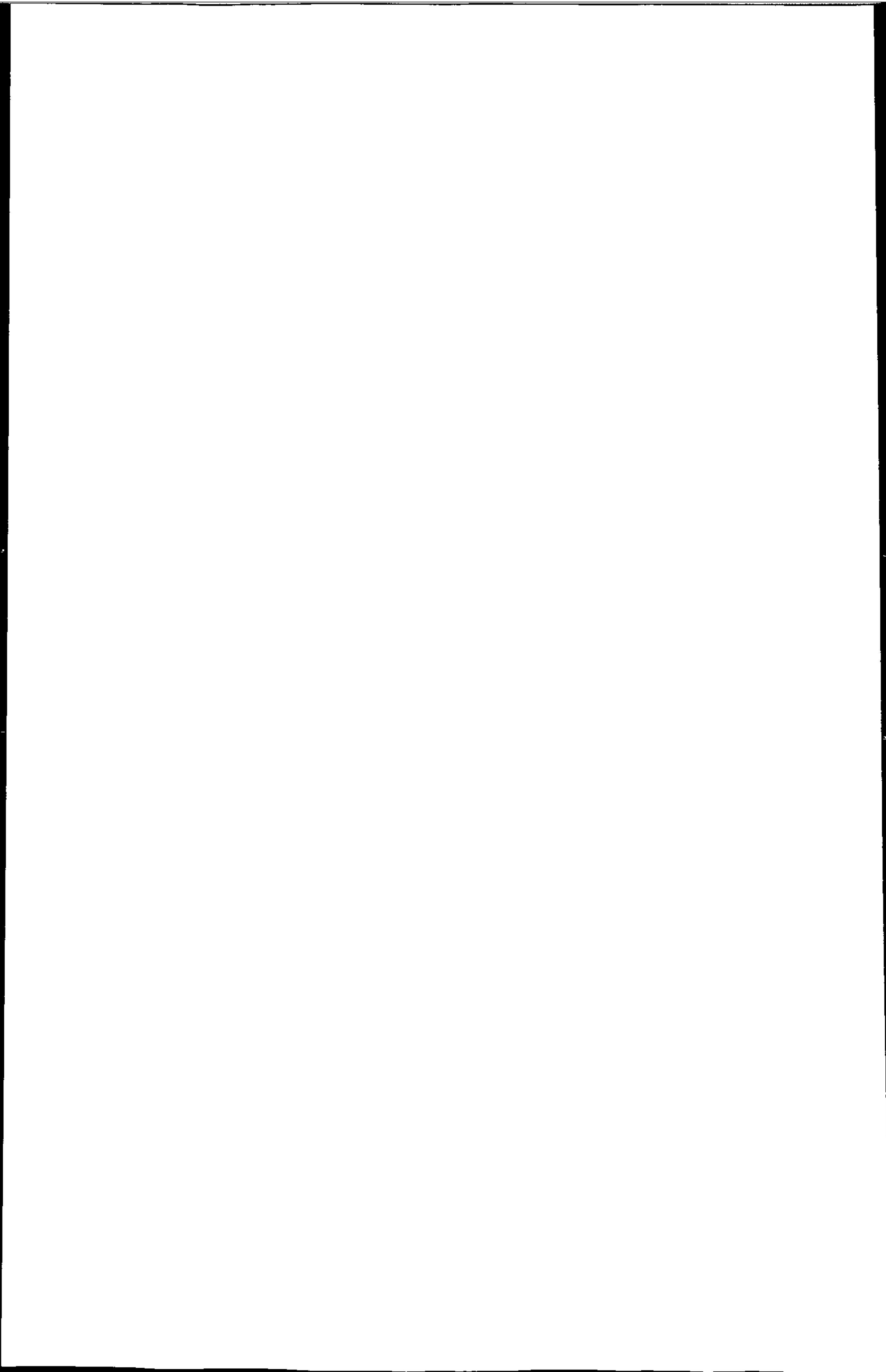
Due to the nature of this problem, most authors analysing field samples using direct fluorimetry, interpret their results in terms of equivalents of particular PAHs fluorescing at specified wavelengths. For example, the fluorescence signal at 290/335 is reported as "naphthalene-type metabolites or equivalents" (Lin *et al.*, 1996), "pyrene equivalents" at 341/383 (Aas *et al.*, 1998, 2000a,b) and "benzo(a)pyrene equivalents" at 380/430 (Lin *et al.*, 1996, Aas *et al.*, 2000a,b). Alternatively, fluorescence at these wavelength pairs can be reported as "2-3 ring", "mainly 4-ring" and "mainly 5-ring" compounds respectively (Aas *et al.*, 2000a). An even simpler approach is to report in terms of exposure to PAHs from petrogenic or pyrolytic sources of contamination. Typically, naphthalene-type metabolites (290/335) are associated with petrogenic and pyrene/benzo(a)pyrene metabolites with pyrolytic sources (Aas *et al.*, 2000a,b, Aas and Klungsoyr, 1998, Lin *et al.*, 1996, Ariese *et al.*, 1993, Krahn *et al.*, 1987). These methods are ideally suited to rapid screening for exposure to groups of PAH compounds at different sites, as opposed to specific identification of individual metabolites. In studies involving fish bile it has been suggested



that 1-OH pyrene could be used as a relative measure for the total uptake of all combustion related PAHs (Ariese *et al.,* 1993, Lin *et al.,* 1994, Aas *et al.,* 1998). Similarly, Ariese *et al.,* (1993) state "strong emission...at shorter wavelengths from hydroxy phenanthrenes would be a first indication of significant exposure to creosote or petroleum-derived PAHs"

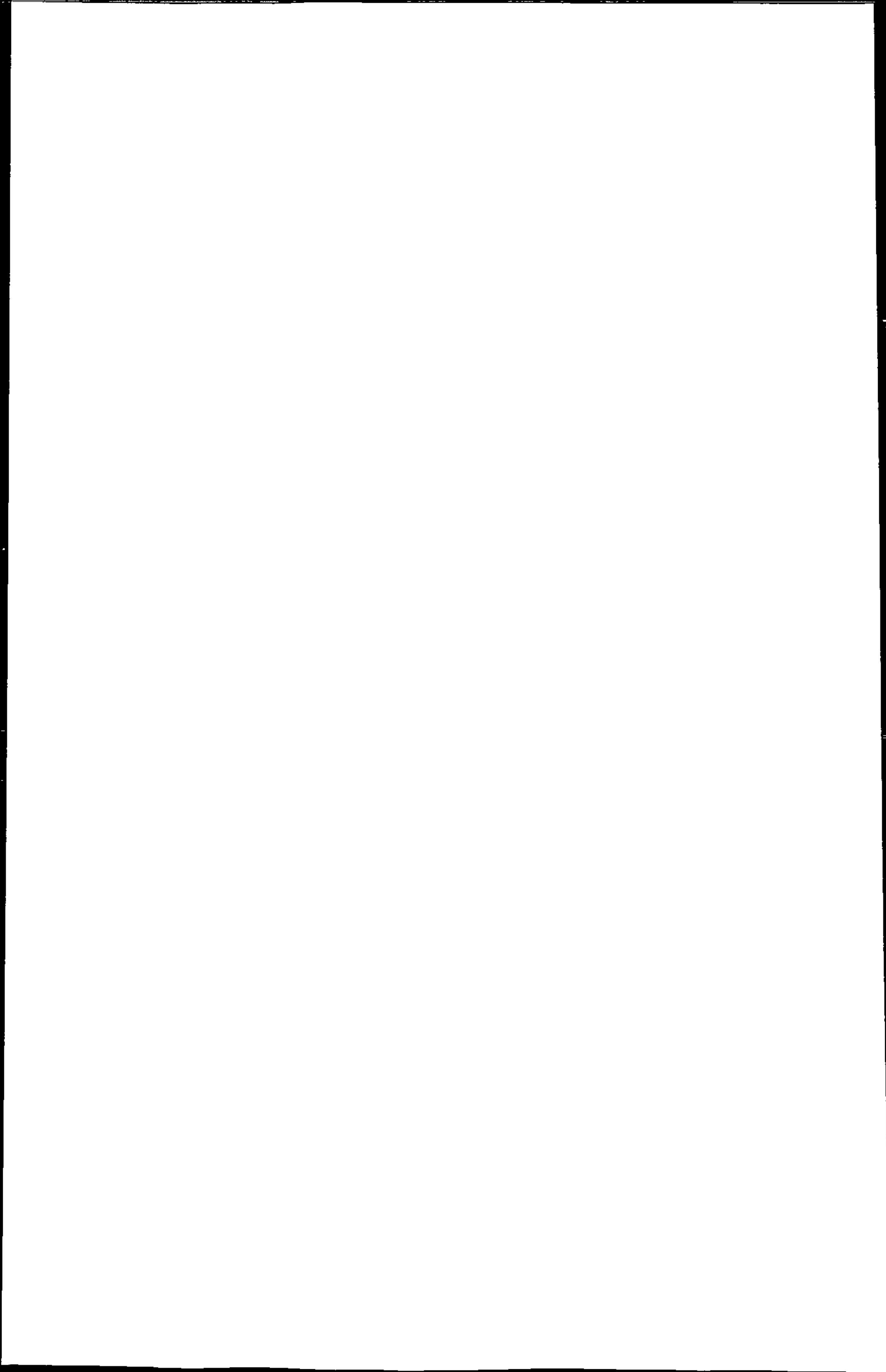
In single contaminant exposures, the above problems are widely avoided although it is useful to consider the possible fluorescence contributors at the specified wavelength (in this case FF345/382 and SFS Em381.4). In addition to pyrene-1-glucuronide, other conjugates of the same metabolite, e.g. sulphates (Ariese *et al.,* 1993) and other metabolites of the parent, e.g. 4-hydroxy, dihydrodiols or dihydroxys might also contribute. Should the present methodology (FF Ex345/Em382nm, or SFS Δλ37nm, Em381.4nm) be applied to screening field samples specifically for pyrenes, it is important to consider fluorescence contribution from the compounds described above, as well as other PAHs which fluoresce at pyrene wavelengths. For example, compounds which possess a pyrene type chromophore such as 7,8,9,10-tetrahydroxy tetrahydro B(a)P (Vahakangas *et al.,* 1985). Lastly, it is possible that certain compounds, produced by normal metabolic processes and excreted in the urine, might also fluoresce at the wavelengths used. Hellou and Upshall (1995) suggest that biogenic compounds such as cholesterol, progesterone and tesosterone can contribute fluorescence which can interfere with the PAH signal. The likelihood of such compounds doing so in crustacea is, however, unlikely, but should not be ruled out.

In light of the above, FF/SFS analyses should be considered semi-quantitative and reporting fluorescence signals in terms of specific metabolites of single PAHs should be avoided, particularly in field studies. However, for the purposes of rapid screening for environmental exposure to PAH, direct fluorimetry analyses of crustacean urine can determine exposure with sufficient resolution to aid environmental mangement decision-making. The technique can categorise crab populations in terms of their overall



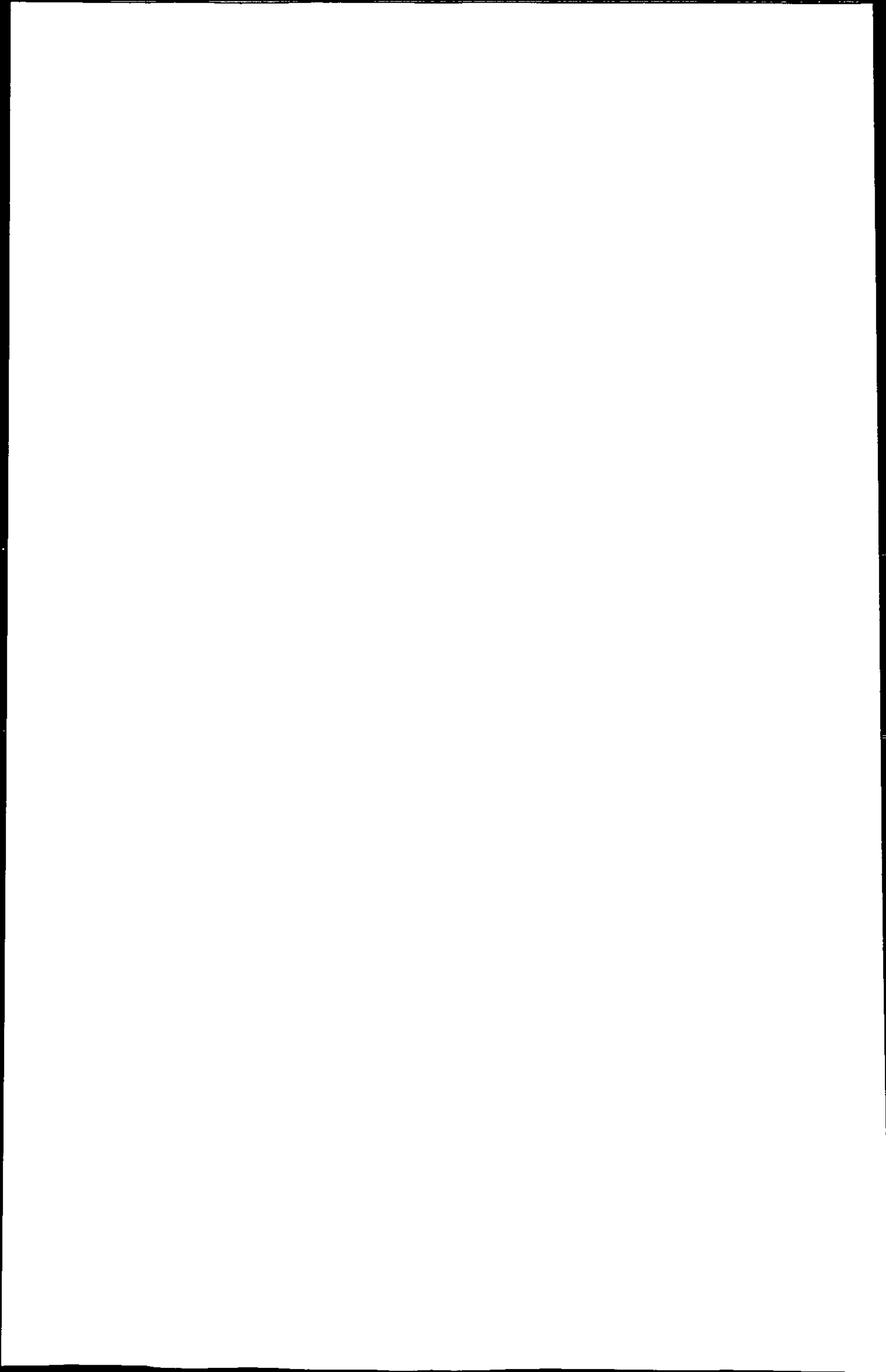
fluorescence (contamination) level. Furthermore, FF can be very useful in discriminating between 2-3, 4 and 5 ring compounds. Using three fixed wavelength pairs it is possible to detect the three most ubiquitous and prioritised groups of PAHs associated with environmental contamination. An SFS method utilising Δλ37nm or Δλ42nm will achieve similar results (Aas *et al.,* 2000a). The use of SFS can also reduce signal interferences and overlap to a certain extent and can greatly simplify spectra obtained from field samples containing large numbers of aromatic compounds.

The detection of pyrene and its metabolites is especially suited to SFS, as illustrated in studies by Lin *et al.,* (1994), Stroomberg *et al.,* (1996), Ariese *et al.,* (1993), Aas *et al.,* (2000a, 1998) and Aas and Klungsoyr (1998). The SFS method preferentially detects hydroxylated pyrene chromophore (pyrene type metabolites) more strongly than other chromophores and parent pyrene (Lin *et al.,* 1994). This is relevant to the present study, as the pyrene standard synchronous peak is clearly distinct from that of 1-OH pyrene but is absent from the urinary and haemolymph spectra of exposed crabs. SFS is therefore capable of discriminating between parent pyrene and its metabolites suggesting *C.maenas* has effectively biotransformed this compound following exposure. This is further supported by data presented in the next chapter and by Fillmann *et al.,* (2004), using HPLC/F, who report that levels of pyrene are negligible in the urine of pyrene exposed crabs. Lin *et al.,* (1994) also note that pyrene-type metabolites estimated by SFS correlate well with B(a)P-type metabolites estimated by HPLC/F, suggesting that pyrene-type metabolites be used as surrogate markers to estimate exposure to B(a)P-type PAHs. Another advantage of fluorimetry is its specificity for PAHs. Unlike biomarkers for general pollutant stress (e.g. haemocyte neutral red retention assay) and enzyme activities (e.g. p450, EROD), the measurement of PAH metabolites is not confounded by other xenobiotics, such as PCBs, dioxins or heavy metals (Ariese *et al.,* 1993). The analysis provides information on the level of exposure to PAHs only and utilises their unique fluorescent properties to do so.



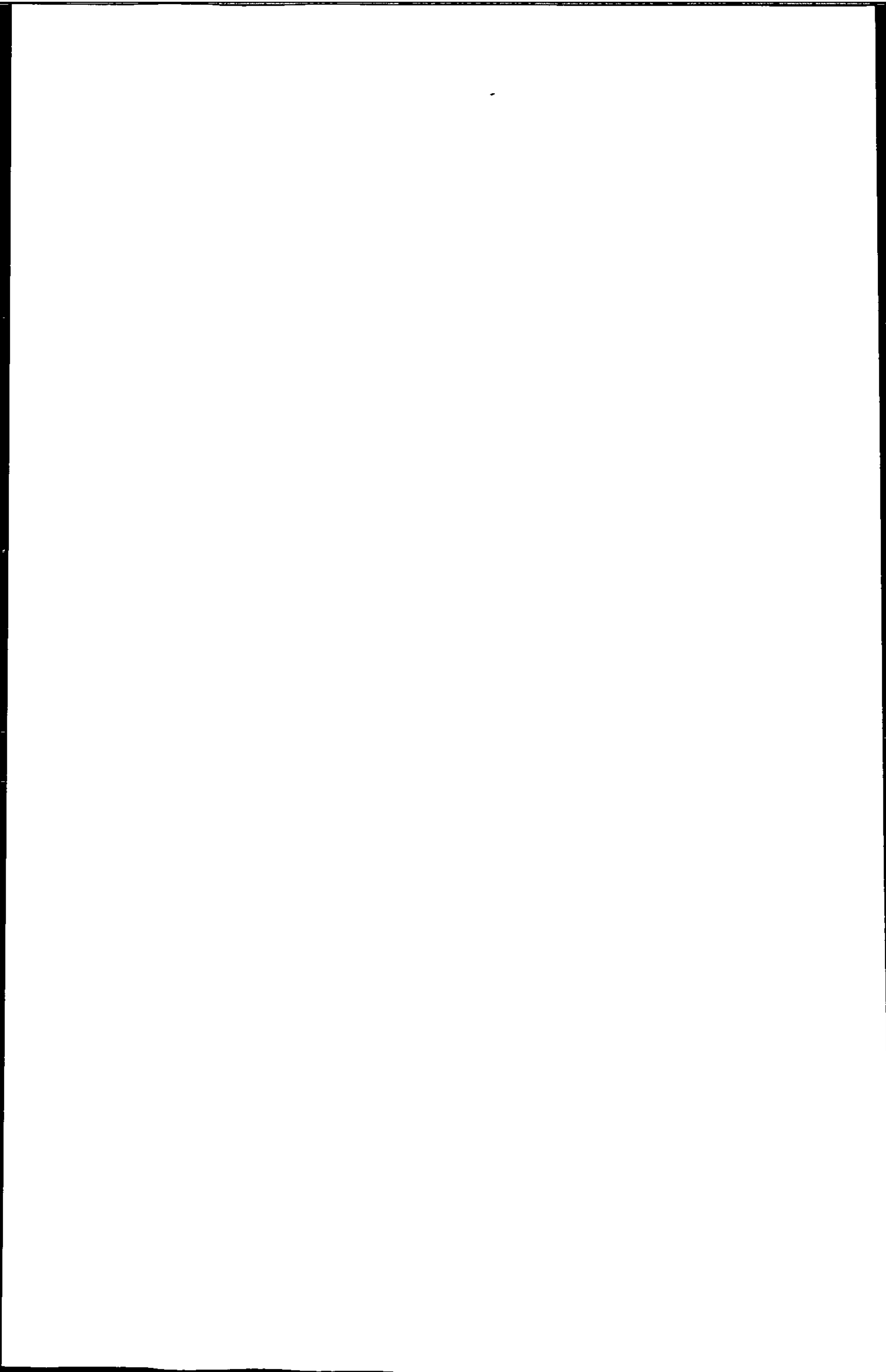
Pyrene is always present in environmental mixtures of PAHs and is often one of the more abundant constituents (Lin *et al.,* 1994, OSPAR, 2002). The physico-chemical properties of pyrene also make it highly bioavailable to aquatic organisms (Landrum, 1989), which is presumably why conjugated 1-OH pyrene is the predominant metabolite in the bile of fish exposed to PAH with 4 or more rings (Krahn *et al.,* 1987, Ariese *et al.,* 1993). The development of techniques for the detection of pyrene equivalents in biological fluids, such as that presented here, is therefore extremely useful for environmental PAH monitoring.

Validation of results obtained with FF or SFS with more powerful techniques is important to ensure these methods are providing accurate results. As mentioned above, results correlate well with HPLC/F (Lin *et al.,* 1994). These authors report that pyrene-type metabolites measured by SFS are appropriate surrogate molecules for estimating B(a)P type metabolites measured by HPLC/F at 380/430nm. SFS measurements of 1-OH pyrenol equivalents in brown bullhead bile samples were highly correlated with B(a)P equivalents measured by HPLC/F. SFS was therefore deemed to be a highly sensitive method for detecting PAH metabolites and could be used confidently in place of HPLC/F to rapidly and inexpensively screen samples for exposure to PAHs in fish. The same is also possible for crustacean urine. If comparable levels of equivalents are detected by both methods, FF/SFS can be used to rapidly and cheaply screen samples to determine whether exposure has occurred and to what degree. If FF/SFS identifies high levels of exposure in certain populations or ecosystems, HPLC/F can then be employed to identify and quantitate the offending compounds to a greater resolution, should such information be required. HPLC/F analysis of urine samples is reported in the following chapter. FF has also produced similar results to gas chromatographic methods. Hellou and Payne (1987) exposed trout to No.2 fuel oil and found that relative biliary concentrations of PAH determined by GC/MS (area of unresolved complex mixtures of hydrolysed and extracted samples) were similar to those obtained by FF (intensity of diluted bile samples at



290/335). In addition to chromatographic methods, other techniques are also applicable. A validation experiment was carried out on the present data using an immunological technique and is reported in the following chapter and also in Fillmann *et al.,* (2002).

It is worth noting here that the present study has dealt strictly with exposure following an acute dose of waterborne pyrene. Whilst analysis of urine has established proof of exposure via this route, waterborne levels of PAH from even severely impacted sites are often very low, given their affinity to bind to sediments or suspended organic material (Onuska 1989, Burns *et al.,* 1990, Lin *et al.,* 1994, Woodhead *et al.,* 1999, Law *et al.,* 1997). In addition to waterborne exposure, ingestion of sediments or suspended particles containing adsorbed PAH can result in PAH being absorbed from the stomach and intestine (James *et al.,* 1995). This route is especially important in epi-benthic omnivores such as lobsters and crabs e.g. *Callinectes sapidus* (Mothershead *et al.,* 1991). The feeding modes of *C.maenas* include scooping sediment into the mouth with the chelipeds (present author observations), which puts this species at risk of being exposed via contaminated sediment. Dietary routes are also important, as organisms may receive PAH by ingesting prey that have taken up PAH themselves (James *et al.,* 1995). Therefore, determination of urinary/haemolymph PAH equivalents following dietary and/or sediment exposure to parent compounds is desirable. Several studies have incorporated different or multiple exposure routes whilst looking at PAH metabolism in crustaceans and fish. James *et al.,* (1995) fed the lobster *Homarus americanus* homogenized shrimp and flatworm mixed with radiolabeled B(a)P, Moese and O'Conner (1985) fed crabs (*C. sapidus*) with mussels exposed to waterborne phenanthrene and Lee (1989) fed shrimp heads containing a variety of ^{14}C-labelled xenobiotics (including BaP) to *C. sapidus*. Exposure to sediments is usually achieved by caging of fish in the field or mesocosm experiments in the laboratory. Flounder (*Platichthys flesus* L.) and Atlantic cod (*Gadus morhua* L.) were caged in a Norwegian fjord (Sorfjorden) adjacent to sediments contaminated with PAHs as well as PCBs and heavy metals (Beyer *et al.,* 1996). A similar study was carried out with



flounder and cod in the same fjord by Aas *et al.,* (1998), and Ariese *et al.,* (1993) kept flounder (*P.flesus*) in large flow through tanks containing sediment taken from PAH contaminated harbours. Future studies should attempt to simulate such environmentally realistic exposure routes to fully evaluate an organism's suitability as an exposure biomarker. However, urine is an excretory pathway for contaminants reaching an organism from all routes (sediment, suspended particles, food and water) and as a result is an integrated measure of exposure. Following chronic exposure to PAH contaminated sediments, water and prey it is reasonable to assume that PAH equivalents would be eliminated in the urine.

The urine and haemolymph data from the dose response experiment shows considerable individual variability, particularly at the higher exposure concentrations. In some cases, levels of urinary 1-OH pyrene equivalents were 3-4 times higher in some individuals compared to others, despite being exposed to the same ambient concentrations of pyrene. While this might be simple to explain in a motile field population where variation in nutritional state, pre-exposure to xenobiotics and contaminant concentration within sites is inevitable (Krahn *et al.*, 1986), it is more difficult to explain in a seemingly homogenous group of organisms (size, sex, moult stage), kept under identical conditions. Variations in the rate and degree to which individual crabs take up, metabolise and excrete waterborne pyrene may partly explain the variability seen. Such variation is genetically predetermined and part of the general variability seen in any species. Variation in rate and degree of metabolism will be largely due to the level of induction and efficacy of the appropriate p450 enzymes, themselves influenced by possible pre-exposure to xenobiotics (Carr and Neff, 1988) and subtle differences in moult stage between crabs. Such phenomena are common in natural systems. Only when subjected to contaminant stress do the latent differences between individuals manifest themselves, giving rise to a range of responses proportional to the magnitude of the stress (Depledge, 1990). Variation caused by differences in urine concentration cannot be discounted. Future studies may require

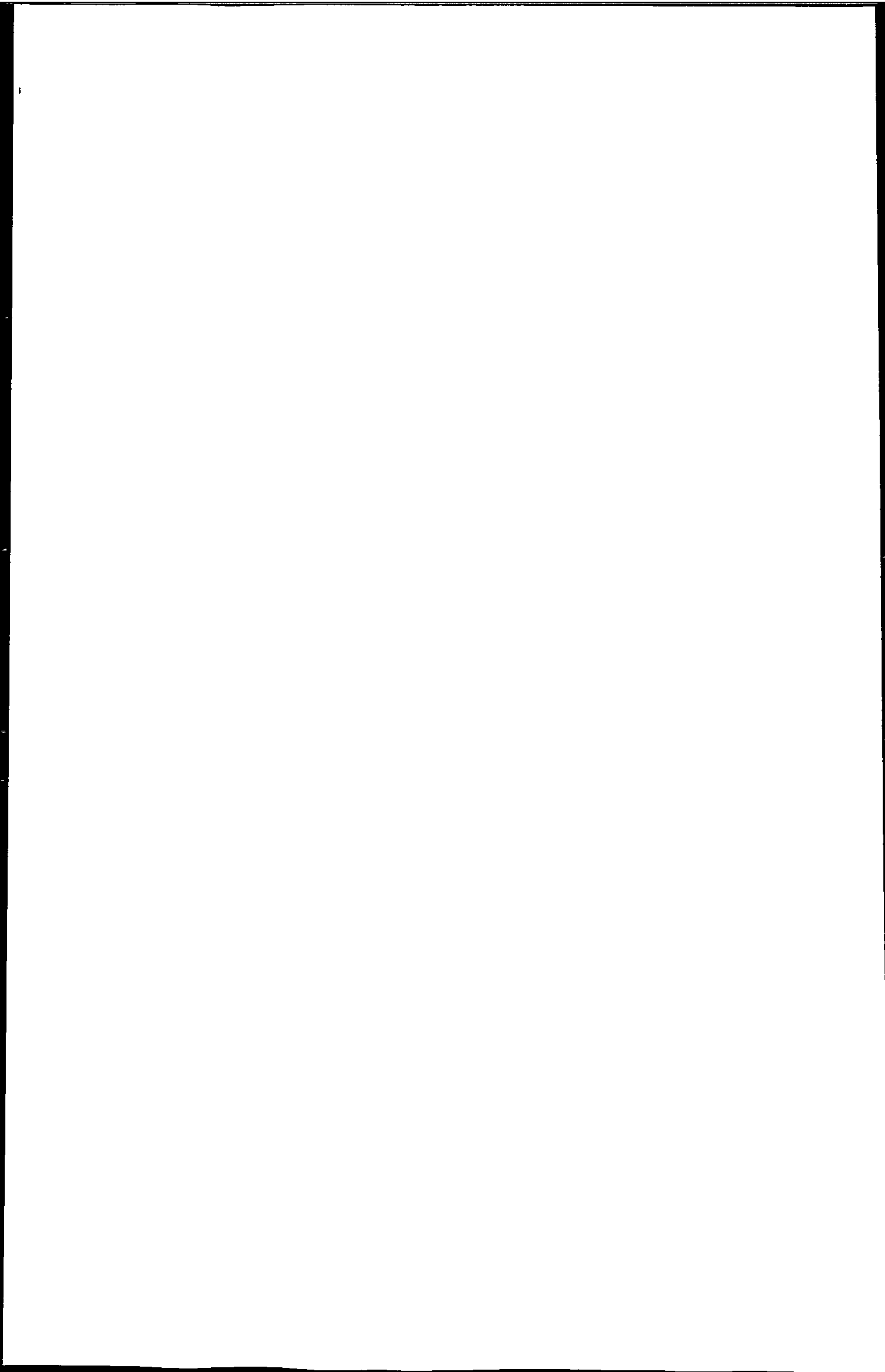
fluorescence measurements to be standardised with respect to urine concentration, in the same way that measurements of bile fluorescence are standardised to biliverdin concentration (Aas *et al.,* 2000a,b).

The present study has tried to minimise any potential moult stage differences in PAH uptake, metabolism and excretion by selecting only green intermoult crabs. However, in the field, suitable numbers of intermoult individuals may not be attainable and samples may need to be taken from individuals at different stages of the moult cycle. It is important to remember in such cases that uptake and accumulation of PAHs has shown to be greatly influenced by ecdysis (Mothershead and Hale, 1992). Moulting and intermoult blue crabs (*C. sapidus*) were placed in wire baskets and deployed in the Elizabeth River, Virginia, whose sediments are some of the most highly PAH contaminated in the United States. Newly moulted crabs showed significantly higher levels of fluorene, pyrene and cyclopenta(def)phenanthrene (indicative of recent creosote spills from a nearby wood preserving facility) in muscle and hepatopancreas. Mean total PAH concentrations of these tissues were almost three fold greater in new moults than intermoults, with the mean concentration in hepatopancreas being almost seven times higher than in muscle in both sets of animals. The authors suggest the elevated levels seen were due to increased water uptake and shell permeability at ecdysis or a decreased level of PAH metabolism during moulting. It is therefore important to avoid using individuals that differ in their moult stage, as ecdysis has a profound effect on exposure levels.

Whilst the present technique is useful in terms of determining exposure to PAHs such as pyrene, it tells us nothing about the potential for deleterious effects such exposure might have. Biotransformation of a parent compound, such as in the present study, can result in its metabolites becoming more toxic and bioactive. Therefore, to fully understand the risks posed to organisms whose biological fluids contain detectable levels of PAH metabolites, it is necessary to combine the present technique with measures of

physiological, histopathological and cellular parameters. One such measure used extensively in fish species is frequency of hepatic neoplasia. Several papers report findings of liver lesions in fish correlating with elevated PAH metabolites in the bile (Krahn *et al.,* 1984, 1986). Benzo(a)pyrene-type metabolites correlated most strongly, consistent with the fact that B(a)P is a potent vertebrate carcinogen. The presence of PAH metabolites in bile has also been correlated with CYP1A induction (Beyer *et al.,* 1996, Miller *et al.,* 1999).

In contrast, studies on the effects of PAHs and their metabolites on crustaceans are less numerous. Increased mixed function oxidase (benzo-a-pyrene hydroxylase) activity and cytochrome p450 content were observed in crabs (*Callinectes sapidus, Sesarma cinerum* and *Uca pugilator*) after exposure to oil, benzo(a)pyrene and PCBs (Lee *et al.,* 1981). Exposure to the water-soluble fraction of crude oil inhibited moulting, reduced growth and prolonged the intermoult period in juvenile *C.sapidus* (Wang and Stickle, 1987). Exposure to crude oil also reduced moulting success in Tanner crabs (*Chionectes bairdi*) (Karinen and Rice, 1974). Naphthalenes, major constituents of the water-soluble fraction of crude oil, have been implicated in retarding ovarian development in swamp crayfish (*Procambarus clarkii*) (Sarojini *et al.,* 1994, 1995). Decreased ovarian index, degeneration of oogonial cells and the presence of avitellogenic oocytes are also reported in the freshwater prawn *Macrobrachium lamerrii* chronically exposed to diesel (Rao *et al.,* 1990). The implications of such exposures for reproductive success in these species are clear. In the estuarine grass shrimp (*Palaemontes pugio*) exposed to 63ppb pyrene, males experienced a significant delay in moulting and in females, despite increased vitellin synthesis, offspring mortality increased (Oberdorster *et al.,* 2000). The authors suggest that pyrene binds to vitellin and is transferred to embryos, resulting in their decreased survival. Exposure to pyrene may cause therefore cause alterations at the population level in this species, due to its effects on moulting and reproduction.

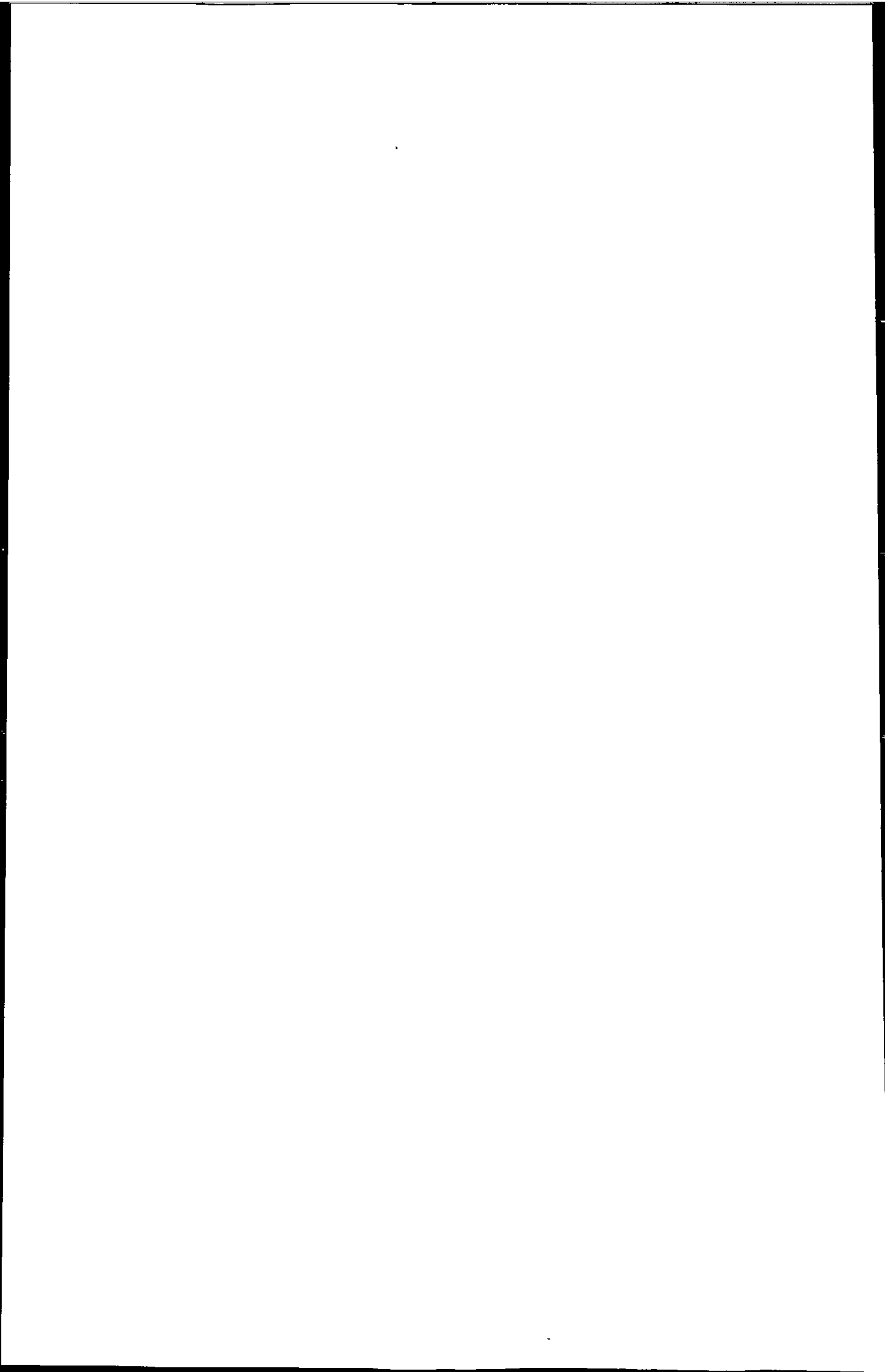


Perhaps due to the limited scope for activating pro-carcinogenic PAHs, occurrence of lesions and tumours in invertebrates have not been reported in the literature (James *et al.,* 1992). Metabolites of BaP have been shown to bind to hepatopancreatic DNA of spiny lobster (*P.argus*), although the authors also show that this organ can efficiently eliminate such adducts (James *et al.,* 1992). It is suggested that this confers a certain degree of resistance to chemical carcinogenesis in this species. It is thought instead that organic pollutants can cause a more general disease state in invertebrates, termed the "Genotoxic Disease Syndrome" or GDS (Kurelec, 1993). GDS is characterised by impairments in enzyme function and general metabolism, altered protein turnover, reduced scope for growth, reduced fitness, suppressed immune function, degenerative processes and atrophy in tissues and organs, faster ageing, impaired reproduction, adaptation and succession and several other effects. The author suggests that GDS may be of much greater biological and ecological significance than the induction of neoplasia. The detection of pyrenes and other PAH metabolites in the urine and blood of crustaceans in the field might be regarded as indicative of potential negative effects, such as GDS. The lack of evidence linking the detection of PAH metabolites with deleterious effects in Crustacea illustrates the need for such studies.

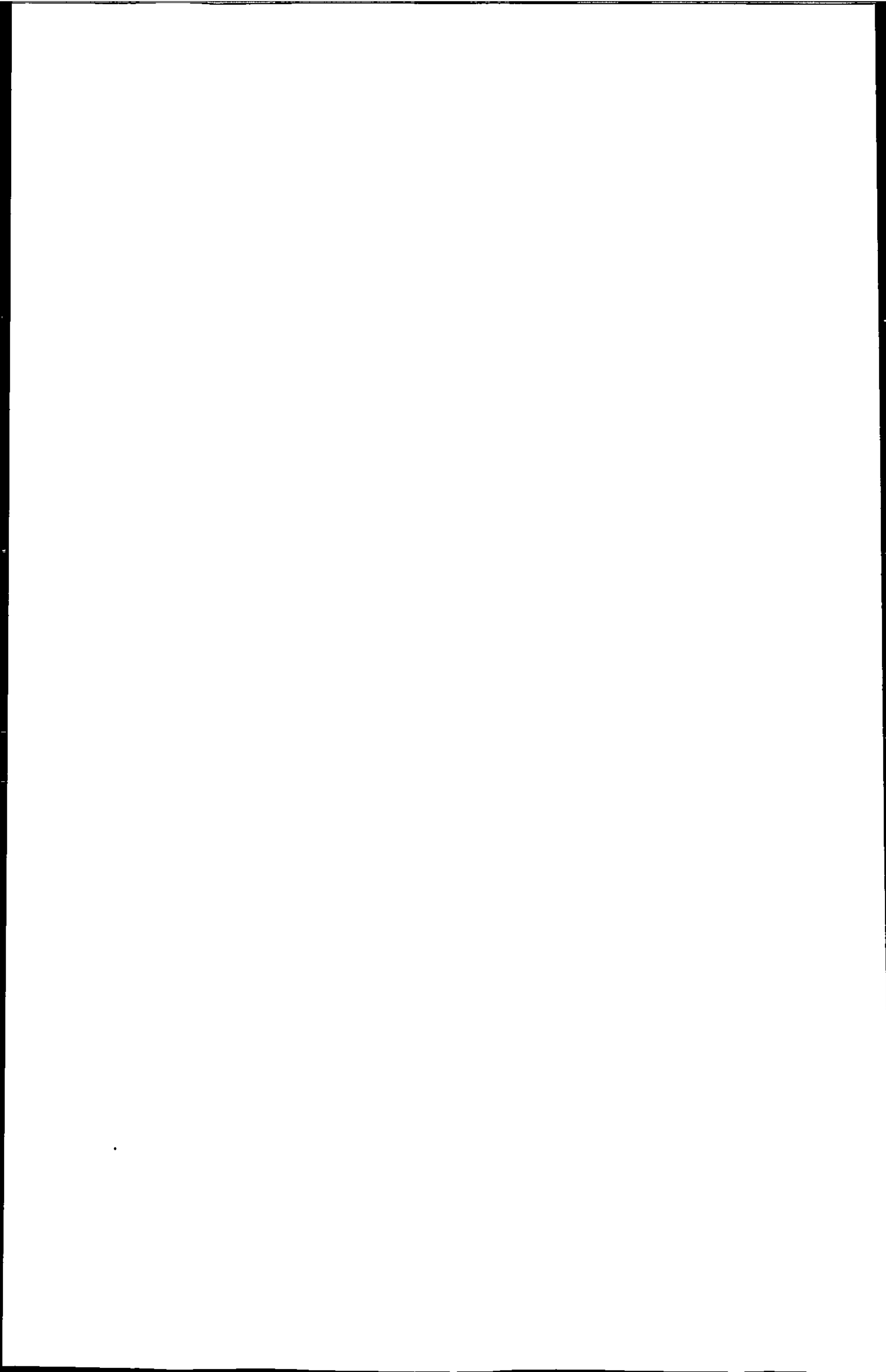
Whilst encouraging, results from laboratory based single contaminant exposures are only the beginning. The primary aim of the present technique, and indeed any biomarker, is application to field situations. To identify impacted sites by analysis of the urinary PAH profile of its crustacean populations is a step towards this method being employed as a robust technique for widespread coastal and estuarine monitoring. To this end, several preliminary field trials were carried out, followed by an extensive trial at a gradient of PAH exposure, and are reported in subsequent chapters.

Following successful field validation, the present technique has the potential to be applied to a variety of ecosytems in both temperate and tropical climates, where it will be

necessary to consider the suitability of the indigenous crustaceans as sentinel species. As discussed above, several decapod crustacean species have the ability to transform PAHs to polar and water soluble metabolites, although limited studies have been performed on their levels in urine. To this author's knowledge, the present study is the first to detect PAH metabolites in the urine of a crustacean using direct fluorimetry with a view to applying the method for environmental monitoring. Preliminary experiments in the sub-tropical environment of Bermuda, using the crab *Callinectes ornatus* (a close relative of the blue crab *C. sapidus*), showed that pyrene equivalents could be detected in the urine using FF and SFS following waterborne exposure to pyrene (authors unpublished observations). *C. ornatus* is similar to *C. maenas* in terms of habitat, occupying shallow, brackish coastal waters and sandy/silty bays (Williams, 1974) and intertidal mud flats and estuarine lagoons (Haefner, 1990a,b), diet (scavenger with preferences for gastropod and bivalve molluscs) (Haefner, 1990b) and feeding habit (often observed scooping sediment into the mouth with the chelipeds) (Haefner, 1990b). It is therefore likely to be exposed to similar xenobiotics *via* similar routes. Urine in this species can be sampled in a comparable fashion to *C. maenas* (present author observations) and in preliminary experiments has been shown to exhibit pyrene equivalent fluorescence following exposure to waterborne pyrene (authors unpublished results). p450 content of the hepatopancreas of a closely related species (*C. sapidus*) is also very similar to that of *C. maenas* - 0.18±0.08 and 0.14 nmol/mg respectively (James, 1989b). It is therefore likely that *C. ornatus* or *C. sapidus* could be used in the place of *C.maenas* to assess bioavailabilty of PAHs. Portunid crabs of the genus *Callinectes* are widely distributed in the Atlantic from southern Brazil to the Caribbean, from Mauritania to Angola in West Africa, the Gulf of Mexico, and the East coast of North America, primarily from the Carolinas to the Maritime Provinces of Canada. In the Pacific they range from southern California to Peru, including Baja California (Williams, 1974). Such wide distribution makes them ideally suited as sentinel species.



Whilst parallels may be drawn between closely related species, there are exceptions that highlight the need for caution when selecting exposure indicators. The rate of hydrocarbon metabolism in crustacea is highly variable between species and it has been shown that whilst the spiny lobster *Panulirus argus* metabolises B(a)P very rapidly, the lobster *Homarus americanus* metabolises it very slowly. (James, 1989b). Similarly, fish differ in their suitability as PAH exposure indicator species. Johnston and Baumann (1989) report that the concentrations of B(a)P equivalents in the bile of carp (*Cyprinus carpio*) were 6 times greater than those in the bile of brown bullhead (*Ictalurus nebulosus*) caught in the Black River, Ohio, an industrially polluted tributary of Lake Erie, whose sediments contain particularly high levels of PAHs, including benzo(a)pyrene. The authors suggest the biliary differences may be due to differing food habits or metabolic pathways. It is therefore important that such inter-specific variation be considered in monitoring programs. Preferably, species short-listed for use in monitoring programs should be evaluated using experiments such as those described above.



2.5 Conclusions

The work in this chapter has demonstrated the uptake and transformation of waterborne pyrene and the urinary excretion of 1-OH pyrene equivalents in *C.maenas*. The detection of urinary metabolites indicates exposure to PAH and the possible risk of deleterious effects from these bioactive compounds. This finding also highlights the potential use of *C.maenas* as a biomonitoring organism to assess PAH contamination of marine ecosystems.

Using fixed wavelength and synchronous scanning fluorescence spectrometry and non-destructive sampling techniques, it has been possible to detect pyrene metaboilite equivalents in crab urine and haemolymph. The levels of these equivalents vary in an exposure dependent manner. Urinary levels of pyrene metabolites also vary in a time dependent manner with the highest level found approximately 48hrs after initial exposure. The nature of the fluorimetric approach and the lack of various metabolite standards meant that identification of specific metabolites was prevented, hence the use of the term "equivalents". Whilst the fluorimetric method lacks this resolution, it's advantages are that it is relatively simple, rapid, and inexpensive and is applicable to a wide range of species. It has great potential as an initial screen to discriminate between clean and contaminated sites, identify PAH hotspots and detect gradients of PAH pollution.

Chapter 3: Refinement and validation of the urinary PAH exposure biomarker and preliminary field trials.

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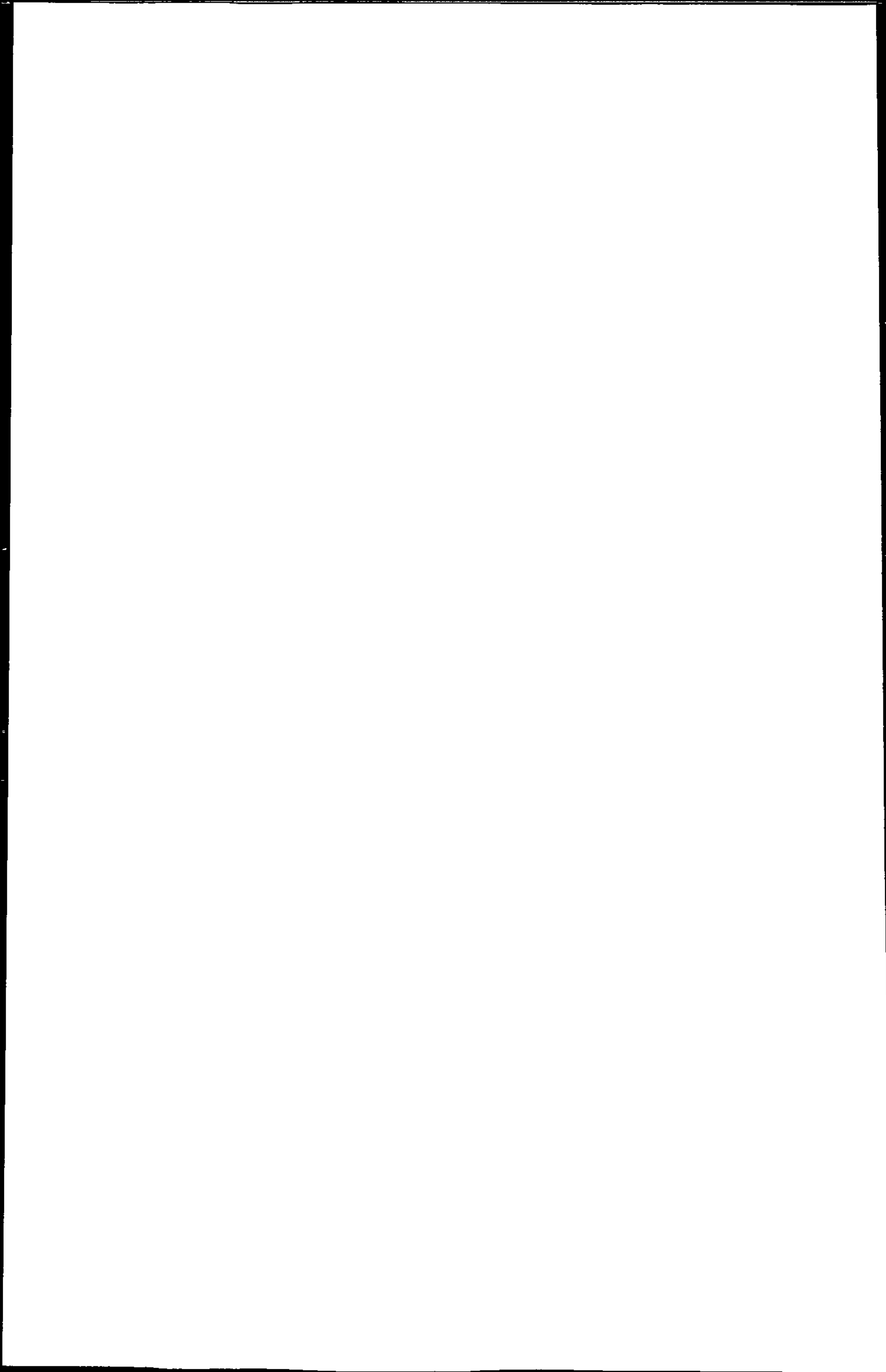
3.1 Introduction

Whilst Chapter 2 describes the genesis and development of the urinary PAH biomarker, certain aspects of the assay still required addressing, in particular the validation of the fluorescence method using more powerful analytical techniques. Evaluation of the assay's applicability to field studies was also needed and information on the fate of the test chemical in the laboratory exposures was desirable to investigate uptake rates.

Chapter 2 reports a shift in peak position between samples and standards. To ensure this is not the result of sample matrix effects, experiments are required to determine if the urinary matrix has any bearing on the fluorescent behaviour of a pyrene metabolite intermediate (1-OH pyrene). Testing the nominal concentrations of waterborne pyrene in a duplicated experiment (with and without crabs) is also necessary to determine the fates and losses from the exposure system due to photolysis, volatilisation, adsorption and uptake.

Identification of the urinary equivalents using HPLC/F seeks to provide analytical evidence for previous claims that the urinary equivalents detected by direct fluorimetry are in fact conjugated metabolites of the 1-hydroxy intermediate. To further substantiate the legitimacy of the results obtained from fluorescence analyses in chapter 2, validation experiments are carried out using two different analytical techniques. Levels of equivalents in selected samples are measured using HPLC/F and by the novel application of an established immunochemical method. Immunochemical methods, such as enzyme-linked immunosorbent assay (ELISA), are routinely used for monitoring of PAH levels in environmental samples (Chuang *et al.*, 2003, Li *et al.*, 2000) and for human PAH exposure monitoring (Knopp *et al.*, 1999). More recently, ELISA techniques have been used to analyse body fluids and excreta of aquatic organisms (Fillmann *et al.*, 2002). ELISAs in which antibody is raised to a 2-3 or 4-5 PAH ring structural motif can provide a total measure of PAH without the need for enzymatic hydrolysis (Fillmann *et al.*, 2002),



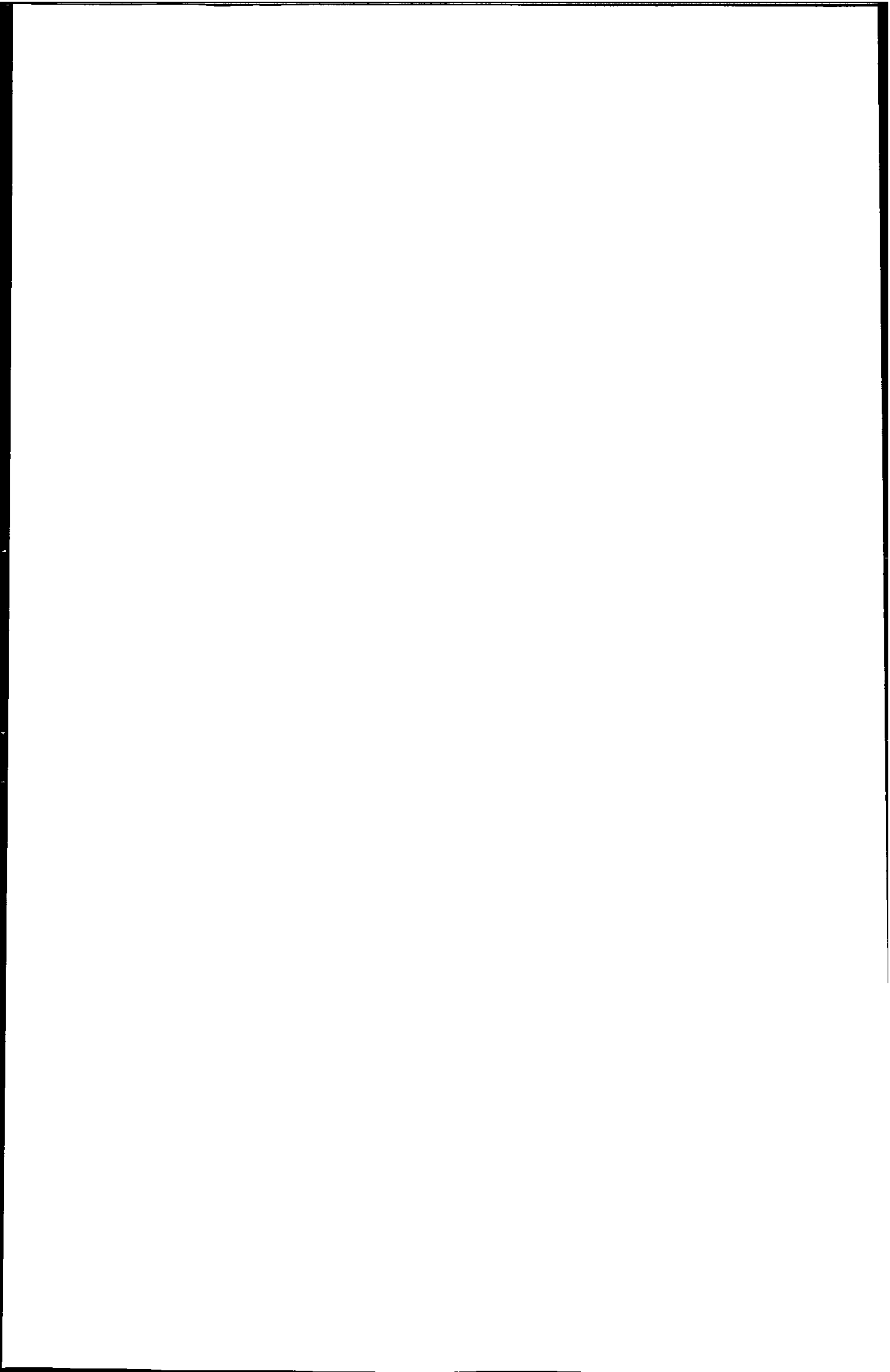
allowing contributions from both petrogenic and pyrogenic sources to be assessed. Additionally, ELISA methods are in general cheaper, quicker and easier to use than conventional chromatographic methods and offer comparable limits of detection whilst being easily adapted for use in the field (Mastin *et al.*, 1998).

Experiments described in chapter 2 confirmed that the urinary assay could be used to detect pyrogenic PAH exposure. Confirmation of its use for determining exposure to petrogenic PAH was also desired. Such an opportunity was provided by experiments carried out as part of a BEEP (Biological Effects of Environmental Pollution in coastal marine ecosystems) project experiment in Akvamiljo research laboratory, Stavanger, Norway, where shore crabs were exposed to crude oil in a continuous flow through system. Positive results in a laboratory exposure illustrate the potential for the technique to be used to evaluate the extent of bioavailability of petrogenic PAH in oil following a spill.

As previously alluded to in chapter 2's discussion, the ultimate goal for any biomarker of exposure and/or effect is application to contamination situations in the field with a view to its use in pollution monitoring programs. With this in mind, it is imperative that biomarkers are thoroughly field-tested before results can be confidently interpreted. Exposures to test chemicals under strictly controlled and constant conditions in the laboratory are rarely representative of field exposure. Exposure to contaminants in the field is characterised by numerous confounding variables relating to the contaminant itself (levels in sediment/water, distribution, speciation, presence of other contaminants), physical factors (salinity, dissolved oxygen, temperature, pH) and biotic factors (sex, developmental stage, nutritional state, reproductive status) that influence bioavailability, uptake and biotransformation. Therefore, extrapolations of laboratory-based findings should be avoided before assays are deployed in field situations. The present chapter presents the results of some preliminary field trials, which paved the way for more extensive fieldwork described in later chapters.

For both the crude oil exposure and field trials, analysis of crab urine samples produced spectra different to those seen in previous experiments. In contrast to the single contaminant exposures described in chapter 2, contamination in the field is characterised by a multitude of different chemicals (including PAH). Similarly, crude oil contains numerous different petrogenic PAH. As a result, the spectra obtained from the urine of crabs exposed to such contamination are more complex and characterised by numerous peaks and areas of broad fluorescence, presenting a greater challenge with regards to interpretation and identification of specific PAH. Spectra are therefore divided up according to the characteristic emission wavelengths of certain PAH groups. Such a system is commonly used for interpreting fluorescence spectra from environmental samples (UNEP, 1992 - figure 3.1).

Finally, given the success of the assay in *Carcinus*, a similar approach is investigated for a biomarker of exposure in the blue mussel, *Mytilus edulis*. An exposure experiment is carried out in this species with comparable levels of waterborne pyrene, to investigate whether a similarly convenient medium for the detection of PAH/metabolites can be found.



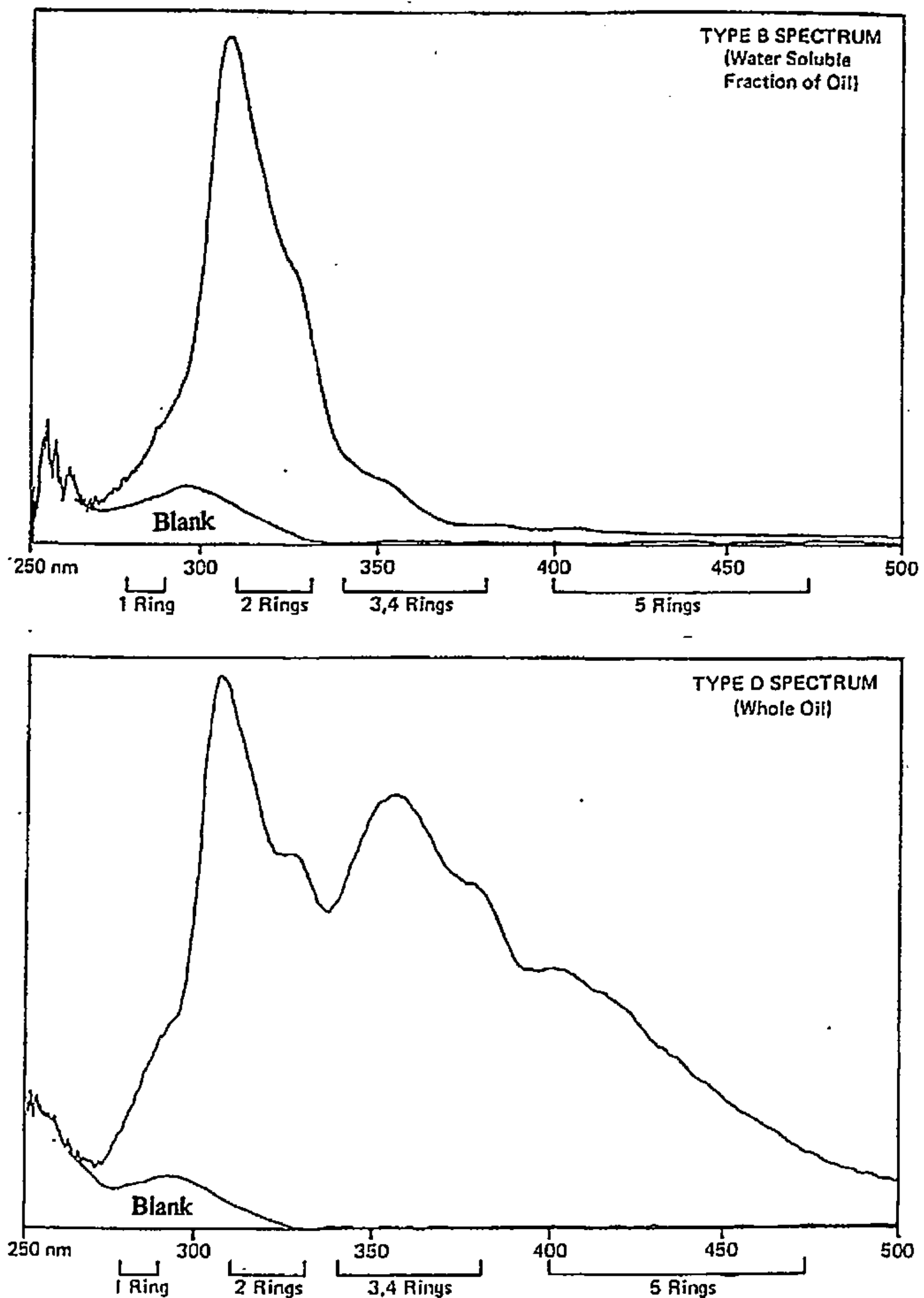
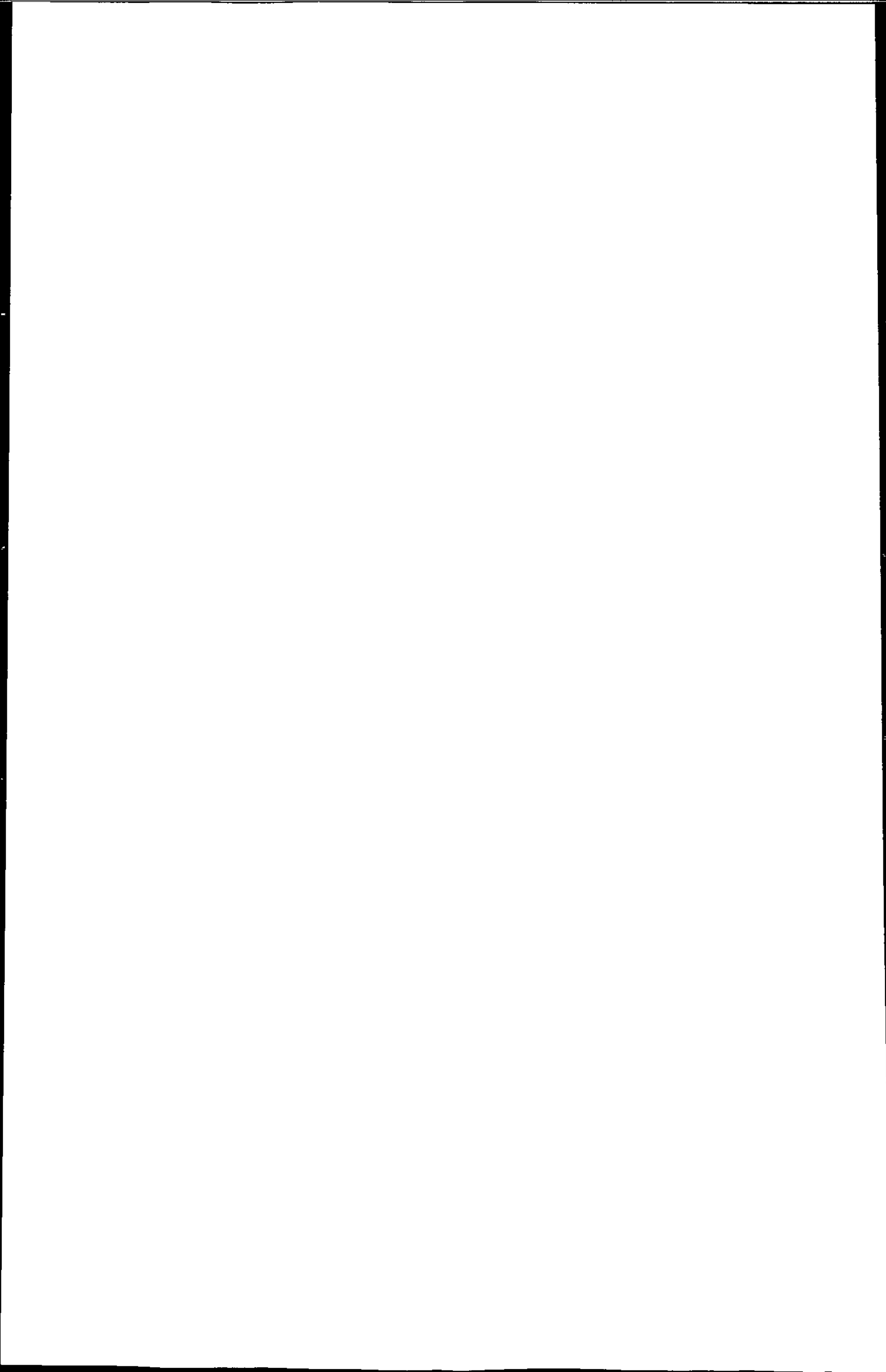


Figure 3.1 Synchronous fluorescence spectra of seawater samples collected following an oil spill (the IXTOC1 blowout) (from UNEP/IOC/IAEA, 1992). These spectra illustrate the relative spectral positions for fluorescence contributed by 1 ring, 2 ring, 3-4 ring and 5 ring PAH compounds. Spectra obtained from urine samples in the present study were divided up and interpreted in a similar fashion.



3.2 Materials and Methods

3.2.1 Spiking of urine samples.

In order to discount any possible urinary matrix type effects on the fluorescence characteristics of the urinary metabolites, a spiking experiment was carried out. This was to determine whether the 5nm observed difference between the hydroxylated standard peak and the urinary metabolite peak was not simply the result of the urinary matrix altering the fluorescence characteristics of an otherwise free- hydroxy metabolite. To do this, urine from control crabs, free from metabolite peaks, was spiked with known amounts of 1-OH pyrene standard (yielding final concentrations of 25, 50, 100 and 200µg l^{-1}). These spiked samples were then analysed as described above. The spectral position of the resulting peak was then compared to that seen in urine from pyrene exposed crabs. The fluorescence results were also plotted against 1-OH pyrene concentration to determine if matrix effects increased with increasing concentration of the metabolite.

3.2.2 GC/MS analysis of water samples

To investigate the uptake of waterborne pyrene by exposed crabs, a series of tanks were set up in duplicate (one containing crabs, the other without) in the same way as for the previous exposure experiments. Ten glass aquaria were set up, containing 10L of filtered (10µm carbon filtered), aerated seawater (34ppt, 15±1°C), under a 12h:12h light: dark regime. 20 adult male intermoult *C.maenas* (carapace width 57-68mm) were collected from Jenkins Quay, Bantham, S. Devon as described above. Groups of 4 crabs were assigned to each of 5 of the aquaria, whilst the remaining 5 aquaria were left without crabs. Tanks were dosed with nominal concentrations of pyrene in duplicate (with and without crabs), using an acetone solvent vehicle. The nominal concentrations of pyrene were the same as for the previous exposure experiments (20, 50, 100 and 200µg l^{-1}). Two tanks were

not dosed and were kept as controls (0μg l^{-1}). Water samples (500ml for 0, 20 and 50μg l^{-1}, 200ml for 100 and 200μg l^{-1}) were taken after 0, 2, 6, 12, 24, 48 and 96 hours, using separate 50ml vacuum pipettes. Samples were then transferred to 1L amber Winchester bottles and stored until analysis.

Water samples were analysed to determine the concentrations of parent PAH in the tanks over the exposure period, with and without crabs, in order to elucidate the fate of pyrene in the exposure system. Water samples were analysed using GC/MS (Hewlett-Packard Model 5890 II Plus GC and a 5972 mass selective detector (MSD) (Palo Alto, CA)). Analytical work was performed by Dr. Gilberto Fillmann at Plymouth Marine Laboratories, Plymouth, Devon, UK.

Briefly, water samples (200-500ml) were spiked with internal standard (pyrene-d_{10}) and concentrated using C_{18} SPE cartridges (IST, Hengoed, UK), which were subsequently eluted (3 times) using 3 ml of ethyl acetate. The eluent was then concentrated down to 1 ml before analyses by GC/MS.

3.2.3 HPLC/F analysis of urinary equivalents and validation of fluorometric results.

HPLC/F analysis was carried out on selected urine samples from control and exposed crabs (from the pyrene exposure experiments in chapter 2) by Dr. Mike Howsam, Vrije Universiteit, Department of Animal Ecology, De Boelelaan 1087, Amsterdam, The Netherlands. The methodology and results are also reported in Fillmann *et al.* (2004).

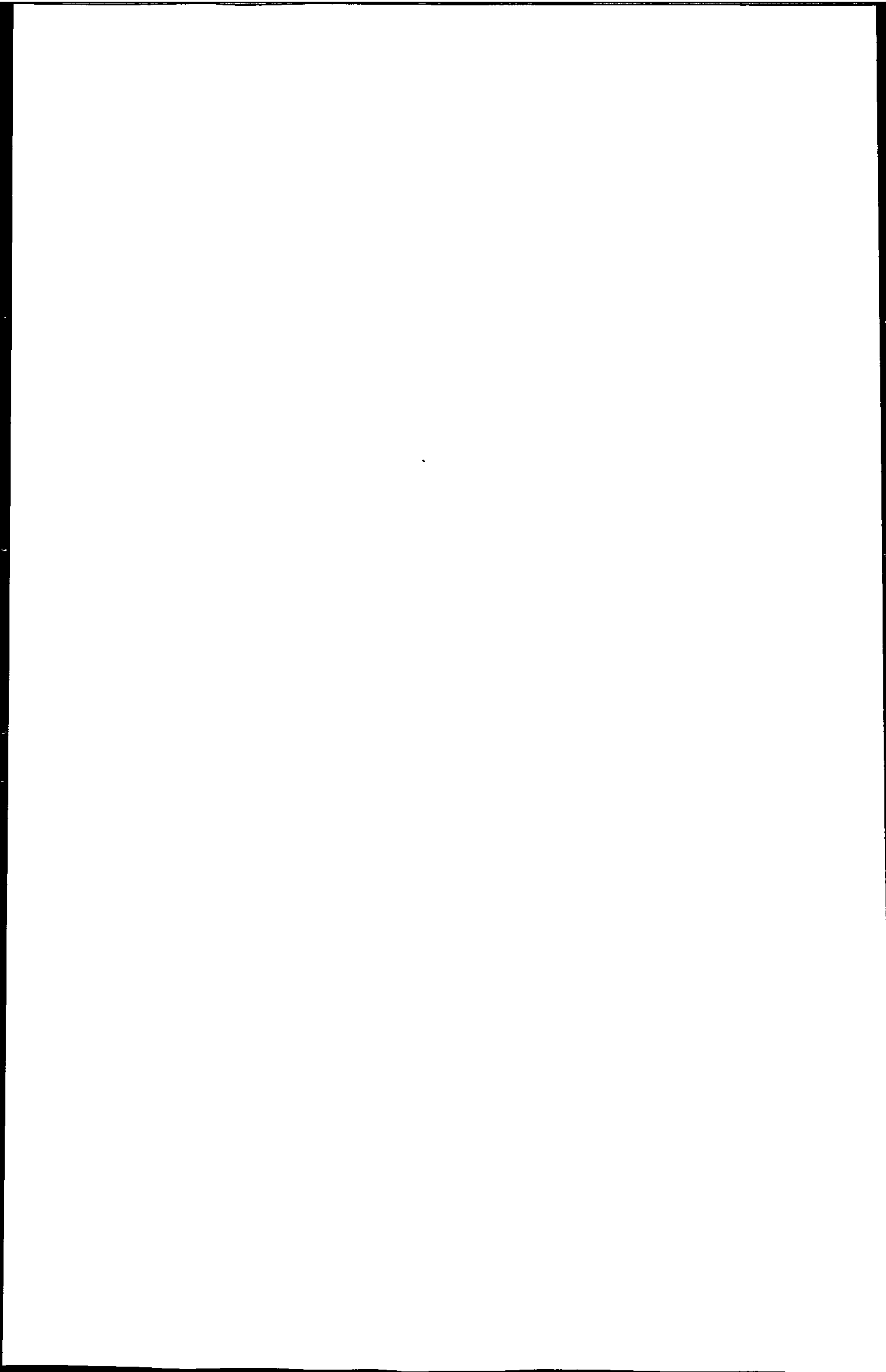
High-performance liquid chromatography was performed using an ion pair elution system under acidic conditions on a reversed phase C_{18} analytical column (Vydac 201TP54 column (250 x 4.6 mm), Hesperia, CA, USA). The column temperature was maintained at 30°C in a column oven. An elution gradient of acetonitrile (CH_3CN) and aqueous buffer (10 mM ammonium acetate, adjusted to ~pH5 with acetic acid / L buffer) was used at a flow rate of 0.5 ml min^{-1} (t = 0 min, 5% CH_3CN; t = 40 min, 90% CH_3CN; isocratic at 90%

CH_3CN for 10 min). The instrument used consisted of two Spectroflow 400 pumps (Applied Biosystems), a Spark-Holland PROMIS II autosampler (20µL injection loop), a GT-103 in-line degasser (Separations, The Netherlands) and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). Fluorescence detection was carried out at $\lambda_{ex/em}$ = 346/384nm for pyrene metabolites (slit widths $\lambda_{ex/em}$ = 18/40nm) (modified from Stroomberg *et al.*, 1999).

Samples were diluted 1:30 with ethanol (modified with 5mg ml^{-1} ascorbic acid) and stored at -20°C. Further dilutions were also made when necessary. Peaks were identified according to their retention times (Stroomberg *et al.*, 1999) and confirmed for the system used in this study (Howsam, pers. comm.). Quantification of 1-OH pyrene was performed using a series of 1-OH pyrene external standards, while the conjugates were quantified using their relative fluorescence efficiencies compared to 1-OH pyrene; pyrene-1-glucoside = 2.0 ± 0.31, pyrene-1-sulfate = 1.23 ± 0.09, pyrene-1-'conjugate' = 1.75 ± 0.18 (Stroomberg *et al.*, 2003). Validation of direct fluorimetric data was also carried out by comparison with total levels of 1-OH pyrene equivalents (conjugates and any free metabolite) determined by HPLC/F (expressed as µg l^{-1} 1-OH pyrene equivalents).

3.2.4 ELISA analysis of urine samples and validation of fluorometric results

Selected urine samples from the original exposure experiments were analysed with a PAH-competitive tube format ELISA (PAH RaPID Assay®), originally designed to detect PAH in water samples (see Fillmann *et al.*, 2002). For further validation (in addition to that provided by HPLC/F), the results obtained from the ELISA were compared with the direct fluorometric results for the same samples (also see Fillmann *et al.*, 2002). Comparison of ELISA and HPLC/F results provides yet another level of validation for the detection of metabolites in urine samples (see also Fillmann *et al.*, 2004).



Immunoassay procedure

PAH RaPID Assay® (SDI, Newark, USA) is a PAH-competitive tube format ELISA employing paramagnetic particles that are covalently coated with anti-PAH antibodies. Kits were supplied by SDI Europe (Alton, UK) and used according to the manufacturers recommendations.

Briefly, the principle of the assay is as follows. During initial incubation, any PAH in the sample competes directly with enzyme labelled PAH analog for binding sites on PAH specific antibodies (attached to paramagnetic particles). A magnetic field is then applied, holding the paramagnetic particles in the tube, along with sample PAH and enzyme conjugated PAH analog in proportion to their original concentrations. Any unbound reagents are washed off and any bound enzyme conjugate then catalyses the conversion of a substrate/chromogen mixture to a coloured product, before the reaction is stopped by the addition of acid. The colour developed, which is inversely proportional to the concentration of PAH in the sample, is then determined by absorbance at 405nm.

All analyses were performed in triplicate with concurrent standard calibrations and blanks. Urine samples (50 μl aliquots) were diluted 1:25 with 50% v/v methanol/buffered solution (SDI diluent). 500μl of antibody-coupled paramagnetic particles and 250μl of enzyme conjugate (PAH-horseradish peroxidase) were then added to 100μl aliquots of diluted samples (or standards) in glass test tubes. The contents were then vortex mixed and incubated at room temperature for 20 minutes (to allow competitive binding to the antibody). Tube contents were then separated (using a magnetic rack to retain the antibodies) and unbound reagents were decanted. 1ml kit washing solution was then added to each tube, and the tubes returned to the magnetic rack for a further two minutes. All tubes were then decanted and the washing step repeated. Tubes were then removed from the separator and 500μl of "colour solution" containing the enzyme substrate (hydrogen peroxide) and chromogen (3,3',5,5'-tetramethylbenzidine) were added. The tubes were

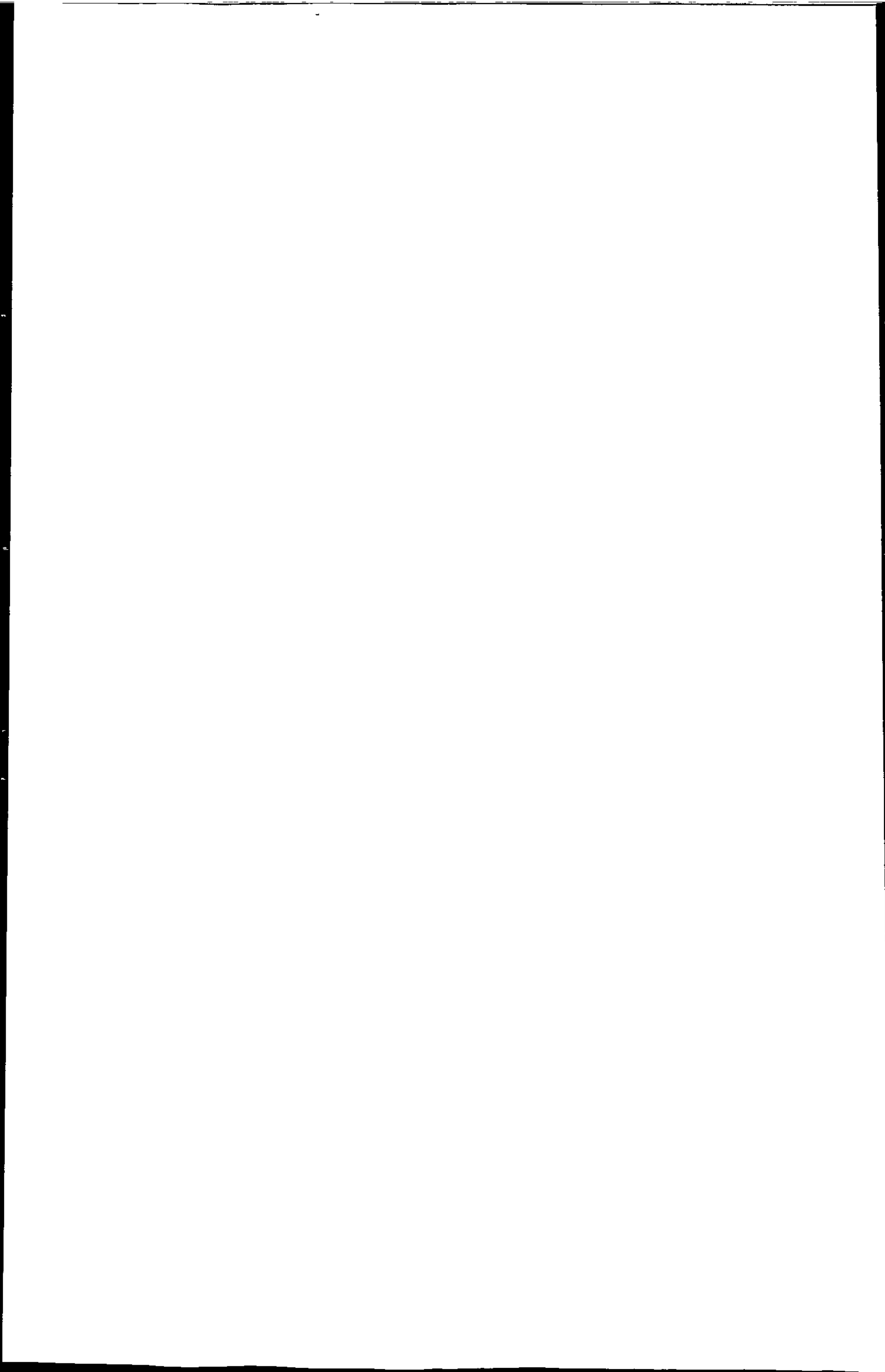
then vortex mixed and incubated at room temperature for 20 minutes. The reaction was then stopped by the addition of 500μl of "stopping" solution (2M sulphuric acid). 150μl aliquots from each tube were transferred to separate wells of a 96 well microplate and the colour produced was measured at 450nm using an Optimax microplate reader (Molecular Devices, Menlo Park, CA). Sample absorbance was compared to a linear regression equation using a logarithm of the concentration versus logit B/Bo standard curve to calculate the final concentration of PAH (where B/Bo is the absorbance observed for a sample or standard divided by the absorbance at the zero standard). Sample concentrations were then corrected for dilution factors. The ELISA was calibrated using 1-OH-pyrene.

3.2.5 Laboratory exposure to crude oil using a continuous flow system

Male shore crabs (carapace width 44-80mm) were exposed to crude oil in a continuous flow through system, previously described by Sanni *et al.*, (1998), as part of a BEEP project WP1 workshop at Akvamiljo research laboratory, Stavanger, Norway. Three treatments were included in this experiment, designed to simulate the conditions experienced by organisms in the vicinity of North Sea oil platforms, where large volumes of waste oil and produced water are discharged.

1. 0.5ppm North Sea crude oil (average droplet size 10μm),
2. 0.5ppm North Sea crude oil + 0.1ppm alkylated phenol (constituent of produced water).
3. Control (seawater only).

Twelve individuals were sampled for urine from each treatment. Urine samples were analysed using SFS (Δλ37nm). Area of broad fluorescence between Em287-377nm was measured as an indication of levels of 2-3 ring petrogenic PAH in the sample. The occurrence of a distinct peak at pyrogenic wavelengths on the spectra prompted the



measurement of intensity at Em387nm. Slit widths for all analyses were 2.5nm and all urine samples were diluted 1:20. No attempt was made to quantify sample fluorescence in terms of $\mu g\ l^{-1}$ of specific PAH. Intensity and integrated peak areas were measured from fluorescence spectra.

3.2.6 Preliminary field trials of the urinary fluorescence biomarker

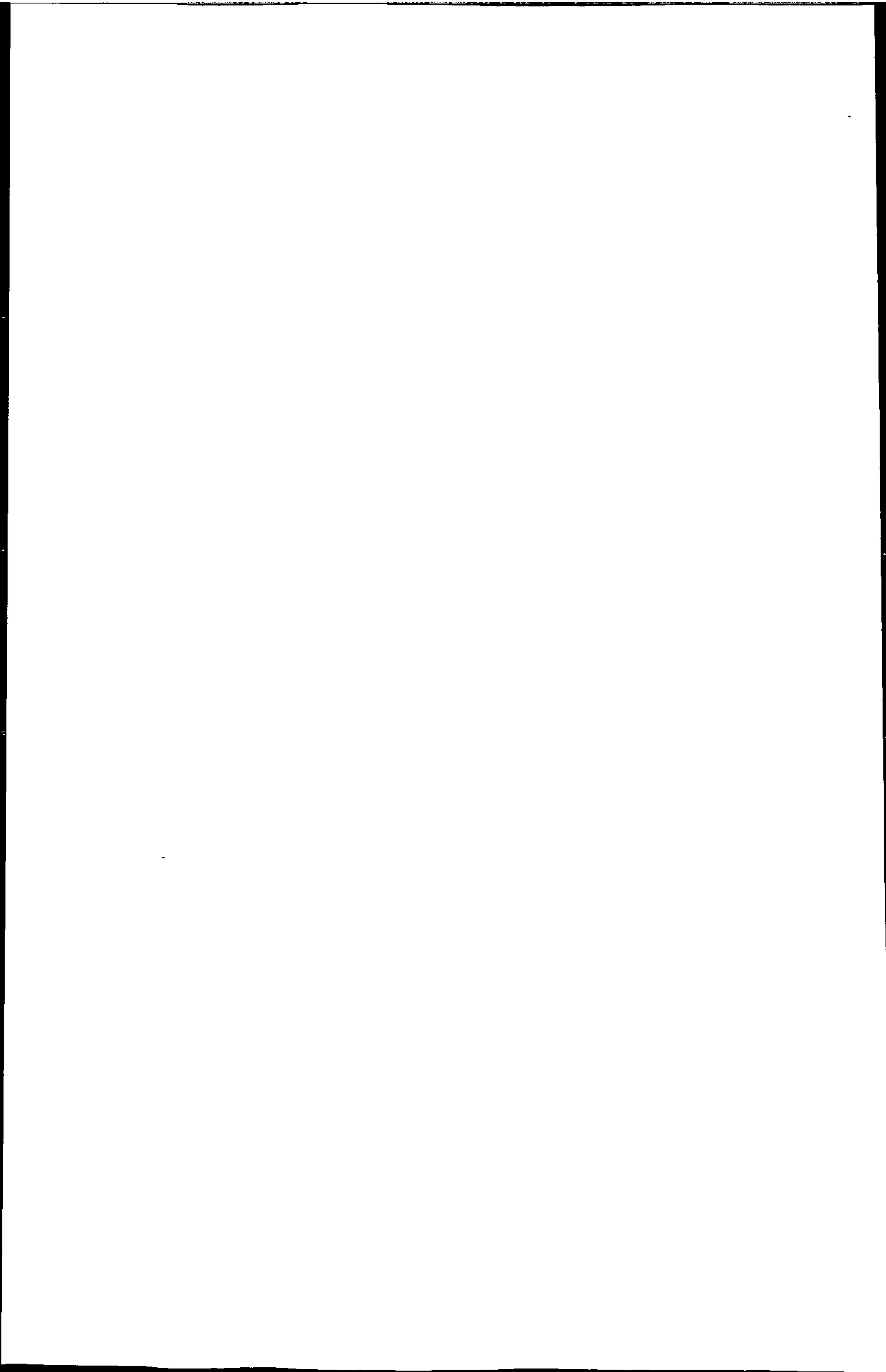
Initial field trials of the urinary biomarker were carried out locally at well characterised contaminated and reference sites. The first chosen site was Sutton Harbour, Plymouth (OS grid reference SX 485543 GB), a highly contaminated body of water subject to multiple inputs from fishing and leisure vessels, as well as urban runoff from the city of Plymouth itself. For comparison, the reference site of Bantham (SX 667438) on the estuary of the River Avon was chosen. Two separate field trials were carried out, the first (October 2000) being a preliminary investigation into the suitability of the contaminated site and its crab population for monitoring purposes. A second field trial was carried out during March of 2001. During both trials and at both sites, actively feeding male *C.maenas* (carapace width 42-80mm) were collected using a baited drop-net. Individuals were kept in well-aerated seawater collected *in situ* until return to the laboratory, where they were sampled for urine within 6 hours.

Urine samples were analysed for a variety of PAH, using both FF and SFS techniques. FF Ex290nm was used to detect naphthalenes, typical constituents of petrogenic contamination. Total area of all peaks between Em310-380nm was used to quantify this typically broad area of fluorescence on each spectrum. The wavelength pairs Ex345/Em382nm and Ex380/Em430nm were used to detect pyrene equivalents and benzo(a)pyrenes respectively, indicative of pyrogenic PAH contamination. Synchronous scanning fluorescence (SFS), which screens for all major PAH groups (2-5rings) in one simplified spectrum, was also performed using wavelength differences ($\Delta\lambda$) of 37 and 42nm. The samples were analysed using two $\Delta\lambda$ in order to compare the results obtained

and to choose the best $\Delta\lambda$ for field samples. Area of broad fluorescence between Em287-377nm was measured as an indication of levels of 2-3 ring petrogenic PAH in the sample. Intensity at Em382nm was measured as an indication of any fluorescent contributions from pyrene equivalents in the sample. Slit widths for all analyses were 2.5nm and all urine samples were diluted 1:50.

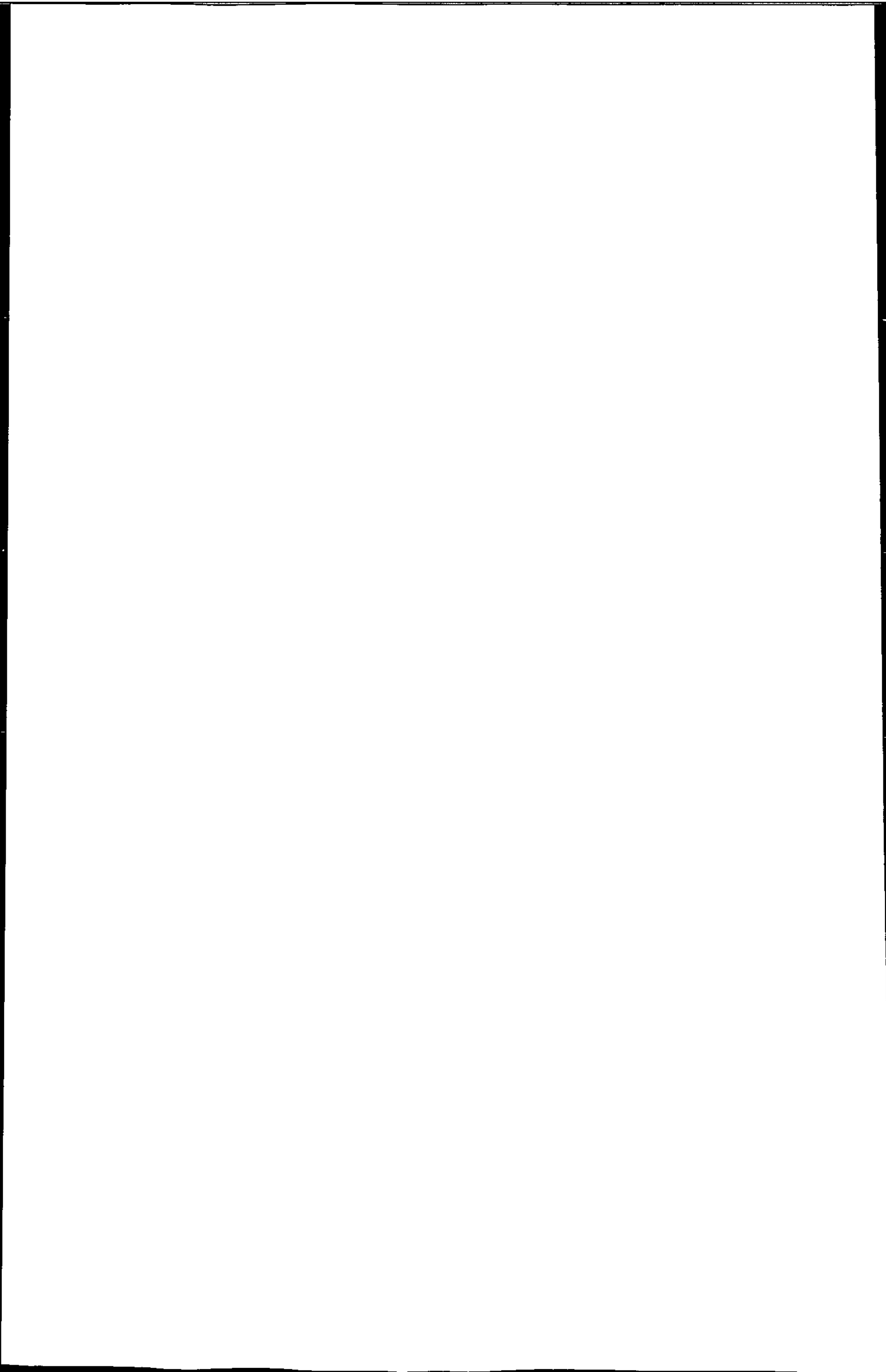
In addition to analysis by direct fluorimetry, urine samples from the second Sutton Harbour and Bantham trial (March 2001) were also analysed using the RaPID ELISA. In light of results obtained from fluorescence analyses, the ELISA was calibrated for petrogenic-type PAH, using 9-OH phenanthrene standards. 9-OH phenanthrene is the primary intermediate during biotransformation of the 3 ring PAH phenanthrene, a commonly occurring petrogenic PAH. Phenanthrene contributes fluorescence at the naphthalene wavelength pair Ex290/Em335, (Aas *et al.,* 2000a,b, Lin *et al.,* 1996), and contributes fluorescence to the SFS spectra in the 2-3 ring area, along with the 2 ring naphthalenes. Use of the readily available, and more soluble, 9-OH standard therefore serves as surrogate measure of total petrogenic PAH. Comparison with fluorescence data (determined by SFS) provides validation of the urinary assay in field samples (see also Fillmann *et al.,* 2002).

A field-sampling trip was also undertaken to selected sites in the North East of England and South Wales during August of 2000. Contaminated sites in both areas were chosen based on their pollution history and measured levels of PAH contamination in sediment and water (Woodhead *et al.,* 1999 and Law *et al.,* 1997 respectively). Three contaminated sites (TA, TB and TC) were sampled near the mouth of the Tees, NE England, an estuary with some of the UK's highest levels of PAH in sediment and water (Woodhead *et al.,* 1999, Law *et al.,* 1997) and home to a large steel manufacturing plant at Redcar. Site TA (NZ 533224) was a short distance upstream of the mouth, beneath derelict pilings opposite an oil refinery. Site TB (NZ 556278) was a jetty at South Gare, within



Tees Mouth, and TC (NZ 547260) was adjacent to an ore terminal for the steelworks. A contaminated site (W) at Whitby Harbour (NZ 900113) was also sampled, as well as a reference site at Robin Hoods Bay (RH) (NZ 953050). In Port Talbot (PT), South Wales (SS 755890), a contaminated site at the entrance to the docks, adjacent to a large steelworks was sampled. Three sites were also sampled in the Milford Haven area, namely Neyland Spit (N) (SM 966047), opposite Pembroke Docks at Hazelbeach (H) (SM 948047) and opposite the oil refinery at Milford Haven (MH) (SM 899053). This heavily industrialised area is near the site of the Sea Empress oil spill, and has been reported as having highly elevated PAH residues in its surface sediments particularly at Neyland, (Woodhead *et al.,* 1999). A reference site at Stackpole Quay (SQ) was also sampled (SR 993957).

Male *Carcinus* (carapace width 40 - 82mm) were collected using a baited dropnet at each site (TA n=5, TB n=7, TC n=8, W n=4, RH n=4, PT n=8, N n=1, MH n=4, H n=2, SQ n=2). Once collected, crabs were kept in 10L buckets containing 5L of aerated seawater from each site. Crabs were immediately sampled *in situ* for urine and samples were then snap frozen in liquid nitrogen for transfer back to the laboratory. Urine samples were analysed for a variety of PAH, using both FF and SFS techniques, as described above.



3.2.7 Rapid assessment of PAH exposure in Mytilus edulis

The advantages of a rapid, inexpensive and simplistic assay for the assessment of PAH exposure in marine organisms are clear, as demonstrated in *C.maenas* in chapter 2. With this in mind, a short experiment was conducted to investigate if PAH (metabolites or parent), could be detected in the haemolymph of mussels exposed to waterborne PAH. Whilst mussel tissues (whole body, digestive gland) have been used extensively for measurement of total burdens of PAH and numerous other contaminants, detection of PAH in untreated haemolymph has not been attempted thus far. The following experiment was therefore designed to test the hypothesis that mussel haemolymph can be used in much the same way as crustacean urine for the fluorometric detection of a model PAH.

Adult *M.edulis* were collected from their natural beds at the clean site of Sharrow Point, Whitsand Bay, on the South coast of Cornwall, in November 2000. Upon return to the lab, they were kept in 10µm carbon filtered, well-aerated seawater (34ppt, 15+/-1°C) for a minimum of 24hrs before the exposure trial began.

Groups of 10 mussels were exposed to the nominal concentrations of 50, 100, 200 and 400µg l^{-1} pyrene (using an acetone solvent vehicle) in 10 glass aquaria, containing 10L of filtered (10µm carbon filtered), aerated seawater (34ppt, 15+/-1°C) alongside control (n=10) and solvent control groups (n=10). Mussels were not fed during the exposure period. After 48hrs and 7 days of exposure, haemolymph samples were taken from all mussels. To collect each haemolymph sample, the valves of the mussel were first prised open and any seawater held within the valves was allowed to escape. Approximately 400µl of haemolymph was then drawn from the posterior adductor muscle into a 1ml syringe and expelled into a 700µl siliconised microcentrifuge tube.

Aliquots of each haemolymph sample were then treated in one of three ways for analysis. First, haemolymph samples were simply diluted 1:50 in 50% ethanol before

analysis. Secondly, the sample was centrifuged (2000rpm for 5 minutes) to spin out the haemocytes, and the supernatant then used for analysis after 1:50 dilution in 50% ethanol. Thirdly, 1μl of a 10% solution of detergent (Triton-X) was added to 50μl of the sample and incubated for 20 minutes, to lyse the haemocytes. Samples were then centrifuged (2000rpm for 10 minutes) to remove cellular debris and the supernatant diluted 1:50 in 50% ethanol.

Analysis of diluted samples was carried out as described previously for pyrene exposed crab urine samples (FF Ex/Em =345/382nm, SFS Δλ37nm Em= 382nm, slit widths 2.5nm).

3.3 Results

3.3.1 Spiking of urine samples

In each case, the peak observed was that of the 1-OH standard (Em~387nm), with no shift to Em382nm. Increasing the concentration of the spiked metabolite did not alter the position of its dominant emission peak, which remained at Em387nm. Fluorescence intensity at Em387nm increased linearly with spiked 1-OH pyrene concentration.

3.3.2 GC/MS analysis of water samples

Recoveries were 95±3% (n=18). Measured concentrations confirmed that the nominal values were within ±12%. Results from tanks with and without crabs are presented in figure 3.2 and reveal that whilst pyrene was lost from the tanks without crabs (most likely through photo-oxidation and adsorption, with some loss through volatilisation), it's levels decreased more rapidly in the water from tanks with crabs.

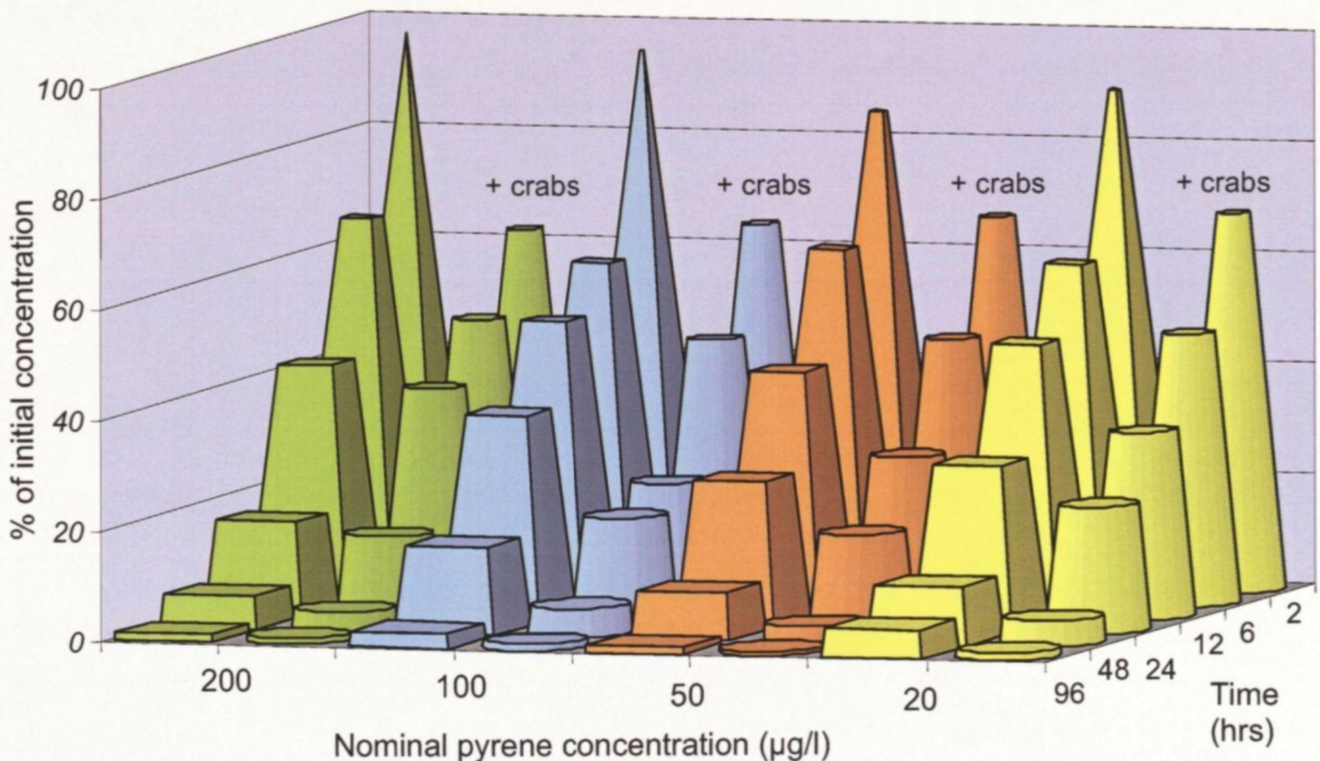


Figure 3.2 Levels of pyrene in exposure water over the experimental period, determined by GC/MS. Levels are expressed as a percentage of the initial concentration (y axis).

For example, after 2 hrs, an average of 8% of the nominal concentrations of parent pyrene was lost from seawater alone, whilst 34% was lost from seawater being filtered over the gills of crabs. After 6 hours of exposure, these losses increased to 37% and 52% of the initial concentration respectively.

3.3.3 HPLC/F analysis of urinary equivalents

The results indicate conjugation of 1-hydroxypyrene into three major metabolites, concurrent with suggestions made in chapter 2 regarding the phase II transformation of the 1-OH pyrene intermediate (see figure 3.3). Two of the conjugates were identified as pyrene-1-glucoside and pyrene-1-sulfate (elution times 28.07min and 26.33min respectively). The identity of one other conjugate (25.25min) is still unknown. Two other peaks on the chromatogram (17.64/18.03min and 24.24min) remain unidentified but their fluorescent properties suggest pyrene-like structures. The relative distributions of conjugates show that pyrene-1-glucoside and pyrene-1-"conjugate" dominated. Comparing the relative amounts of 1-OH-pyrene and its conjugates, it was found that an average of 38.9% is present as pyrene-1-glucoside, 9.7% as pyrene-1-sulfate, 47.7% as the unknown conjugate and 3.7% as non-conjugated 1-hydroxy-pyrene (39.15min). The identity of the unknown pyrene-1-"conjugate" is still undetermined, but is not a glucuronide conjugate nor (derived from) a glutathione conjugate (Stroomberg, pers. comm.). The chromatogram also shows that pyrene, which elutes last (47.72min), was negligible in exposed samples.

Comparison of urinary equivalent levels determined by HPLC and fluorescence analyses (figure 3.4) showed a good correlation (FF r^2=0.9039, SFS r^2=0.8724).

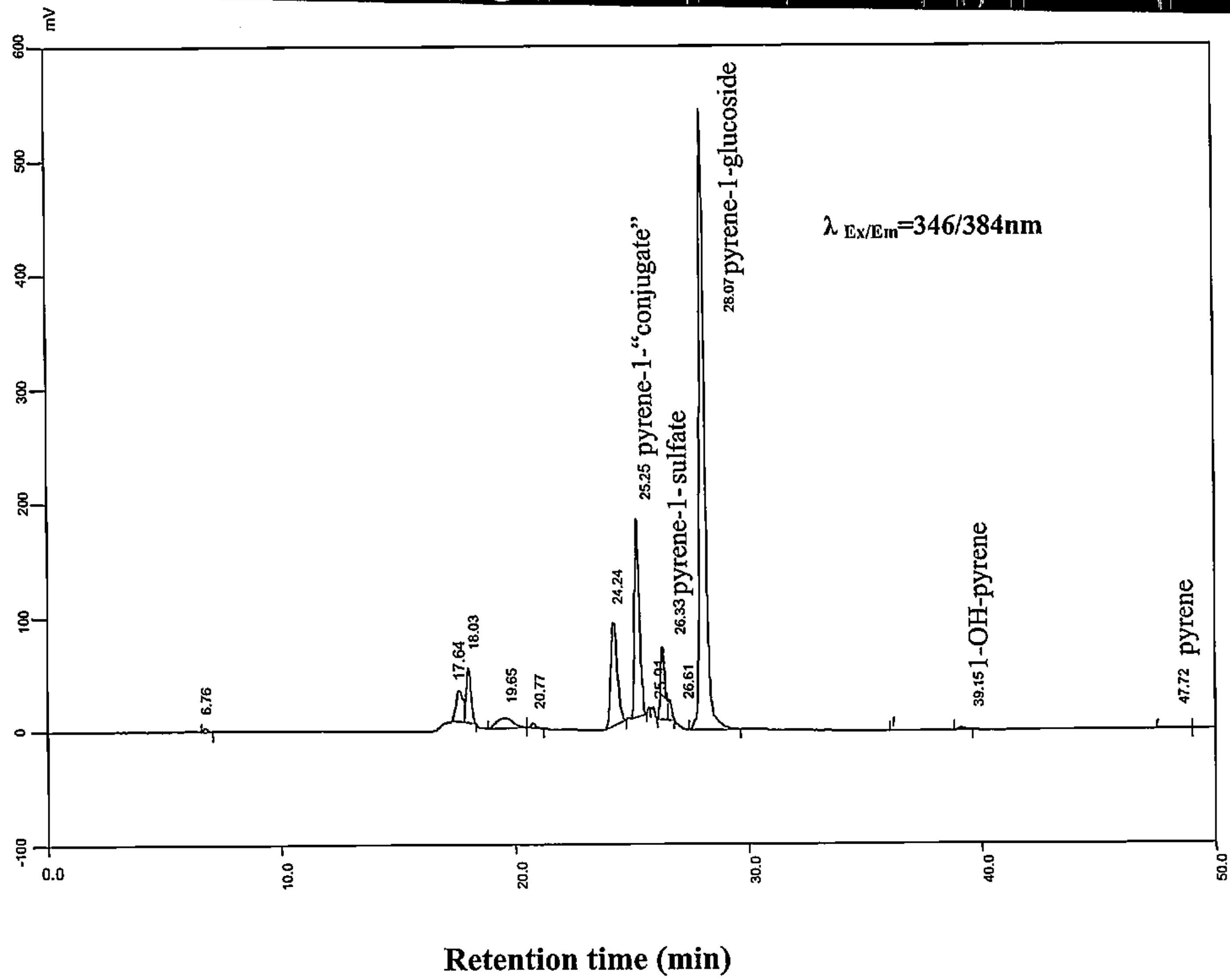
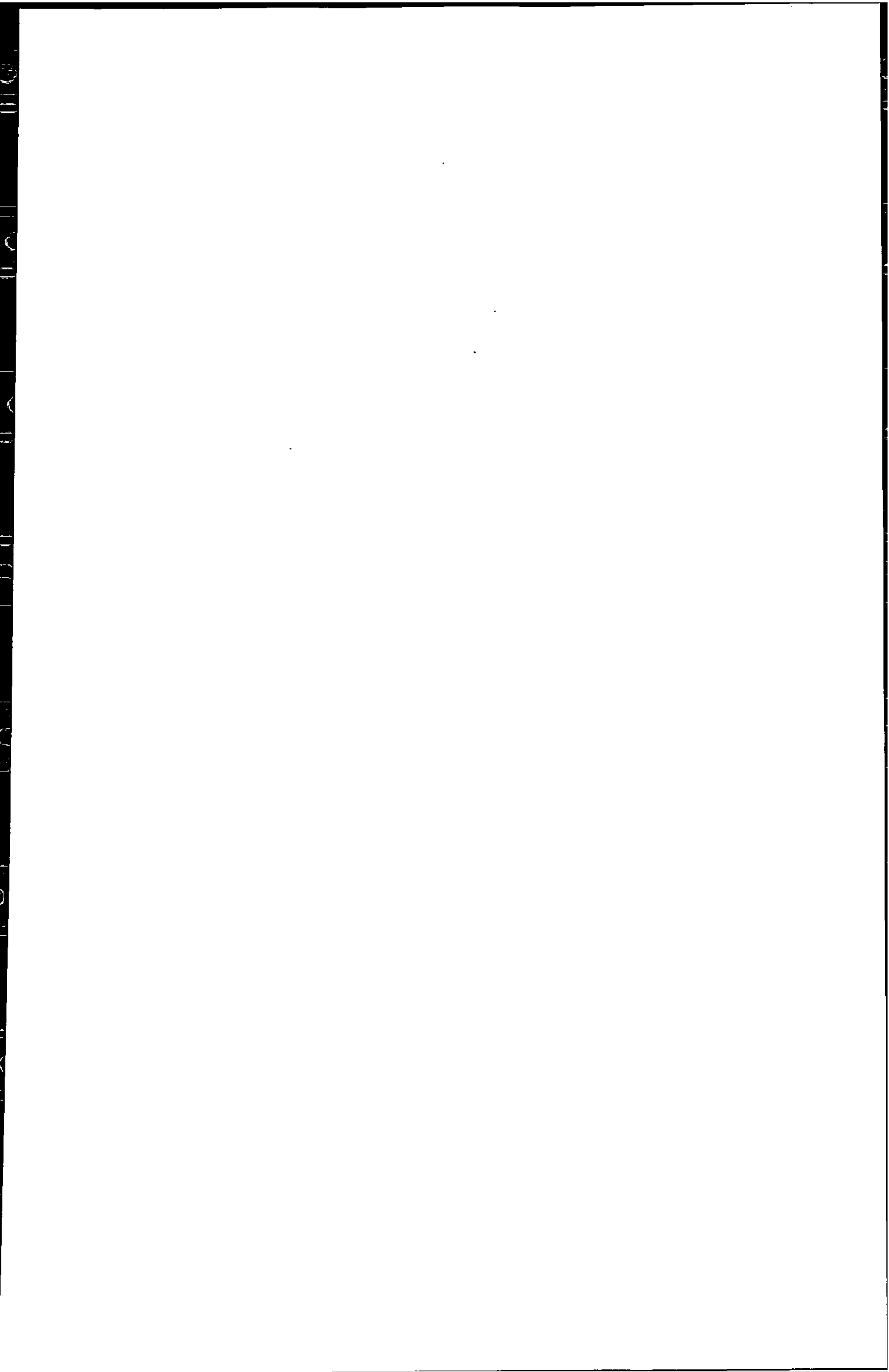
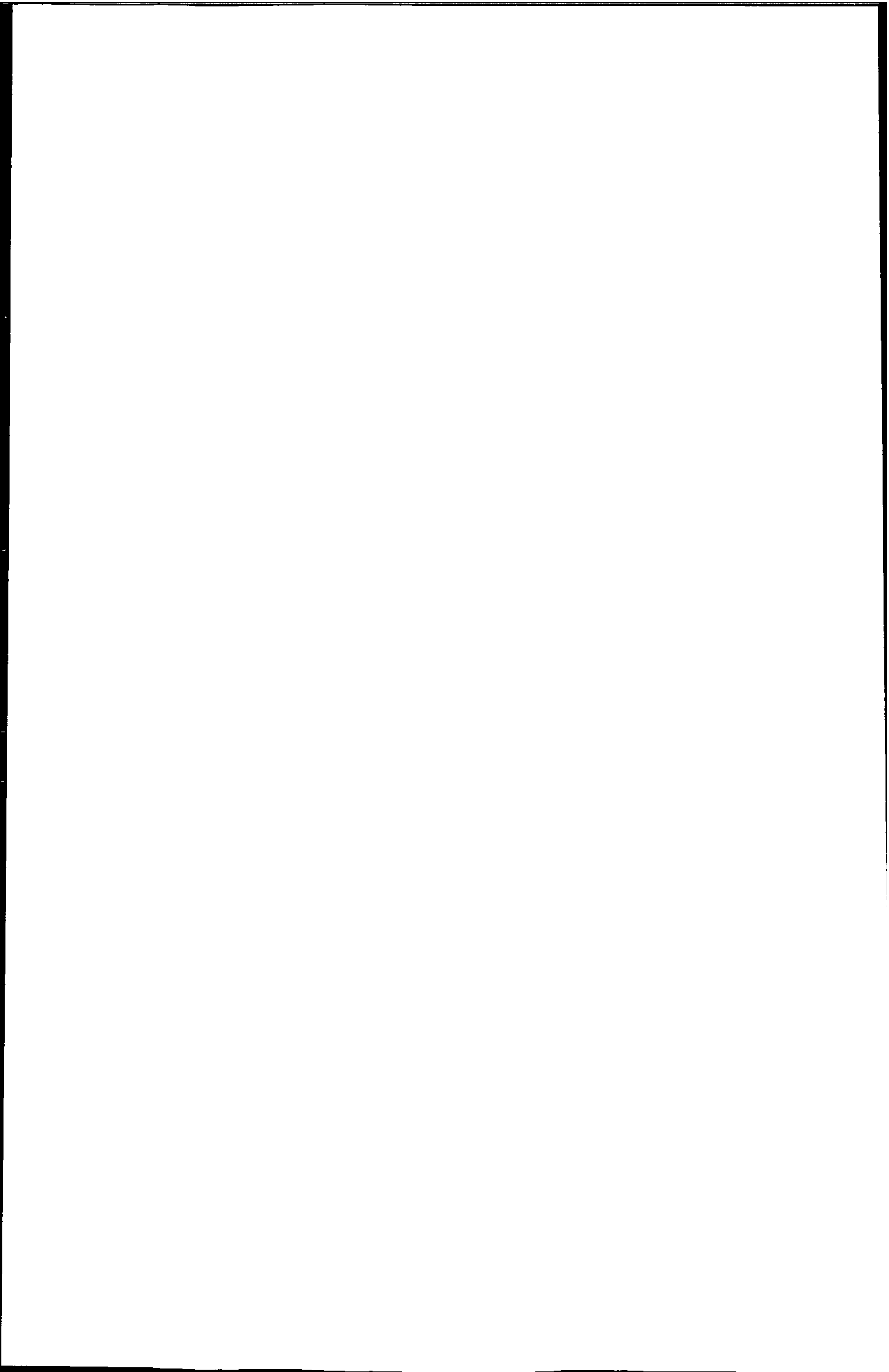


Figure 3.3 Representative HPLC-fluorescence chromatogram ($\lambda_{Ex/Em}$ = 346/384 nm) of urine from crab exposed to 200µg l^{-1} waterborne pyrene.





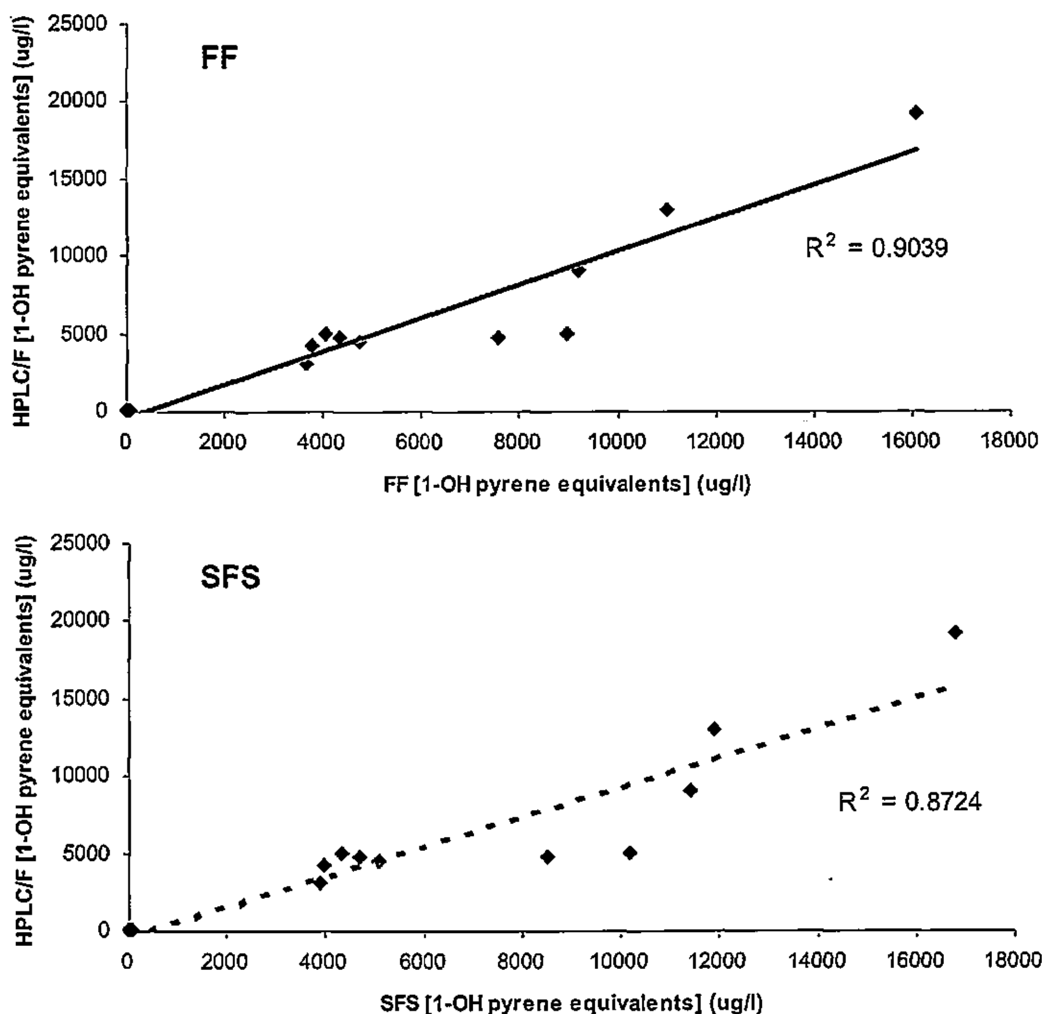
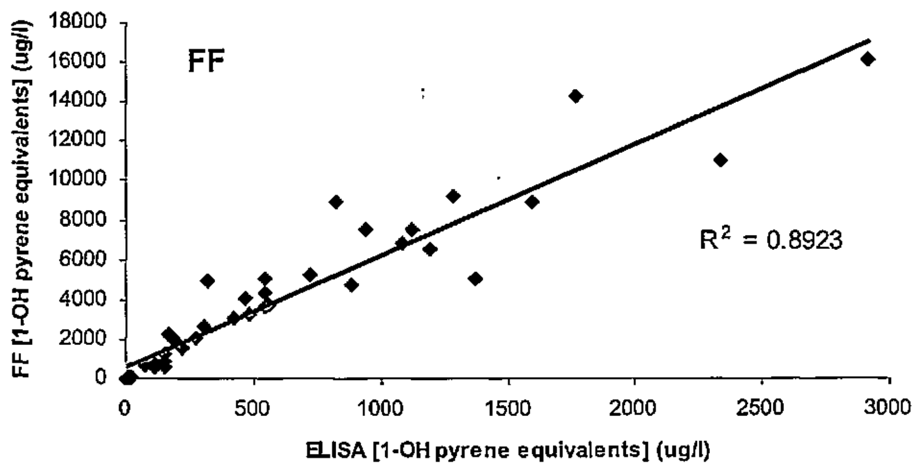


Figure 3.4 Regression plots comparing levels of 1-OH pyrene equivalents (μg l^{-1}) in urine samples determined by direct fluorescence (FF and SFS) and HPLC/F.

3.3.4 Validation of fluorometric results using PAH ELISA

Results obtained from the PAH ELISA were dose dependent and showed that this technique was suitable for detecting PAH in biological samples. Comparison of fluorescence results with those obtained from the ELISA showed a strong positive correlation (FF r^2 =0.8923, SFS r^2 =0.8404) between the two techniques (figure 3.5) (See also Fillmann *et al.,* 2002). ELISA and HPLC results also showed very good agreement (r^2 =0.9382) (figure 3.6).



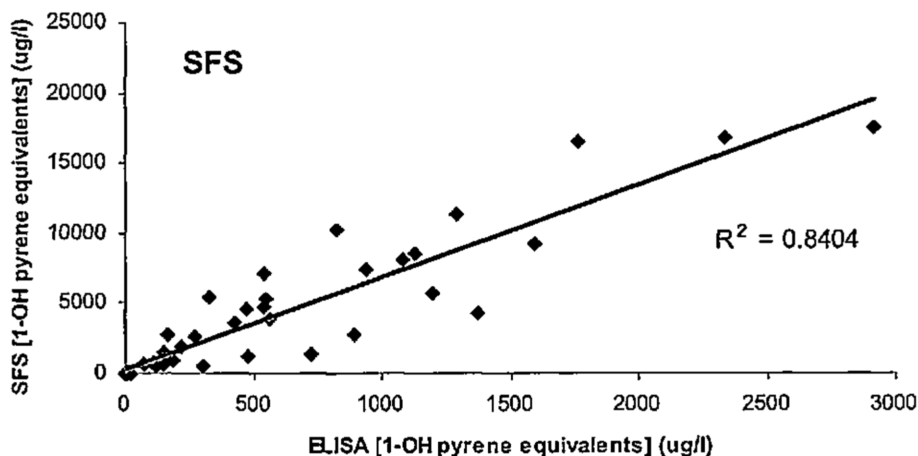


Figure 3.5 Regression plots comparing levels of 1-OH pyrene equivalents (μg l^{-1}) in urine samples determined by immunoassay (ELISA) and direct fluorescence (FF and SFS).

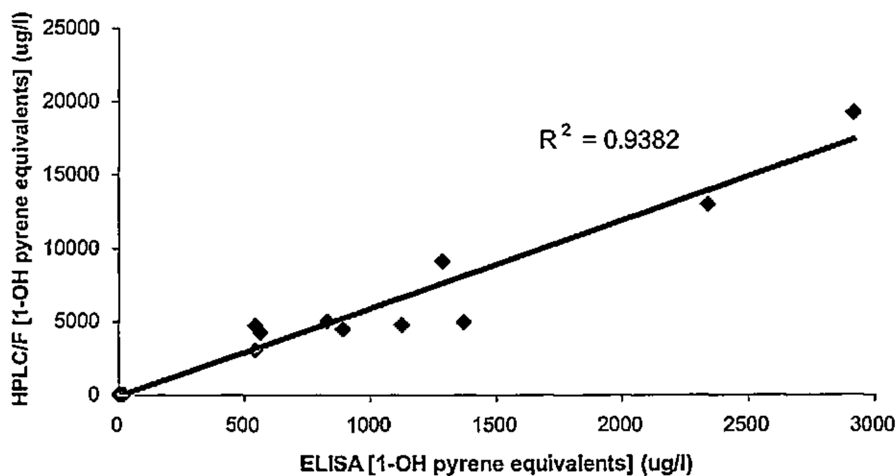
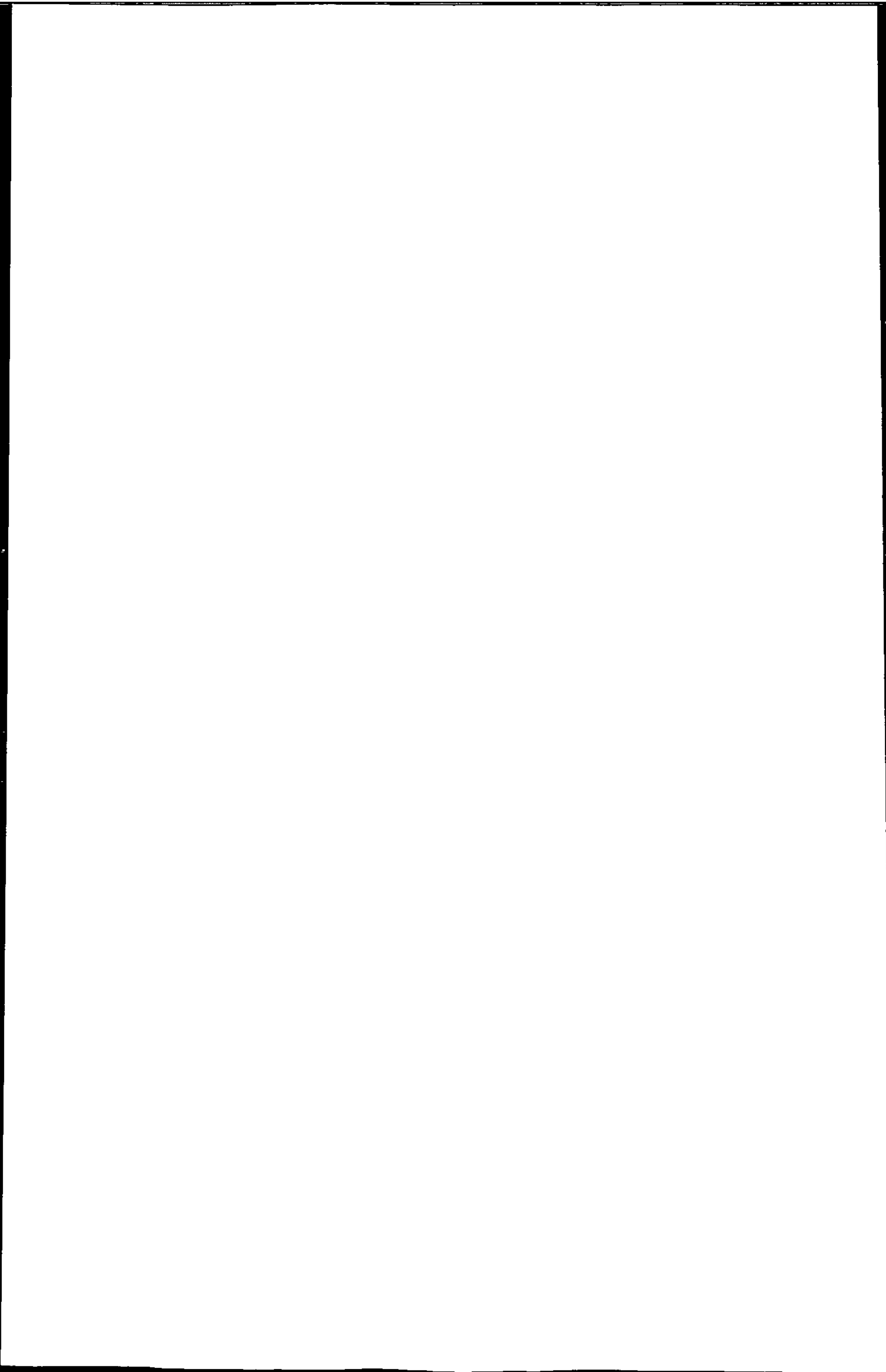


Figure 3.6 Regression plot comparing levels of 1-OH pyrene equivalents (μg l^{-1}) in urine samples determined by ELISA and HPLC/F.



3.3.5 Laboratory exposure to crude oil using a continuous flow system

Overlaid spectra from each treatment in this exposure trial are shown in figure 3.7. In both crude oil and crude oil/alkylated phenol exposed crabs, urine samples contained compounds that contributed broad fluorescence at 2-3 ring wavelengths, indicative of petrogenic PAH. No significant differences were observed between the treatments (KW $p>0.05$). However, samples from control individuals also contained comparable levels of 2-3 ring fluorescence and their spectra were qualitatively similar to those of exposed crabs. As a result, controls were not statistically significantly different from exposed crabs (KW $p>0.05$). This unexpected result is discussed below.

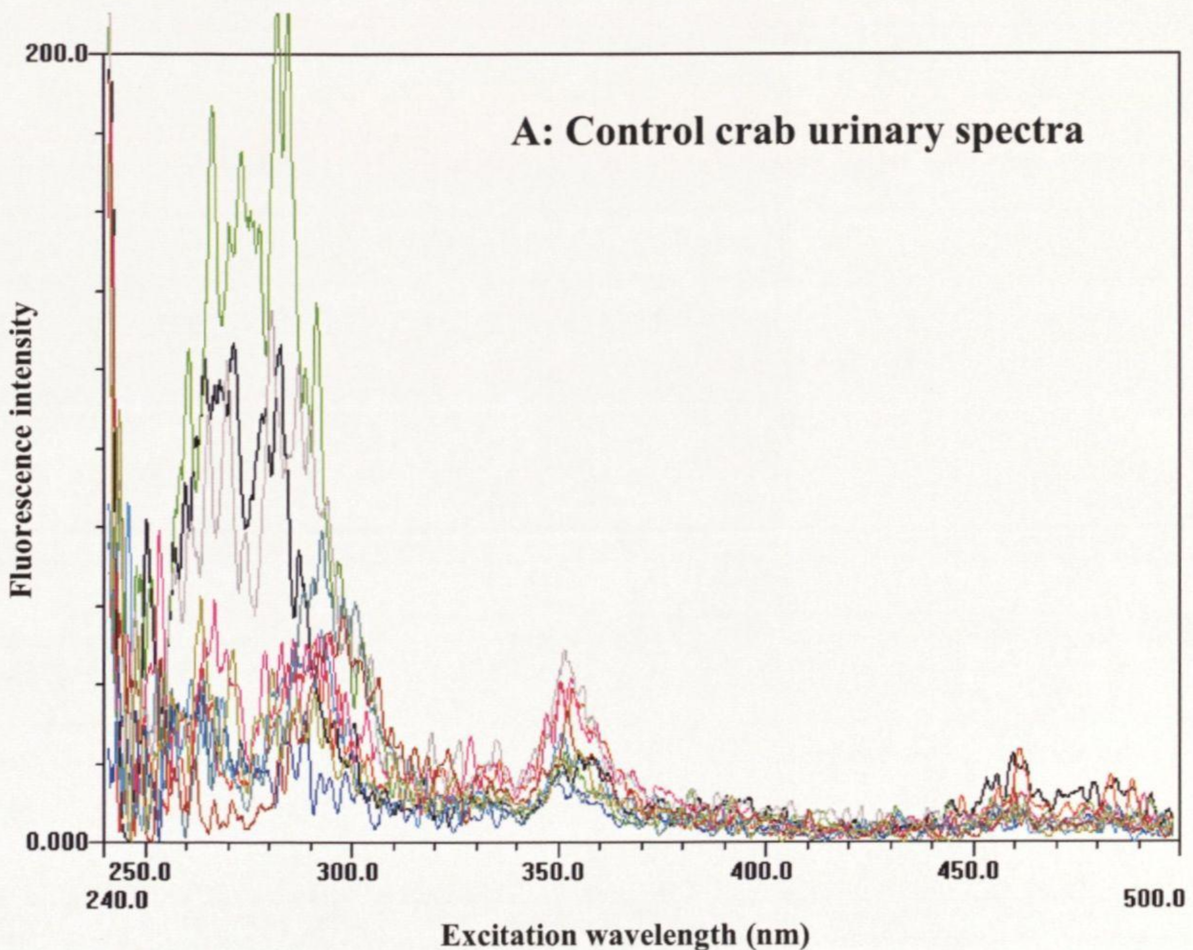
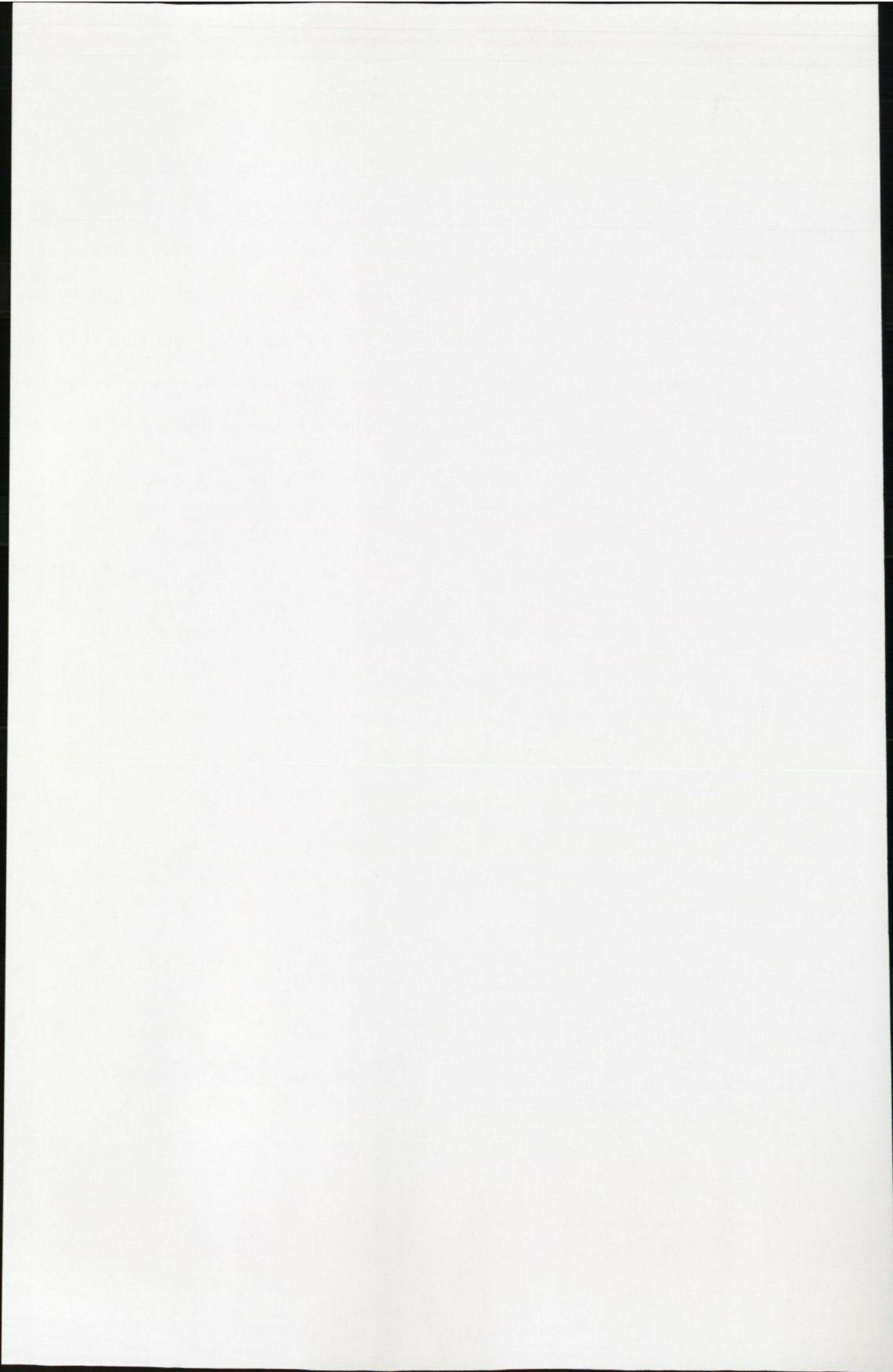
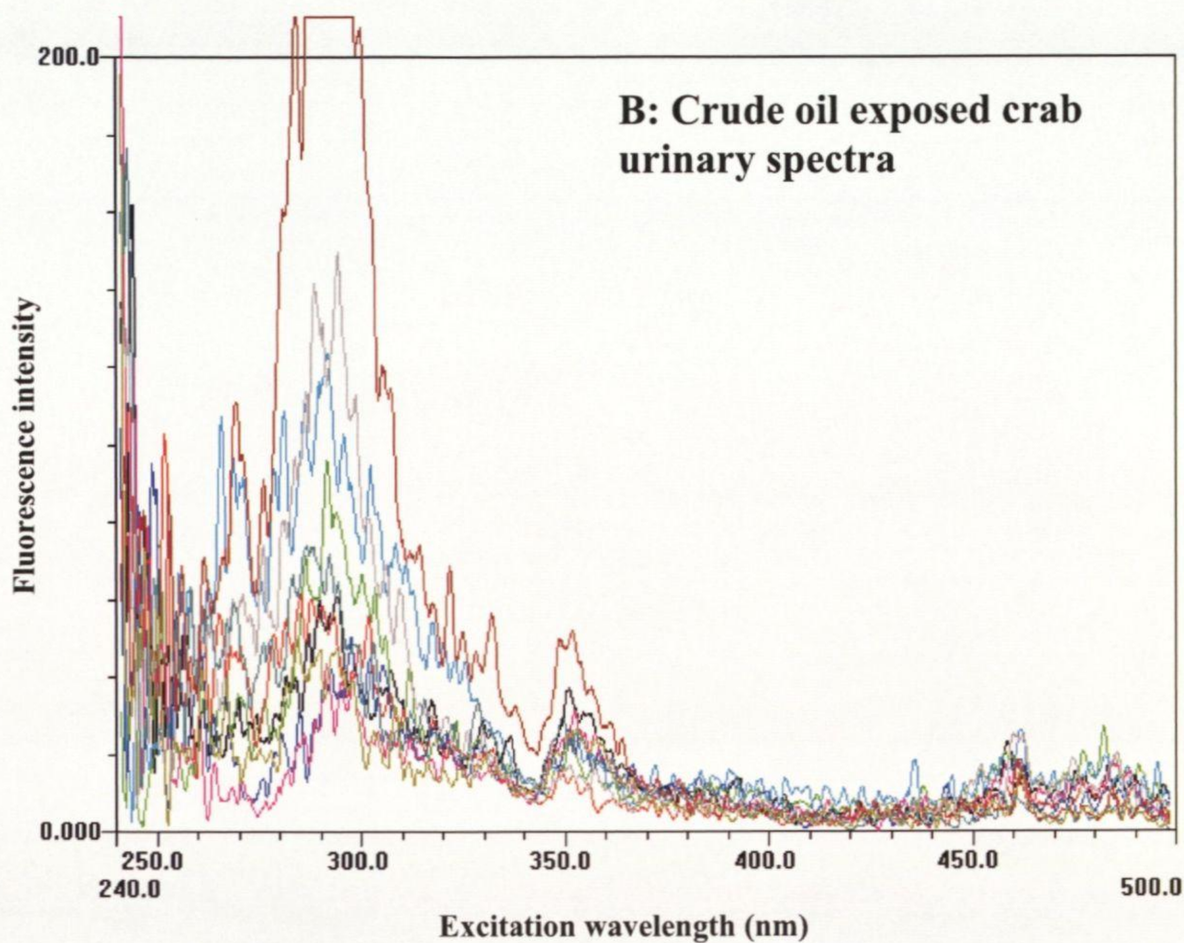


Figure 3.7 Overlaid urinary spectra obtained from control crabs in the continuous flow system experiment.





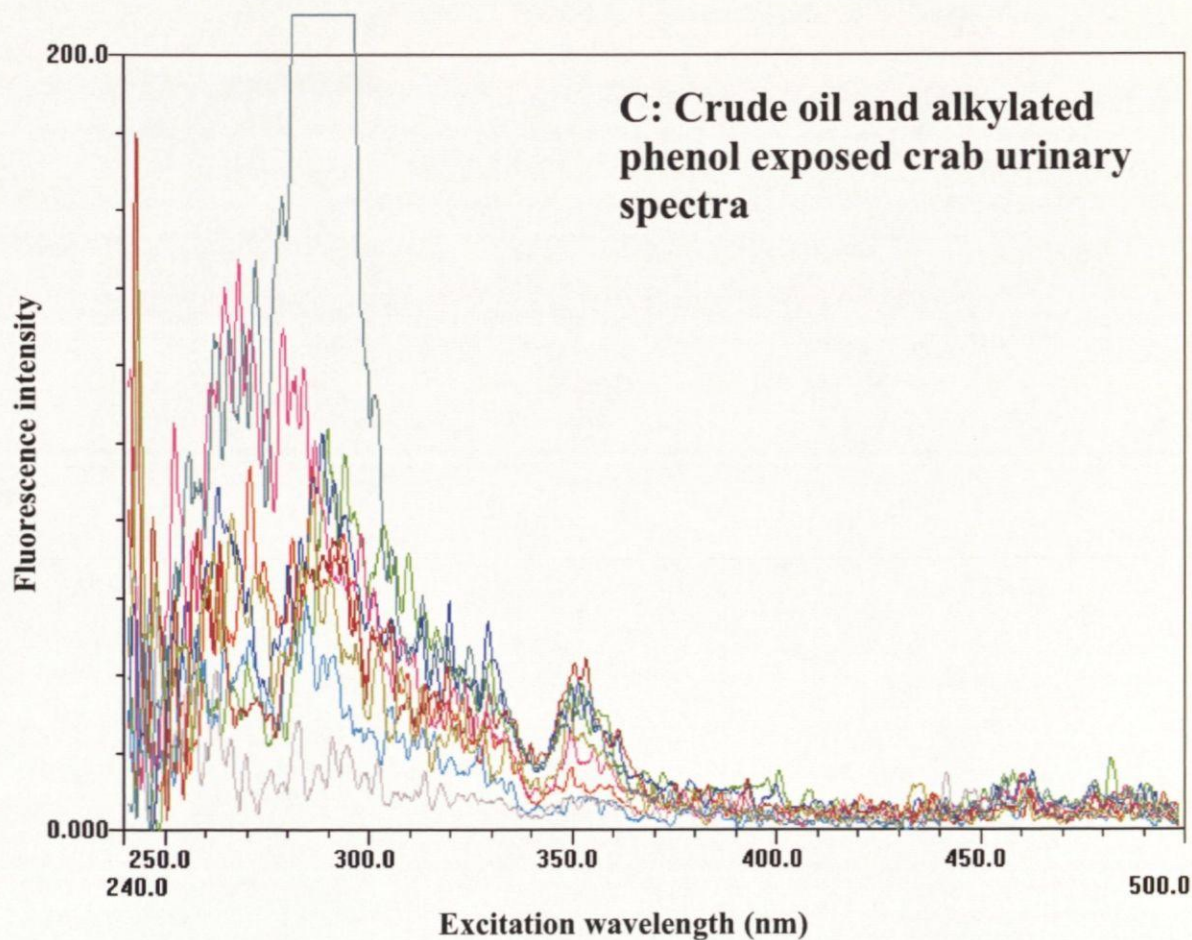
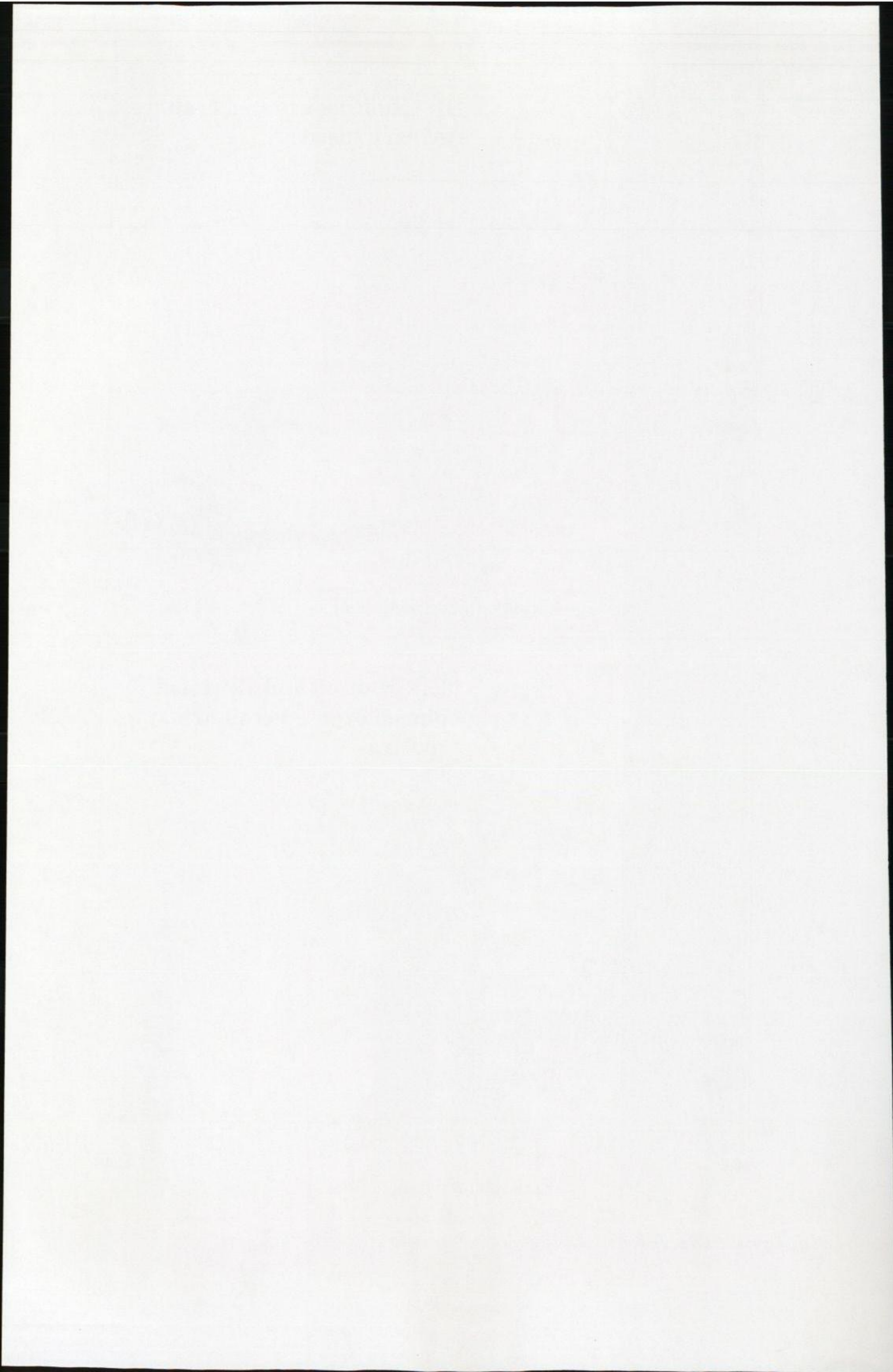


Figure 3.7 (cont'd) Overlaid urinary spectra from exposed crab samples.

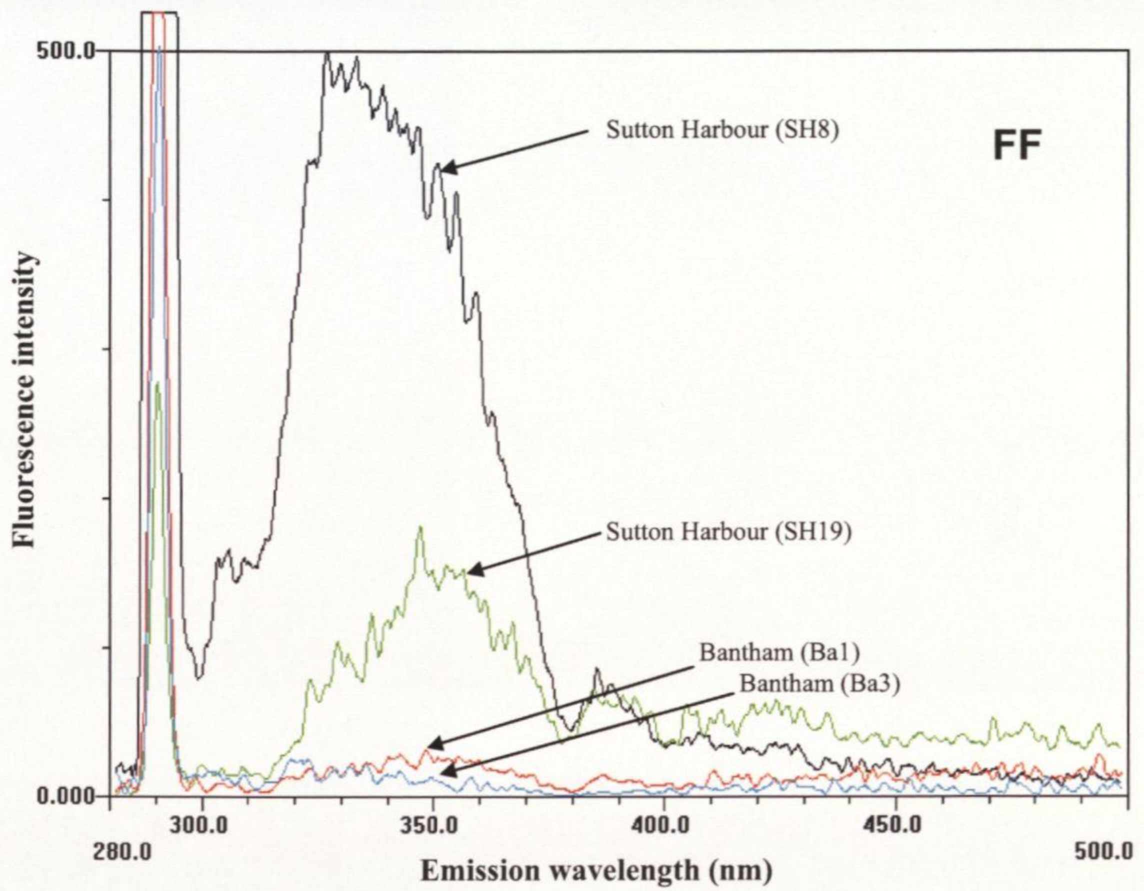


3.3.6 Preliminary field trials of the urinary fluorescence biomarker.

Both $\Delta\lambda$ (37 and 42nm) were appropriate for synchronous analysis of field samples. 1:50 dilution was sufficient to produce clearly resolved fluorescent signals, without reducing intensity below the limits of detection.

The PAH exposure biomarker proved sensitive enough to discriminate between samples from contaminated and clean sites, but variability was high. Positive samples from Sutton Harbour were found to contain broad fluorescence at petrogenic-type (naphthalene) wavelengths (FF) (figure 3.8). SFS spectra also showed broad 2-3 ring petrogenic fluorescence in several samples. Samples from reference crabs at Bantham contained minimal, but not entirely negligible, fluorescence at such wavelengths. Low-level broad fluorescence seemed to be a feature of urine sampled from crabs at this supposedly clean environment. Fluorescence intensity of diluted samples at specified wavelengths or sum of intensity (area) of all peaks in diluted samples between specified wavelengths was used to generate data from the spectra. No attempt was made to quantify sample fluorescence in terms of $\mu g\ l^{-1}$ of specific PAH, as these studies were simply intended to preliminarily test the applicability of the urinary biomarker in the field. The data presented are those for 2-3 ring petrogenic PAH, which dominated the fluorescence spectra (figure 3.9).

The high level of variability in the Sutton Harbour data set unfortunately obscures any meaningful differences between the sites. Levels of 2-3 ring fluorescence were broadly similar between sites, with only a few samples from Sutton Harbour showing highly elevated 2-3 ring PAH in their urine. Whilst these high levels were only found in Sutton Harbour crabs, providing compelling evidence of higher levels of exposure, they were too infrequent in the sample set to generate significant differences between the sites.



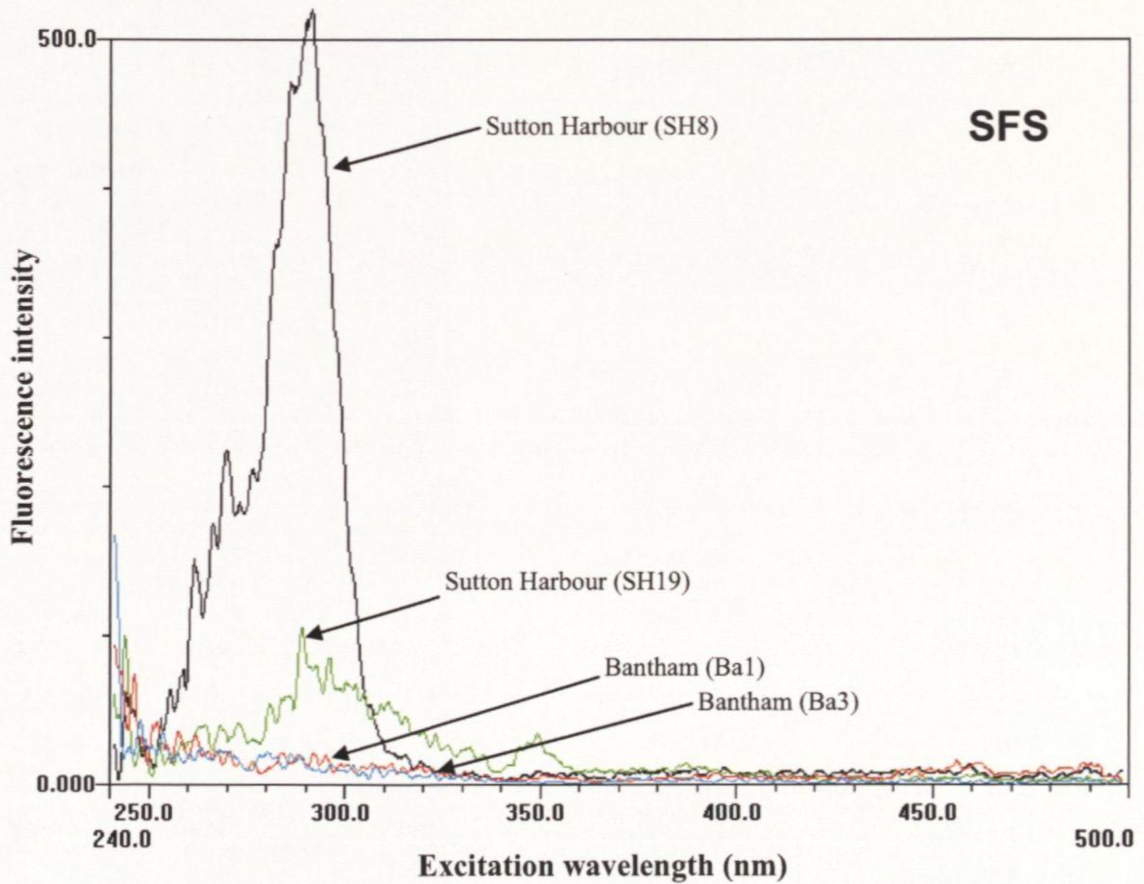
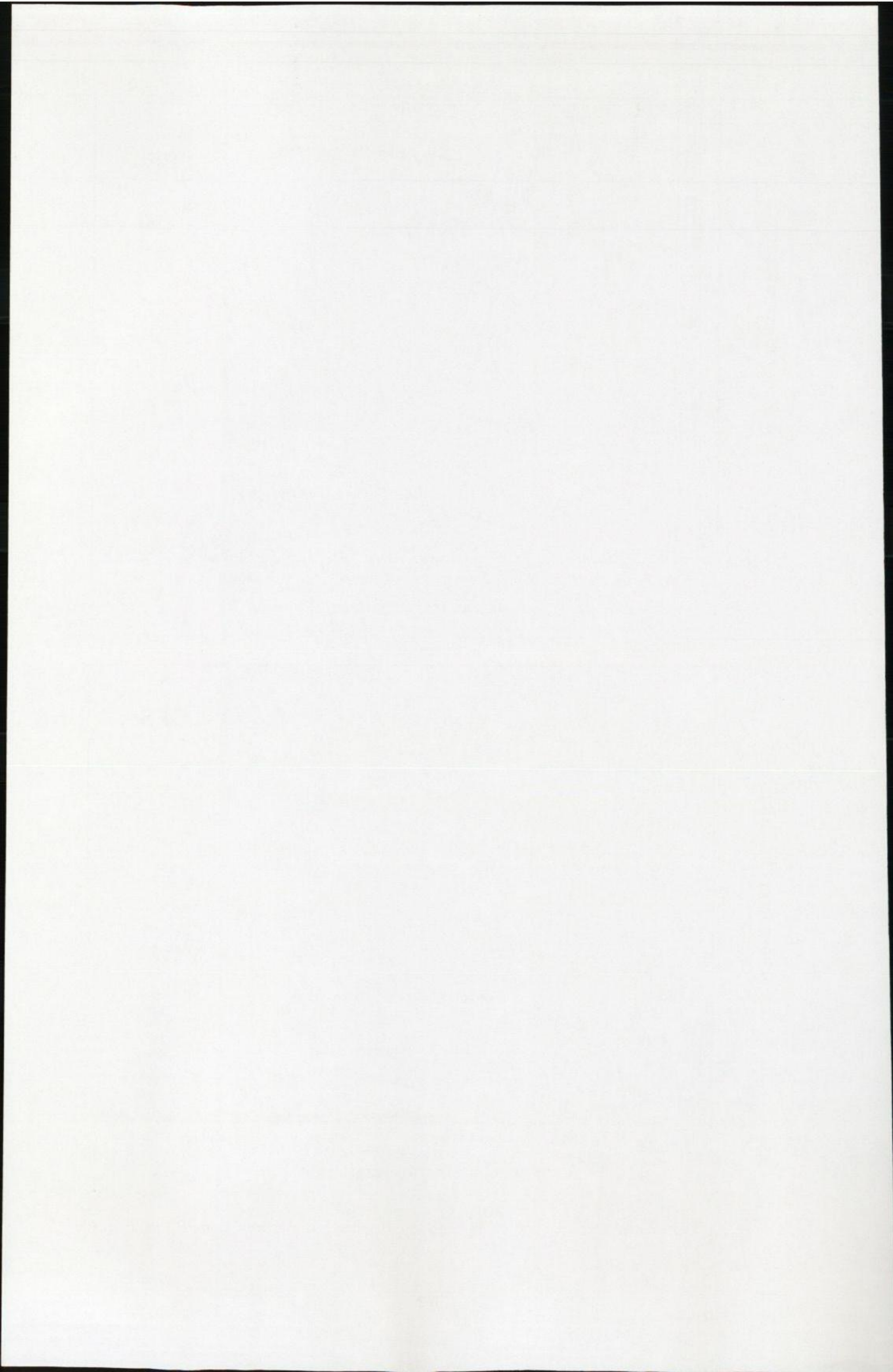


Figure 3.8 Representative urinary spectra from Sutton Harbour and Bantham crabs (FF naphthalene wavelength λ_{Ex}=290nm, SFS $\Delta\lambda$=42nm).



Petrogenic data (FF Ex290nm, SFS Δλ37nm) were not normally distributed, even following log transformation, so results were statistically analysed by comparison of medians by the non-parametric Kruskal-Wallis test (95% confidence level). Whilst no statistically significant difference (FF p=0.7906, SFS p=0.4257) was observed between Sutton Harbour and Bantham crabs during the October 2000 sampling (n=20, n=8 respectively), medians were statistically significant different (FF p=0.00119, SFS p=0.00039) during the March 2001 sampling (n=15, n=8 respectively) (figure 3.9). Unfortunately, this SSD was not particularly compelling, despite clearly elevated petrogenic PAH in the urine of a number of Sutton Harbour crabs.

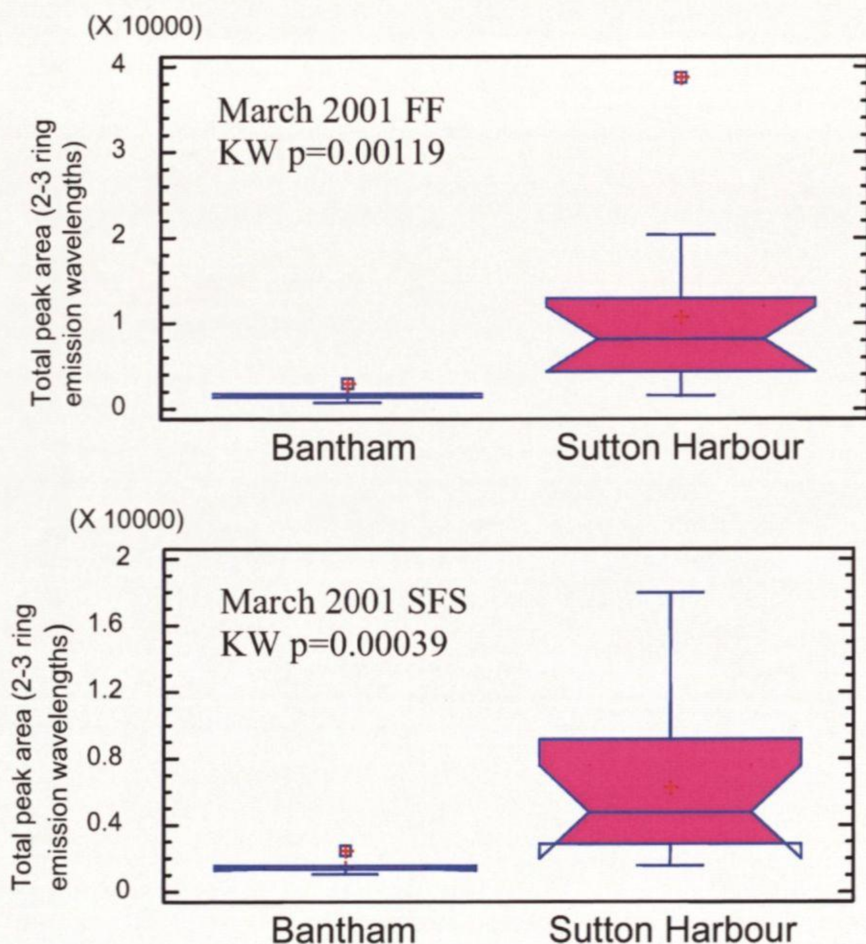


Figure 3.9 Notched box and whisker plot of total peak area of 2-3 ring PAH in diluted (1:50) urine of Bantham and Sutton Harbour crabs, determined by FF and SFS.

Fluorescent results for pyrenes in the October 2000 Sutton Harbour/Bantham trials showed no statistically significant differences (Kruskal Wallis FF p=0.2219, SFS

p=0.1674) (data not shown). Fluorescent intensities measured at these pyrene wavelengths were in fact at basal levels, suggesting that pyrogenic PAH were not present in detectable amounts. However, this information is included to illustrate the nature of contamination affecting Sutton Harbour (i.e. largely petrogenic rather than pyrogenic). Similarily, in the March 2001 study, pyrogenic PAH were not detectable above the fluorescence baseline and did not differ between the sites. Fluorescence data for B(a)Ps were not included, since fluorescence at FF Ex380/Em430nm was negligible in all samples for both sites.

Fluorescence results from the March 2001 trial were validated using the PAH RaPID ASSAY® ELISA kit. This technique detected petrogenic type PAH, indicative of the kind of pollution that characterises the Sutton Harbour site. Results from the ELISA were compared with fluorescence results (SFS $\Delta\lambda$42nm) and the agreement between them was good (figure 3.10, r^2=0.84).

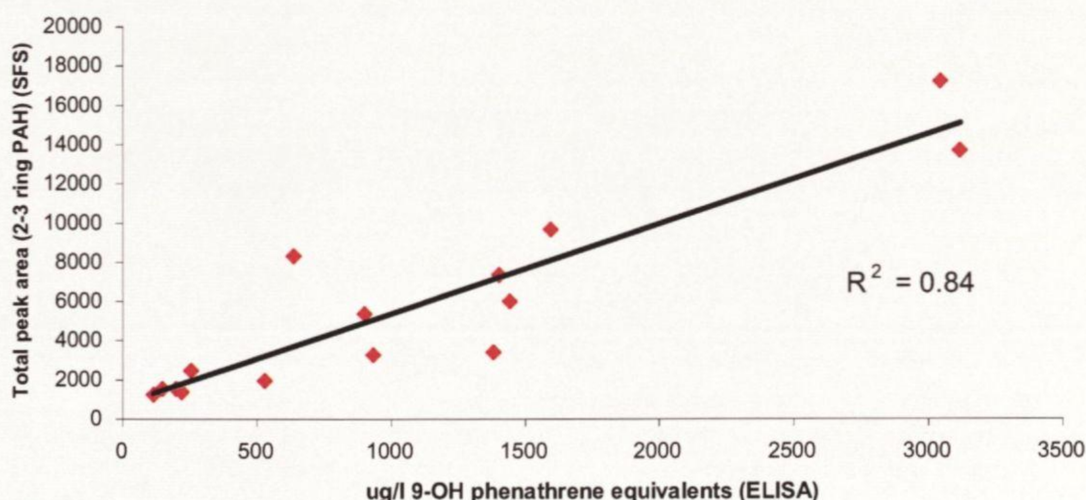
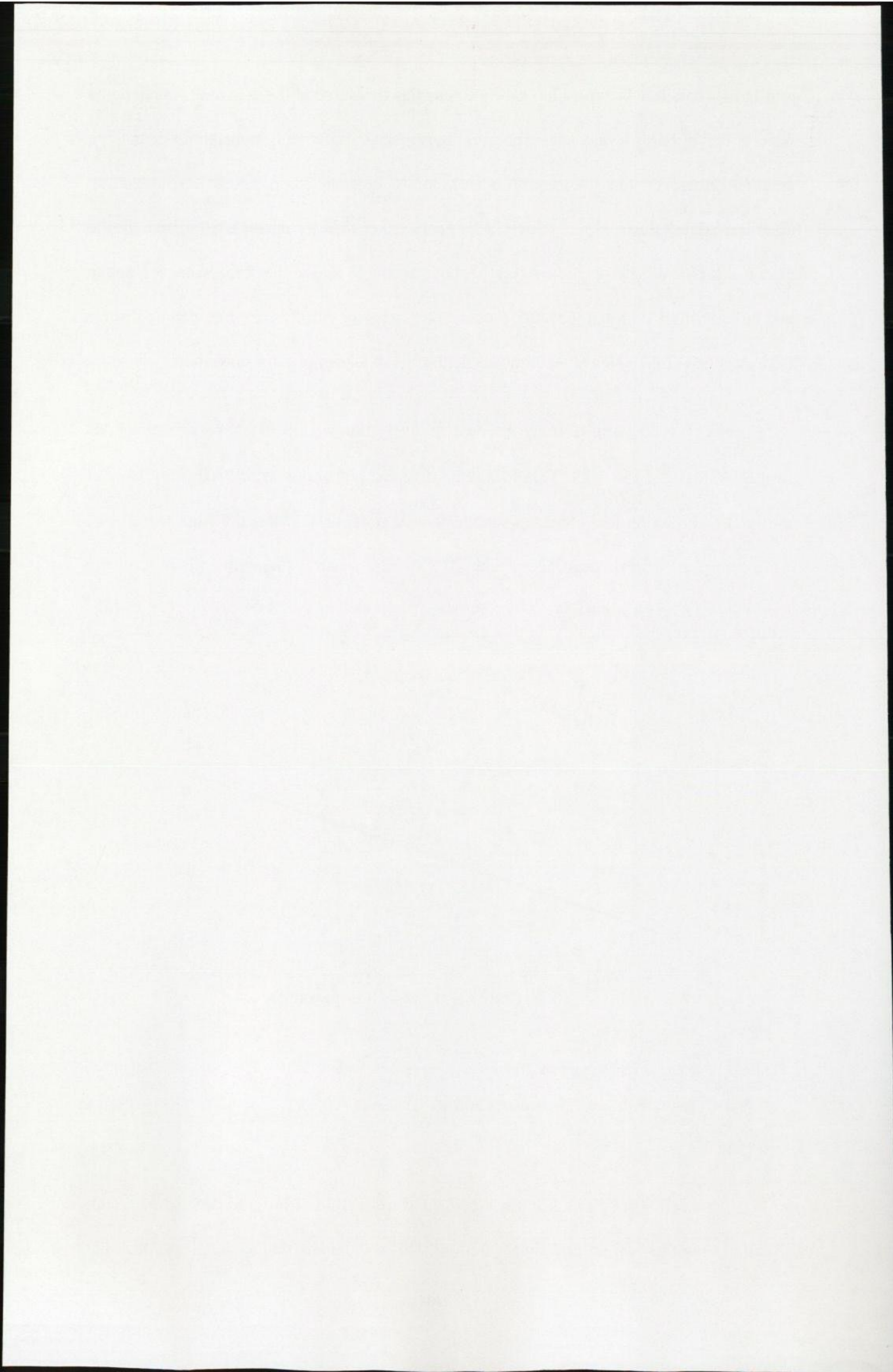


Figure 3.10 Regression plot comparing total fluorescence peak area of 2-3ring PAH (SFS $\Delta\lambda$=42nm) with μgl^{-1} 9-OH phenanthrene equivalents determined by immunoassay in the urine of Sutton Harbour and Bantham crabs.

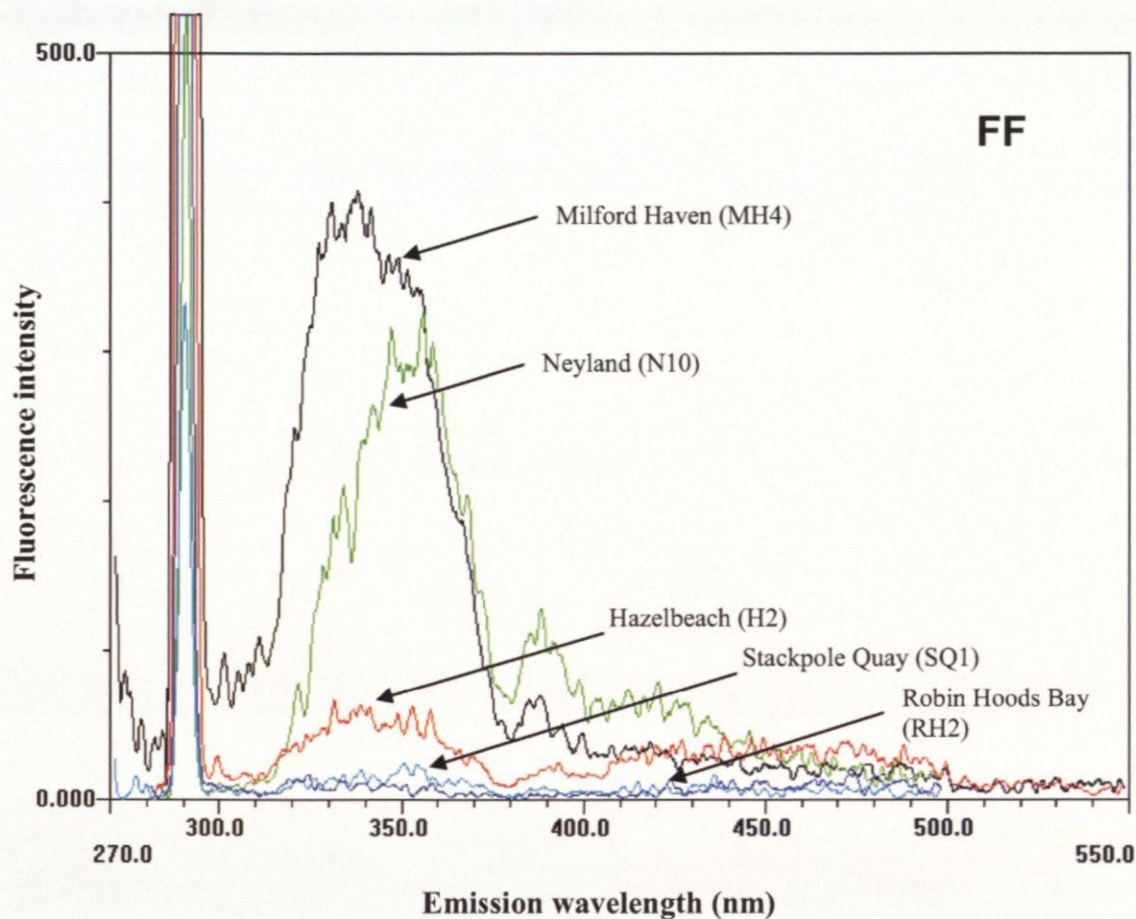
The Tees Estuary and South Wales field trials did not provide conclusive results. Replicate numbers for several sites were low. This was largely due to sampling constraints



and limited success in collecting suitable numbers of crabs. Often, even when sufficient numbers of crabs were collected, urine sampling proved fruitless, as urinary bladders were empty. Fluorescence analyses were inconclusive, with the majority of samples showing no discernible fluorescence (above a noisy baseline) at the wavelengths used. Broad 2-3 ring petrogenic type fluorescence was observed to varying degrees in only 3 samples, namely N1, MH4 and H2 (figure 3.11). In contrast, the remaining samples, regardless of their site of collection, showed negligible fluorescence at test wavelengths. Samples from reference sites appeared qualitatively virtually identical to those from contaminated sites. No data is therefore presented as meaningful differences could not be observed between sites.

3.3.7 Rapid assessment of PAH exposure in Mytilus edulis?

Analysis of prepared haemolymph samples yielded no discernible peaks on the emission spectra, which could be attributed to pyrene or any of its metabolites. This was consistent for all samples, irrespective of their pre-treatment, and spectra from exposed samples were indistinguishable from those from control individuals. Spectra from all samples also showed negligible differences to 50% ethanol blanks. Since levels of pyrene equivalents were below the limits of detection for this assay, data are not presented.



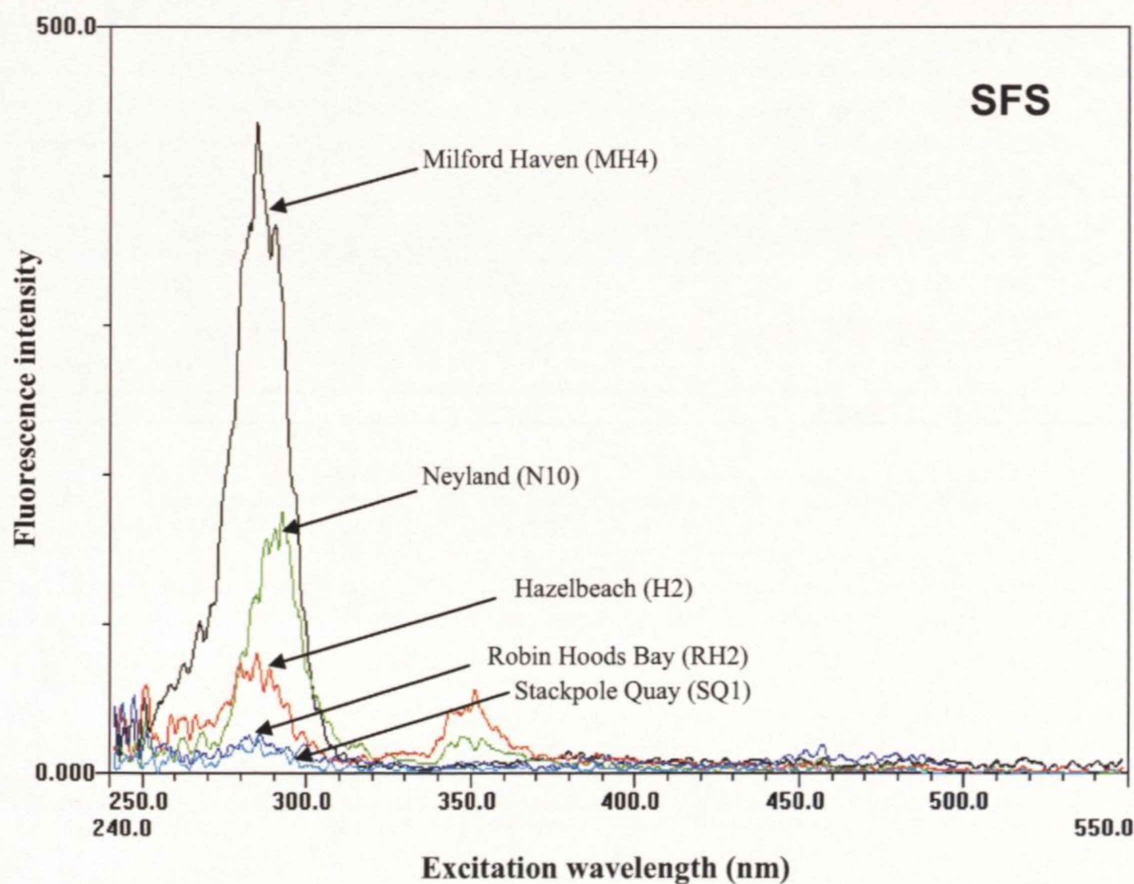
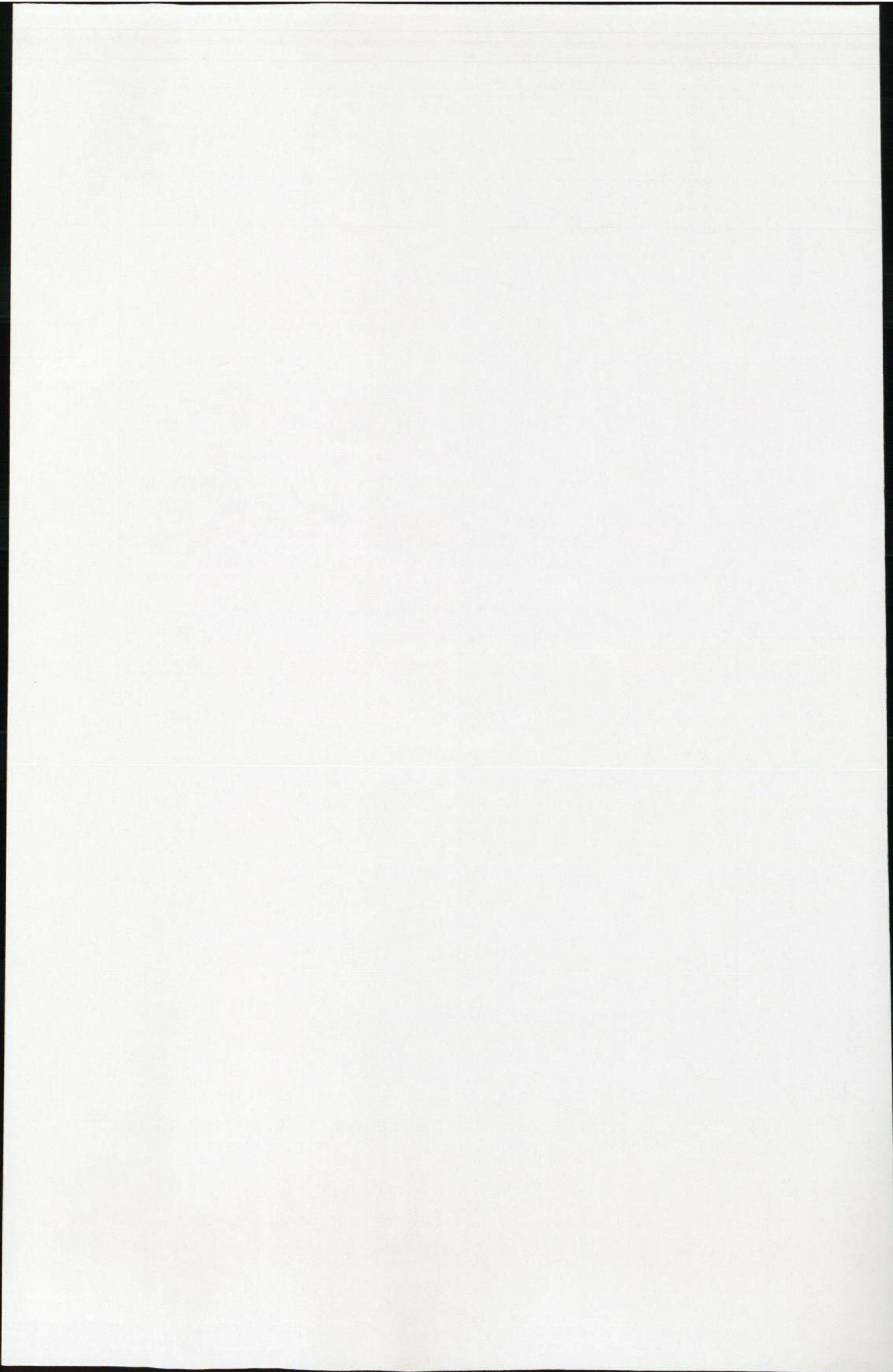


Figure 3.11 Representative urinary spectra from Neyland, Milford Haven, Hazelbeach, Stackpole Quay and Robin Hoods Bay crabs (FF naphthalene wavelength λ_{Ex}=290nm, SFS $\Delta\lambda$=37nm).



3.4 Discussion

GC/MS analysis confirmed that exposed crabs are removing pyrene from their exposure water by uptake over their gills. Physical and chemical processes do account for substantial losses from the system, but rates of loss from tanks with crabs are elevated over those without, particularly in the initial stages of the exposure period. Besides uptake by crabs, sorption (K_{OW}=4.98, solubility 133μg l^{-1}) and photo-oxidation (Lehto *et al.*, 2000) processes are believed to be largely responsible for losses from exposure water (Onuska, 1989, J. Readman, pers. comm.). Evaporation and volatilisation are likely to be minimal (<5%), due to the low vapour pressure of pyrene (9.1×10^{-8}) (Onuska, 1989).

The spiking experiment revealed that the urinary matrix is not responsible for any peak shift, suggesting that the peak seen in urinary spectra is distinct from the free hydroxylated peak. Final confirmation of this was provided by the HPLC analyses. HPLC results show that *Carcinus maenas* is capable of conjugating the intermediate phase 1 metabolite 1-hydroxypyrene into three major metabolites (pyrene 1-glucoside, pyrene 1-sulphate and an "unknown") following waterborne exposure to parent pyrene. These findings are concurrent with suggestions made in chapter 2 regarding phase II biotransformation of the 1-OH pyrene intermediate. It is now clear that these compounds contribute the bulk of the observed fluorescence at λEm382nm on the urinary spectra from Chapter 2's experiments. Since non-conjugated 1-OH pyrene only accounts for 3.7% of the equivalents eliminated in the urine, it is clear that the conjugation rate is high, at least in this excretory pathway. This negligible contribution from non-conjugated 1-OH pyrene also explains the lack of a distinct peak at Em387nm (characteristic of 1-OH pyrene fluorescence) on urinary spectra. The ratio of glucoside to sulfate conjugate illustrates that glucosidation is more extensive than sulfation.

The identity of the unknown conjugate is undetermined, but it is not a glucuronide (as it did not co-elute with a known pyrene 1-glucuronide standard). Indeed, none of the

metabolites co-eluted with this conjugate, suggesting glucuronidation is not occurring to any significant degree. Whilst, glucuronides are common phase 2 metabolites in fish (Ariese *et al.*, 1993, Aas *et al.*, 1998, Law *et al.*, 1994, Solbakken *et al.*, 1980), they are seemingly absent in crustaceans. A possible explanation is that in crustaceans, the preferred carbohydrate co-substrate is UDP-glucose, forming the glucosides, whereas in vertebrates, the preferred cosubstrate is UDP-glucuronic acid, giving rise to glucuronides (James, 1987, Li and James, 1993). The chromatogram also shows that pyrene, which elutes last, is negligible in exposed samples, confirming the findings of the fluorescent studies in Chapter 2. Almost complete biotransformation of pyrene therefore seems to be occurring through this excretory route. It is important to remember however that metabolism of pyrene is limited to one major intermediate (1-OH pyrene), restricting the number of final conjugates. Metabolism studies on benzo(a)pyrene in *C.maenas* (Lemaire *et al.,* 1993) have shown that a much greater number of final conjugates are formed, via several intermediates.

Comparison of urinary equivalent levels determined by HPLC and fluorescence analyses show a good correlation (FF r^2=0.9039, SFS r^2=0.8724) (see also Fillmann *et al.*, 2004). This is important as it shows that the rapid fluorescence assay produces results that are largely the same as those obtained from a much more powerful and sophisticated technique. This validation further strengthens the case for the use of the rapid assay as an effective biomonitoring tool.

Results obtained from the PAH ELISA show that this technique is suitable for detecting PAH in biological samples. Although the immunoassay antibodies supplied with the PAH RaPID Assay® kit were raised against phenanthrene (Waters *et al.*, 1997), the assay has proved capable of detecting other PAH i.e. the conjugated 1-OH pyrene metabolites. Such cross reactivity (despite differential sensitivity) still provides an effective monitoring tool for environmental pyrogenic PAH exposure. Whilst comparison

with fluorescence results showed a strong positive correlation (FF r^2 =0.8923, SFS r^2 =0.8404), ELISA results are consistently lower than those obtained by fluorescence. This is most probably a result of differential reactivity of the antibody (raised against the parent), with the diverse range of conjugates and metabolites formed during biotransformation. Nonetheless, the strong correlation proves once again that direct fluorimetry is a reliable and robust technique, producing good results. The results also show that the ELISA is an effective tool for measurement of environmental pyrogenic PAH exposure in biological samples. The ELISA could therefore be used for biomonitoring in the absence of any analytical capability for fluorescence measurements. Lastly, comparison of ELISA and HPLC results also show very good agreement (r^2 =0.9382) providing another level of validation for the original results.

Urine samples from the pyrene exposure experiments have therefore been analysed by no less than three different techniques, all of which have detected the same relative levels of metabolites in pyrene exposed and control animals. It is now clear that whichever method is used, detection of PAH metabolites in the urine of *C.maenas* is reliable and proven indicator of PAH exposure.

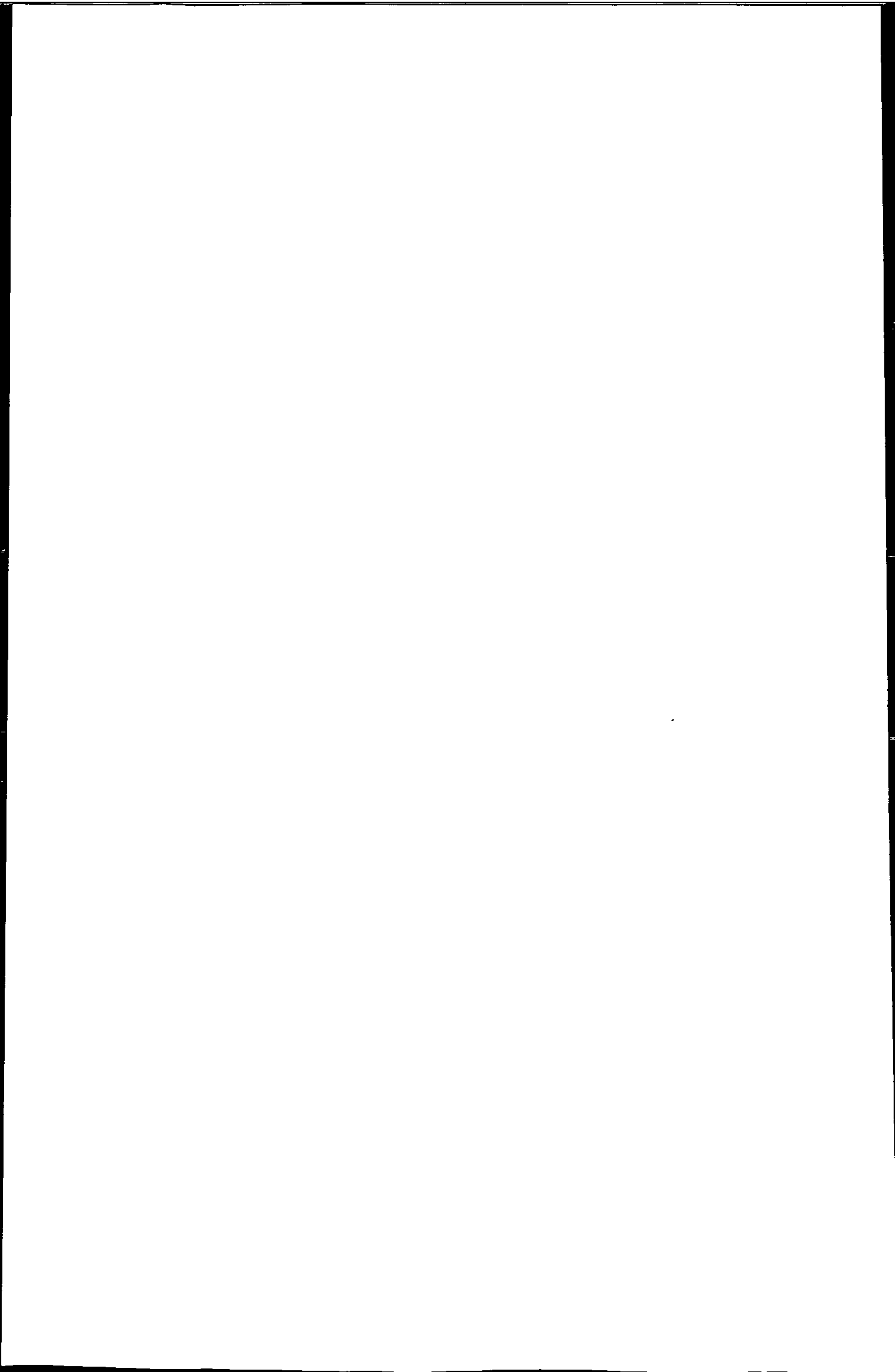
Fluorescent techniques are not limited to the detection of pyrogenic PAH only, but confirmation of the urinary elimination of 2-3 ring petrogenic PAH was desired to ensure that the urine assay could confidently be used to detect exposure to PAHs other than pyrene. The continuous flow exposure experiment provided this confirmation, as samples taken from crabs exposed to crude oil exhibited broad fluorescence at 2-3 ring wavelengths, indicative of the petrogenic type of PAH found in crude oil. Urinary spectra also showed small contributions from pyrogenic PAH, which are often present in petrogenic type contamination situations (UNEP, 1992). This result provides compelling evidence that the assay is suitable for determining exposure to petrogenic as well as pyrogenic PAH in shore crabs. This is clearly of great importance when applying the

biomarker to field situations, a fact made clear by the results from the preliminary field trials presented in this chapter, which were characterised to a large extent by 2-3 ring PAH.

Controls from this experiment revealed, unexpectedly, broad fluorescence at 2-3 ring wavelengths, comparable to the levels seen in crude oil exposed crabs. Qualitatively, urinary spectra from control crabs looked no different to those from crude oil exposed crabs. In this authors opinion, this is not a background level of fluorescence present in all individuals and does not invalidate the results obtained from exposed crabs, but is simply the result of inadvertent contamination of the control treatment system with crude oil, or perhaps experimental error i.e. crabs believed to be controls were in fact taken from the wrong treatment group. The former explanation is supported by a general health biomarker (neutral red retention time) measured in mussels (*M.edulis*) kept in the same test system, which indicated significant impacts in controls, indistinguishable from those of exposed mussels (D. Lowe, personal communication).

Results from the Sutton Harbour field trial were encouraging and the PAH exposure biomarker has proved sensitive enough to distinguish between a contaminated and clean site. To this authors knowledge, this is the first time a urinary PAH exposure biomarker has been applied successfully in the field on wild populations of crustacean. The majority of PAHs detected were those attributable to petrogenic sources (2-3 ring PAHs), seen as broad fluorescence at the beginning of synchronous emission spectra. For reasons described previously, SFS is more suitable for analysis of environmental samples and the simplified spectra can present peaks representative of the main PAH groups of concern (2, 3, 4 and 5 rings). Despite the fact the assay was established using pyrene, the field trials have indicated that the assay is equally applicable to petrogenic type pollution. A more extensive field test, along a gradient of PAH pollution, is reported in a later chapter.

Field samples were validated using the PAH ELISA, detecting levels of petrogenic PAH that correlated well with the fluorescence results (r^2=0.84). The assay was calibrated



for field samples using the petrogenic PAH metabolite 9-OH phenanthrene and since the kit antibodies are raised against phenanthrene, it is perhaps unsurprising that the response was good.

The relative success of the Sutton Harbour trial was unfortunately not repeated on the Tees estuary and South-Wales field trials. Due to low replicate numbers, and poor sample quality, results were inconclusive. Fluorescent spectra from both contaminated and reference site samples were broadly similar with only very few (3) notable exceptions. Whilst environmental 2-3 ring PAH exposure was clearly evident in these few individuals, larger numbers of such positive samples are required before significant differences can be established between sites. Given the reported levels of PAH in sediment and water at the sites (Woodhead *et al.*, 1999, Law *et al.*, 1997), the apparent absence of PAH exposure is perhaps surprising. PAH might not be very bioavailable in these areas, despite elevated water and sediment concentrations. Conversely, if bioavailability were not limiting uptake, the lack of urinary PAH levels might be explained by the fact that PAH levels, whilst comparatively high compared to clean sites, are not high enough to result in detectable levels of metabolites in the urine of exposed crabs. Higher sample quality and number of replicates would have undoubtedly improved the data set, as it is clear that individuals with detectable levels of PAH in their urine are less frequent than those without. Sutton Harbour proved to be more suitable for preliminary investigations with the assay. Whilst the Sutton Harbour data set shows a comparable level of variability, 2-3 ring PAH are more prevalent and occur with greater frequency in the individuals tested. It is possible that this is due to the enclosed nature of Sutton Harbour, whose waters are not subject to the high levels of exchange and flushing seen in the large Tees and Milford Haven estuaries. Combine this with the pollution history of the harbour and it is likely that PAH are more liable to accumulate in the sediments and be more readily available to resident organisms. PAH contamination is also more localised and the crab population more confined than in a large dynamic estuarine system. This could conceivably lead to a higher, more persistent level of

chronic exposure, resulting in the levels observed in crab samples. Dredging of sediments in the harbour might also re-suspend previously sediment bound PAH into the water column, making them freely available to the resident crab population.

The *M.edulis* exposure suggests that mussel haemolymph is not suitable for the rapid evaluation of PAH exposure. Levels of pyrene equivalents were below the limits of detection in haemolymph samples. The lack of metabolites is however, not sufficient evidence to dismiss the occurrence of PAH exposure. It is well documented that sessile suspension feeding mussels such as *M.edulis*, which filter large volumes of ambient seawater over their gills, bioaccumulate lipophilic organic pollutants such as PAH (Baumard *et al.*, 1998, Dadamo, 1997, Widdows and Donkin, 1992). Despite the occurrence of p450 enzymes in digestive gland microsomes (James, 1989a, Stegeman and Lech, 1991, Snyder, 1998), it is thought that metabolism of accumulated PAH is much less active and relatively slow when compared with fish, crustacea and polychaetes (Farrington, 1991, Baumard *et al.*, 1998). Detection of metabolites at the levels seen in *C.maenas* is therefore unlikely, although detection of unmetabolised parent is almost certain in the correct compartments of the organism, as evidenced by numerous field studies (e.g. Naes *et al.*, 1995, Baumard *et al.*, 1998). In the present study parent pyrene was not found in the haemolymph of exposed mussels, but this was not taken as evidence of lack of exposure. Measurement of tissue burdens might provide such proof, but measurements were beyond the scope of the study.

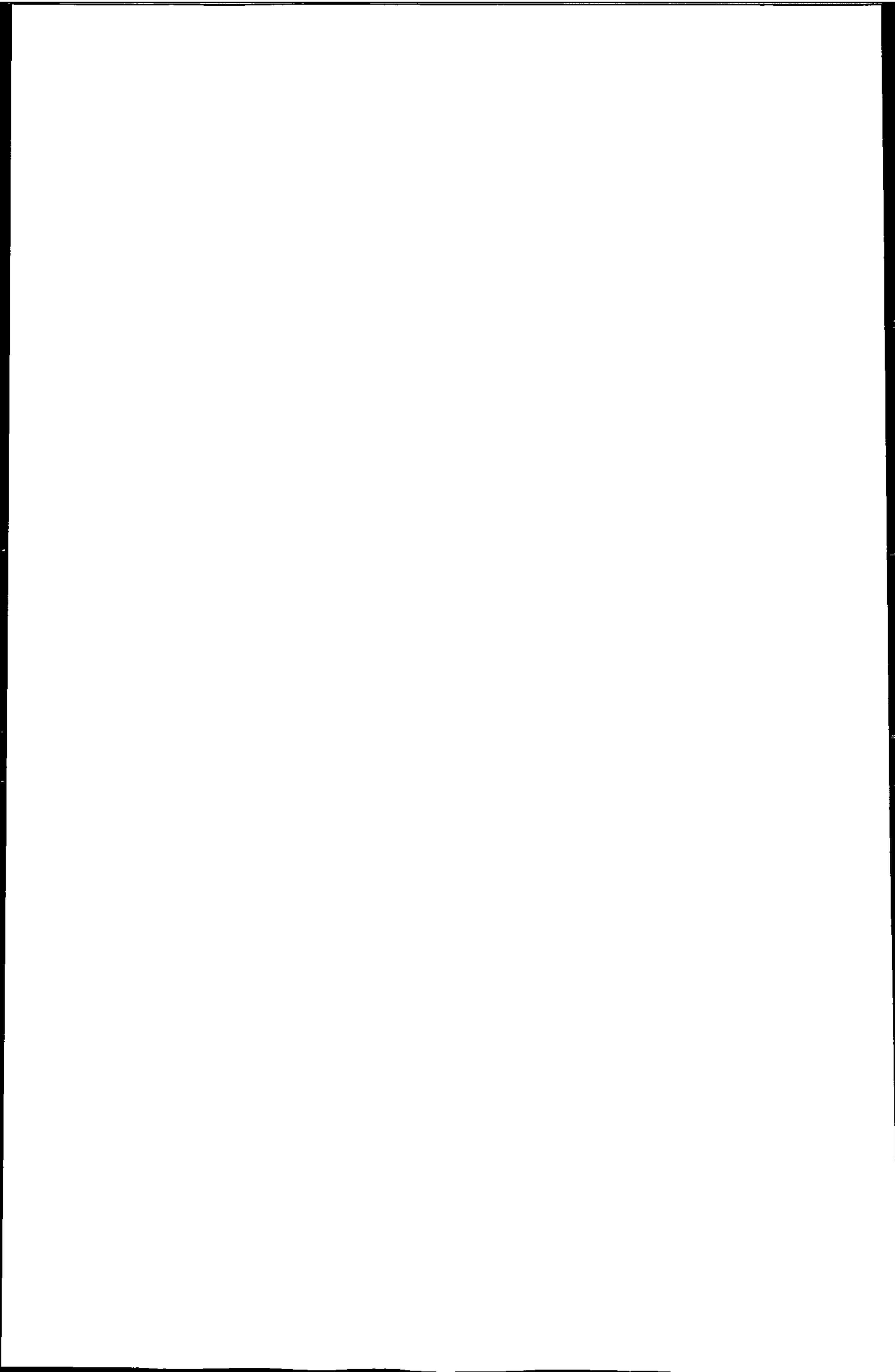
Following rapid uptake by the gills from either the truly dissolved, colloid bound or organic particulate bound phases, PAH is transported in the circulation to storage lipid reserves (Farrington, 1991). Residence time in the haemolymph may therefore be short, and re-circulation unlikely, since it has been suggested that release of PAH from lipid rich compartments is very slow (Lee, 1984). Similar findings have been reported for crustaceans, where the highly lipophilic PAH B(a)P was rapidly cleared from the

haemolymph (James *et al.*, 1989). These authors therefore suggest that haemolymph levels may not accurately predict total body burdens. Accumulation in the gill tissue, to concentrations several orders of magnitude higher than those in water (Lee, 1984) might also limit the amount of freely circulating organic contaminant available for detection. Despite PAH being known to accumulate in lysosomes (Grundy *et al.*, 1996), lysis of cellular membranes did not improve the fluorescent signal attained. However, untreated haemolymph samples from *C.maenas* showed characteristic pyrene equivalent fluorescence (chapter 2), which suggests that lysosomal binding in crustacean haemocytes, if it is occurring, does not necessarily eliminate a fluorescence signal. A more likely explanation is simply that circulating levels are negligible since PAH rapidly accumulate in lipid rich tissues after uptake by the gills. Analysis of digestive gland, gill tissue or whole body homogenates might therefore yield more conclusive results, but this would involve extensive sample preparation and would not be suitable for rapid and inexpensive monitoring work.

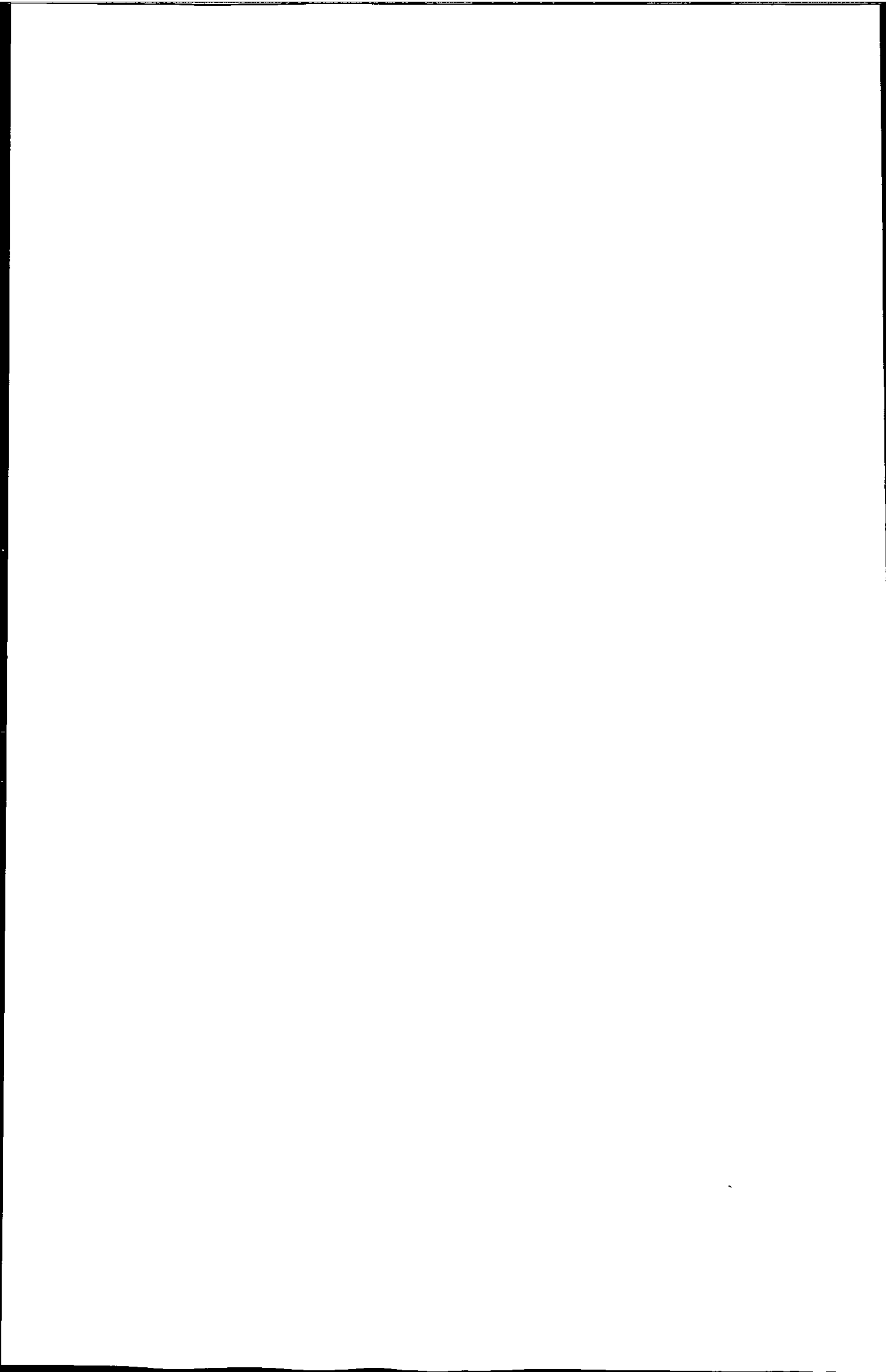
3.5 Conclusions

Experiments in this chapter have tested several aspects of the urinary biomarker to ensure that it is providing meaningful results and can be used confidently in the field. Matrix effects of the urine have been shown to be negligible, with the observed differences in peak position attributable to fluorescent differences between the free metabolite and its conjugated forms. GC/MS analysis of water samples has provided evidence of uptake and demonstrated that PAH gains access to the crab's internal environment. HPLC analyses have proven that *C. maenas* is capable of phase II metabolism and eliminates conjugated metabolites of the 1-OH pyrene intermediate. HPLC results also demonstrate that parent pyrene is negligible in exposed crab urine samples, suggesting complete biotransformation via this route. Validation of fluorescence results, a prerequisite if the technique is to be adopted as a biomonitoring tool, was carried out by two analytical techniques (HPLC and ELISA). Results from both validation techniques consistently showed very good agreement with fluorescence data, as well as correlating well with each other. This validation proves that the urinary assay is a robust and reliable measure of PAH exposure in this species.

The continuous flow experiments demonstrated that 2-3 ring PAH could be detected in the urine of crabs following exposure to crude oil. This conclusion establishes that the urinary assay can be confidently used to determine exposure to the full range of environmentally relevant PAH, which is clearly of great value in biomonitoring applications. Preliminary field trials produced interesting results, with a local field site proving to be a good test bed for the detection of 2-3 ring, petrogenic PAH in the urine. The field trials provide the first compelling evidence for the assay's use in the field. Validation with an immunochemical technique strengthens these findings further.



Attempts to replicate the inexpensive and rapid nature of the PAH assay in the haemolymph of a bivalve mollusc proved unsuccessful. The more traditional analysis of tissue PAH burden provides evidence of exposure in this organism, but is not compatible with the aims of the rapid, inexpensive biomarker approach.



Chapter 4: Application of the urinary exposure biomarker to a field gradient of PAH contamination in a Norwegian fjord.

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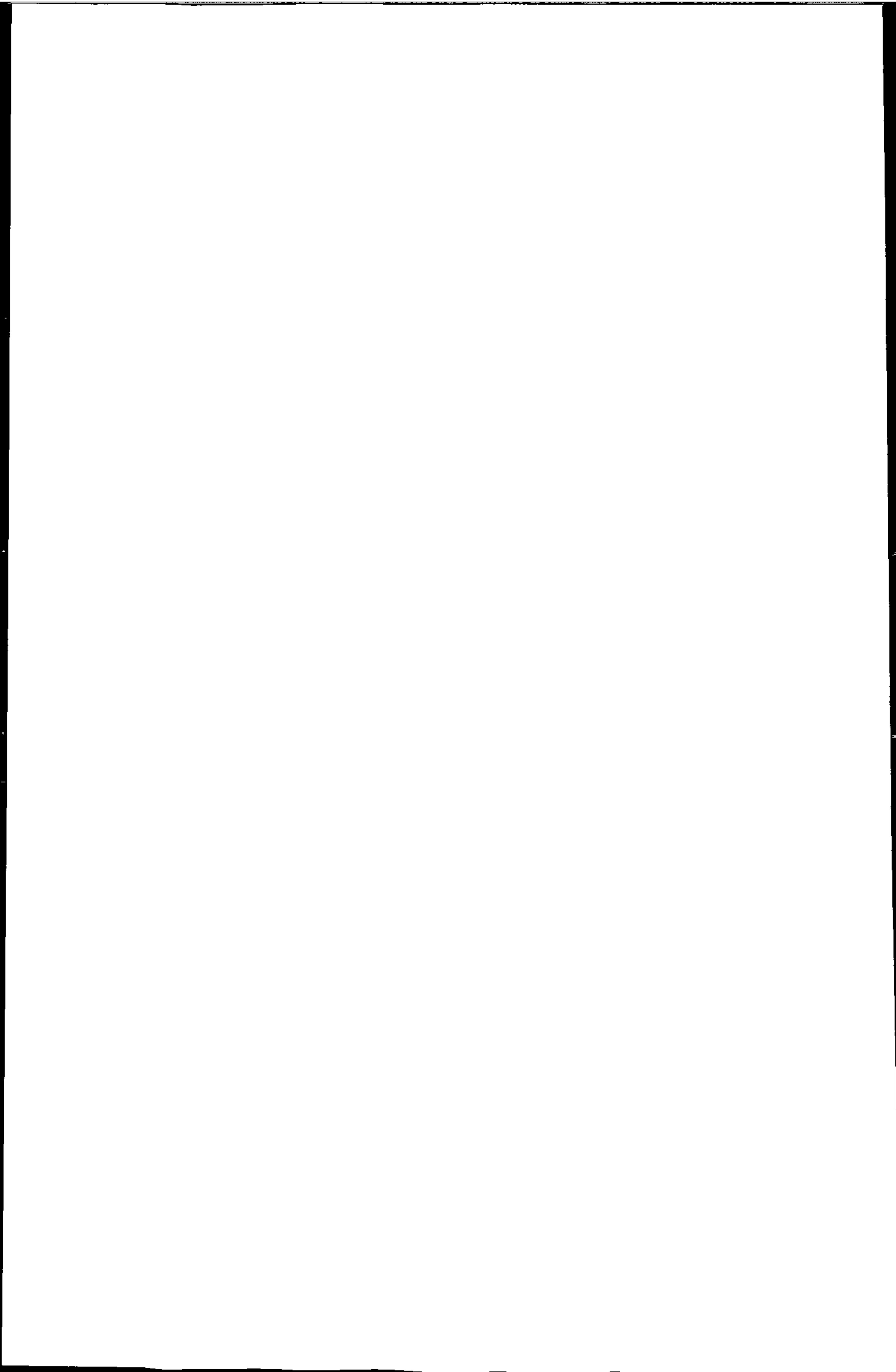


4.1 Introduction

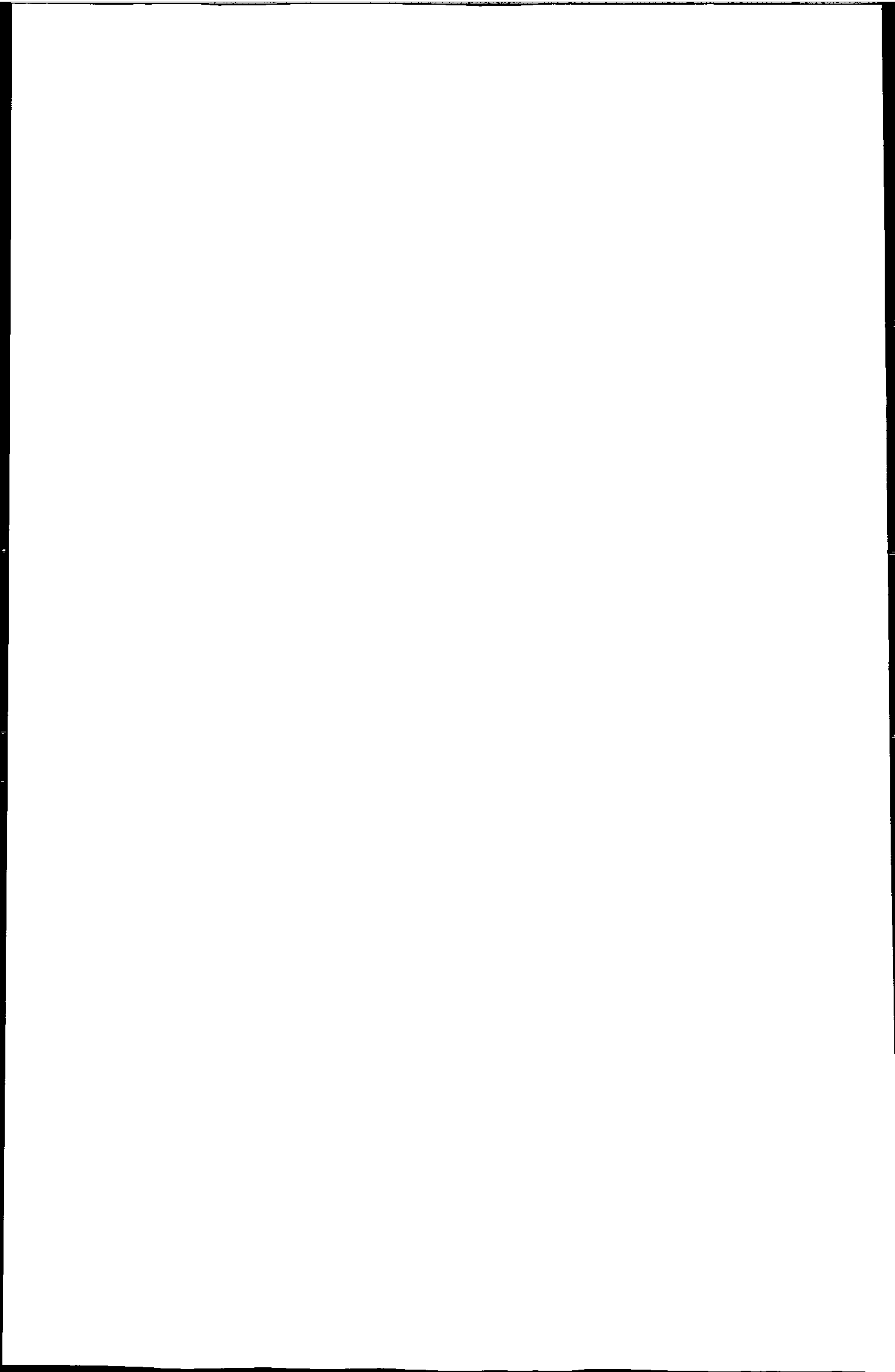
As previously discussed in Chapter 3, successful field application of a biomarker is invaluable to evaluate contaminant exposure and/or effect in the organism of interest. Only then can it be used for environmental monitoring purposes. It is therefore imperative that all novel biomarkers are thoroughly field tested and evaluated before being used in monitoring programmes (Sanchez-Hernandez *et al.*, 1998). With this in mind, a field trial was carried out at a well-characterised gradient of PAH contamination, originating from aluminium smelter discharges in a Norwegian fjord.

Norwegian fjords and coastal waters have received, and in some areas still receive, large amounts of PAH from anthropogenic sources (Naes *et al.*, 1995). Emissions generated by the primary (electrolytic) production of aluminium are the most significant sources of pyrogenic PAH (Naes *et al.*, 1999). Aluminium production is very energy intensive and aluminium plants are therefore often situated in areas with abundant supplies of inexpensive energy, such as hydroelectric power (IAI - International Aluminium Institute, 2003). Due to its extensive hydropower resources, Norway is a major world producer, with a volume of over 1 million tonnes of aluminium annually from seven smelters (Oye, 2003).

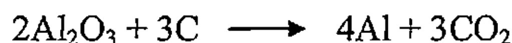
4.1.1 Electrolytic production of aluminium

The electrolytic process of aluminium production is outlined briefly below (all information from IAI, 2003).

All modern primary aluminium smelting is based on the Hall-Héroult Process. Alumina, refined from bauxite ore, is dissolved in an electrolytic bath of molten cryolite (sodium aluminium fluoride) and heated to a temperature of approx. 980°C, in a large carbon or graphite lined steel container known as a “cell” or “pot”. A low voltage, high



amperage (typically 150,000A) electric current is passed through the electrolyte and flows between an anode (positive), made of petroleum coke and pitch, and a cathode (negative), formed by the thick carbon or graphite lining of the pot. Aluminium ions are reduced to produce molten aluminium metal at the cathode and oxygen is produced at the graphite anode and reacts with the carbon to produce carbon dioxide.



$$2Al_2O_3 + 3C \longrightarrow 4Al + 3CO_2$$

Smelting is a continuous process and molten aluminium deposited at the bottom of the pot is siphoned off periodically. A typical aluminium smelter consists of around 300 pots, producing approximately 125,000 tonnes of aluminium annually with 15.7 kWh of electricity used to produce each kilogram of aluminium.

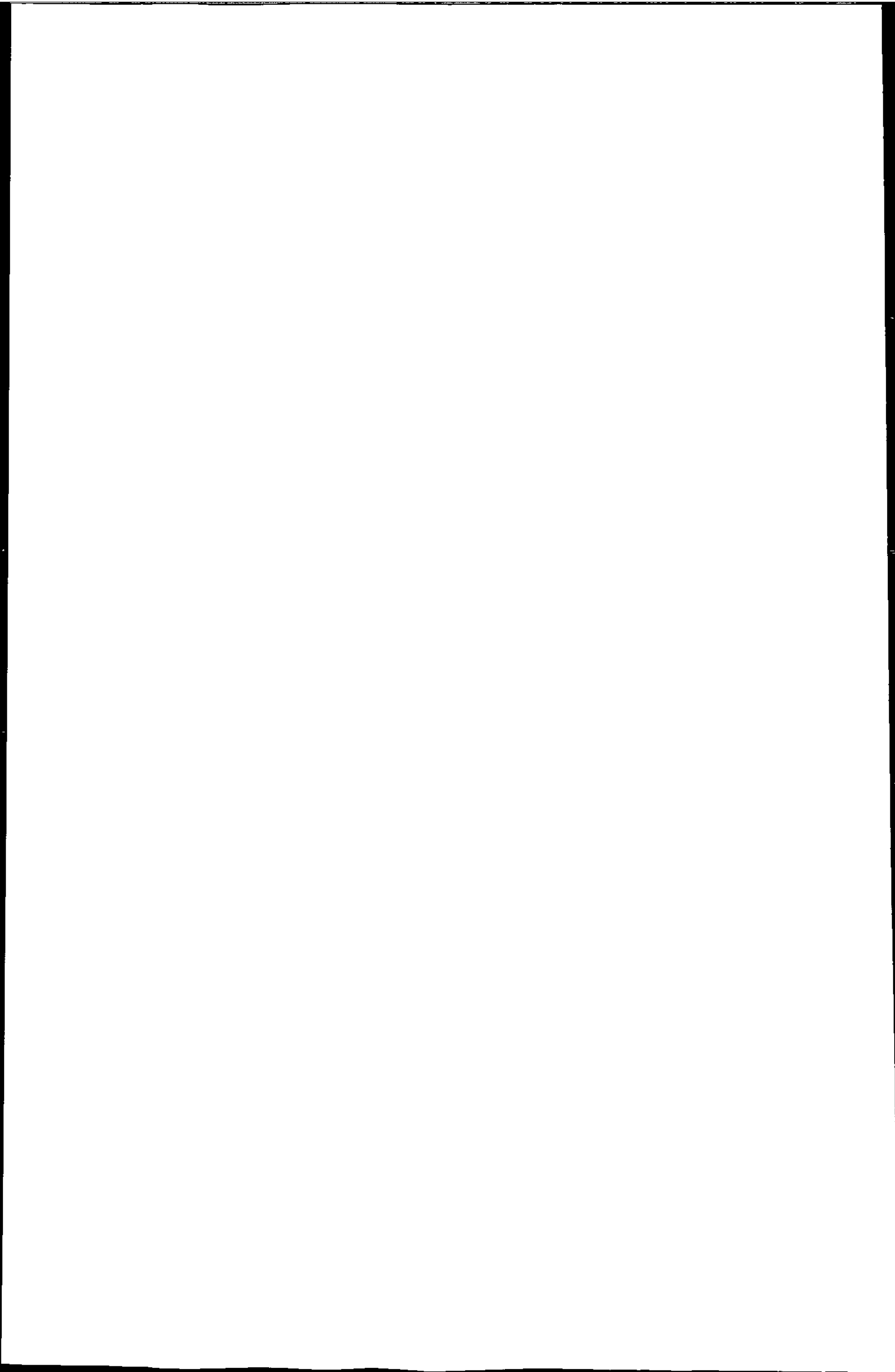
Carbon anodes used in the Hall-Héroult process are consumed during electrolysis and are of two main types - "Søderberg" and "Pre-Bake". Søderberg technology uses a continuous anode, delivered to the cell in the form of a paste, baked by the heat from the molten electrolyte and is continuously consumed. Pre-Bake anodes are made separately (using coke particles bonded with pitch and oven baked) and are consumed then periodically changed. Pre-bake technology uses multiple anodes in each cell with the anodes attached to rods and suspended in the cell. All new plants and most plant expansions are based on pre-bake technology. The newest primary aluminium production facilities use a variant on pre-bake technology, Centre Worked Pre-bake Technology (CWPB), whose emissions are very low (less than 2% of the total generated emissions). The remainder of the emissions are collected inside the cell itself and carried away to very efficient scrubbing systems that remove particulates and gases.

Despite removal of 96-99% of all pot emissions by powerful scrubbing equipment, emissions from smelters still contain a variety of compounds, including sulphur dioxide, carbon dioxide, inorganic fluorides, perfluorocarbons (PFCs) and PAHs. Of primary

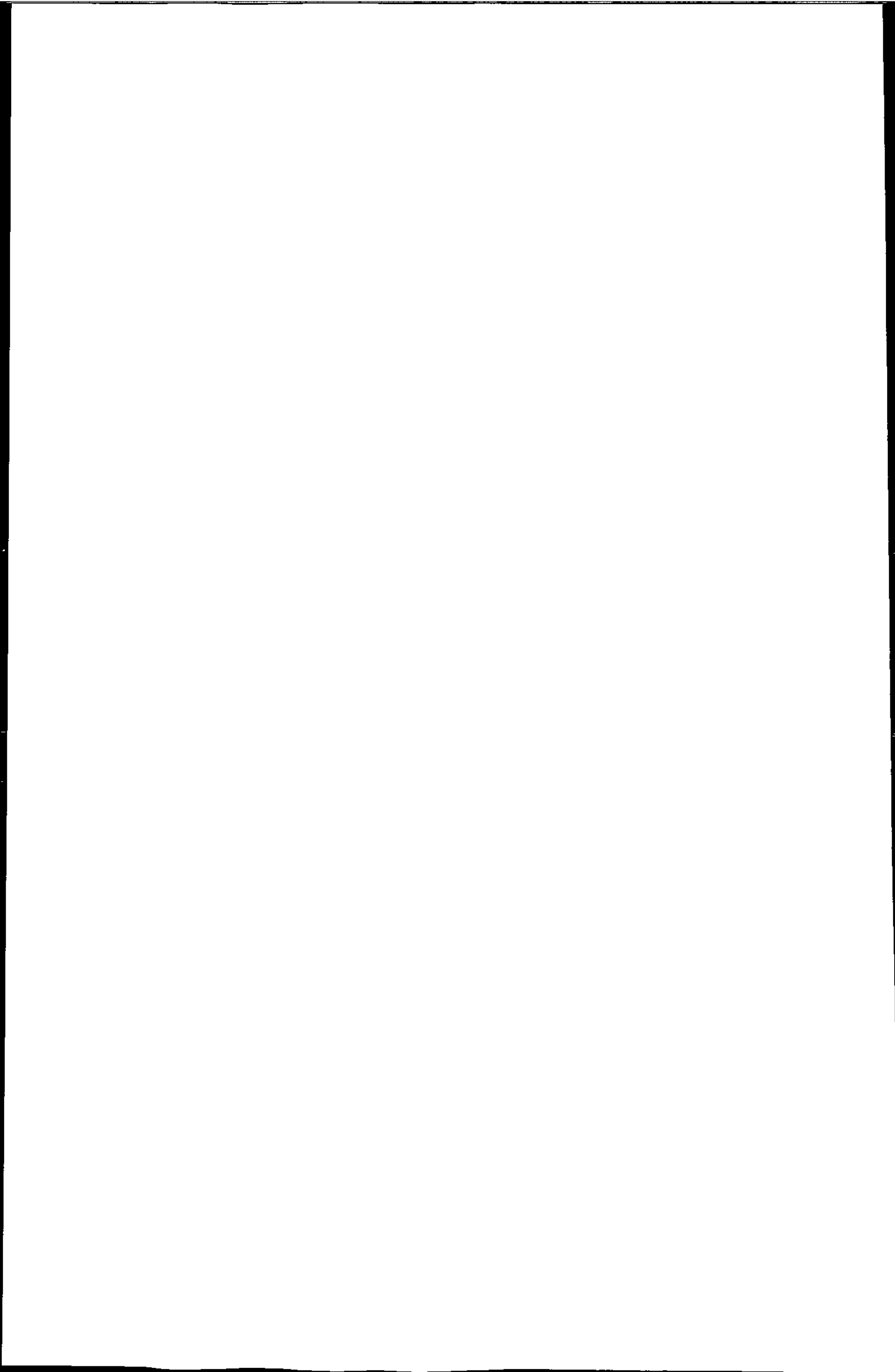
interest to the present study are the PAHs, produced during the manufacture of anodes for pre-bake smelters, and during the electrolytic process itself in older Søderberg facilities. Current air emission levels of PAH from pre-bake plants average approximately 0.05 kg per tonne. In contrast, emissions to air from Søderberg pot rooms of total PAH (particulate and gaseous) averaged 0.638kg per tonne (OSPAR, 2002). In recent years levels have been reduced considerably, with modern pre-bake plants emitting less than 0.01kg per tonne. PAH emissions from Søderberg facilities have been dramatically reduced by the introduction of dry anode technology at many locations.

4.1.2 Smelter derived PAHs and contamination of adjacent waters

Despite recent reductions, PAH discharges from five of the seven main aluminium smelters in Norway average 1-10 tonnes or more per year (Naes *et al.,* 1995). PAH contamination mainly originates from the seawater scrubbing (washing) of furnace off-gases and ventilation air produced when using Søderberg electrode technology (Naes *et al.,* 1999). This PAH is either discharged directly to fjordal and coastal waters as slurry or greatly diluted in scrubber water effluent via settling ponds (Knutzen, 1995). Discharges to water from the three Norwegian smelters with seawater scrubbing facilities (Lista, Mosjøen and Karmøy), were on average 84, 72 and 17g/tonne Al respectively (OSPAR, 2002). PAH also originates from the production/consumption of anodes (Naes *et al.,* 1995), volatilisation of pitch/tar anode binder, handling of pitch and coke on site (Simpson *et al.,* 1996, 1998) and via leakage from waste deposits or runoff from contaminated local catchment areas (Knutzen, 1995). Dust from anode production is often mixed with scrubber waste and discharged directly to receiving waters in the fjord (Naes *et al.,* 1995). Smelters are typically situated at the head of the fjord and a significant proportion, if not all, of the scrubber and associated waste is discharged into shallow water (Naes *et al.,* 1995).



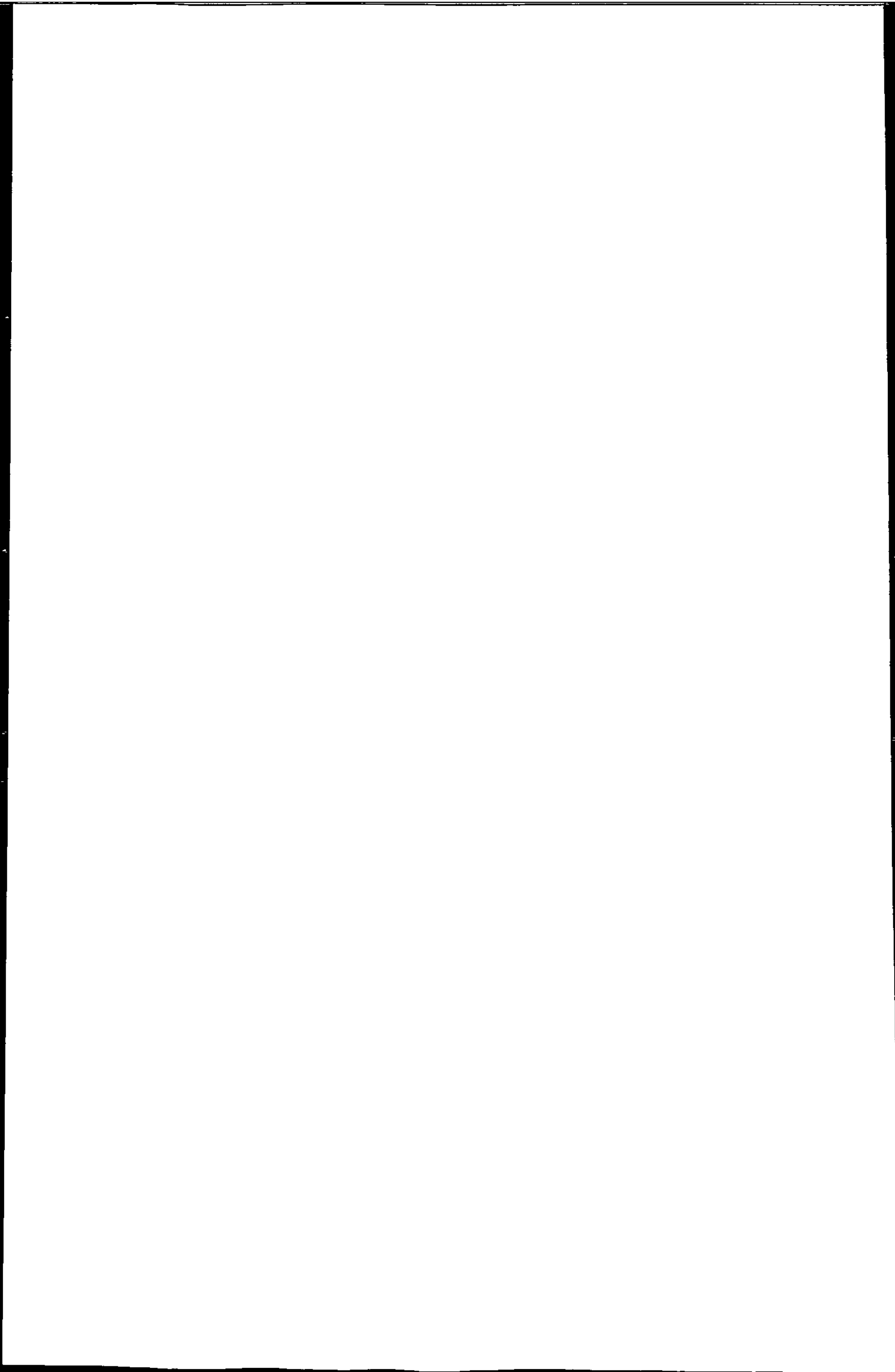
The discharge of large amounts of PAH has resulted in pronounced contamination of sediments around aluminium works, with PAH levels 100-1000 times higher than background concentrations, particularly in the immediate vicinity of smelter outfalls (Naes *et al.*, 1995, Paine *et al.*, 1996, Oug *et al.*, 1998). PAH concentrations in sediment typically decrease rapidly with distance from aluminium smelters, with spatial gradients for surface sediments being very steep (Naes *et al.*, 1999, Paine *et al.*, 1996, Simpson *et al.*, 1996, Knutzen, 1995). However, PAH concentrations elevated over background levels can be observed tens of kilometres away from smelter point sources, a phenomenon thought to be due to the extensive transport of PAH bound to small soot particles, characteristic of scrubber wastes (Naes *et al.*, 1995). Several authors report that this binding to these organic carbon rich particles limits the bioavailability of PAH and therefore any potentially deleterious effects (Knutzen, 1995, Paine *et al.*, 1996, Naes *et al.*, 1999, Simpson *et al.*, 1998, Naes and Oug, 1997, 1998). This highlights the importance of the binding characteristics of the PAH and it's associated matrix. In comparison to PAH derived from petroleum, smelter derived PAH associated with particles are less desorbable and more persistent in sediments even after transport over significant distances (Simpson *et al.*, 1996). Smelter derived PAHs in sediments are also more resistant to transformation and degradation processes (Naes and Oug, 1997). The suspected lack of bioavailability clearly has implications for any biomonitoring studies. This has been suggested as an explanation for the lack of associated effects in bioindicator organisms, despite elevated sediment concentrations (Paine *et al.*, 1996). However, other authors have shown that smelter discharges of PAH are bioavailable to indicator organisms (Eickhoff *et al.*, 2003a,b). Clearly a distinction must be made between exposure and effect. While levels of particulate associated PAH might produce responses in exposure biomarkers (Eickhoff *et al.*, 2003a), but not effect biomarkers (Paine *et al.*, 1996), this does not mean that the PAH are not bioavailable. The present study aims to use the previously described urinary exposure biomarker to determine if PAH from a Norwegian smelter are bioavailable.



4.1.3 Hydro Aluminium Karmoy in Karmsund Strait, Norway

The field trial in the present study was carried out in Karmsund strait, a shallow fjordal strait adjacent to Karmoy Island on the south-west coast of Norway (figure 4.1). Hydro Aluminium Karmoy (HAK) aluminium works, the largest in Northern Europe, is located within Karmsund strait between the sites of Hogevarde and Austvik. Production is based on Søderberg technology (50%) in the oldest part of the plant and cleaner pre-bake technology (50%) in recently expanded facilities, although by 2010 the entire plant will be pre-bake (Jonny Beyer, pers. comm.). Annually, HAK uses approximately 60,000t of organic anode material (Beyer *et al.,* 1998) and in 1998, aluminium production stood at approximately 270,000t with discharges of PAH estimated to be around 1.9t (Aas *et al.,* 2001). Almost all discharges originate from the older Søderberg part of the works (Jonny Beyer, pers. comm.). Annual Søderberg based production stands at approximately 118,000 tonnes and emissions to air (both gaseous and particulate) were 1191, 923 and 723g PAH/tonne of aluminium produced in 1996, 1997 and 1999 respectively (OSPAR, 2002). Following scrubbing of ventilation air, discharges of PAH to water were 12, 17 and 14g PAH/tonne of aluminium produced in 1997, 1998 and 1999 respectively (OSPAR, 2002). In the particulate fraction of discharged effluent, the dominating PAH components were phenanthrene, fluoranthene, pyrene and benzo(bjk)fluoranthene, accounting for approximately 50-70% of the 16 PAH routinely monitored (OSPAR, 2002). In the dissolved fraction, (which has been shown to account for 50-70% of PAHs in the discharge water), phenanthrene, fluoranthene and pyrene dominated, accounting for some 90% of the total PAH (OSPAR, 2002).

Additional PAH inputs arise from a number of smaller land based industries and heavy ship and small boat traffic along the length of the strait. Contamination may also originate from the resuspension of older sediment bound PAH (Aas *et al.,* 2001). Sediment studies have shown highly elevated PAH loadings in the vicinity of the aluminium works



(Grosvik *et al.*, 1999, Naes *et al.*, 1995) and levels of total body burden PAH in the edible crab *Cancer pagurus* are also elevated near the smelter (O-K. Andersen, pers. comm.). Previous studies in Karmsund strait with Atlantic Cod and corkwing wrasse have reported elevated levels of largely pyrogenic PAH metabolites in bile with increasing proximity to the smelter effluent release points (Aas *et al.*, 2001, Beyer *et al.*, 1998). A similar gradient of PAH exposure has been observed in *M. edulis* (O-K. Andersen, pers. comm.). HAK also discharges 19t per year of biocide (C-Treat 6= N-trimethylendiamine acetate) used to prevent fouling in the seawater pipe system (O-K. Andersen, pers. comm.).

4.1.4 Biomonitoring within Karmsund Strait

The present study aims to establish whether *C.maenas* could be used as a bio-indicator of PAH exposure in this fjordal system. Despite being a motile predator it is usually restricted to localised populations in fjords (O-K. Andersen, pers. comm.), making it an excellent sentinel species for evaluating spatial contamination differences. It has already been established in Chapters 2 and 3 (and Watson *et al.*, in press) that *C. maenas* is capable of PAH uptake, biotransformation and elimination, providing dose dependent information on PAH exposure. Although crab species have been used previously for field-monitoring purposes (e.g. Eickhoff *et al.*, 2003a,b, Law *et al.*, 2002, Paine *et al.*, 1996, Hellou *et al.*, 1994, Mothershead *et al.*, 1991), to this author's knowledge the present study is the first to analyse crustacean urine to indicate PAH exposure in the field. Urinary data were also compared with total PAH body burdens of mussels and edible crabs from selected sites within Karmsund strait (provided by research colleagues from Akvamiljo Marine Laboratory in Randaberg, Norway).

In addition to the urinary biomarker, carboxylesterase activity in *C.maenas* haemolymph is measured along the PAH gradient. Carboxylesterase (CbE) and acetylcholinesterase (AChE) belong to a family of enzymes known as the "B" esterases, which are a large group of serine hydrolases, inhibited by organophosphate (OP) e.g.

paraxon and carbamate pesticides (Thompson, 1999). Inhibition of AChE has long been used as an indicator of exposure to these pesticides (Thompson, 1999) and inhibition of CbE activity is also reported (Galloway *et al.,* 2002a). CbE has traditionally been used alongside AChE activity to assess exposure of marine organisms to biocides and OP/carbamate pesticides, which can enter coastal and estuarine waters as a result of intensive agricultural practices and industrial processes (Galloway *et al.,* 2002a, Sanchez-Hernandez *et al.,* 1998, Escartin and Porte, 1997). Recent studies have shown that CbE activity is higher in *C.maenas* haemolymph than AchE and is therefore a more sensitive indicator of exposure in this species (T. Galloway, pers. comm.). Recent fish and mammalian studies have suggested that carboxylesterases also play an important role in the biotransformation and detoxification of many drugs, lipophilic compounds and hydrophobic organic xenobiotics (Barron *et al.,* 1999, Hodgson and George, 1998, Tang and Sun, 1996, Hinson and Forkert, 1995). A recent study with mussels has also shown that the PAH benzo(a)pyrene is capable of a suppressing AchE in gill tissue (Akcha *et al.,* 2000) and studies on crustaceans have suggested that PAH may inhibit esterase activity (Narbonne *et al.,* 1995). In light of all the above, combined with the fact that biocide is also discharged from the smelter, it was considered pertinent to measure CbE activity in crab haemolymph to identify any adverse affects on this important enzyme group.

Thirdly, heart rate is measured in *C.maenas* as an indication of overall physiological competence. Physiological biomarkers such as this are indicative of an organism's integrated response to pollutant exposure. Impairment of physiological function reduces the survival potential of the organism (Lundebye and Depledge, 1998, Depledge *et al.,* 1995) and therefore physiological biomarkers are of great ecological relevance. Previous studies have shown heart rate in this and related species to be affected by numerous different contaminants including PAH and trace metals (Bamber and Depledge, 1997a,b, Hebel *et al.,* 1997, Fossi *et al.,* 2000, Depledge and Lundebye, 1996, Lundebye and Depledge, 1998, Depledge *et al.,* 1995). Typically heart rate is elevated in response to

physical stress and this capacity for rapid increases in respiration rate is presumably critical to the survival of crabs attempting to avoid predation of physical disturbances (Bamber and Depledge, 1997b). Any contaminant-induced impairment of cardiac function therefore has potential for adverse effects on the individual.

4.2 Materials and Methods

The field trial was carried out as part of the EU BEEP (Biological Effects of Environmental Pollution) programme, Work Package 4 (WP4) sampling mission in Norway during September of 2001. In total, 10 sites were sampled, characterised by varying levels and types of predominant contamination. Seven of these sites were used for comparison of a multitude of biomarker responses investigated by various European laboratories. Six sites, three of them exclusive to the present study, were sampled to investigate a PAH exposure gradient already established in mussels and fish.

4.2.1 Sampling sites and collection of animals

Six sites within Karmsund Strait were selected (Hogevarde, Austvik, Koppervik, Krokaneset, Salvoy and Bukkoy) with increasing distance south of the smelter (with the exception of Bukkoy and Salvoy), based on previous studies (Aas *et al.*, 2001, Beyer *et al.*, 1998) (figure 4.1).

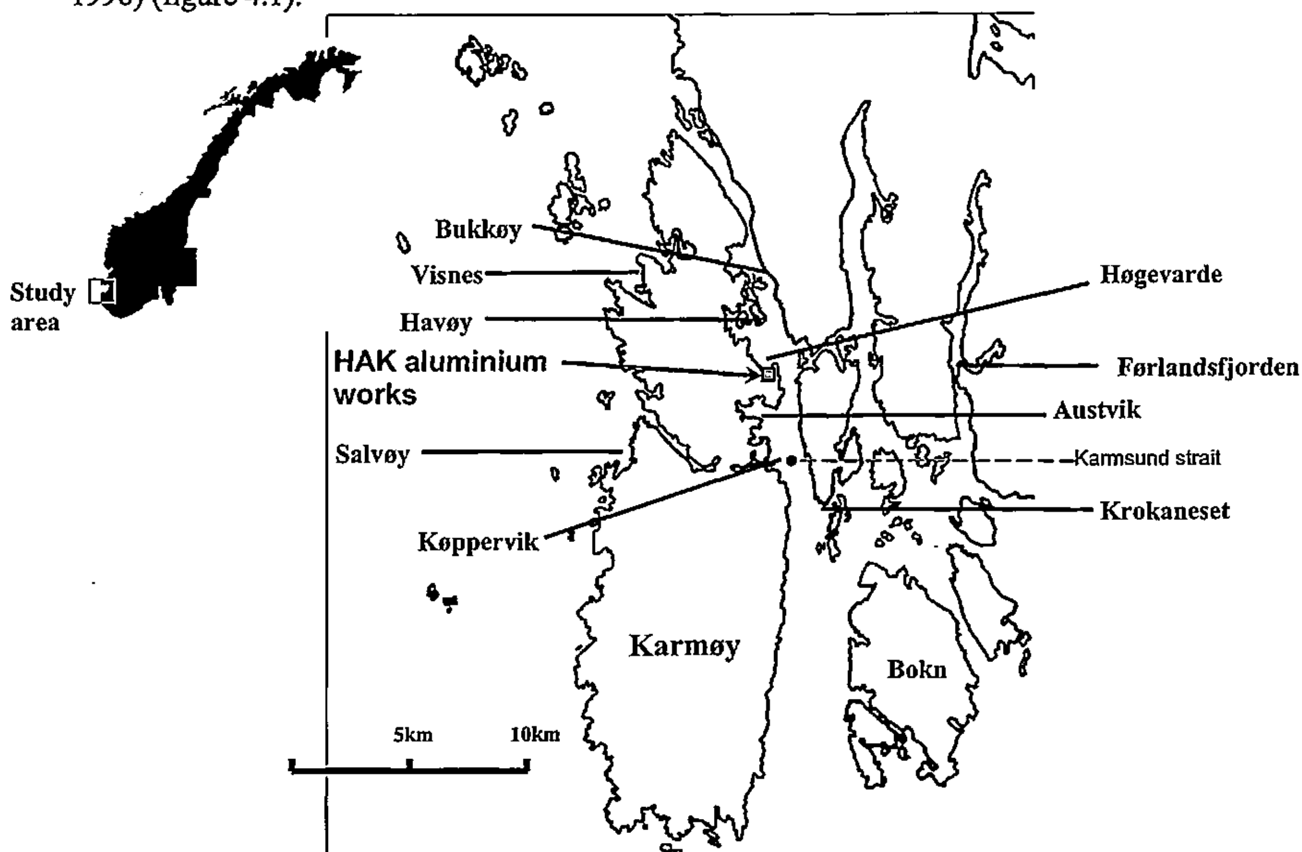
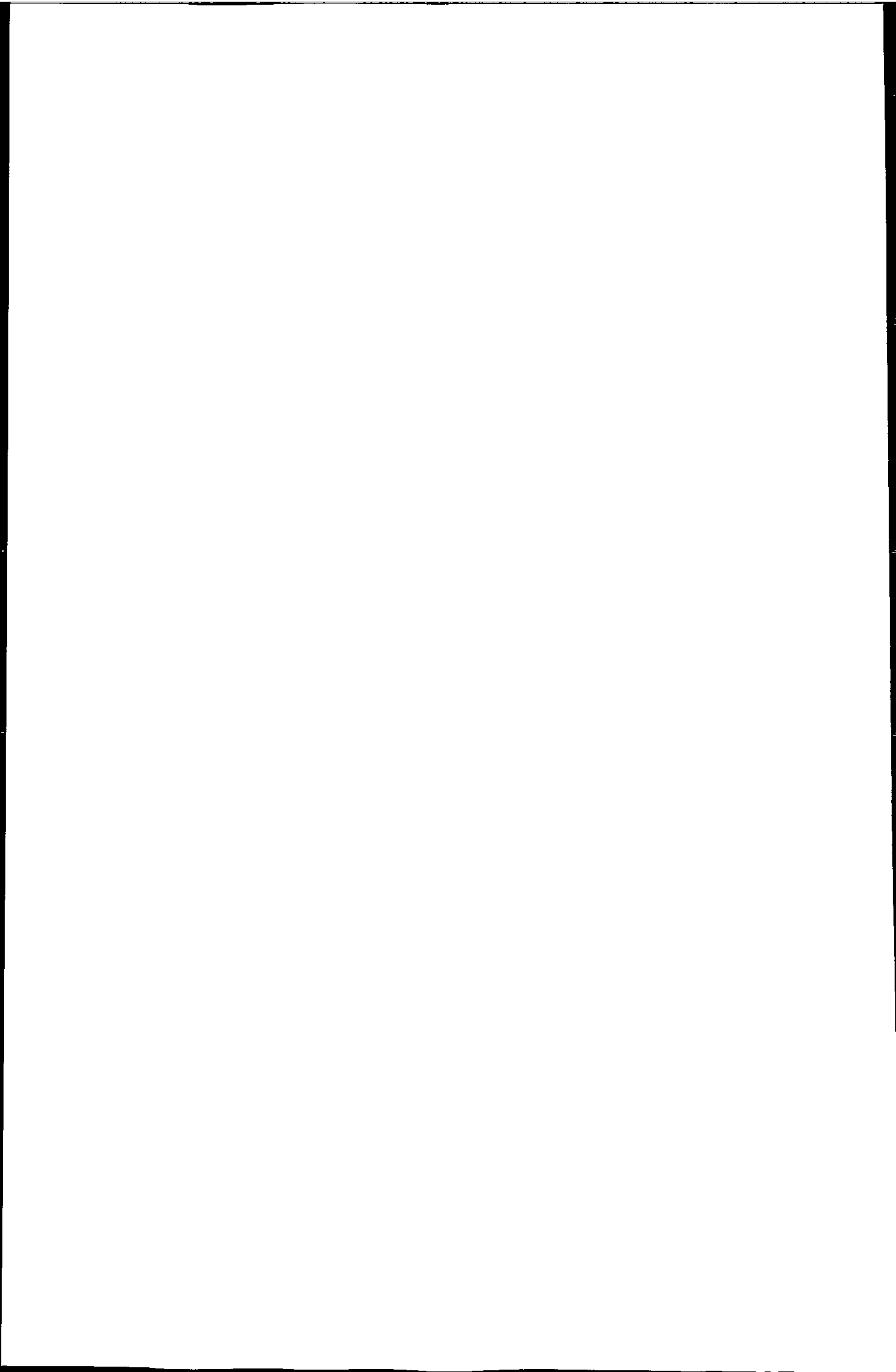


Figure 4.1 Karmoy Island, Karmsund strait and sampling sites.



Hogevarde is adjacent to the largest point of discharge for the aluminium works' scrubber effluent and Austvik is next to a smaller discharge point (Aas *et al.,* 2001). Koppervik is situated 2.5km south of the aluminium works, with Krokaneset 5.5km south in a relatively pristine environment (Aas *et al.,* 2001). Bukkoy is 6 km north of the smelter and the reference site at Salvoy is on the western side of Karmoy island. Male *C.maenas* were collected by local fishermen using crab pots at each site on the day of sampling and stored in aerated seawater from each site until sampling could be performed.

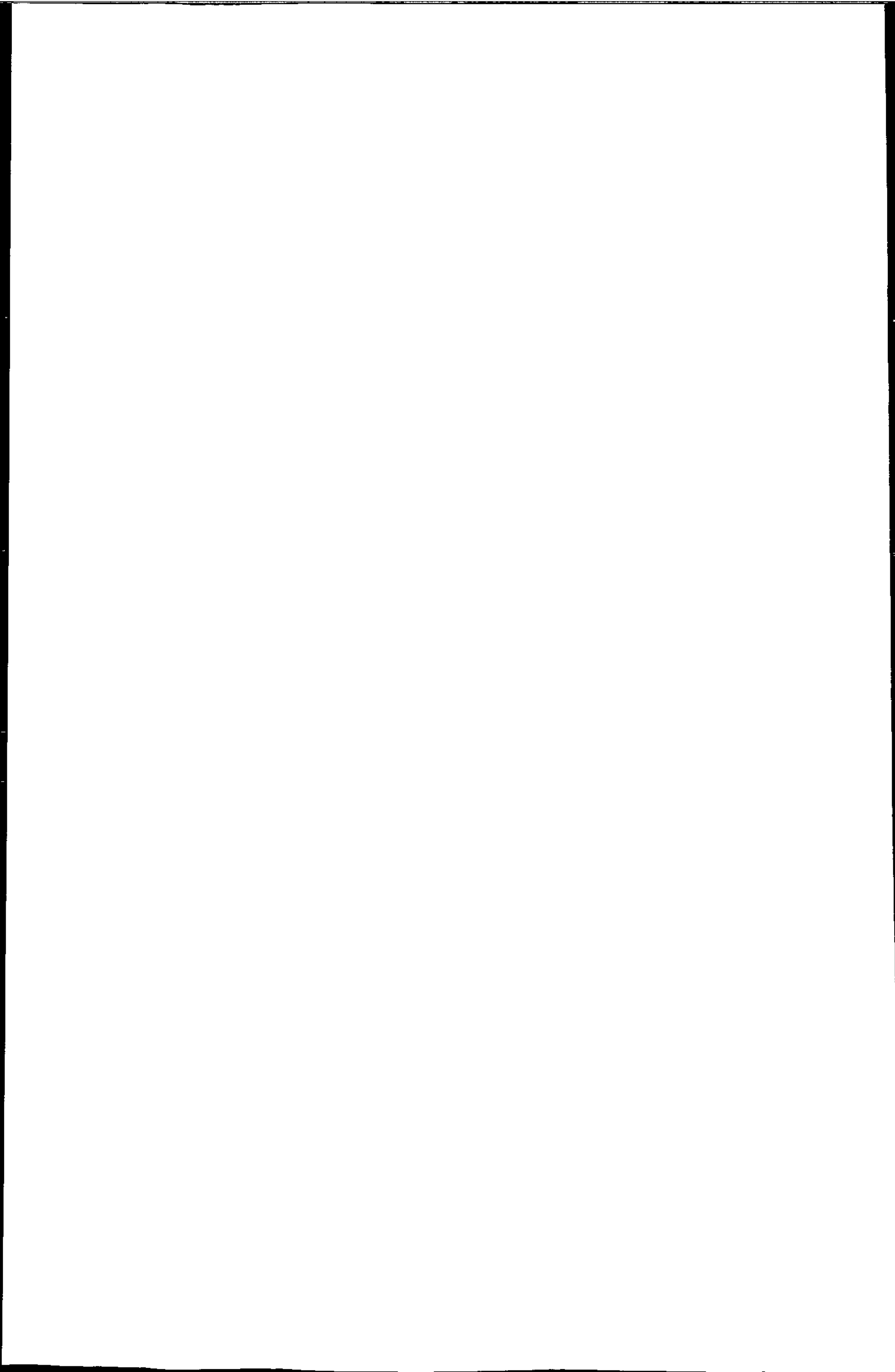
4.2.2 Sampling of animals

Urine and haemolymph were sampled *in situ* from male intermoult crabs at each site (n=12, Koppervik, n=8) as described previously. Samples were immediately snap frozen in liquid nitrogen and kept frozen on dry ice for transportation and then at - 80°C until analysis.

4.2.3 Analysis of urine samples

Previous experience with analysing urine samples led to a refinement of the wavelength regimes used to analyse field samples. For the present study, the following regime was employed to determine levels of 2-3 ring (largely petrogenic) derived fluorescence and pyrene "equivalent" (pyrogenic) fluorescence in urine samples, since it is established that the fjord receives both PAH contamination from both sources (Aas *et al.,* 2001, O-K, Andersen pers. comm.).

Urine samples were analysed using both FF and SFS techniques. Intensity at the FF wavelength pair Ex290nm/Em335nm was used to determine fluorescence contributions from naphthalenes (2 rings). The wavelength pairs Ex345/Em382nm and Ex380/Em430nm were used to detect pyrene "equivalents" and benzo(a)pyrene respectively, indicative of pyrogenic PAH contamination. Screening for all major PAH groups (2-5 rings) with synchronous scanning fluorescence (SFS Δλ37nm) was also performed. Total area of all



peaks between Em310-370nm was used to quantify the typically broad area of fluorescence contributed by 2-3 ring PAH (naphthalenes, phenathrenes, anthracene) (UNEP, 1992), which are typically associated with petrogenic contamination. No attempt was made to quantify this broad fluorescence with respect to standards, and instead area was used as a comparative measure of contributions from 2-3 ring compounds at each site.

Intensity at Em382nm was measured as an indication of any fluorescent contributions from 1-OH pyrene equivalents in the sample. It should be noted that this emission wavelength is different from that described in chapter 2, where intensity at Em381.4nm was used for calibration with standards. Differences in intensity between the two wavelengths are negligible and the modification was made to simplify the method. Slit widths for all analyses were 2.5nm and all urine samples were diluted 1:50.

4.2.4 Quantification of sample peaks

Urinary equivalent peaks (FFEx345/Em382nm, SFS Em382nm) were quantified with respect to a series of 1-OH pyrene standards (400, 200, 100, 75, 50, 25, 10, 5μg l^{-1}), analysed at the fixed wavelength pair of Ex345/Em387nm (FF) and Em387nm (SFS). Results are corrected for dilution and reported in terms of μg l^{-1} of 1-OH pyrene equivalents.

4.2.5 Carboxylesterase assay on haemolymph samples

Carboxylesterase activity was assayed using a method modified from that of Ellman *et al.,* (1961). A thioacetate derivative (phenylthioacetate) is hydrolysed by carboxylesterase to yield thioacetate. Combination with DTNB then forms the yellow anion 5-thio-2-nitrobenzoic acid, which absorbs strongly at 412nm (Galloway *et al.,* 2002a). The present study uses absorbance at 405nm, which is equally well suited to quantify colour development as it is still within the absorbance peak of the chromophore.

Haemolymph samples were thawed fully and centrifuged in a precooled (3-4°C) centrifuge at 10,000rpm for 5 minutes to spin out haemocytes. Supernatant was then transferred to clean, siliconised microcentrifuge tubes. 20µl from each sample was aliquotted into a separate tube for determination of total protein. 30µl of sample or blank (50mM phosphate buffer, pH 7.4) were loaded into appropriate wells of a 96 well microplate. 150µl of 270µM DTNB (5,5-dithiobis-2-nitrobenzoic acid, diluted with 50mM sodium phosphate buffer) was then added to each well and mixed well. The mixture was then left to incubate and any endogenous reaction recorded for 2x5 minutes (or until the reaction plateaued out), by measuring absorbance @405nm at 25°C. Measurement of enzyme activity was then initiated by the addition of 50µl of the substrate phenylthioacetate (3mM, in phosphate buffer) to each well. Colour development was measured by reading absorbance @405nm for 5 minutes at 25°C, with readings taken every 30 seconds. Carboxylesterase activity was calculated as follows,

$$\textbf{CbE activity} = \frac{\Delta A \times Vol_T \times 1000}{1.36\times10^4 \times LP \times Vol_S \times [\text{protein}]}$$

$$= \mu\text{mol PTA min}^{-1}\text{ mg protein}^{-1}$$

ΔA = change in absorbance per minute

Vol_T = Total assay volume (DTNB + sample) + substrate (ml).

Vol_S = volume of sample (ml)

1.36×10^4 = molar extinction coefficient of coloured anion

[protein] = mg/ml protein in haemolymph/tissue extract.

LP = Lightpath (microplate well depth=1cm).

Results were calculated as µmol substrate hydrolysed min^{-1} mg^{-1} protein. Total haemolymph protein was determined by the BioRad method with bovine serum albumin as standard.

4.2.6 Measurement of a physiological biomarker: Heart rate

Adult male *C.maenas* (n=8) of carapace width 47-84mm, were collected by local fishermen at Hogevarde, Forlandsfjorden and also at Visnes, a BEEP 2001 site heavily impacted by tailings and runoff from a disused copper mine, on the west side of Karmoy island. Crabs were kept at ambient temperature in individual light-tight 2.5L plastic aquaria containing 2L of well-aerated seawater from their site of collection.

Cardiac activity (heart rate) was measured using a non-invasive infrared light system (Computer Aided Physiological Monitoring - CAPMON), according to the method described by Depledge and Andersen (1990). Briefly, a plastic collar, into which a coupled infrared transmitter-detector unit is attached, was super-glued onto the dorsal surface of each crab, directly above the heart. A square indentation on the carapace, formed by attachment points for the heart's suspensory ligaments, signifies this position. As the conformation of the heart changes with each cardiac cycle, the intensity of the light reflected back to the detector fluctuates (Bamber and Depledge, 1997a). This detected signal is fed to an analogue-to-digital converter and then to a computer, where a custom designed software program displays the cardiac cycles continuously. The software is calibrated to identify each cardiac cycle and these are then counted using a cumulative counter. Cumulative cardiac cycle counts (i.e. heart rates) are downloaded at 1 minute intervals.

Crabs were connected to the CAPMON system and then left to acclimate overnight with the minimum of physical disturbance, so as to attain a stable and representative resting heart rate. The following morning, heart rate of resting crabs was recorded for 6 hours and the data saved. Counts were also recorded visually from the software display for 10 minutes, by way of a backup should the downloading process fail. The results presented are those recorded from the system by the software.

4.2.7 Measurement of PAH in sediment within Karmsund strait

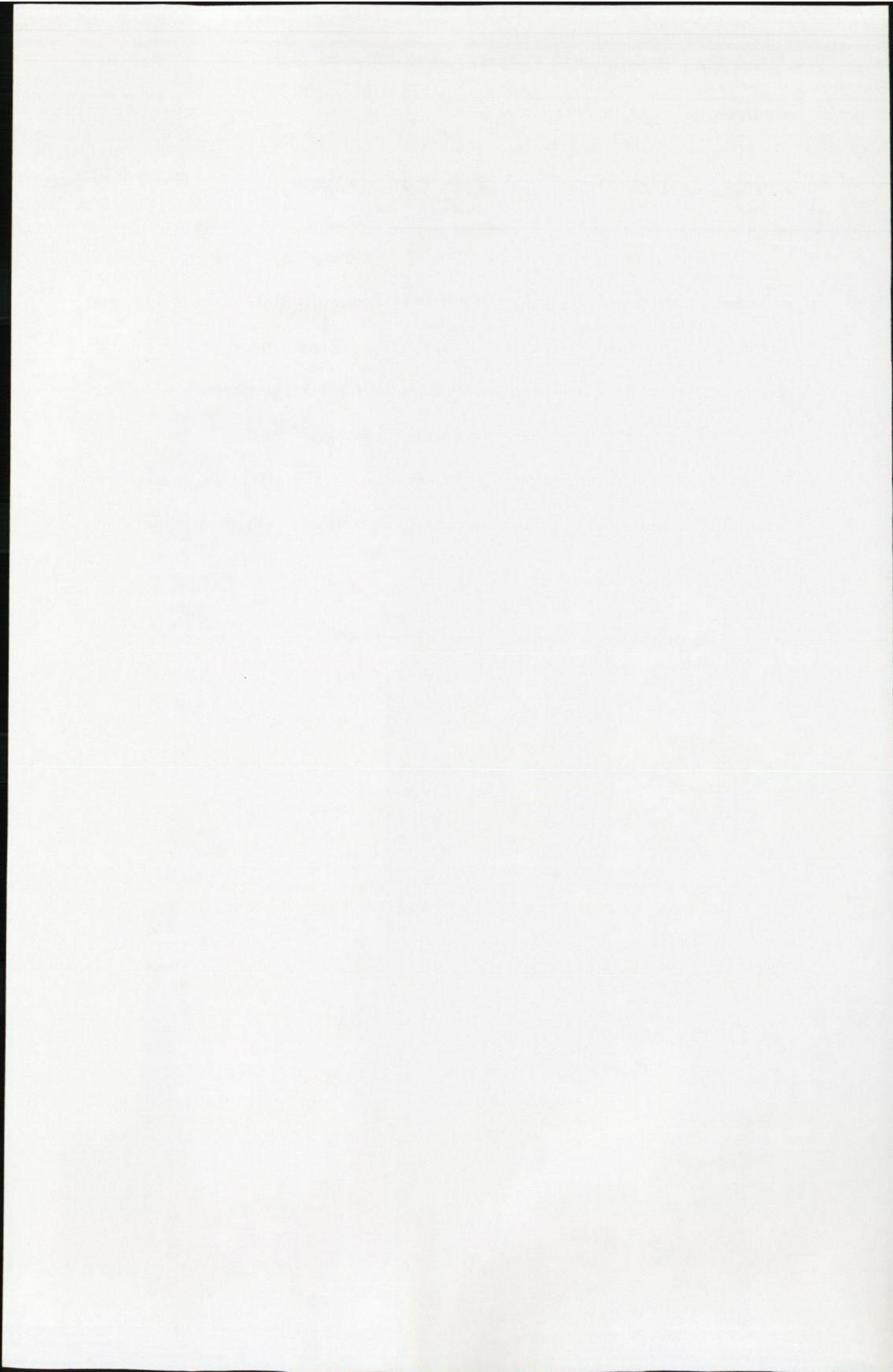
Levels of total PAH in sediment at selected sites within Karmsund strait have been routinely monitored over several years. Data relevant to the present study for Krokanes (reference) and 2 sites at Hogevarde (Hogevarde itself and a site 500m to the south), were generously provided by Anne Bjornstad (Akvamiljo marine laboratory, Rogaland Research, Stavanger, Norway) from a study by Grosvik *et al.*, (1999). PAHs in sediment samples were assayed by GC/MS using standard protocols. This study did not provide sediment PAH data for Koppervik, Salvoy, Austik or Bukkoy, although total PAH levels in sediment at Havik (Hogevarde), Austvik and Salvoy are reported in Beyer *et al.,* (1998). Therefore, only sediment data (µg/kg) for HAK, 500m south and at the mouth of Karmsundet can be included here.

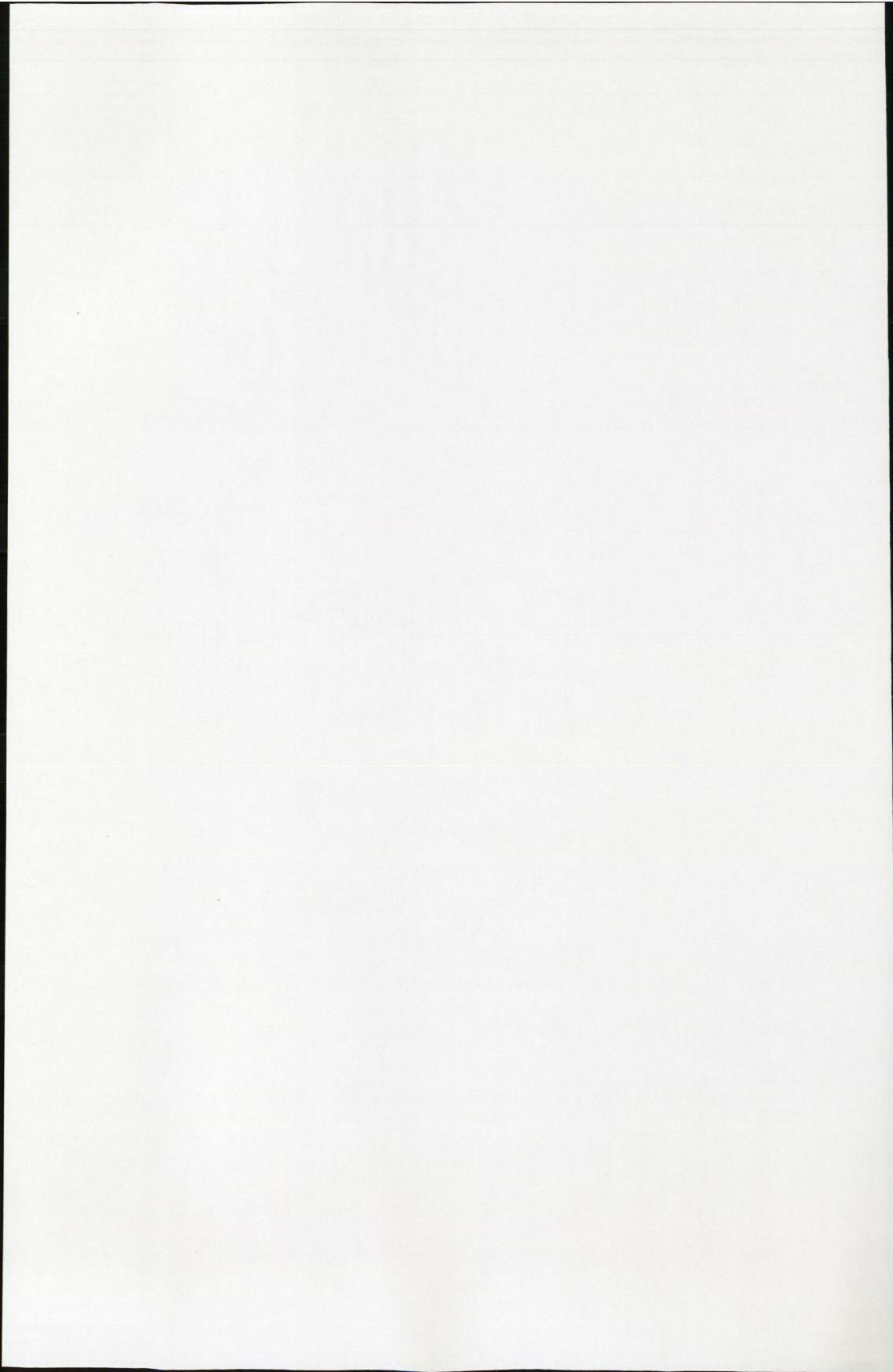
4.2.8 Measurement of PAH in mussel and edible crab within Karmsund strait

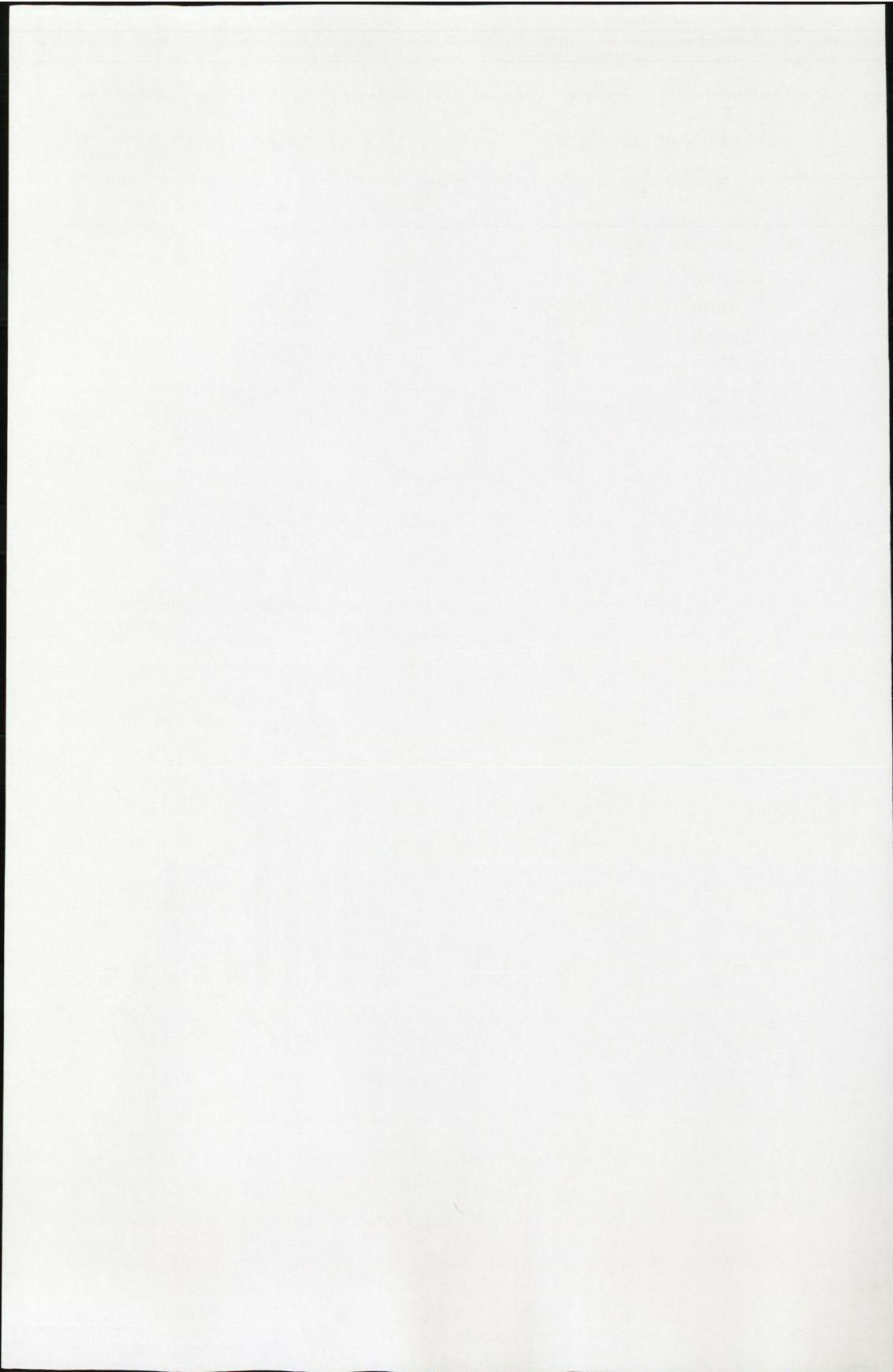
Levels of total PAH in mussel (*Mytilus edulis*) and edible crab (*Cancer pagurus*) were measured at selected sites within the fjord by Anne Bjornstad and Odd-Ketil Andersen, at Akvamiljo marine laboratory, Rogaland Research, Stavanger, Norway in June, 1999. PAHs in tissue samples were assayed by GC/MS using standard protocols.

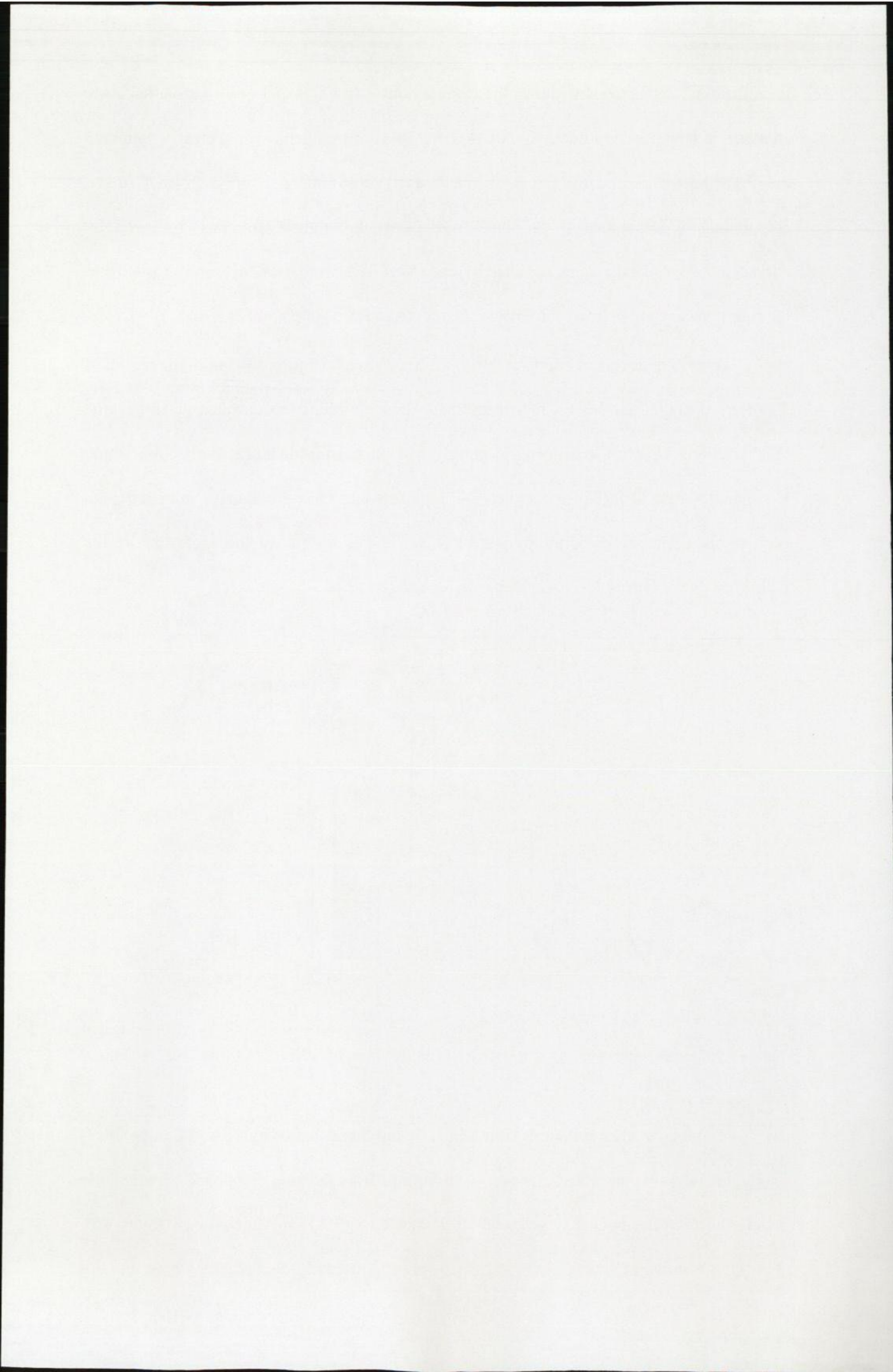
Mussel (n=20) PAH body burdens (µg/kg wet weight) were measured at various sites, with Hogevarde, Austvik, Koppervik, Krokanes and Bukkoy being the most relevant for the present study. Mussels from the reference site at Salvoy were not measured during the study, but an alternative reference site in a nearby fjord (Forlandsfjorden) was utilised. Forlandsfjorden is an extremely sheltered fjord, with no major activities impacting it, save for some minimal small boat traffic, runoff from some small local farms and a mussel farm in its waters (O-K, Andersen pers. comm.). Edible crab (n=5) whole body PAH burdens (µg/kg wet weight) were measured at Hogevarde, Austvik, Krokanes and Bukkoy.

Krokanes, at the mouth of the fjord, was designated as the reference site during this study and an additional site north of the smelter at Havoy, was also sampled.

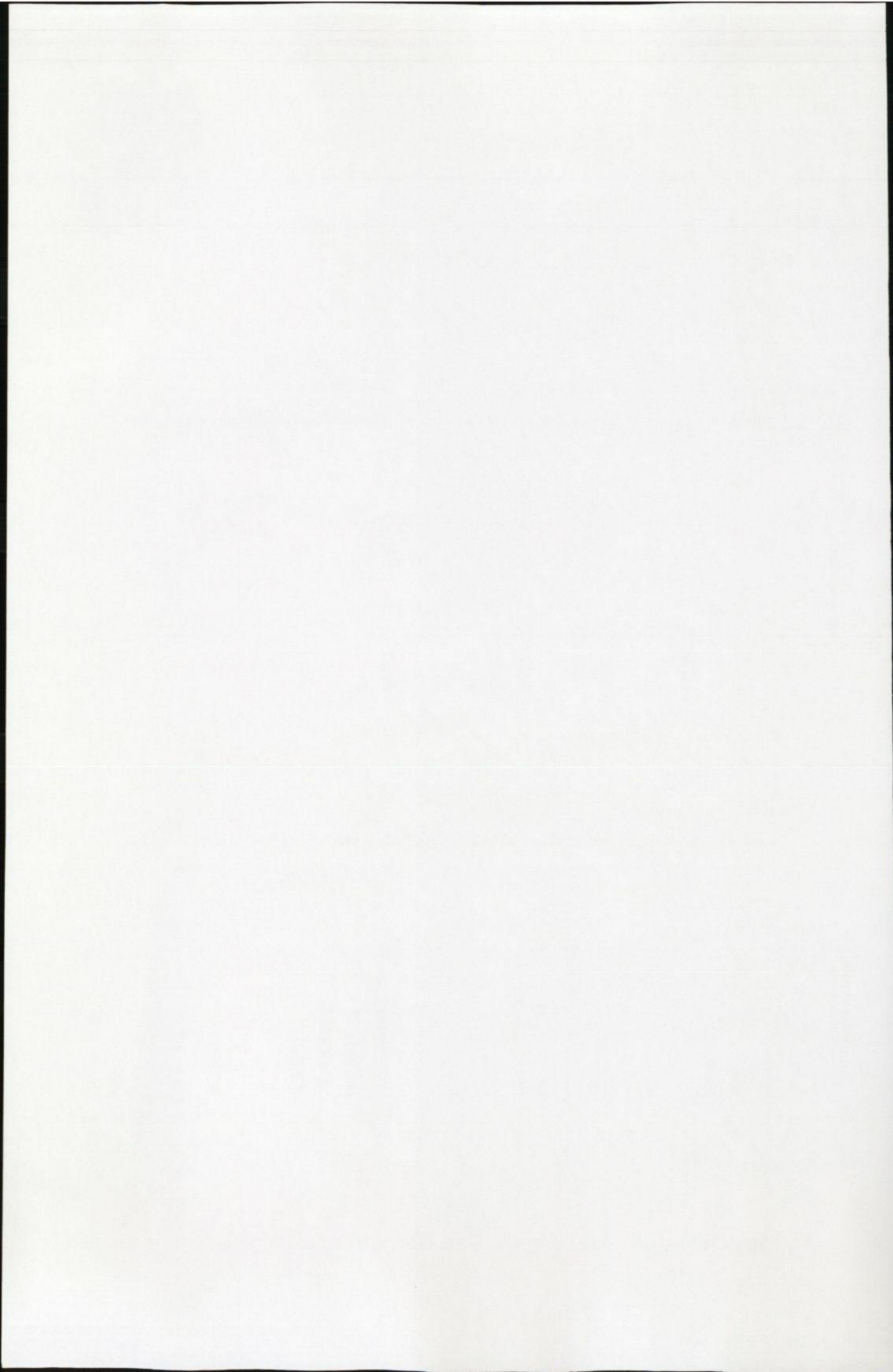


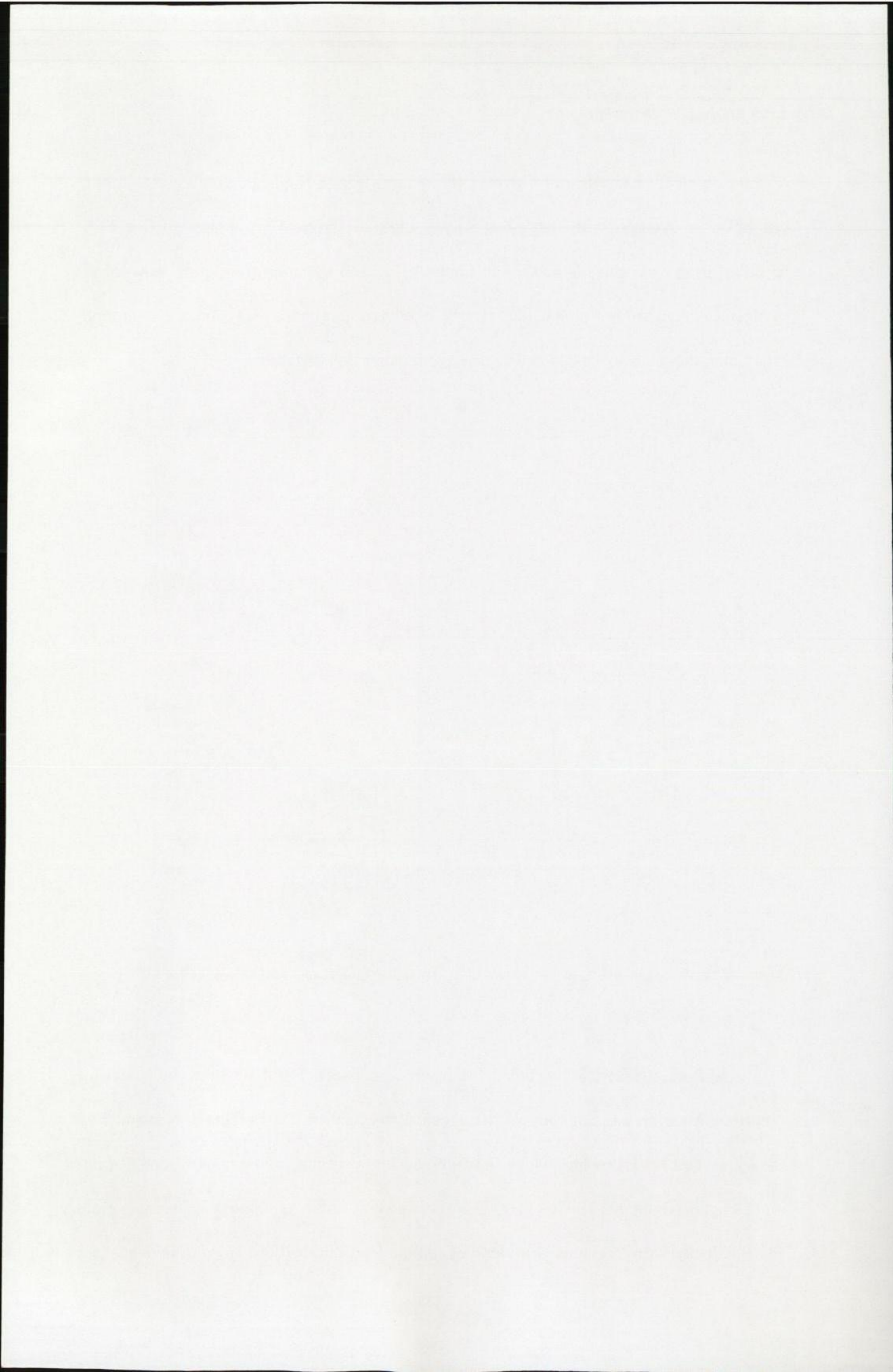


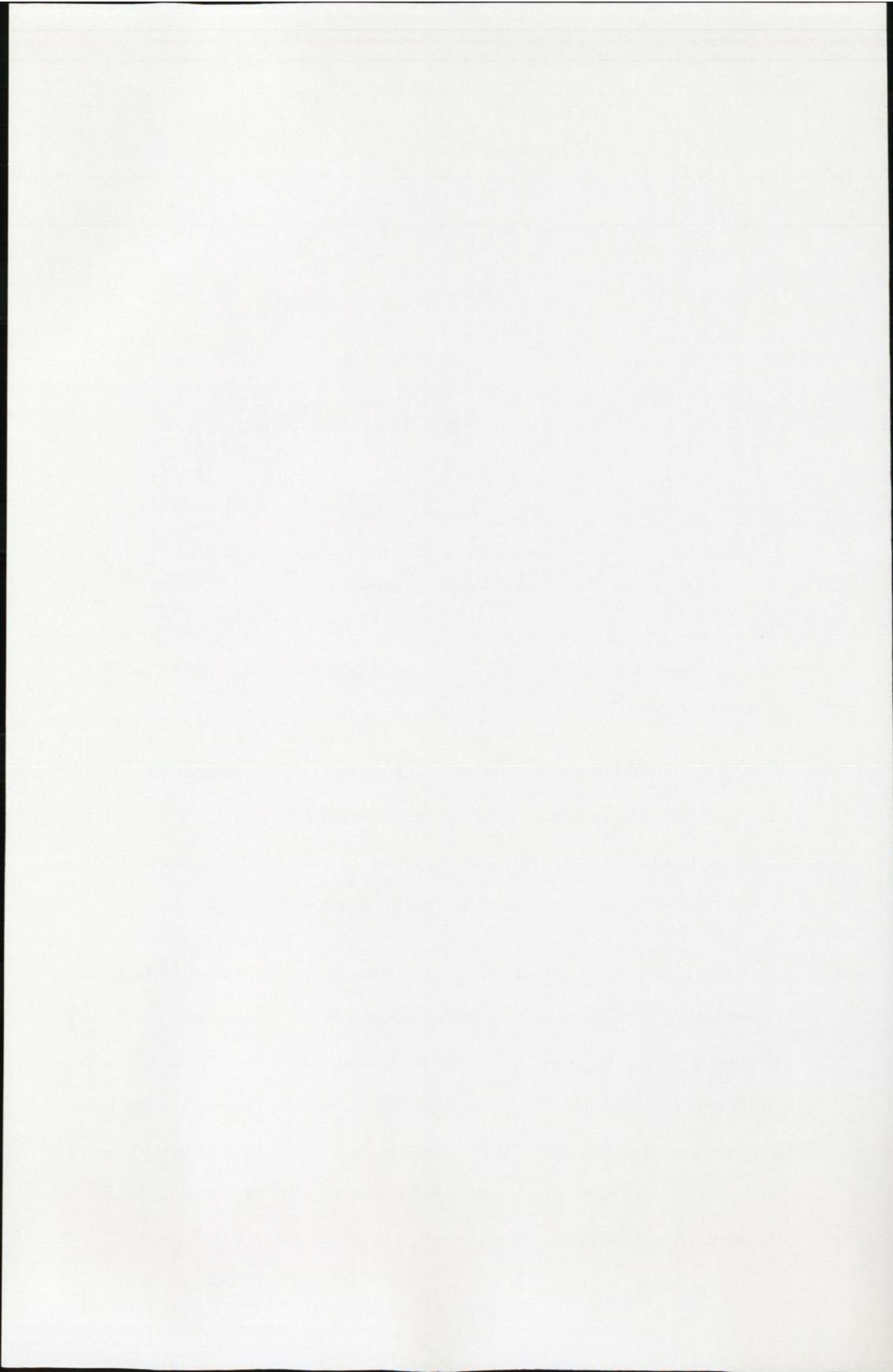




from 4-6 ring PAH. The two data sets also differ in that no detectable levels of PAH were found in mussels at Krokanes, but mean ΣPAH in edible crabs at this site was 24.7μg/kg. The 2-3 ring domination of body burden in edible crabs at Bukkoy and Havoy is largely attributable to naphthalenes (figures 4.8 and 4.9), whereas at Hogevarde 2-3 rings dominate due to high levels of phenanthrene and C1-phenanthrene/anthracene (figure 4.10). Fluoranthene and phenanthrene are largely responsible for the elevated total PAH level at Hogevarde (figure 4.10) and 4 ring PAH are generally higher here than at the other sites. 4 ring PAH at Austvik are also higher, due to elevated fluoranthene, pyrene and benzo(a)anthracene and chrysene in tissues.







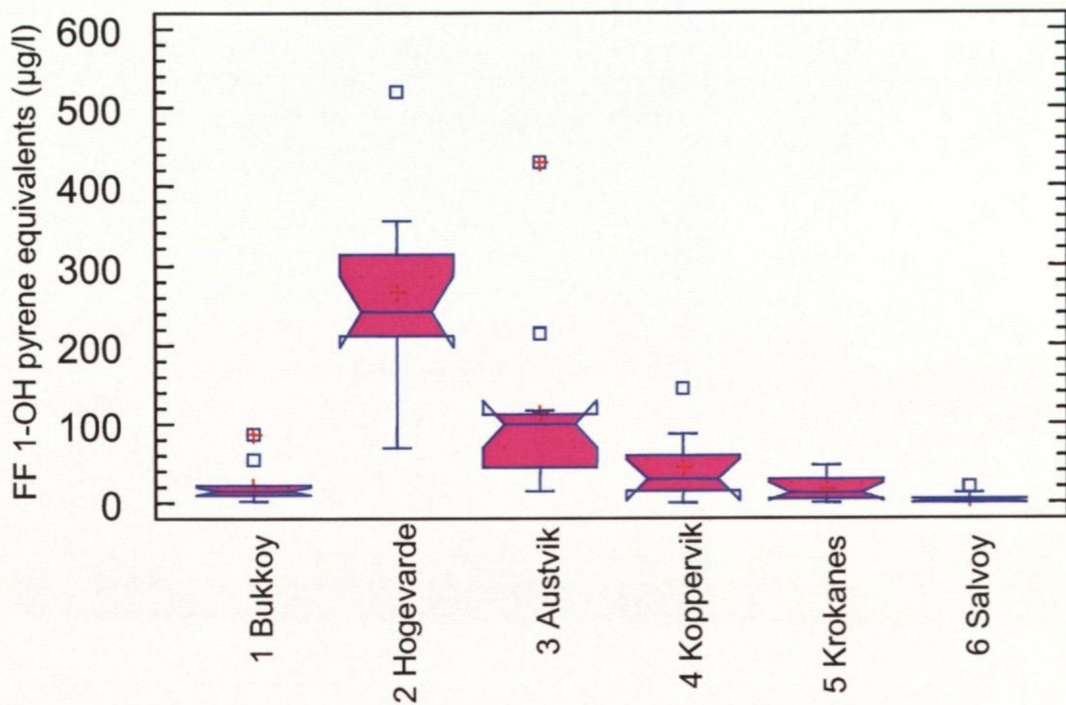


Figure 4.14 Notched box and whisker plots showing levels of 1-OH pyrene equivalents ($\mu g l^{-1}$) in the urine of crabs collected from sites within Karmsund strait (FF).

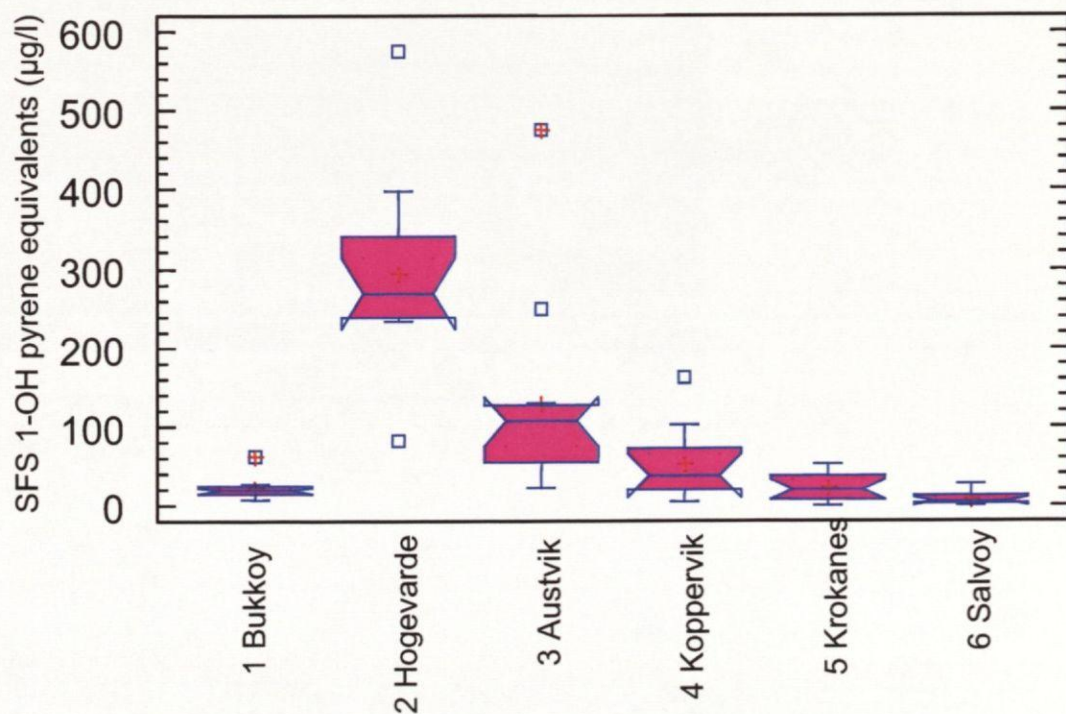
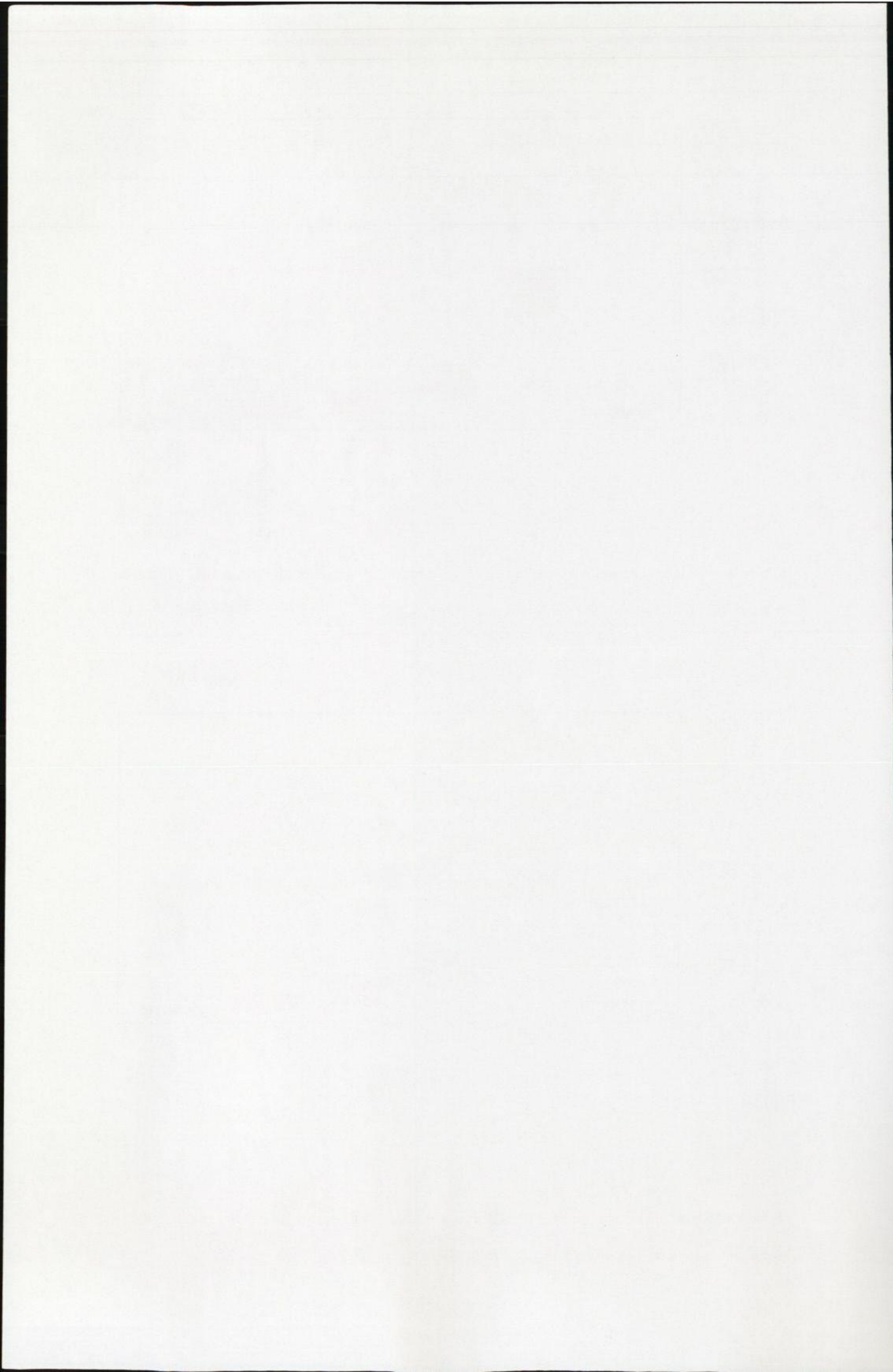
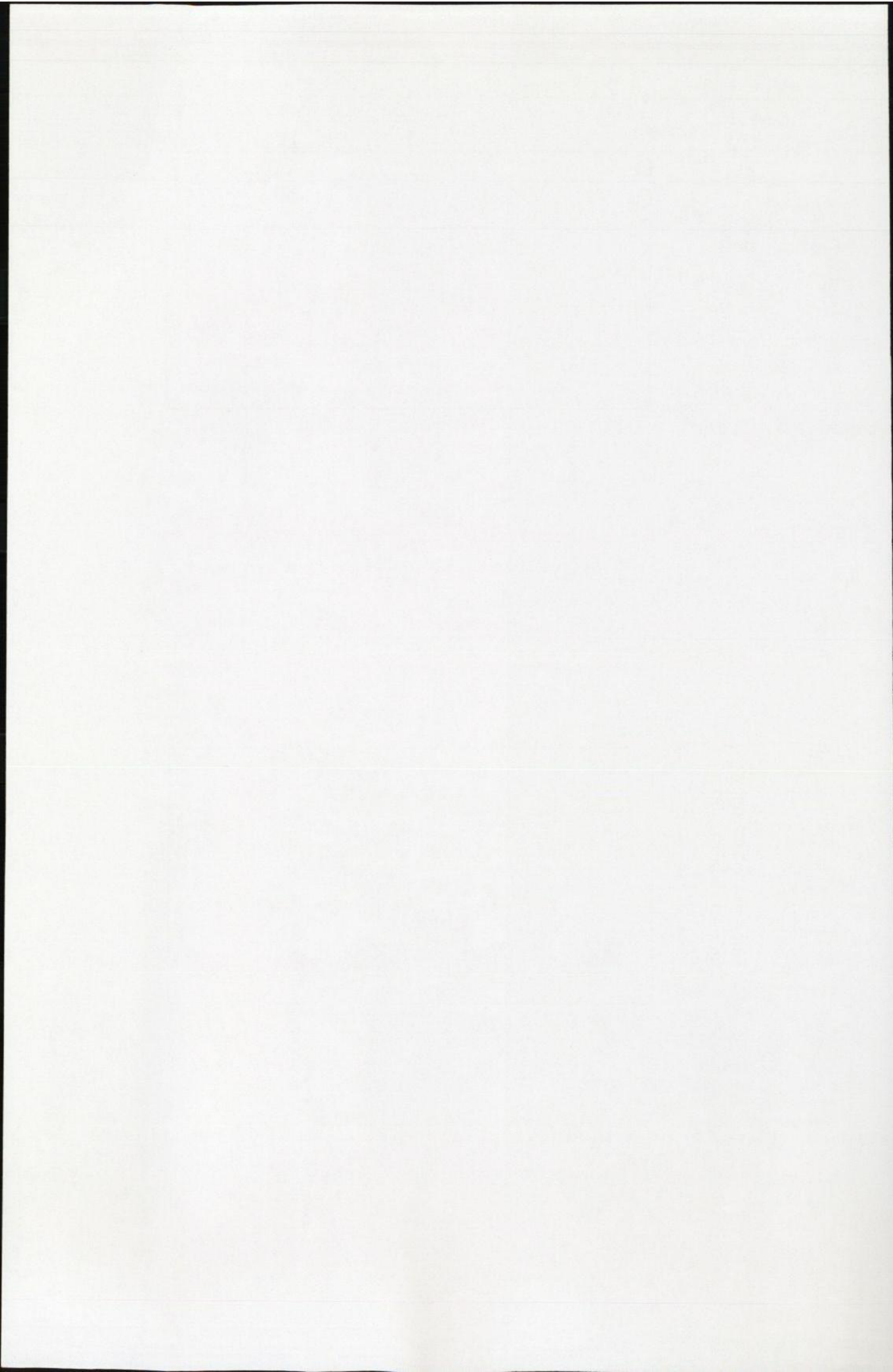


Figure 4.15 Notched box and whisker plots showing levels of 1-OH pyrene equivalents ($\mu g l^{-1}$) in the urine of crabs collected from sites within Karmsund strait (SFS).



SFS data at 2-3 ring wavelengths were variance checked and found to be normally distributed. One-way analysis of variance was therefore applied to the data to compare the means (figure 4.16), which were found to be statistically significantly different ($p=0.0005$, ANOVA). A multiple range test revealed that levels of 2-3 ring petrogenic-type fluorescence at Hogevarde were elevated over Koppervik, Krokanes, Bukkoy and Salvoy. Levels at Hogevarde and Austvik did not differ significantly, nor did levels at Austvik and Koppervik, but levels at Austvik were elevated over Krokanes, Bukkoy and Salvoy. The 2-3 ring results followed a similar pattern to the 1-OH pyrene equivalents, decreasing with distance from the smelter, but the spatial gradient was less steep. Interestingly, FF results from the naphthalene specific wavelength pair showed greater similarity between sites (figure 4.17, $p<0.05$ KW), i.e. the gradient is less pronounced when looking at levels of naphthalenes in urine samples. Only levels at Krokanes and Salvoy show statistically significantly differences from the sites adjacent to the smelter. When 3 ringed compounds are included in the analysis, the spatial gradient becomes steeper. Overall, there was large inter-individual variability in urinary levels of 1-OH pyrene equivalents and 2-3 ring PAH, increasing with proximity to the smelter.



4.3.4 Carboxylesterase assay on haemolymph samples

Haemolymph carboxylesterase activity and total protein results are shown in figure 4.18. Carboxylesterase activity is elevated in Hogevarde and Austvik crabs compared to the other sites, but these differences are not statistically significantly different (p=0.1506, ANOVA). Despite a p value of 0.0302 (KW), only crabs at Hogevarde and Koppervik are statistically significantly different with respect to haemolymph total protein.

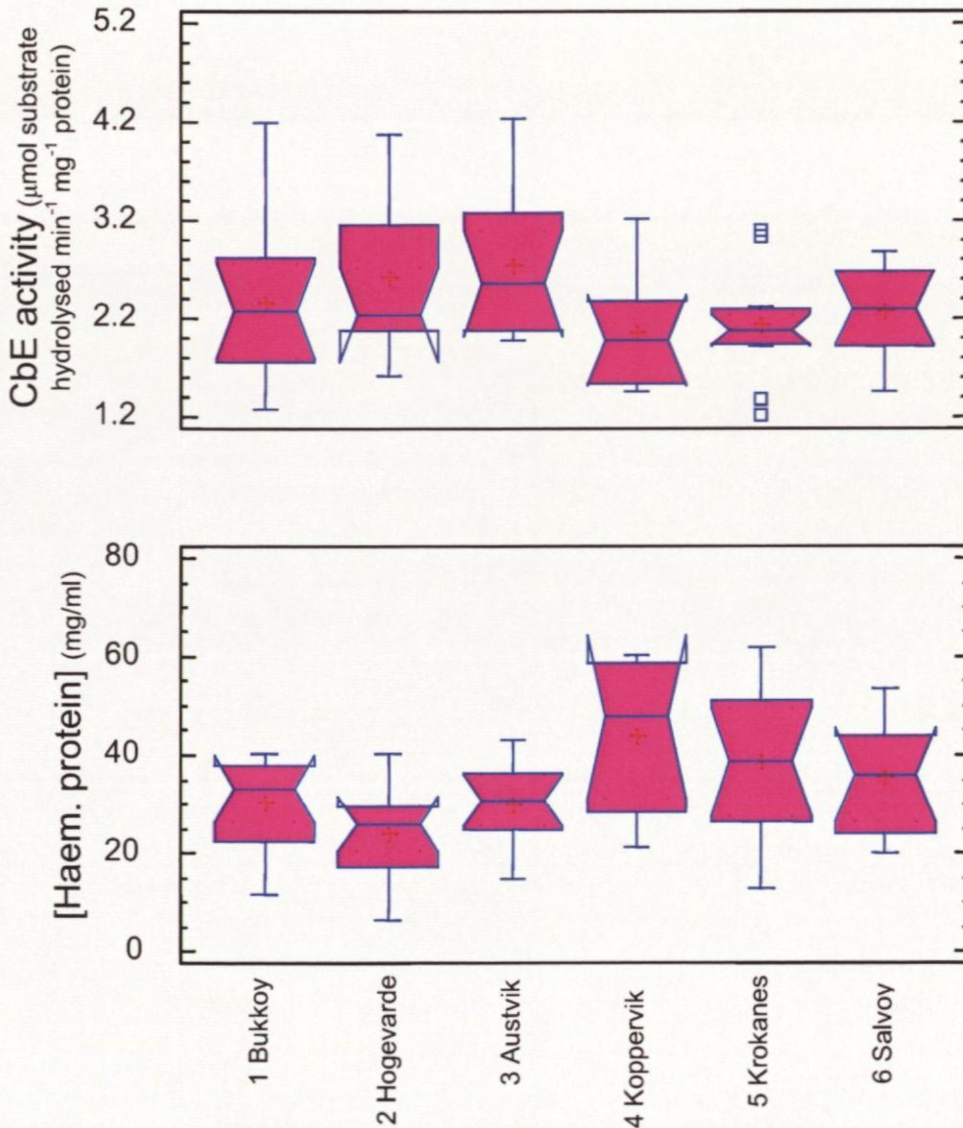
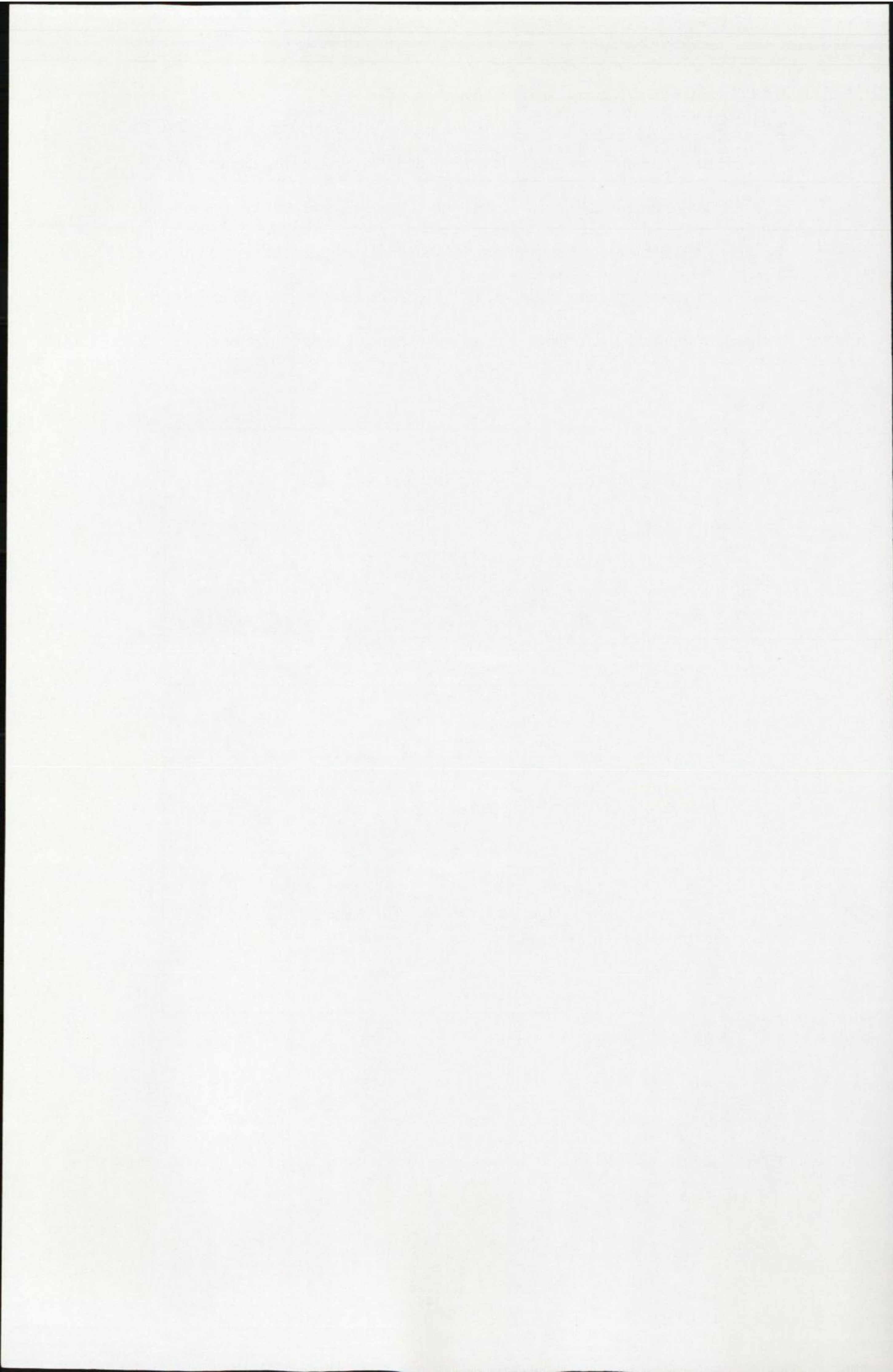


Figure 4.18 Carboxylesterase activity and total protein concentration in the haemolymph of crabs at sites within Karmsund Strait.



4.3.5 Measurement of a physiological biomarker: Heart rate

Heart rate in Hogevarde crabs was significantly elevated over Forlandsfjorden crabs (figure 4.19) ($p<0.05$, ANOVA). Heart rates of Visnes crabs were also significantly elevated over reference crabs ($p<0.05$, ANOVA), but were not significantly different from those of Hogevarde crabs.

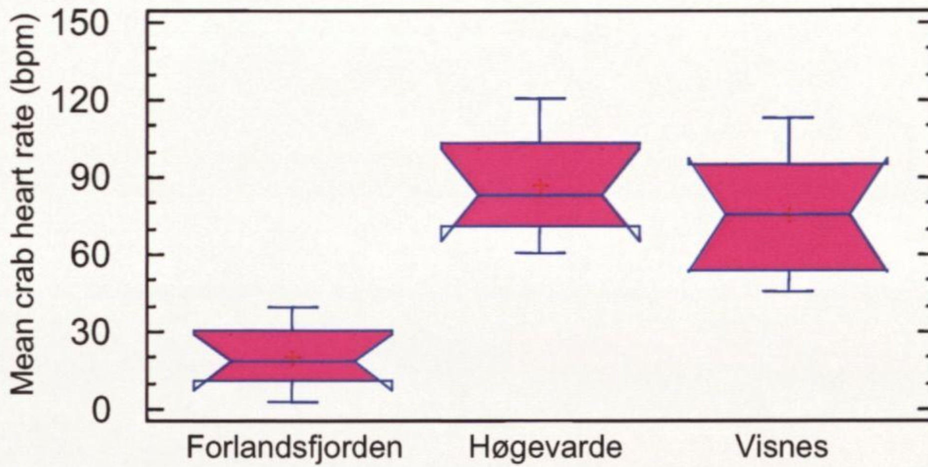
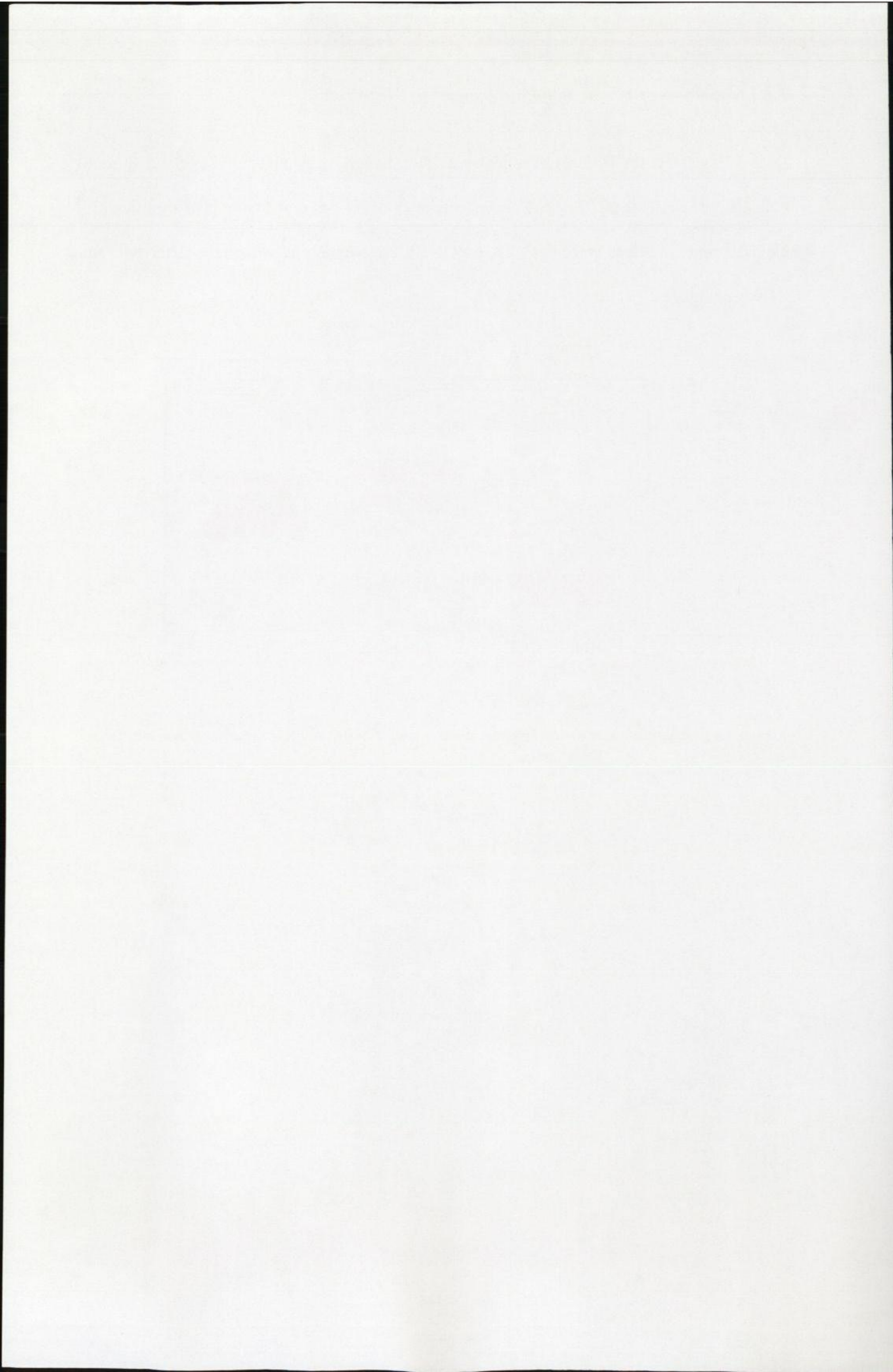


Figure 4.19 Mean heart rate (bpm) of crabs at Høgevarde (PAH), Visnes (copper) and Førlandsfjorden (reference).



4.4 Discussion

Crab urine analyses have identified a steep southwards gradient of PAH exposure within Karmsund Strait. This exposure gradient is most pronounced when measuring fluorescence contributed from pyrogenic (4+ rings) type PAH. The gradient is less steep with respect to 2-3 ring petrogenic type PAH, and is least pronounced when contributions from only 2 ring naphthalenes are taken into account. These results suggest that contamination in the fjord is primarily pyrogenic PAH with lesser contributions from petrogenic sources. This is supported by the results of sediment analyses, which showed a predominance of 4+ ringed, pyrogenic PAH (particularly fluoranthene, pyrene and chrysene) in the vicinity of the smelter. The source of pyrogenic PAH is scrubber wastes released from two settling pond discharge points adjacent to the smelter (at Hogevarde and Austvik). Sources of petrogenic PAH are likely to be more diffuse than point source, with other land based industrial discharges, local run off and heavy shipping traffic in the strait contributing 2-3 ring PAH to Karmsund waters. An exposure gradient also exists to the north, with urinary pyrene equivalent levels at Bukkoy, 6km north of the smelter, being similar to those at Krokanes (5.5km south), despite less water exchange in this region (O-K. Andersen, pers. comm.). Evidence of exposure to pyrogenic PAH in Karmsund Strait has been demonstrated in previous studies on fish bile (Beyer *et al.*, 1998, Aas *et al.*, 2001) and an exposure gradient has also been detected (Aas *et al.*, 2001). The results presented here support these findings and provide compelling evidence of the applicability of the urinary biomarker to field situations.

Chemical analysis of the total and individual PAH residues in mussel tissues showed a steep gradient of pyrogenic exposure (4-6 ring) from the smelter in both a north and south direction, supporting the crab urine data. Conversely, the 4-6 ring PAH profile of Hogevarde mussels (fluoranthene>chrysene>benzo(a)anthracene>pyrene) appears to contradict the results obtained from crab urine, where the strong fluorescence signal was at

wavelengths previously established to be specific for pyrene. However, as discussed previously, a range of PAHs (and metabolites) can contribute fluorescence at a wavelength pair specific for one PAH, even if these wavelengths are not optimal. For example, it has been reported that chrysene and its metabolites contribute fluorescence at the wavelength pair for pyrene (Aas *et al.,* 2000a). This phenomenon is highly likely to be occurring here, as the mussel data show that numerous different PAH are present in Karmsund strait, the most abundant of which (fluoranthene) is capable of contributing fluorescence at pyrene wavelengths. A predominance of fluoranthene and chrysene in the sediment at Hogevarde supports this hypothesis. Camus *et al.,* (1998) also report that phenanthrene will contribute fluorescence at pyrene wavelengths, which is relevant since broad fluorescence on urinary SFS spectra suggests 3-ring PAHs are present.

Contributions from different PAH aside, the prevalent signal at pyrene wavelengths may be attributable to the fact that detection of pyrene and its metabolites is especially suited to SFS (Lin *et al.,* 1994, Stroomberg *et al.,* 1996, Ariese *et al.,* 1993). The SFS method preferentially detects hydroxylated pyrene chromophore (pyrene type metabolites) over other chromophores and parent pyrene (Lin *et al.,* 1994) and 1-OH pyrene produces a very strong fluorescent signal (Aas *et al.,* 1998, Ariese *et al.,* 1993, Eickhoff *et al.,* 2003a). The physico-chemical properties of pyrene also make it highly bioavailable to aquatic organisms (Landrum, 1989) with reports that the uptake rate of pyrene by flatfish is much higher than that of other 4 or 5 ring PAH (Ariese *et al.,* 1993). The large signal produced at pyrene wavelengths is also likely to be the result of less complicated metabolite pattern. In contrast to B(a)P, which is metabolised to more than 20 different metabolites (IARC, 1983), 1-OH pyrene is the only major intermediate of pyrene biotransformation (Lin *et al.,* 1994). Conjugated 1-OH pyrene is the predominant metabolite in the bile of fish exposed to PAH with 4 or more rings (Krahn *et al.,* 1987, Ariese *et al.,* 1993, Lin *et al.,* 1994) and constitutes the vast majority of 1-OH pyrene equivalent fluorescence in pyrene exposed crab urine (see chapter 3). Due to the differing optimal wavelengths for different

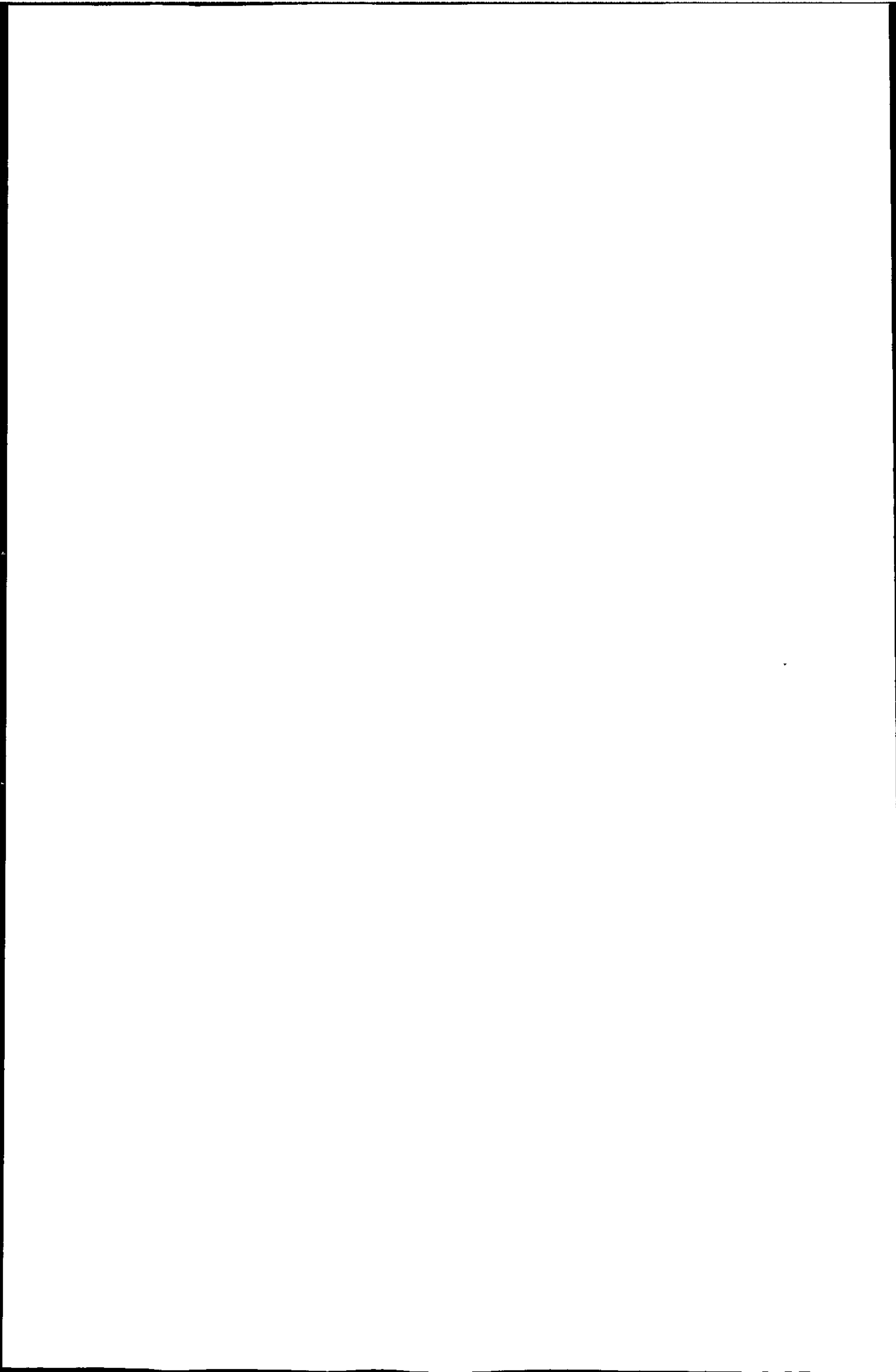
metabolites, selected wavelength pairs (FF or SFS) might only detect a small proportion of all metabolites in a sample. When the majority of metabolites are of one type, as in the case of the 1-OH pyrene chromophore, most of the potential signal is measured (Lin *et al.*, 1994), giving rise to strong fluorescent signals on sample spectra.

Clearly, it would be advantageous for the metabolite profile of the urine to be resolved using HPLC/F, as has been done previously. Identifying the metabolites eliminated in the urine would provide invaluable information on the bioavailability of PAH to *C.maenas* in Karmsund strait. Regrettably, such analysis was beyond the scope of this PhD. However, it is likely that exposure to pyrene in smelter discharges would result in the elimination of pyrene metabolites of the kind identified in chapter 3.

Urine data showed large inter-individual variability, increasing with proximity to the smelter. This phenomenon was also a feature of the results obtained from lab exposed crabs in chapter 2 and is reported for levels of pyrene metabolites in the haemolymph of Dungeness crabs collected from near an aluminium smelter in Kitimat Arm, BC (Eickhoff *et al.,* 2003a). These authors suggest that the variability in their data might result from the random deposition of PAH contamination (soot, coal dust, tar balls, spillage of pitch) within Kitimat Arm. Levels of exposure at each site in Karmsund Strait may not be completely homogeneous either. Random distribution and/or localised movements of individual *C.maenas* would therefore reflect any differing sediment PAH concentrations at each site. Variability in the rate and degree to which individual crabs take up, metabolise and excrete bioavailable PAH may also be responsible. Such variation is genetically predetermined and part of the general variability seen in any species. Variation in rate and degree of metabolism will be largely due to the level of induction and efficacy of the appropriate p450 enzymes. Considerable interindividual variation is seen in cytochrome p450 content of hepatopancreas microsomes from feral crustacean species, ranging from 0.5 to 1.3nmol mg^{-1} in spiny lobster (see James and Boyle, 1998 for review). These authors

report that such variation may be related to hormonal, nutritional or moulting status. Indeed, subtle differences in moult stage between crabs may prove to have considerable influence, since ecdysone is a competing substrate for cytochrome p450, which converts it to the active moulting hormone 20-hydroxyecdysone (James and Boyle, 1998, Mothershead and Hale, 1992).

Mussel body burdens are over two orders of magnitude higher than those of edible crabs at the same sites (µg/kg w.w). In terms of PAH profile, data from edible crab is more similar to shore crab urine data. Whilst the gradient exists for total PAH and 4-6 ring PAH, contributions from 2-3 ring petrogenic PAH also reveal a gradient, albeit less marked (figure 4.7). In mussels, although a southward gradient does exist for 2-3 ring PAH (figure 4.5) it is dwarfed by extremely high levels of 4-6 ring PAH. Pyrogenic PAH are highly elevated in sediment around the smelter (figure 4.3), and therefore it is unsurprising that the biota contain high levels of these compounds. However, this alone cannot account for the enormous difference between levels observed in mussel and crab. A possible explanation might be due to the greater lipophilicity (K_{ow}) of the higher molecular weight PAH which have a greater tendency to bioaccumulate (McElroy *et al.,* 1989, Lee, 1984). While 4-6 ring compounds bind to lipid rich tissues following uptake, the more soluble, less hydrophobic 2-3 ring PAH are more effectively eliminated by mussels. This has been shown in recent laboratory exposure studies with *M.edulis* (Rantamäki, 1997). Following exposure, elimination of naphthalene and methylnaphthalene was very rapid (<24hrs) whereas clearance of fluoranthene and pyrene from tissues was still not complete after 2 weeks (Rantamäki, 1997). The author attributes this result to the higher relative solubility and volatility of naphthalene and methylnaphthalene. An organism's capacity to biotransform accumulated PAH is also very important and plays a major role in influencing its body burden. Suspension feeding bivalves such as *M.edulis* filter large volumes of ambient seawater over their gills and as a result, bioaccumulate lipophilic organic pollutants such as PAH (Baumard *et al.,* 1998, DAdamo *et al.,* 1997, Widdows and



Donkin, 1992). This, combined with much slower rates of metabolism and elimination compared to fish, crustacea and polychaetes (Farrington, 1991, Baumard *et al.*, 1998) results in highly elevated PAH body burdens in mussels inhabiting contaminated waters (Baumard *et al.,* 1999). It has also been suggested that larger, more electronegative PAHs are more readily biotransformed in *M.edulis* than the smaller, less reactive 2-3 ring structures (Hellou *et al.*, 2002, Varanasi and Stein, 1991).

Another possible explanation is the nature of PAH/particulate associations. It has been stated previously (section 4.1.2) that pyrogenic PAH from smelter discharges are associated with soot like particles (Knutzen, 1995, Paine *et al.,* 1996, Naes *et al.,* 1999). Filter feeding bivalves such as *M.edulis* are exposed to both truly dissolved and particulate forms in the water column (Baumard *et al.,* 1999) and are therefore highly likely to bioaccumulate such PAH to high levels. Clearly, exposure to contaminants from different environmental compartments will result in different organism contaminant residues (Baumard *et al.,* 1999).

In the edible crab, high levels of fluoranthene, pyrene, benzo(a)anthracene and chrysene in Hogevarde and Austvik are consistent with the smelter derived pyrogenic source of PAH. However, crabs tissues are also enriched with phenanthrene and alkylated phenanthrenes (particularly C1) at these sites. Mussel tissues also contain higher levels of parent and alkyl substituted phenanthrenes, although proportionally their levels are much lower. Thirdly, broad fluorescence on urinary spectra suggests contributions from 3-ring PAH. Whilst smelters are characterised by their high temperature generation of 4-6 ring pyrogenic PAH, high temperature combustion has also been shown to produce 3-ring phenanthrenes in comparable amounts to fluoranthene, pyrene and chrysene (Law and Biscaya, 1994, Readman *et al.,* 1996). Smelter effluents contain substantial amounts of low-molecular weight PAH Naes *et al.,* (1998b) and Søderberg electrodes in particular are responsible for prominent discharges of phenanthrene (Solbakken and Palmork, 1981,

OSPAR, 2002). Recent studies have demonstrated elevated phenanthrene and alkylated phenanthrenes in sediments (Naes and Oug, 1997, Simpson *et al.*, 1996, 1998), crabs (Eickhoff *et al.*, 2003b) and mussels (Naes *et al.*, 1995) near aluminium smelters. Phenanthrene levels were in fact greater than higher molecular weight PAH in tissues of Dungeness crab (*Cancer magister*) in Kitimat Arm (Eickhoff *et al.*, 2003b). These authors suggest the greater water solubility of the lower molecular weight PAH makes them more available in the water column, increasing exposure and potential for bioaccumulation. In the present study, emissions from the older Søderberg technology at HAK (OSPAR, 2002) have clearly contributed phenanthrenes to the recipient waters around the smelter, based on the residue data in sediment and organisms.

One interesting aspect of the crustacean data deserves mention here, as it highlights an important point worth considering for monitoring purposes. While levels of PAH were undetectable in mussels or sediment at the reference site of Krokanes, mean ΣPAH in edible crabs was 24.7μg/kg and urinary 1-OH pyrene equivalents were detectable in the urine of shore crabs. This result suggests that edible/shore crab populations within Karmsund strait might be more mobile than was first thought, since PAH residues in sediments and mussel suggests contamination from the smelter does not extend to this site. This may be at least partly responsible for the variability observed in the urinary results, since mobile individuals may not be restricted to specific locations and are therefore exposed to PAH to varying degrees. This would appear to contradict suggestions made in the introduction regarding the suitability of *C.maenas* as a sentinel species, but in this authors opinion does not preclude its use for biomonitoring, since a clear gradient of exposure to bioavailable PAH has been identified.

No statistically significantly differences in haemolymph carboxylesterase activity or total protein levels were observed between sites, despite increased CbE activity in Hogevarde and Austvik crabs. Elevated activity might be expected in response to increased

levels of hydrophobic organic xenobiotics, since it has been reported that carboxylesterases have a role to play in their detoxification (Barron *et al.*, 1999, Galloway *et al.*, 2002a). Conversely, it has been suggested that exposure to pyrogenic PAH can decrease esterase (including CbE) activities in *Carcinus* species (Fossi *et al.*, 2000). Haemolymph total protein is also reportedly affected by contaminant stress (Fossi *et al.*, 1997, Depledge *et al.*, 1995), and has been shown to decrease following exposure to B(a)P (Fossi *et al.*, 1997). However, no such results were observed in the present study.

Measurement of resting heart rate revealed that crabs from Hogevarde were significantly different ($p<0.05$, KW) from Forlandsfjorden crabs. Increased heart rate following laboratory exposure to a pyrogenic PAH has been previously reported (Fossi *et al.*, 2000), but to this authors knowledge has not been observed in a chronically PAH exposed field population. Significant increase in crab heart rate ($p<0.05$, KW) from the copper impacted site at Visnes also corroborated results seen in exposures to copper in the laboratory (Bamber and Depledge, 1997a, Lundebye and Depledge, 1998). These results indicate that contaminant loadings at these two impacted sites are having detrimental effects on this important physiological process in resident crab populations. Whether this will directly affect survival is hard to establish, but it is fair to assume that pollution induced physiological impairment in a significant proportion of a population is likely to have direct relevance on the population as a whole (Depledge *et al.*, 1995).

Evidence of PAH exposure in Karmsund strait is now well established in shore crabs and edible crabs (this study), mussels (this study), periwinkle (Naes *et al.*, 1995), Atlantic cod (Aas *et al.*, 2001, Beyer *et al.*, 1998) and corkwing wrasse (Aas *et al.*, 2001). There is also a large body of evidence for exposure of biota in other fjordal systems and coastal waters impacted by aluminium smelter discharges (Beyer *et al.*, 1996, Paine *et al.*, 1996, Eickhoff *et al.*, 2003a,b, Naes *et al.*, 1999, Naes *et al.*, 1998a, Naf *et al.*, 1994, Axelman *et al.*, 1999). The PAH levels in these bioindicator organisms largely correlate

well with sediment levels. Several authors have suggested however, that PAH derived from smelter discharges has limited bioavailability, due to its association with carbon-rich, soot like particles (see 4.1.2), and therefore has little biological effect on biota (Naes *et al.,* 1999, Paine *et al.,* 1996, Knutzen, 1995). There is, however, some disagreement in the literature. Whilst bioavailability might be limited after deposition in sediments, it seems that particle-associated PAH in the water column is highly bioavailable to fish (Beyer *et al.,* 1998). Paradoxically, effects seem to be negligible in some studies. For example, Naes *et al.,* (1999) report that despite increased levels of PAH metabolites in the bile of cod and flounder collected near an aluminium smelter in a Norwegian fjord, hepatic DNA adducts were below the limits of detection. Knutzen *et al.,* (1995) report that fish caught in PAH impacted waters in Norway showed no evidence of tumours. Conversely, Aas *et al.,* (2001) report higher levels of hepatic DNA adducts in cod and wrasse collected near Hydro Aluminium Karmoy compared to reference sites. Such conflicting results are surprising, given the well-documented incidence of neoplasia, DNA adducts and cancerous tumours in PAH-exposed fish in previous studies (Krahn *et al.,* 1986, Malins *et al.,* 1988).

Another reason for apparent lack of effects in indicator organisms could be their metabolic capacity. The limited capacity of invertebrates, particularly molluscs, to biotransform PAH protects them from the carcinogenic and mutagenic metabolites formed during phase I and II reactions (Knutzen, 1995). Indeed, many invertebrates, including molluscs and crustaceans accumulate PAH without obvious detrimental effects (Naes *et al.,* 1995, Law and Biscaya, 1994, Paine *et al.,* 1996). In such instances, evidence of exposure by elevated body burdens or equivalents in the urine is not a necessary indication of deleterious effects. PAH do however have the potential to cause adverse affects in Crustacea and numerous studies are reported in the literature and have been previously discussed in chapter 2. Such studies highlight the risks PAH pose to the health of aquatic organisms such as Crustacea.

Similar to the situation in fish, studies on community structure and benthic diversity in PAH-impacted Norwegian fjords have provided contrasting results. In some studies, significant species changes in soft-bottomed macrofauna were found along PAH gradients, associated with feeding mode (Oug *et al.,* 1998). Greatly reduced benthic diversity is observed in the immediate vicinity of two Norwegian smelters (Lista and Årdal) although the authors admit to difficulties in attributing these effects solely to PAH discharges from the smelters (Knutzen, 1995). Conversely, Paine *et al.,* (1996) report that despite highly elevated sediment concentrations (<10,000mg/kg total PAH) within 1km of the smelter, no significant effects on benthic communities could be observed. These authors postulate that since such levels have previously been shown to cause significant effects on benthic community structure, that the bioavailability of the PAH must be limited, consistent with the soot-particle association hypothesis. It seems PAH from smelter discharges can cause deleterious effects in some cases, but not to the extent expected considering levels in sediments (Knutzen, 1995).

4.5 Conclusions

Elevated levels in invertebrate tissues and crustacean urine in the present study suggests particle-associated PAH in Karmsund is still bioavailable to these organisms. It is possible that this is only a fraction of the PAH which could potentially be available, but does not alter the fact that biota are exposed. In this respect, the urine assay has identified levels of exposure at each site, which is a function of the bioavailable portion of the smelter discharges. The urine assay has also illustrated that a distinct gradient of exposure to PAH exists within the fjord. Aside from heart rate data obtained from Hogevarde crabs, no biomarkers of effect were measured in *C.maenas* and any adverse effects of PAH exposure cannot be discussed. More studies are therefore needed to determine the relevance of PAH exposure to the overall health of crustaceans in Karmsund strait and other fjordal systems.

Importantly, this work has shown that the responses of *C.maenas* following exposure to PAH in the field are comparable to those measured in the laboratory (dose dependent uptake, phase I/II biotransformation and elimination). Despite the presence of a multitude of environmental contaminants (include numerous PAH) and naturally fluctuating environmental conditions, *C.maenas* is able transform and excrete environmental PAH effectively following exposure.

Chapter 5: Understanding organismal responses following exposure to multiple contaminants.

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5.1 Introduction

The present chapter uses biomarkers to investigate the responses of *C.maenas* following exposure to mixtures of contaminants. As discussed in Chapter 1, natural aquatic environments are contaminated with numerous different classes of contaminants and exposure to single contaminants in isolation rarely occurs (Moore, 2002). Knowledge of how contaminants in such mixtures influence each other's uptake, biotransformation and toxicity is lacking (Moore, 2002), and a present priority requirement for ecotoxicologists is to assess the risks presented by combinations of chemicals (Walker, 1998). Most chemical toxicity tests and many biomarker studies have been conducted using single contaminant exposures, increasing our knowledge of specific single biomarker responses (Livingstone, 1993). In contrast, little is known regarding the behaviour of biomarkers and the responses of organisms when exposed to more than one contaminant, particularly in natural populations subject to fluctuating environmental conditions.

Simulating environmentally realistic conditions in the laboratory in order to investigate such problems is fraught with difficulty and in practical terms is often not feasible. It is impossible to represent every single possible class of environmental contaminant and reproduce natural fluctuations in photoperiod, water temperature, salinity, predator stress, food availability, contaminant distribution and availability. However, the presence of more than one contaminant is an improvement on the single chemical exposure approach and a step towards more realistic environmental conditions (Verrhiest *et al.*, 2001). Such a solution can involve applying a suite of biomarkers to a multi-contaminant system in the laboratory. The biomarkers chosen can be used to detect the integrated impact of the contaminants and to reveal any possible changes in response that could be indicative of exposure to contaminant mixtures. Which, if any, of the observed processes will be compromised for the maintenance of another? The use of a suite of biomarkers also allows evaluation of the validity of each response in a multi-contaminant system. Can

biomarkers designed to reveal exposure to or the effects of one specific contaminant still provide such relevant information when other contaminants are present?

In recent years, authors have started to address the problems associated with complex mixtures (e.g. Viau, 2002, Klerks, 1999) and propose explanations for the results obtained in biomarker studies that use them. Viau (2002) suggests that when interactions occur, they often result from competition between two or more substances for the same biotransformation enzymes. Using exposure to PAHs and metals, Klerks (1999) demonstrated that acclimatisation to contaminant stress in the grass shrimp (*Palaemonetes pugio*) becomes less likely as the number of contaminants increases. The author offers several mechanistic explanations for this, suggesting that one contaminant may inhibit the detoxification of another, or that the energetic requirements (for repair/detoxification) for exposure to one contaminant may compete with those for another.

The work in the present chapter is of a similar nature and aims to determine, using biomarkers, the nature of *C.maenas'* responses to multiple contaminants. For example, MT and the PAH exposure biomarker can be applied to observe the responses of crabs exposed to both a trace metal and a PAH. Will MT levels fall to allow metabolic energy to be shunted to the metabolism and excretion of the PAHs, or will PAH metabolism be less of a priority than chelating the offending metal? Similarly, is the energetic investment in antioxidant systems maintained to minimise oxidative stress, or is the detoxification and excretion of oxyradical generating pollutants increased to reduce oxidative damage? The experiments investigate if the level of genotoxic damage is altered by the presence of more than one contaminant, or if DNA repair mechanisms are sacrificed for the maintenance of p450 systems. In the case of organophosphorous pesticide exposure, the use of biomarkers aims to determine if an additional organic contaminant, competing for biotransformation enzymes, alters the level of PAH metabolism and concentrations of excreted PAH equivalents. Alternatively, is it possible for PAH induced increases in levels of detoxifying

CbE enzymes to compensate for the inhibition of esterase activity by a pesticide? The experiments will also investigate alterations in biomarker responses upon exposure to more than one contaminant. For example, to see if the CbE assay still provides meaningful information when an organism has been exposed to more than just an esterase inhibiting pesticide.

The experiments described below aim to address such questions by investigating any inter-relationships between biomarker responses following exposure to combinations of three specific contaminants (PAH, metal and pesticide). Biomarkers for exposure to PAH and esterase inhibitors (CbE) have been described previously. Also employed are biomarkers of metal exposure, genotoxic damage and antioxidant stress, namely metallothionein induction, frequency of micronuclei in haemocytes and total antioxidant status. Total haemolymph protein is included as one indicator of physiological health.

5.1.1 Metallothionein

Metallothioneins (MTs) are widely distributed low molecular weight (6-8Kda for vertebrates), cysteine-rich (20-30%), inducible metal binding proteins, present in most if not all animals (Viarengo *et al.*, 1997, George and Olsson, 1994). MTs are thought to have several functions due to their high metal affinity (7-9g atom mol^{-1} thionein) (Viarengo *et al.*, 1999) and ability to chelate essential (Zn, Cu) and non-essential metals (Cd, Hg, Ag). These functions include intracellular regulation of endogenous metals and detoxification of excess levels of pollutant metals (Roesijadi, 1992). Other important roles include general stress responses e.g. temperature stress and also free radical scavenging (Kling *et al.*, 1996, Livingstone, 1993). MTs are inducible proteins (Viarengo *et al.*, 1999). Increased heavy metal concentration in cells stimulates *de novo* synthesis of MTs by metal–mediated transcriptional activation of MT genes (Roesijadi, 1992). MTs then bind the free metal cations in a non-toxic form, reducing their deleterious effects (Roesijadi, 1992). The dual roles of MTs result in their being regarded as two intracellular pools represented by basally

synthesised proteins involved in essential metal regulation and induced proteins involved in metal detoxification (Engel and Roesijadi, 1987). MTs are induced in response to an increase in cellular concentrations of metal ions and this forms the basis for the use of this biomarker of metal exposure (Viarengo *et al.*, 1999, Roesijadi, 1992). In molluscs, particularly mussels, MTs are involved specifically in responses to metals and induction is considered a biomarker of exposure to heavy metal pollution (Viarengo *et al.*, 1997). MTs have also been used as biomarkers in crustaceans such as *C.maenas* (Legras *et al.*, 2000, Astley *et al.*, 1999, Pedersen *et al.*, 1997, Pedersen and Lundebye, 1996) and Cd, Zn and Cu have been shown to induce the same identical MT isoform MT1a (Pedersen *et al.*, 1998).

MT synthesis can be induced by conditions other than elevated metal concentrations and their levels are not solely influenced by exogenous metal burdens. In rainbow trout, MT levels fluctuate according to the reproductive status of the individual (Olsson *et al.*, 1987) and glucocorticoids have been shown to induce MT synthesis (Olsson *et al.*, 1990). Various kinds of stress such as cold, heat and extreme exercise can stimulate MT synthesis (Viarengo *et al.*, 1999) and laboratory handling stress has been shown to do the same (Baer and Thomas, 1990). Certain polar organic contaminants e.g. paraquat, may also induce metallothionein (Baumann *et al.*, 1991). MT levels in mussel tissues can be influenced by environmental fluctuations (temperature, oxygen and salinity) and have shown seasonal variations (Viarengo *et al.*, 1997). Although it is generally accepted that metal contamination of the environment is associated with MT induction in organisms, the above illustrates that caution should be exercised when interpreting MT data for evidence of metal exposure in biomonitoring programs (Mouneyrac *et al.*, 2002, Legras *et al.*, 2000, Viarengo *et al.*, 1997). The choice of tissue for MT determinations is also important as levels of MT in certain tissues reflect exposure to trace metals more strongly than others. For example, MT concentrations in the midgut gland of *C.maenas* from the chronically copper-contaminated Fal estuary were shown to reflect the gradient for this metal only

weakly, whilst MT levels in the gills correlated well with copper exposure (Pedersen *et al.*, 1997). For this reason, the present work uses levels of gill MT to evaluate exposure of *C.maenas* to copper.

5.1.2 Frequency of micronuclei: a biomarker of genotoxic damage

Micronuclei are small intracytoplasmic masses of chromatin with the appearance of small nuclei, arising at cell division from chromosomal breakages (Burgeot *et al.*, 1995). Other micronuclei arise from chromosome lagging at anaphase, due to spindle dysfunction (Wrisberg and Rhemrev, 1992). Counting cells with micronuclei to evaluate cytogenetic damage was initially developed in dividing mammalian cells (Schmid, 1975) and frequency of micronuclei has proved to be a reliable indicator of chromosomal mutations due to aneugenic and genotoxic pollutants (Burgeot *et al.*, 1995).

A genotoxin is defined as a substance that interacts with genetic materials such as chromosomes and DNA (Newman, 1998). Examples include PAHs, aflatoxin and vinyl chloride. In the case of PAH, oxidative metabolism produces reactive intermediates that elicit the genotoxic effects (Walker *et al.*, 2001). Exposure to benzo(a)pyrene increases the frequency of micronuclei in mussel haemocytes (Venier *et al.*, 1997) and metals have also been shown to cause genotoxic damage (Bolognesi *et al.*, 1999). Incidence of micronuclei is frequently used to evaluate the genotoxicity of chemicals to aquatic organisms in the laboratory and genotoxic impacts of contaminants in the field. Induction of micronuclei in fish is a widely used cytogenetic assay (Rao *et al.*, 1997). It has also been extensively used in haemocytes and gill cells of many mollusc species, with micronuclei frequency in bivalves such as the oyster *Crassostrea gigas* (Burgeot *et al.*, 1995), the Mediterranean mussel *Mytilus galloprovinciallis* (Scarpato *et al.*, 1990) and the blue mussel *M.edulis* (Scarpato *et al.*, 1990, Wrisberg and Rhemrev, 1992, Bolognesi *et al.*, 1999) used to investigate the effects of genotoxins in the laboratory and the field. The assay has also been

applied to crustaceans (*Carcinus* spp) in gill cells (Fossi *et al.*, 1997) and haemocytes (Fossi *et al.*, 2000) in biomarker studies.

5.1.3 Antioxidant status

Xenobiotics have the potential to cause oxidative stress (an increase in free oxyradicals) and oxidative damage (deleterious interactions with biomolecules by oxyradicals) in the cells and tissues of aquatic organisms (Newman, 1998). Aerobic metabolism generates free radicals such as the superoxide radical ($O2^{\bullet -}$) and the hydroxy radical ($OH^{\bullet}$) that can damage proteins, lipids, DNA and other biomolecules (Newman, 1998). Xenobiotics may cause oxidative stress indirectly, by interfering with antioxidant defence systems and can also participate directly and produce oxyradicals themselves. For example, phase 1 biotransformation of PAHs produces free radical species (Livingstone *et al.*, 1990) and has been shown to cause oxidative damage in mussels (Cheung *et al.*, 2001). PAH and other organic contaminants such as paraquat can induce massive free radical damage as they undergo redox cycling (Di Guilio *et al.*, 1989); they are first reduced to free radical species by single electron reduction (via P450 reductase) and then donate their electron to molecular oxygen, producing superoxide and regenerating the parent compound. As the contaminant exits the cycle in its original form it is available to recycle many times and produce large amounts of oxyradicals (Newman, 1998). Copper exposure has also been shown to increase lipid peroxidation (attack of lipids, in particular those of cell membranes, by free radical species) in mussel (*M.galloprovincialis*) tissues (Viarengo *et al.*, 1990).

Changes in antioxidant enzymes (e.g. superoxide dismutase, catalase or glutathione peroxidase) associated with elevated levels of oxidative stress caused by xenobiotic contaminants can be useful biomarkers of exposure to such compounds (Torres *et al.*, 2002). Total antioxidant levels of specific tissues, biological fluids or whole body homogenates can also be used as an indication of oxidative stress. The total oxyradical

scavenging capacity (TOSC) assay measures the biological resistance to various kinds of oxyradicals, providing useful indications to predict oxyradical-mediated adverse effects on the physiological condition of organisms (Regoli, 2000). Total antioxidant status is another parameter that can be applied to the same ends and is measured in the current study using a commercial assay kit.

5.1.4 Choice of contaminants

The contaminants chosen were representative of three classes of chemical, namely PAHs, trace metals and pesticides.

Pyrene was chosen as the model PAH as extensive work has been carried out using this compound previously and its metabolite pattern in *C.maenas* following laboratory exposure has been well documented (see previous chapters). Pyrene is invariably present in environmental PAH mixtures (Lin *et al.*, 1994), and is particularly abundant in areas subject to pyrogenic PAH contamination (Naes *et al.,* 1999). Detection of pyrene-type metabolites in biota can be used as a surrogate measure of exposure to benzo(a)pyrene (Lin *et al.*, 1994), an EU priority pollutant and potent vertebrate carcinogen following bioactivation (IARC, 1983).

The organophosphate pesticide chlorfenvinphos was chosen as a test compound because it is a priority pollutant under current EU legislation and is used extensively in the UK for the control of cabbage root fly (DEFRA, 2002). Recent public concerns regarding the possible human health and environmental hazards associated with the use of OP pesticides in general has prompted reviews into the use of these chemicals in the UK (Thompson, 2002). As a result many OP pesticides have been withdrawn from use in the UK, although chlorfenvinphos has been granted an extension of approval for its use on swedes and turnips until July 2003 (Thompson, 2002). Chlorfenvinphos is relatively resistant to hydrolysis and volatilisation and this results in an aquatic half-life of several

days to weeks (WHO, 1972). Consequently, residues have been detected in the environment, with levels as high as 31.6μgl^{-1} reported in river basin surface waters (Cerejeira *et al.*, 2003). It has also been shown to bioconcentrate in and cause adverse effects in marine mussels (*M. galloprovincialis* and *M.edulis* respectively) (Serrano *et al.*, 1995, Rickwood and Galloway, 2003). Investigation into its effects on non- target species is therefore desirable.

Copper is a trace metal contaminant commonly found in the environment (Bamber and Depledge, 1997a,b) and can reach high levels (<2500μg g^{-1}) in sediment of chronically polluted aquatic environments (Pedersen and Lundebye, 1996). Its effects on various processes in marine organisms, including crustaceans, are well documented and it has been used in numerous exposure and biomonitoring studies (Lundebye and Depledge, 1998, Bamber and Depledge, 1997a, Canli *et al.*, 1997, Pedersen *et al.*, 1997, Pedersen and Lundebye, 1996). Extensive background knowledge on this contaminant provides a good basis for comparing observed biomarker responses. Despite being an essential trace element in crustaceans and playing a key rôle in growth and oxygen transport, copper is also highly toxic to crustaceans (Hebel *et al.*, 1997), so investigations into organismal responses following exposure are highly valuable.

5.2 Materials and Methods

Urinary PAH equivalents, carboxylesterase inhibition, metallothionein, micronucleus, total antioxidant status and total haemolymph protein were measured in *C.maenas* following waterborne exposure to mixtures of copper, pyrene and chlorfenvinphos. These biomarker assays are described below. Assays described previously are referenced.

5.2.1 Collection of experimental animals and laboratory conditions

Male, intermoult *C.maenas* (carapace width, 54-72mm) were collected using a baited drop-net on incoming tides from Jenkins Quay on the Avon estuary at Bantham, South Devon, England. On return to the laboratory, they were maintained in holding tanks containing filtered (10μm carbon filtered), well aerated 34ppt, 15+/-1°C seawater, under a 12h light: 12h dark regime for one week to acclimatise prior to experiments. Crabs were fed twice weekly to satiation with irradiated cockles. Water was changed within 12h of feeding.

5.2.2 Exposure chemicals

Pyrene (98%, cat. no: 18, 551-5) and copper chloride ($CuCl_2$) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Chlorfenvinphos (trade name Sapecorn®) was obtained in the form of an emulsifiable concentrate (90% v/v chlorfenvinphos) from Ciba-Geigy Agrochemicals (Cambridge, UK). Acetone (GPR™) was obtained from BDH Laboratory Supplies (Poole, Dorset, UK).

5.2.3 Exposure experiments

Crabs were exposed to a combination of waterborne contaminants; a PAH, pyrene, a metal, copper and a pesticide, chlorfenvinphos, in three different treatment sets and at

two different nominal concentration ranges ([high], and a more environmentally realistic [low]) for a total of 14 days. The high exposure concentrations were 100μg l^{-1}, 1mg l^{-1} and 1μg l^{-1} and the low exposure concentrations were 10μg l^{-1}, 0.1mg l^{-1} and 0.1μg l^{-1} for pyrene, copper and chlorfenvinphos respectively. High and low exposures were run consecutively. For both high and low exposure trials, treatment 1 was pyrene singly, treatment 2 was pyrene and copper and treatment 3 was pyrene, copper and chlorfenvinphos. Solvent (acetone) and seawater controls were run alongside these three treatments in both trials.

Crabs were transferred to glass aquaria containing 10L of filtered (10μm carbon filtered), well-aerated seawater (34ppt, 15+/-1°C), under a 12h: 12h light: dark regime. For both high and low exposure trials, animals were randomly assigned to one of 10 groups of four in separate aquaria, with a total of 8 crabs exposed to each of the five treatments. Animals were fed twice a week with irradiated cockle during the exposure period. Exposure water was changed and re-dosed within 12h of feeding.

5.2.4 Waterborne exposure of contaminants

To yield the nominal concentrations above, pyrene was added to seawater in an acetone carrier (at a ratio of 1:1, w:v, pyrene/acetone), copper was added as a solution of copper chloride and chlorfenvinphos was added as a commercial formulation using a solution diluted from a stock solution (240mgl^{-1}) supplied by the manufacturer. Solvent controls were exposed to acetone only, equivalent to the amount used to deliver pyrene to each treatment group. Controls were not exposed to any waterborne chemicals. In order to limit the volatalisation of contaminants, and in particular the release of pesticide vapour, tanks were sealed with parafilm, following dosing of seawater.

5.2.5 Urine, haemolymph and tissue sampling

Urine and haemolymph samples were taken from each crab after 48h, 7 days and 14 days of exposure, using techniques described previously. Samples were expelled into siliconised microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C until analysis. Test animals were returned to their respective aquaria immediately after sampling.

For analysis of haemocyte micronuclei, an additional small sample of haemolymph (20μl) was also taken from each crab following 48hr, 7d and 14d sampling and spread evenly onto microscope slides coated with a 1:10 solution of poly-L-lysine (to encourage haemocyte adhesion).

At the end of the exposure period (14d), crabs were sacrificed by destruction of the thoracic ganglion and following removal of the carapace, their internal organs were sprayed with a protective buffer (pH 7.25, 50mM K_2HPO_4, 0.75M sucrose, 1mM EDTA, 0.5mM DTT, 400μM PMSF, 10μM leupeptin, 1μM pepsatin, $1mg^{-1}$ aprotinin) to avoid degradation of tissues by proteolytic enzymes. Gill and hepatopancreas were carefully dissected out and placed into siliconised centrifuge tubes containing small amounts of protective buffer. Samples were then frozen in liquid nitrogen and stored at -80°C until analysis.

5.2.6 Analysis of urine samples

Levels of pyrene equivalent fluorescence in urine samples were determined using both FF and SFS techniques. FF Ex345/Em382nm and SFSΔλ37nm (Em382nm) were used to detect pyrene equivalents. Slit widths for all analyses were 2.5nm and all urine samples were diluted 1:50. Urinary pyrene equivalent peaks (FFEx345/Em382nm, SFS Em382nm) were quantified with respect to a series of 1-OH pyrene standards (200, 100, 75, 50, 25, 10 and $5\mu gl^{-1}$), analysed at the fixed wavelength pair of Ex345/Em387nm (FF) and Em387nm

(SFS). Results are corrected for dilution and reported in terms of μgl^{-1} of 1-OH pyrene equivalents.

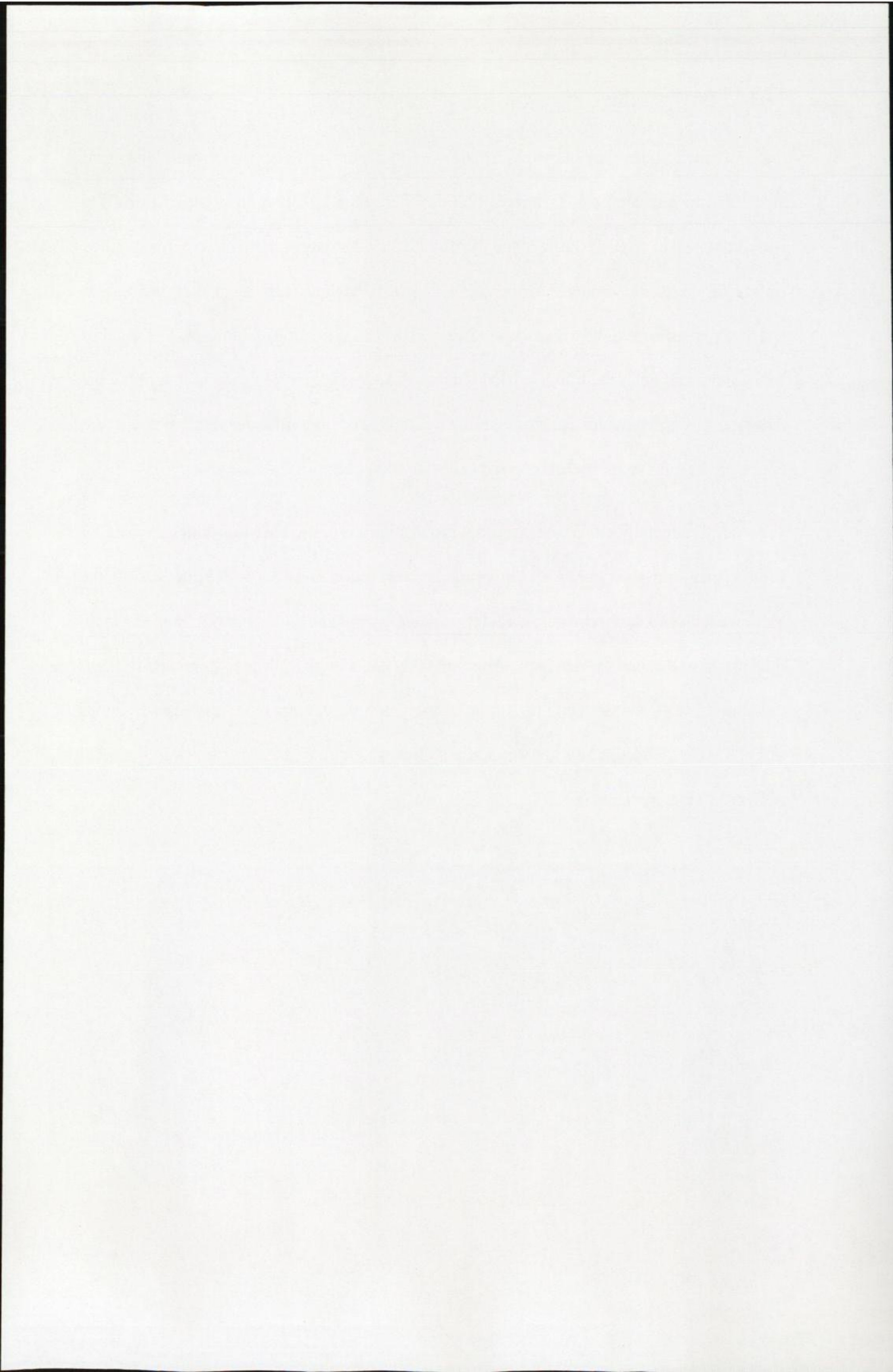
5.2.7 Carboxylesterase assay

Carboxylesterase activity in haemolymph samples was assayed as described in chapter 4. Results are expressed as μmol substrate hydrolysed min^{-1} mg^{-1} protein.

5.2.8 Total haemolymph protein

Total haemolymph protein was determined by the BioRad method with bovine serum albumin (BSA) as standard (0.21-1.4mg/ml). Haemolymph samples were thawed and centrifuged for 5 minutes at 10,000g to remove haemocytes and any clot formation. Samples were then diluted 1:50 with distilled water and vortex mixed. 1:50 dilution is based on the assumption that the average protein concentration of crab haemolymph is 40mg/ml. A 1:50 dilution would therefore yield a protein concentration of 0.8mg/ml from such a sample, which is well within the standard range.

A blank (20μl dH_2O), 20μl of each standard and 20μl of each diluted sample were then pipetted into a 1.5ml microcentrifuge tubes. 1ml of BioRad (Bradford) protein reagent, diluted 1:4 with dH_2O, was then added to each tube and all tubes were vortex thoroughly. Samples, standards and blank were left for 30 minutes at room temperature for colour development, after which 200μl aliquots of each were pipetted out in triplicate into appropriate wells of a 96 well microplate. Absorbance of all wells was then read at 595nm and samples were compared to the standard curve to yield protein concentrations in mg/ml. Values were multiplied by 50 to account for prior dilution. Any samples lying outside the standard range were analysed again, following greater or lesser dilution accordingly.



5.2.10 Total antioxidant status

Digestive gland (hepatopancreas) samples were thawed and homogenised on ice in 1 part wet weight of tissue: 3 parts homogenisation buffer (0.01M Tris-HCl pH 7.6, 0.25M sucrose, 0.03% Triton X-100 detergent). The homogenate was then sonicated for 15 seconds (40% power output) and centrifuged for 30 minutes at 15,000g, 4°C. The resulting supernatant was aliquotted into vials and stored at -80°C. Analysis was carried out within 2 weeks of crab sacrifice.

Total antioxidant status in digestive gland tissue was measured using a Total Antioxidant Status Assay kit (catalogue number 615700) supplied by Calbiochem (Calbiochem-Novabiochem Corporation, San Diego, CA). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2'-Azino-di-[3-ethylbenz-thiazoline sulphonate]) to $ABTS^{\bullet +}$ by metmyoglobin (a peroxidase). The amount of $ABTS^{\bullet +}$ produced can be monitored by reading the absorbance at 600nm. Under the reaction conditions used, the antioxidants in the sample inhibit the production of $ABTS^{\bullet +}$ (and therefore reduce the level of absorbance at 600nm) to a degree which is proportional to their concentration in the sample. The kit consists of buffer (phosphate buffered saline), chromogen (metmyoglobin and ABTS), substrate (stabilized hydrogen peroxide, H_2O_2) and standard (1.73mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The chromogen and substrate are diluted with buffer and the standard diluted with distilled water before use. Diluted chromogen and substrate were equilibrated to 37°C before running the assay.

The assay was run according to the kit protocol. Sample supernatant aliquots were thawed and 5μl was added in duplicate to the appropriate wells of a 96 well microplate. 5μl of distilled water (blank) and 5μl of standard were also added in duplicate to appropriate wells of the microplate and then 250μl of chromogen was added to all loaded wells. The plate contents were then thoroughly mixed and absorbance at time zero (A_0) was read at

600nm at 37°C using an Optimax microplate reader (Molecular Devices, Menlo Park, CA). 50μl of substrate was then added to each well and absorbance at 600nm was re-read after exactly 3 minutes at 37°C (A).

Antioxidant activity of the samples was then calculated using the equation below.

$$\textbf{Antioxidant activity (mM)} = \frac{\textbf{1.65mM*}(\Delta A\ \textbf{Blank} - \Delta A\ \textbf{Sample})}{(\Delta A\ \textbf{Blank} - \Delta A\ \textbf{Standard})}$$

ΔA = change in absorbance ($\Delta A = A - A_0$)

1.65mM = antioxidant concentration of the standard

5.2.11 Protein assay for digestive gland supernatant

Total protein concentration of digestive gland supernatant samples was measured using an adapted semi-microplate method based on the Hartree (1972) modification of the Lowry protein assay (Lowry *et al.*, 1951).

Supernatant samples were diluted 1:50 with distilled water, mixed well and 1ml of each diluted sample was added to a 5ml scintillation tube. 1ml of distilled water (blank) and 1ml of each of five standards (40, 80, 120, 160, 180μg/ml BSA in dH_2O) was also added to separate tubes. 0.9ml of reagent A (7mM sodium potassium tartrate, 0.81M sodium carbonate, 0.5M NaOH) was added to each tube, vortex mixed and incubated at 50°C for 10 minutes. Tubes were then cooled to room temperature and 100μl of reagent B (70 mM sodium potassium tartrate, 40 mM copper sulfate, 1N NaOH) was added to each tube and mixed. Tubes were incubated at room temperature for 10 minutes and then 3ml of reagent C (1 volume Folin-Ciocalteau reagent diluted with 15 volumes of distilled water) was added to each. Tubes were again vortex mixed and incubated at 50°C for 15 minutes. Tubes were then cooled to room temperature and 200μl was added in triplicate to appropriate wells of a 96 well microplate. Absorbance was read at 630nm. Results for

samples were compared with the standard curve and corrected for prior dilution to provide a value for total protein concentration (μg/ml or mg/ml).

5.2.12 Metallothionein

Metallothionein in gill samples was measured according to methods modified from Viarengo *et al.*, (1997) and Astley *et al.*, (1999). MT levels were quantified by using a partially purified metalloprotein fraction obtained by acidic ethanol/chloroform fractionation of the tissue homogenate. The assay avoids the oxidation of sulphydryl groups, contamination by low molecular weight thiols and enzyme protein degradation during sample preparation by using rapid extraction steps and the addition of a reducing agent (DTT) and an antiproteolytic agent (PMSF). In the final extract, the concentration of metallothionein, denatured by low pH and high ionic strength, is quantified spectrophotometrically following reaction with DTNB.

All equipment was thoroughly acid-washed prior to performing the assay. Pestles and mortars were cooled in a - 80°C freezer for 24 hours prior to use.

Sample preparation

Gill tissue was ground up in liquid nitrogen with an ice-cold mortar and pestle. 0.75g of tissue was then transferred into a 10ml beaker and 1.9ml of ice-cold DTT (dithiothreitol) solution and 22.5μl ice cold PMSF (phenylmethylsulphonyfluoride in ethanol) were added. The beaker was kept on ice and the contents were thoroughly mixed. The mixture was then sonicated (3 x 15 seconds, 40% duty cycle, output control 4) using a sonication probe and another 30μl ice cold PMSF was added. The beaker remained on ice whilst the contents were mixed. 2.5ml of tissue mixture was then transferred to Beckmann centrifuge tube and centrifuged at 55,000 rpm, 4°C for 70 min in a Beckmann TL100 centrifuge. 500μl of supernatant was then transferred to each of three microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C until MT purification.

MT purification

Samples were thawed and to each 500μl supernatant, 500μl of ice-cold (-20°C) ethanol and 40μl chloroform were added. Tubes were vortex mixed and then centrifuged at 7000 rpm for 10 min in a microcentrifuge. The supernatants were then poured into large labelled Nalgene centrifuge tubes and 3ml of -20°C ethanol was then added (pellets were discarded). Tubes were vortex mixed and then frozen (@-20°C) for 1 hour. Samples were then centrifuged at 6000g, 4°C for 12 minutes and the resulting supernatant was discarded. The pellet and sides of the tube were washed with 2x1ml of -20°C washing buffer (500μl chloroform, 6ml 20mM Tris-sucrose in 50ml of absolute ethanol). Tubes were vortex mixed and then centrifuged again at 6000g, 4°C for 12 minutes, after which the supernatant was poured away. Each pellet was then dissolved in 300μl Tris-EDTA buffer (5mM:1mM Tris-EDTA in dH_2O, pH 7) and vortexed thoroughly. 4.2ml DTNB solution (DTNB in Na_2-P buffer, 0.2M, pH 8) was then added to all samples and pre-prepared standards (GSH, glutathione in Tris-EDTA, 8 – 256$\mu g l^{-1}$), all tubes were vortex mixed and then left to incubate at room temperature for 15 minutes. During incubation, 200μl of each sample/standard was transferred in duplicate to appropriate wells of a flat-bottomed 96 well microplate. Absorbance was read at 412nm after exactly 15 minutes.

Results were expressed as μg MT/g wet weight of tissue, according to the following equation,

$$\text{Wet weight of tissue in sample (g/ml)} = \frac{\text{Initial weight of sample (g) x volume of sample purified (0.5ml)}}{\text{Initial volume of buffer added (1.9ml)}}$$

$$\text{[MT] (}\mu\text{g/g wet weight of tissue)} = \frac{\text{MT concentration from GSH standard curve (}\mu\text{g/ml)}}{\text{Wet weight of tissue in sample (g/ml)}}$$

5.2.13 Statistical analysis

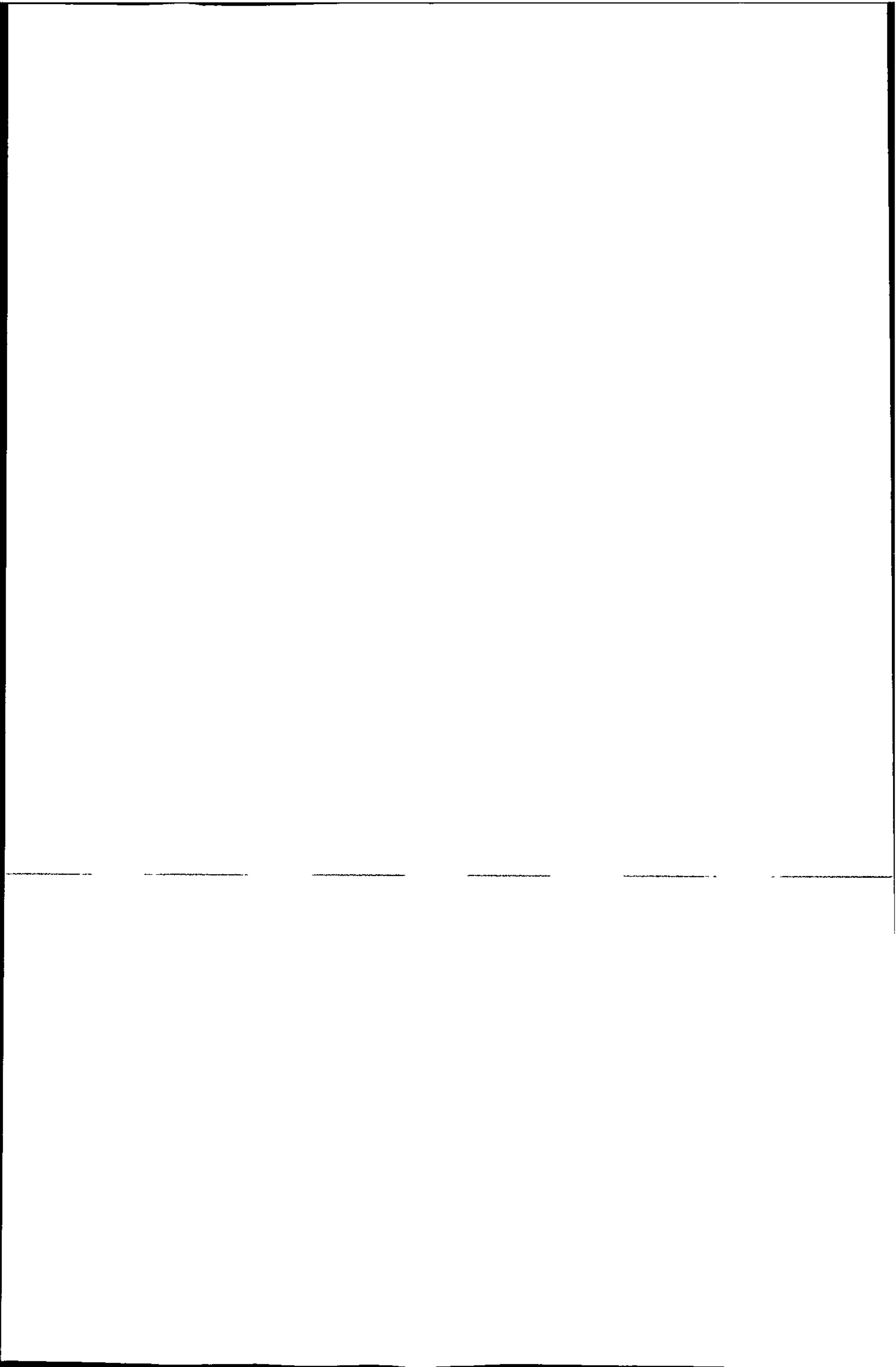
Data were analysed using Statgraphics Plus 5.1. Variance checks were carried out on all data sets. Normally distributed data were subject to a one-way analysis of variance (ANOVA) to compare means. Data not normally distributed were first log transformed and variance checked again. If transforming the data resulted in them being normally distributed then analysis was performed by one-way ANOVA. If data transformation did not improve the variance, data were analysed using the non-parametric Kruskal-Wallis test to compare medians.

Simple regression analysis was performed on all complete data sets to determine if relationships existed between biomarker responses at both exposure concentrations.

Additional analysis using the multivariate statistics program PRIMER 5.0 (Plymouth Routines In Multivariate Ecological Research) was carried out to observe if any relationships could be established between treatments, exposure concentrations and biomarker responses.

Due to the large differences in values between assays (for example, from 0.2mM/mg protein for total antioxidant concentration to 348µg/g wet wt tissue for MT) data were square-root transformed and standardised before analysis. A similarity matrix was first generated for each data set (48hr, 7d, 14d HIGH and 48hr, 7d, 14d LOW). Cluster analysis and MDS (Multi Dimensional Scaling), analyses that compare the level of similarity between the biomarker responses at each treatment, were then performed. Cluster analysis generates a similarity dendrogram based on how similar individuals are to each other. MDS generates a 2 dimensional map of the individuals, where the distance between any two individuals is proportional to the dissimilarity between them. Due to the nature of PRIMER analyses, the program stipulates complete individual data sets only, with no gaps (empty cells) in the data. The lack of urinary data for certain individuals (due

to empty antennal bladders) therefore meant that the data for the remaining biomarkers would have to be omitted, reducing replicate numbers for each treatment significantly. It was therefore considered judicious to omit the PAH urine data from the analysis. Due to the all-or-nothing nature of the urinary assay, inclusion of urine data in preliminary analyses polarised the results to such an extent that differences between PAH exposed individuals were obscured anyway. This result reinforced the decision to omit the urinary data in the final analysis.



5.3 Results

In order to display the full range of biomarker responses at each treatment, all data are presented as notched box and whisker plots.

5.3.1 Urinary PAH biomarker

There was no statistically significant difference (SSD) between results obtained by FF or SFS (KW, $p>0.05$). Results presented and described below are therefore only the SFS data (figures 5.2 and 5.3).

In both high and low exposure trials, 48h data show a statistically significant difference between pyrene-exposed crabs and controls in terms of urinary pyrene equivalent levels (low, KW $p=0.00026$, high, KW $p=0.00075$). No significant differences in urinary levels are apparent between the exposed treatments in each trial, aside from slightly elevated levels of the PAH and PAH/metal groups in the [high] treatment. After 7 and 14 days, one way ANOVA of log-transformed data shows a SSD between the PAH-exposed and control groups in both high and low exposure trials (log ANOVA $p=0.000$). No significant differences exist between the exposed treatments, regardless of the number of contaminants in the treatment, with the exception of the PAH/metal/pesticide group in the [high] trial which is statistically significantly higher than the PAH and PAH/metal group after 14 days (KW $p=0.0038$).

Throughout the exposure period, levels of equivalents are significantly higher in urine from crabs exposed to the high PAH concentration (48h KW, $p=1.1 \times 10^{-7}$, 7d and 14d log ANOVA $p=0.0$).

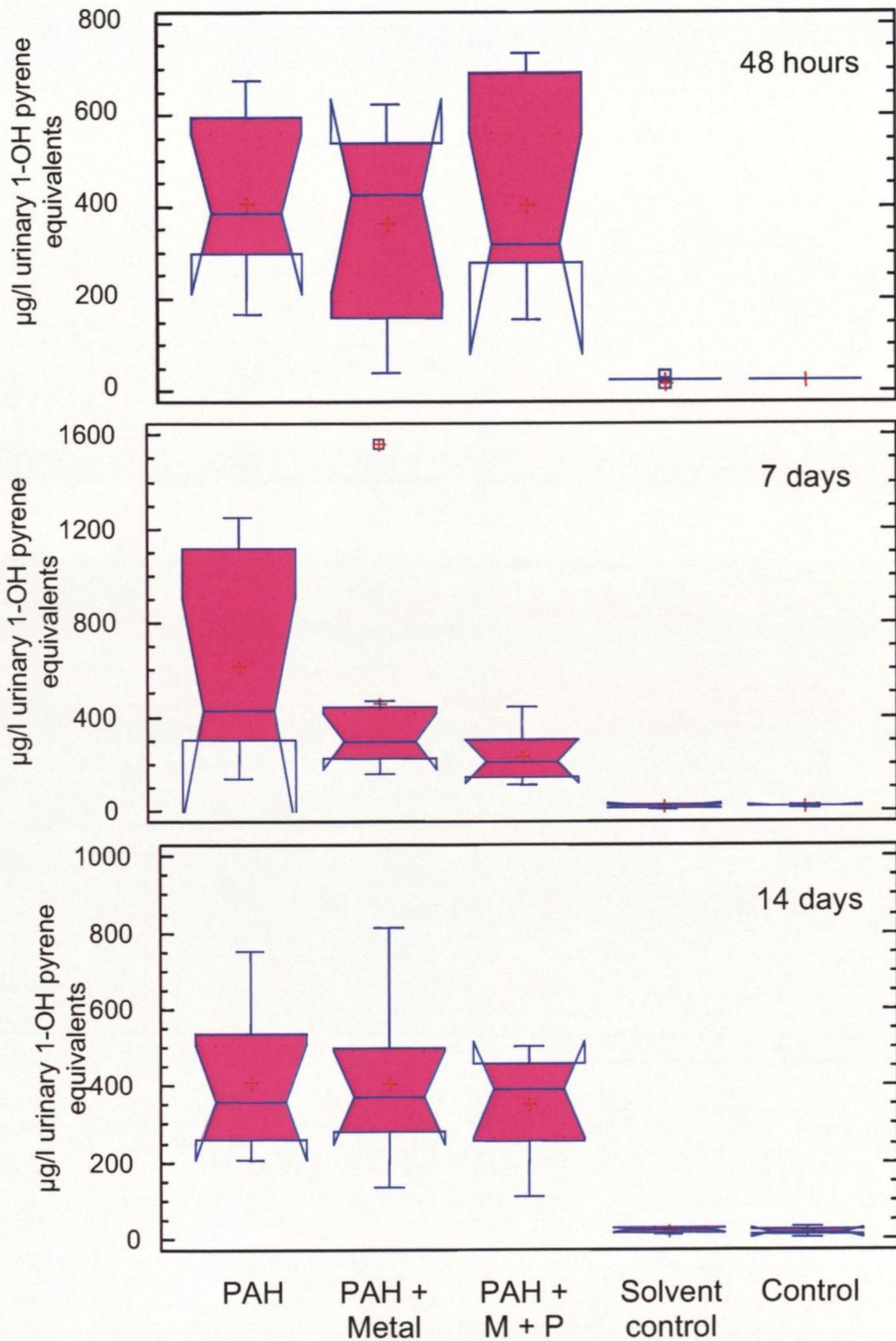
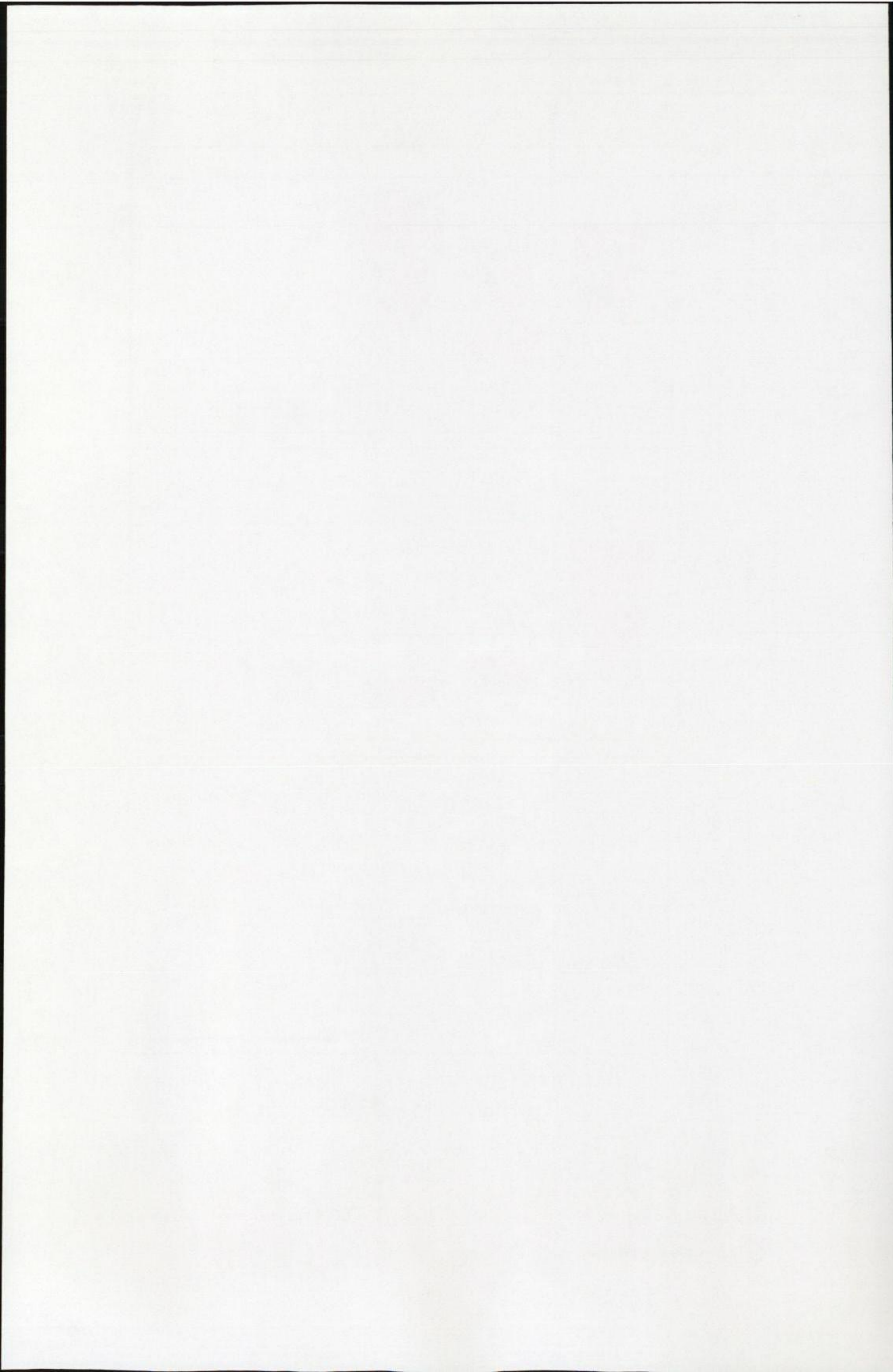


Figure 5.2 Notched box and whisker plots of urinary levels of 1-OH pyrene equivalents from crabs exposed at the LOW exposure concentration.



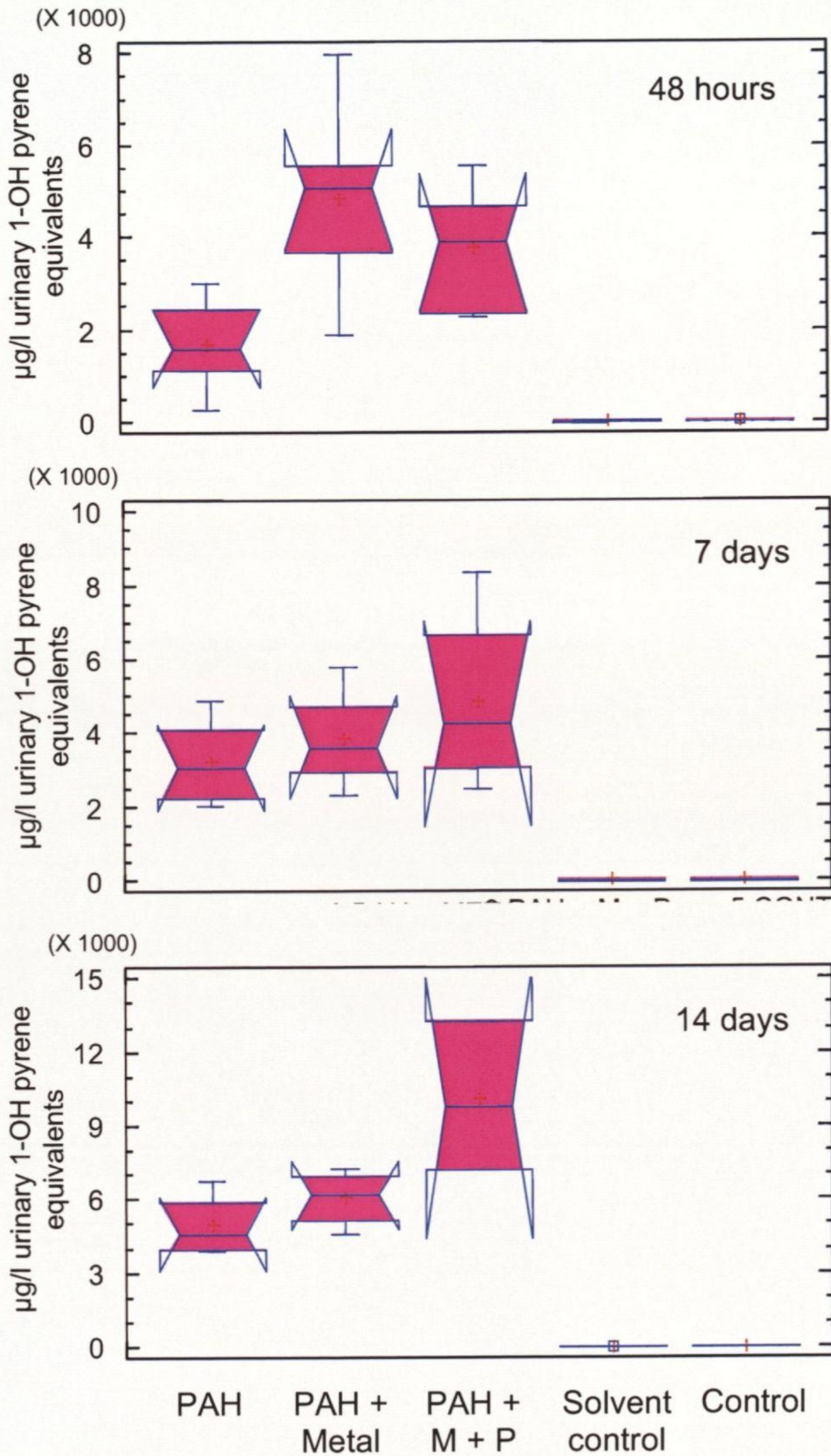


Figure 5.3 Notched box and whisker plots of urinary levels of 1-OH pyrene equivalents from crabs exposed at the HIGH exposure concentration.

5.3.2 Carboxylesterase activity

Results for both the high and low trials are presented in figure 5.4. Statistically significant differences in carboxylesterase activity exist between chlorfenvinphos exposed crabs and the remaining treatment groups after 48h (KW, 0.00092), 7 days (KW, p=0.00081) and 14 days (log ANOVA, p=0.000) in the high exposure trial. There were no significant differences in activity between treatment groups not exposed to the pesticide. Similarly, in the low exposure trial, after 7 and 14 days exposure, significant differences exist between crabs exposed to chlorfenvinphos and those not exposed (log ANOVA, p=0.000). Conversely, no significant differences exist between treatments after 48 hours in the low trial (ANOVA, p=0.0772), despite a reduction in activity in the pesticide-exposed crabs. Throughout the exposure period, activity is reduced to a greater extent in crabs exposed to the higher concentration of chlorfenvinphos, and this difference is statistically significant (48hrs log ANOVA p=0.000, 7d KW p=0.00001404, 14d log ANOVA p=0.000).

5.3.3 Total haemolymph protein

Results for both the high and low trials are presented in figure 5.5. In the high exposure trial, no statistically significant differences were apparent between the treatments after 48h, 7 days or 14 days (KW, p=0.887, 0.782, 0.279 respectively). Results from the low trial reveal the same, with no differences in haemolymph total protein between treatments throughout the exposure period (KW, p=0.679, 0.485, 0.544 for 48h, 7d and 14d respectively). However, variability in total protein levels was noticeably reduced in the crabs exposed to the lower concentrations of contaminants. When protein values are averaged, a clear decrease is observed over 14 days in crabs exposed to the high concentration of contaminants, compared to controls (figure 5.11). This decrease is most marked in the PAH/metal/pesticide group.

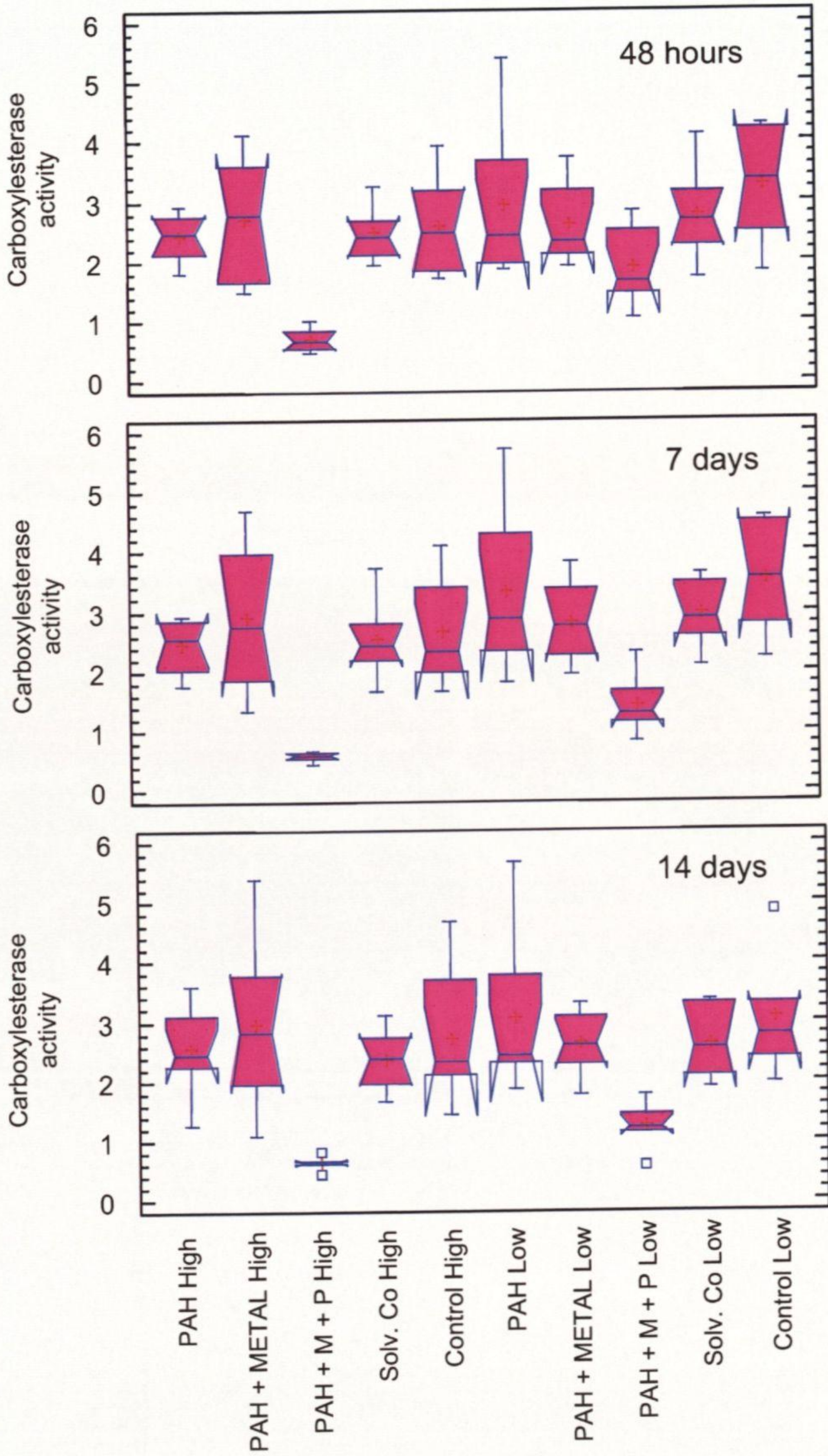
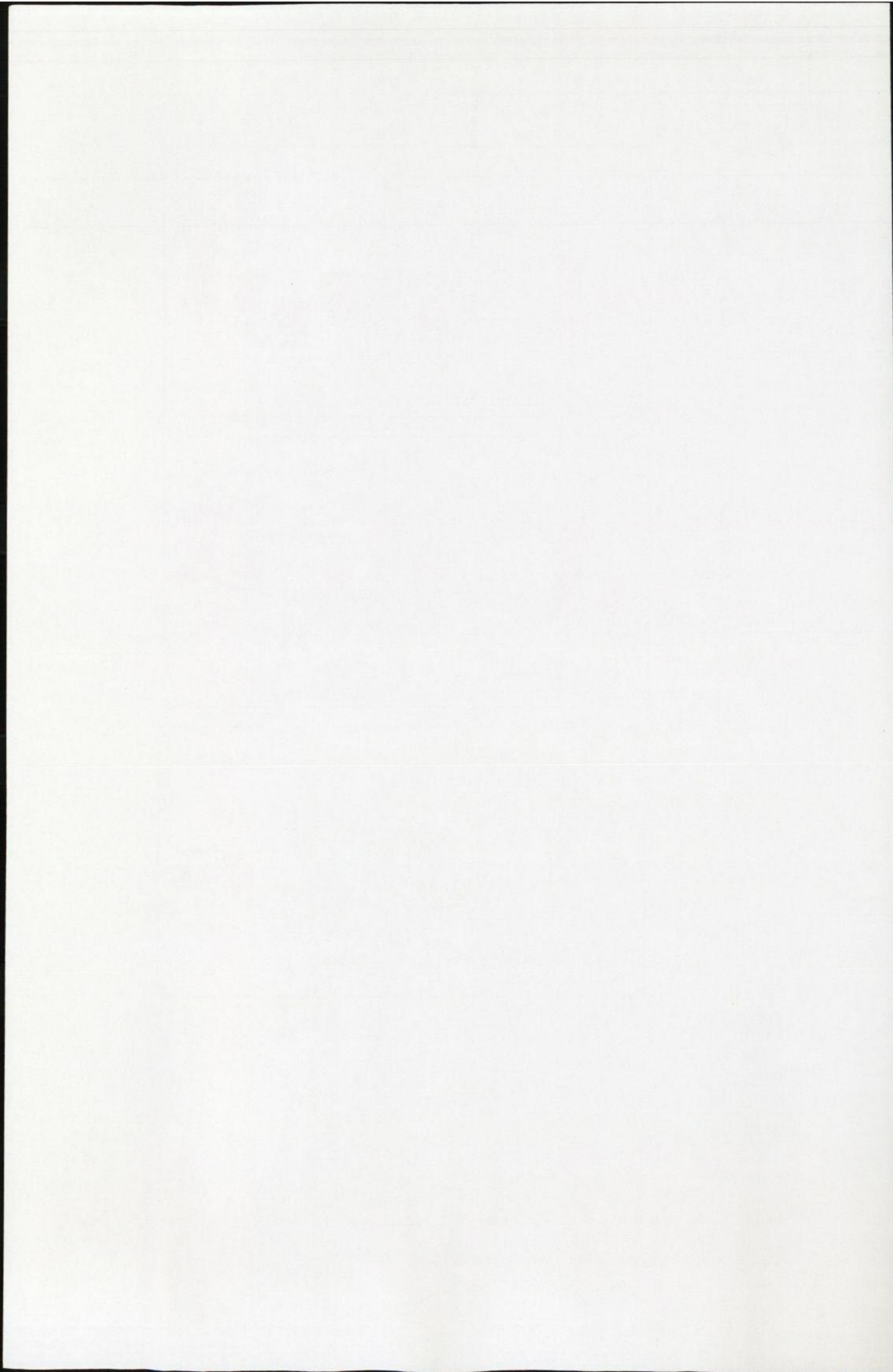
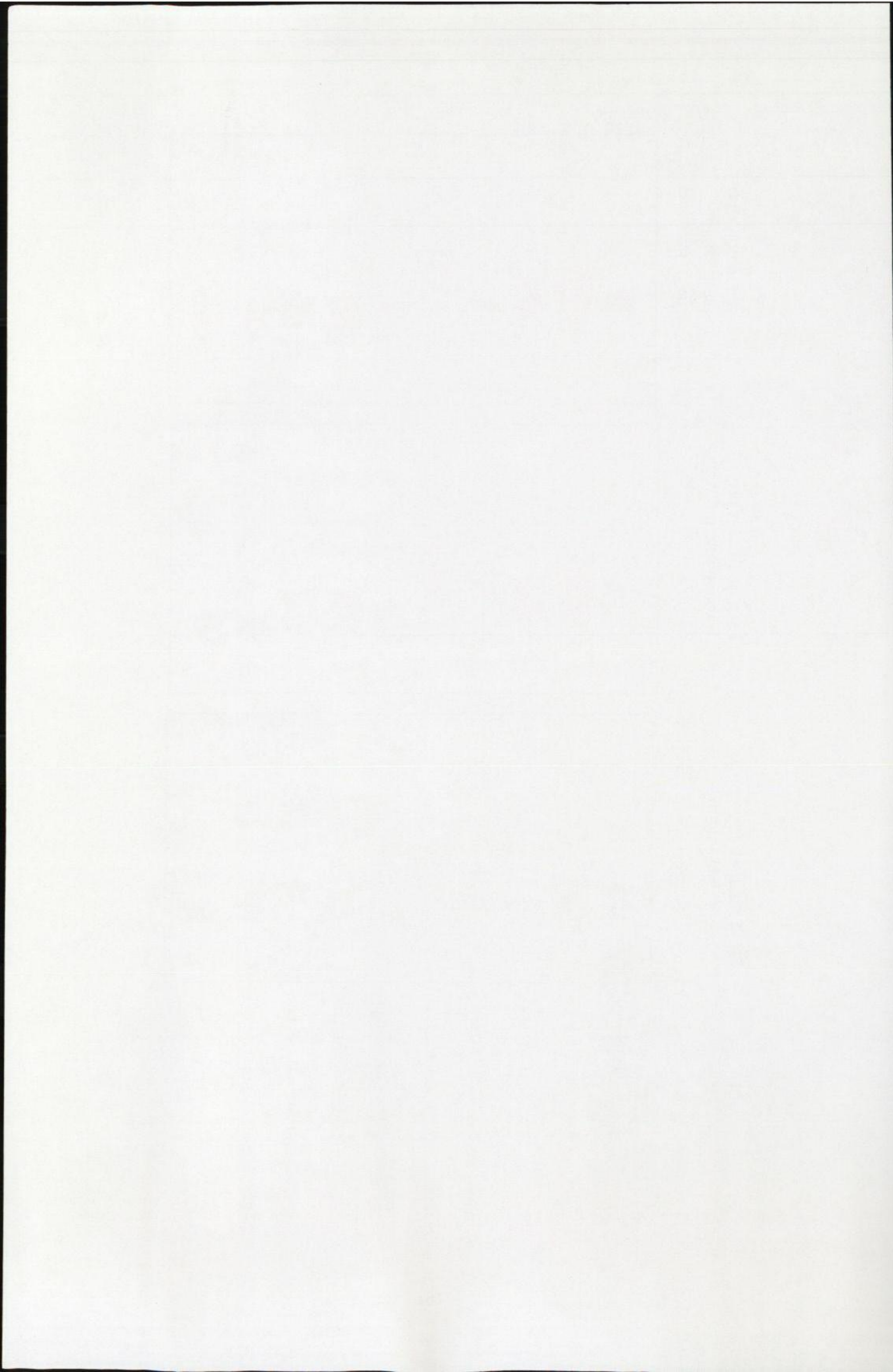


Figure 5.4 Haemolymph carboxylesterase activity (μmol substrate hydrolysed min^{-1} mg^{-1} protein) of crabs exposed to both high and low contaminant concentrations.





5.3.4 Frequency of micronuclei

No statistically significant differences were observed between treatments after 48h, 7 days and 14 days of exposure in the high trial (ANOVA, p=0.986, 0.508, 0.921 respectively) (figure 5.6). Scoring of the slides from the low trial was therefore considered counter productive and was omitted.

5.3.5 Total antioxidant status

Despite slightly depressed levels in metal and chlorfenvinphos exposed individuals (high trial), no statistically significant differences in total antioxidant status were observed between treatments after 14 days of exposure (high exposure ANOVA, p=0.4204, low exposure ANOVA, p=0.0820 at 95% confidence level) (figure 5.7).

5.3.6 Metallothionein

Levels of MT in the gill of exposed crabs showed no statistically significant differences between treatments in the high exposure trial (KW, p=0.0752) (figure 5.8). Crabs exposed to PAH alone exhibited a mean MT concentration below that of controls and the remaining treatment groups, but this difference was not significant. In light of the lack of induction, analysis of samples from the low trial was omitted.

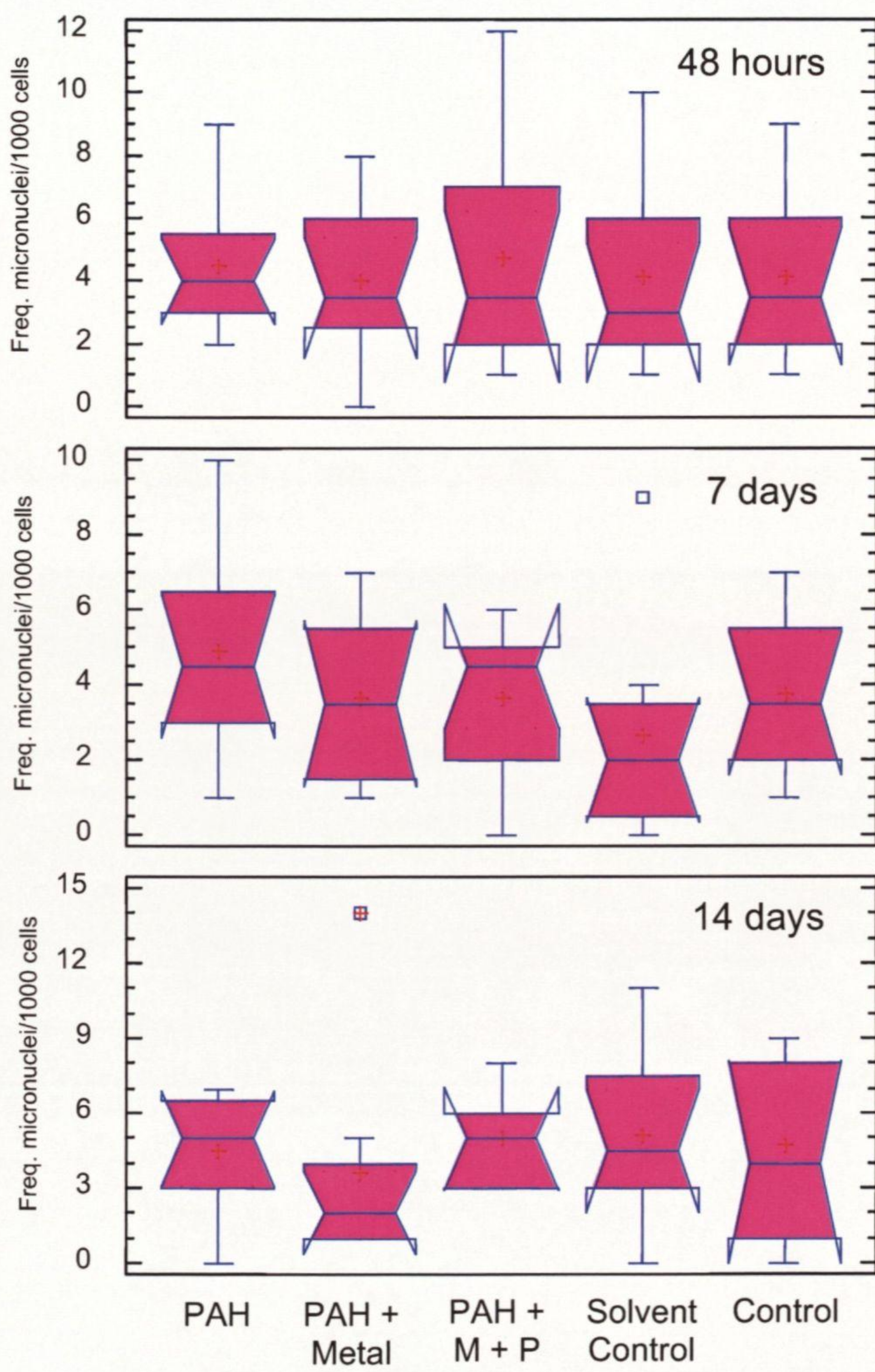
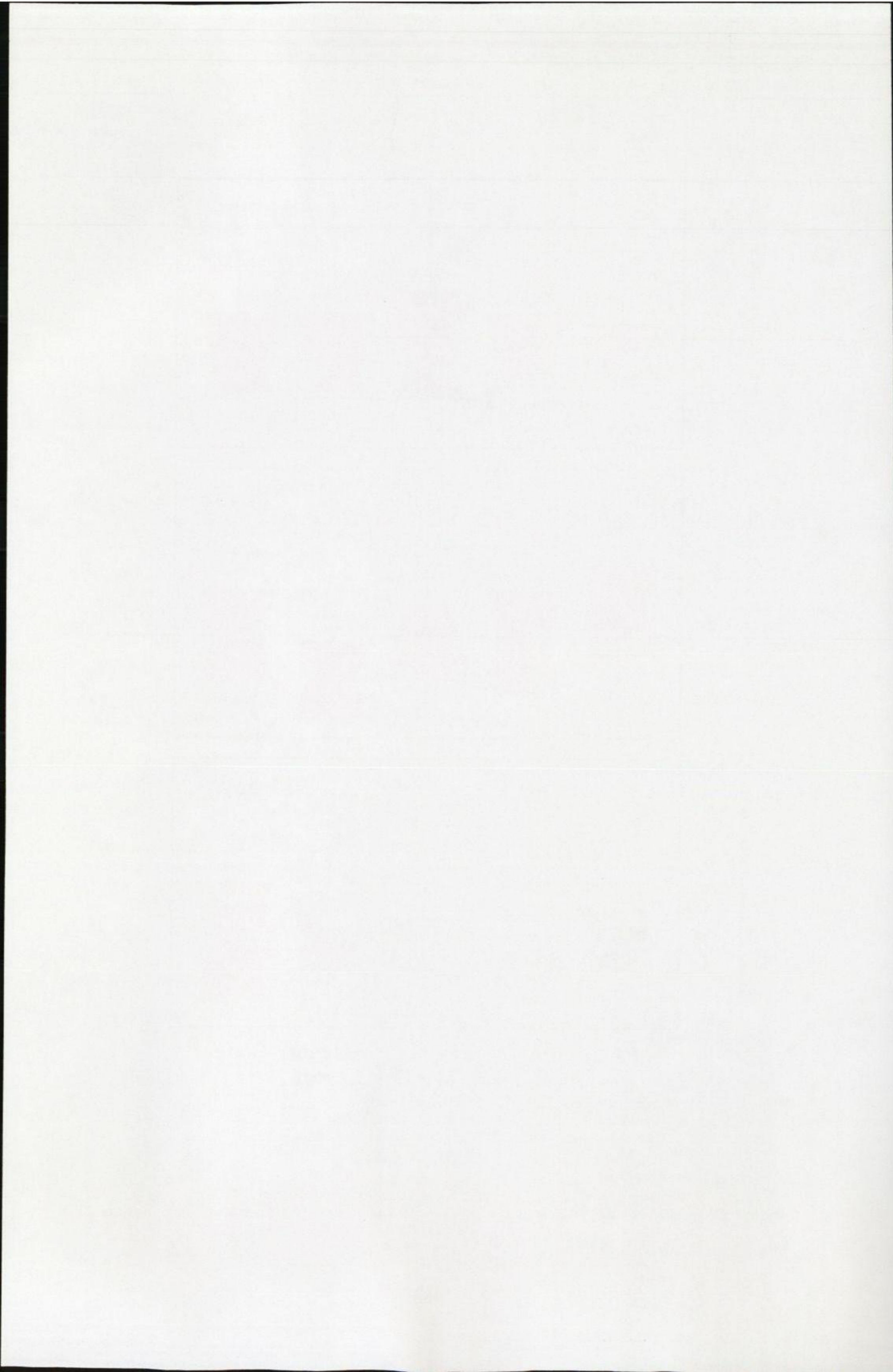


Figure 5.6 Notched box and whisker plots of micronucleus frequency /1000 haemocytes in crabs from the high concentration exposure trial.



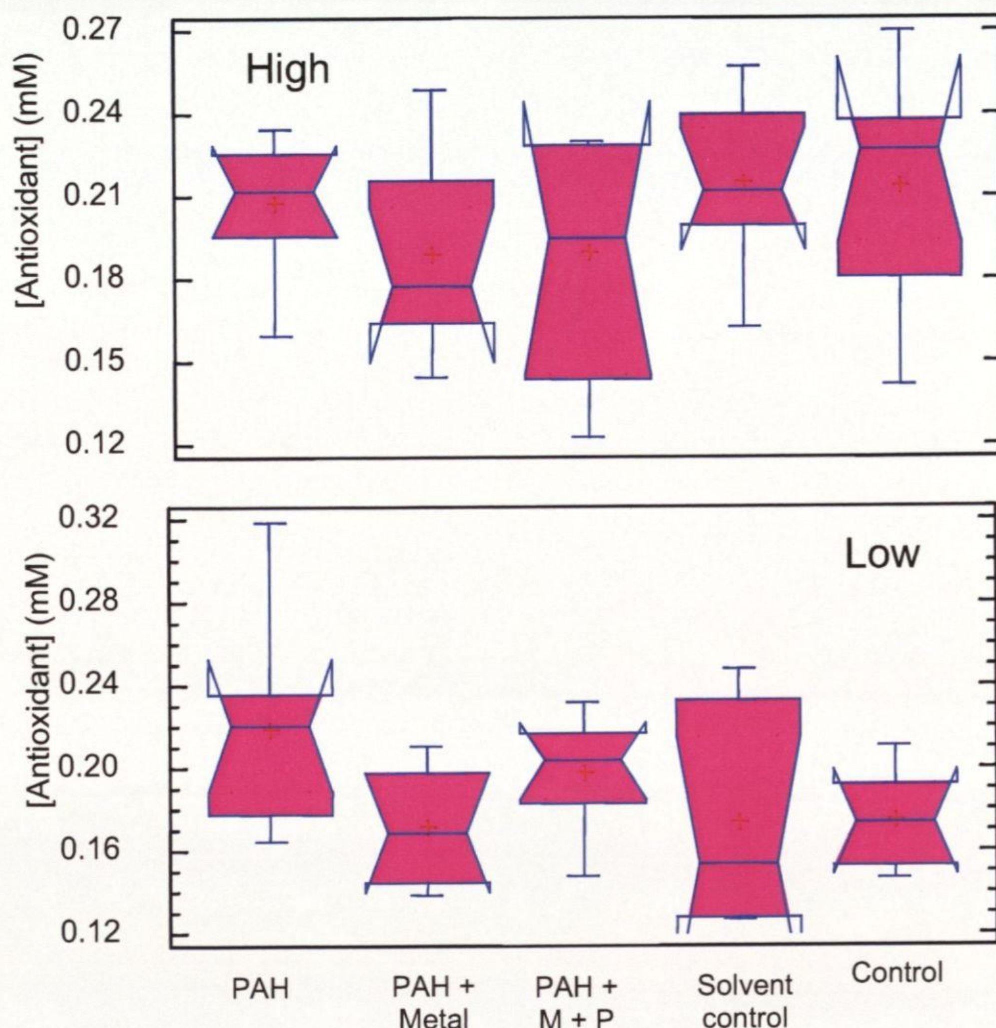


Figure 5.7 Notched box and whisker plots of antioxidant levels in the digestive gland (hepatopancreas) of crabs from the high and low exposure trials.

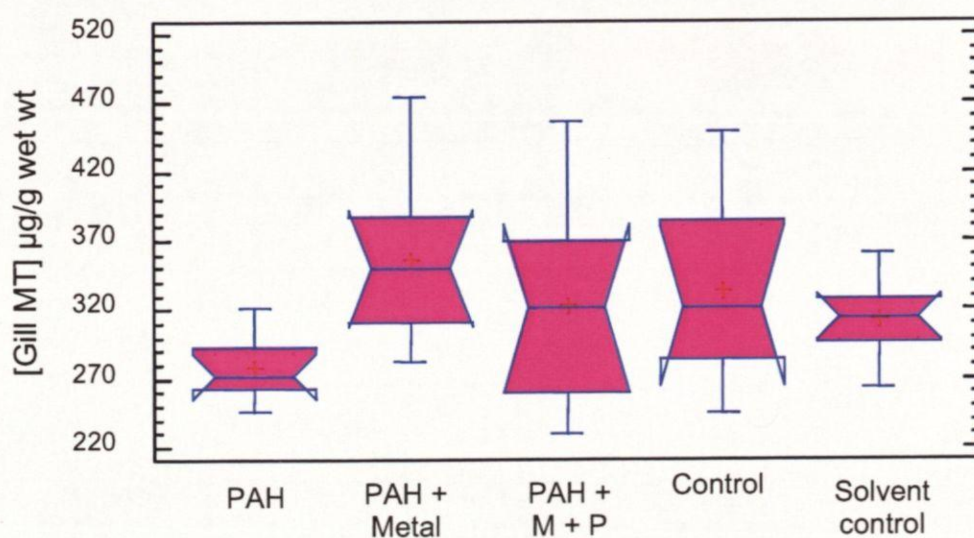
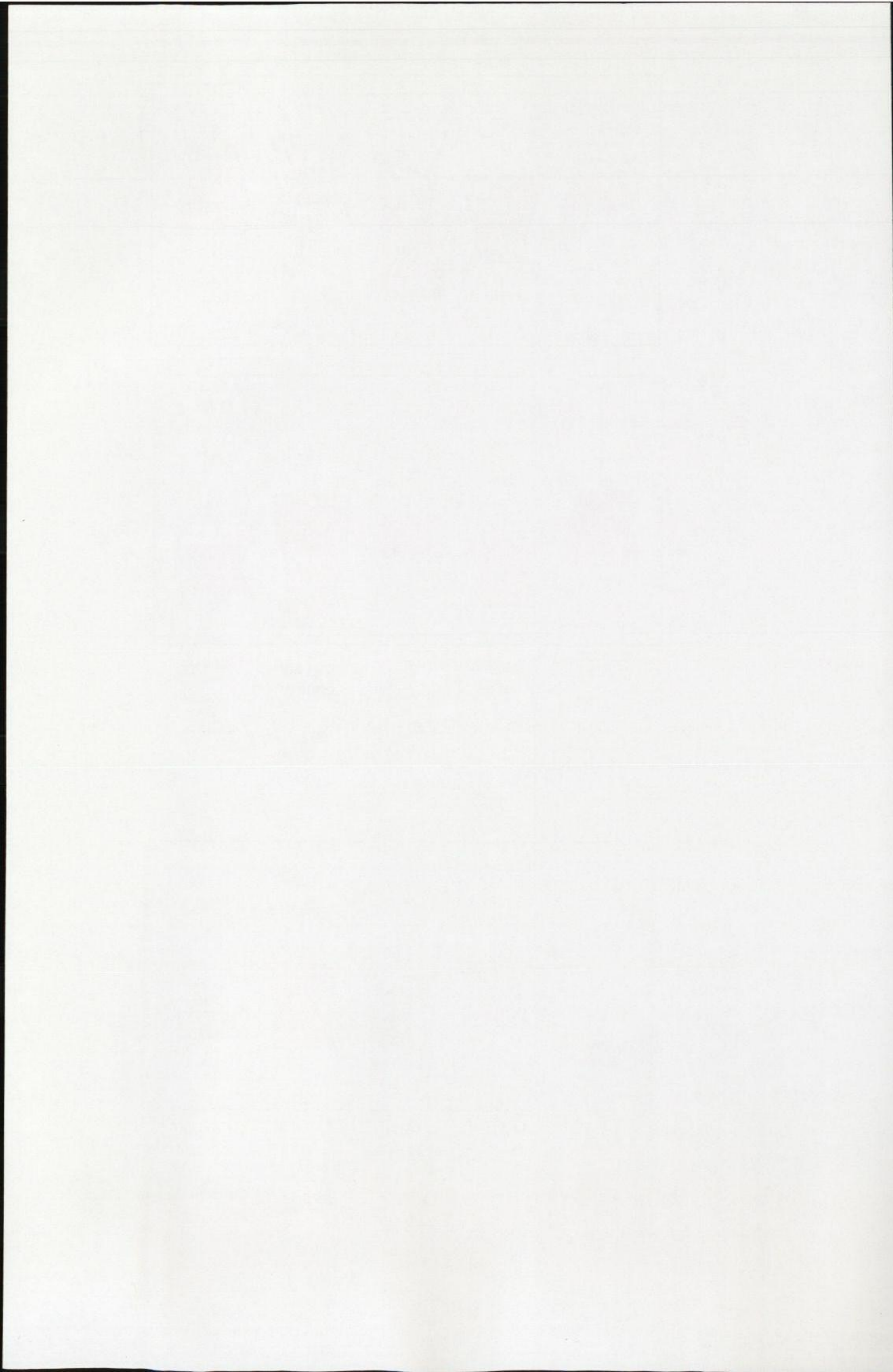


Figure 5.8 Notched box and whisker plots of metallothionein levels (µg/g wet wt tissue) in the gills of crabs from the high exposure trial.



5.3.7 Regression analysis and PRIMER analysis

Simple regression plots comparing biomarker results (data not shown) did not reveal any significant relationships, due primarily to the lack of significant differences between the treatments with respect to all but two of the biomarkers investigated.

Multivariate analysis using PRIMER 5.0 revealed very few discernible patterns in the data and levels of similarity between individuals in exposed treatments (high and low) were very high. Example plots for Cluster and MDS analyses (14d, high exposure trial) can be seen in figures 5.9 and 5.10, illustrating the general similarity in biomarker responses.

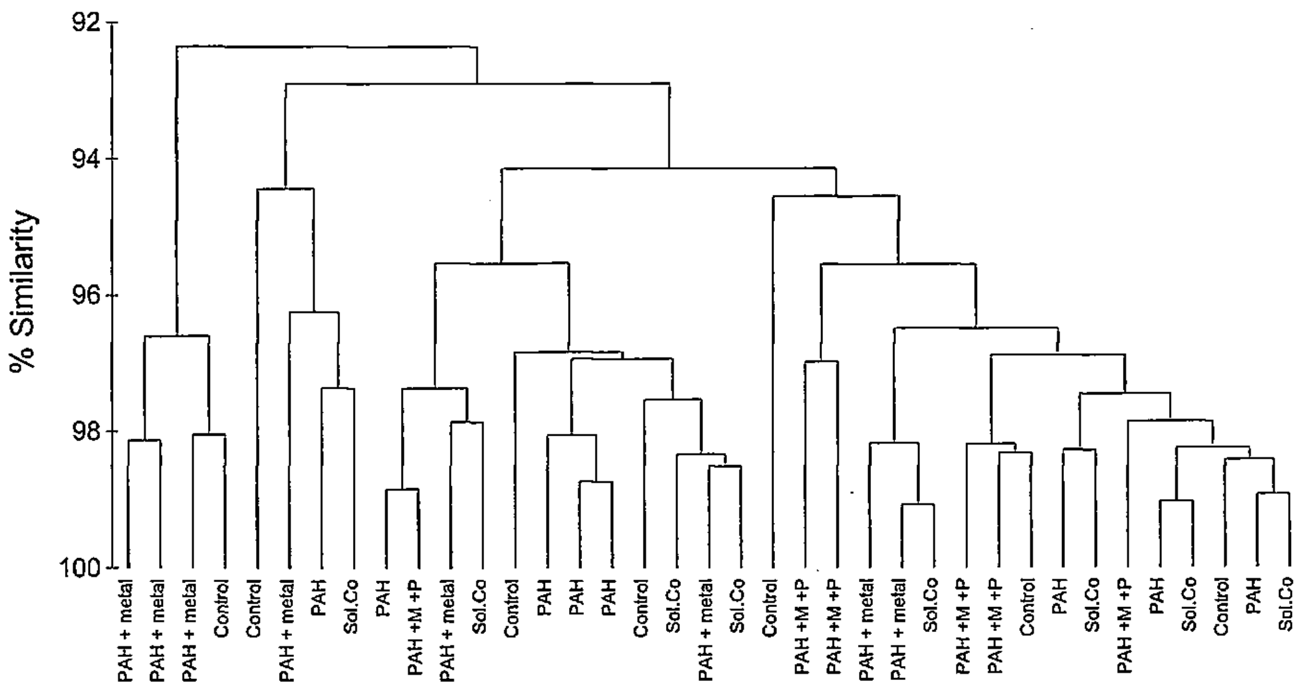


Figure 5.9 Similarity dendogram (PRIMER CLUSTER analysis) comparing the responses of all biomarkers after 14 days in the high concentration exposure trial.

Figure 5.10 2D scatter plot (PRIMER Multi Dimensional Scaling [MDS] analysis) comparing the responses of all biomarkers after 14 days in the high concentration exposure trial. Plotted distance between individuals exposed to different treatment is proportional to their similarity. Markedly dissimilar responses in measured biomarkers due to different treatments would result in tightly packed, separated clusters of individuals from those treatments.

Aside from a loose, separated clustering of individuals from the pyrene/copper/chlorfenvinphos group (most probably due to the pesticide induced inhibition of CbE activity, absent from the other groups), there are no visually distinct treatments.

5.4 Discussion

The lack of positive response in several of the biomarkers prevents a comprehensive investigation into the nature of *C.maenas*' response when faced with contaminant mixtures. However, results did show that the response of each biomarker was largely unchanged when different contaminants were added to the exposure system. For three biomarkers (MT, micronuclei, antioxidant status) there was no SSD between any of the treatments (control or exposed individuals). Exposure to copper at the high concentration used in this study did not induce MT synthesis over baseline levels and induction of micronuclei did not occur in haemocytes. Contaminant exposure did not induce oxidative stress sufficient to cause any significant increase in antioxidant levels.

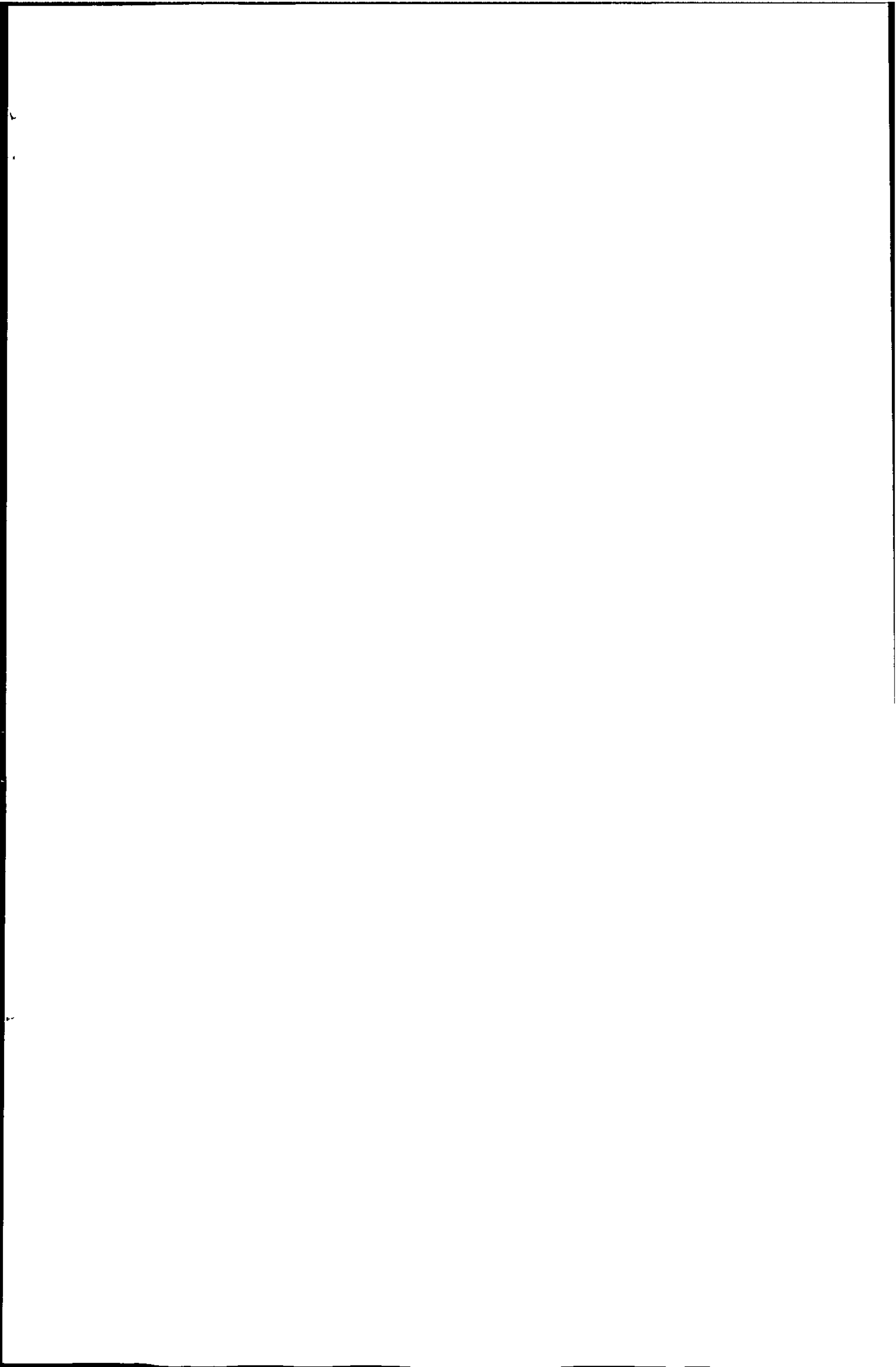
Significant differences were seen for two biomarkers for specific classes of contaminants (PAH and organophosphate pesticides). Levels of urinary equivalents in PAH exposed crabs were significantly elevated over controls but the presence of additional contaminants (copper and/or chlorfenvinphos) had no significant effect on these levels, with one exception after 14 days in the high trial (see below). Carboxylesterase activity was reduced significantly following exposure to chlorfenvinphos but remained at control levels upon exposure to copper and pyrene. The lack of response of three biomarkers (MT, micronuclei, antioxidant status) suggests that exposure levels were not sufficient to induce metallothionein synthesis, cause significant genotoxic damage or affect antioxidant systems. Contaminants were, however, taken up by the exposed individuals, as evidenced by clear responses in the exposure (urine assay) and exposure/effect (CbE inhibition) biomarkers.

In the case of micronuclei, it is possible that genotoxic damage has occurred but repair mechanisms were effective enough to eliminate or repair damaged nuclear material. It has been suggested that aquatic organisms exposed to genotoxins my continually eliminate blood cells including those carrying micronuclei, in a continuous process of

renewal (Wrisberg and Rhemrev, 1992). The inducibility of micronuclei has also been questioned, with suggestions that it is limited under laboratory conditions (Carrasco *et al.*, 1990, Wrisberg and Rhemrev, 1992). However, increased micronuclei frequencies have been observed in laboratory exposures with mussels (Bolognesi *et al.*, 1999) and crabs (Fossi *et al.*, 1997, 2000). Waterborne copper increased micronuclei in the gills of the mussel *Mytilus galloprovincialis*, with frequencies reaching 15 MN per 1000 cells. In exposure experiments using benzo(α)pyrene, PCBs and methylmercury, basal and maximal micronuclei frequencies in the haemolymph of *Carcinus aestuarii* were similar to levels observed here (ranging from 4 to 16 micronuclei per 1000 haemocytes), but significant differences were observed between treatments (Fossi *et al.*, 1997, 2000). However, the contaminants used were arguably more potent inducers of genotoxic damage and micronuclei formation than those used in the present work.

No significant changes to antioxidant systems occurred in any of the three exposed treatments. This result suggests that saturation of oxyradical scavenging pathways did not occur and any reactive oxygen species generated by contaminant exposure were efficiently sequestered by antioxidant systems. Similar results have been reported for the Arctic spider crab, *Hyas araneus*, exposed in the laboratory to oil contaminated sediment (Camus *et al.*, 2002) and *C.maenas* from Basque estuaries contaminated with PAH and PCBs (Orbea *et al.*, 2002). Orbea *et al.* (2002) suggest that seasonal factors might affect biomarker responses to a greater extent than pollution variations, highlighting the need for natural fluctuations in response to be elucidated before using such biomarkers to detect oxidative stress in the field. Carrying out investigations in winter and summer would be a step towards this.

Lack of gill MT induction due to copper exposure may be related to the role copper and MT both assume in the normal biological functions of crustaceans, and it is for this reason that some authors have previously advised against the use of MT in crustaceans for



environmental monitoring (George and Olson, 1994). Copper is an essential metal in crustaceans and is a normal cell constituent during metal homeostasis (Bryan, 1968, Legras *et al.*, 2000). Copper is also central to the metabolic functioning of haemocyanin, the oxygen binding respiratory pigment in the haemolymph (Hebel *et al.*, 1997). MT is involved in homeostatic regulation of copper and other essential metals in crustaceans (Legras *et al.*, 2000), in the synthesis of haemocyanin (Brouwer *et al.*, 1989) and CuMT donates Cu(I) to apohaemocyanin to activate it to its oxygen binding form (Brouwer *et al.*, 1986). MT is also involved in regulation of intracellular levels of copper during the moult cycle (Engel and Brouwer, 1987) and modulation of tissue metal concentrations between soluble and insoluble forms (George and Olson, 1994). It has been suggested that high basal levels of MT synthesis and MT bound copper may obscure any response due to exogenous copper (Roesijadi, 1992). With the above in mind, it is possible that basal levels of MT were sufficient to sequester the exogenous copper burden in the present exposures, without the need for *de novo* synthesis of MT. No overloading of the normal systems involved in cellular trafficking of metals has occurred and so additional MT is not required (Handy *et al.*, 2003). Therefore, MT concentrations in copper exposed crabs were not elevated over controls. George and Olson (1994) advise that in choosing suitable sentinel species for trace metal monitoring, those with alternative systems competing with MT for sequestration of metals should be avoided. However, confounding of results due to moult cycle influences on MT levels (Engel and Brouwer, 1991, 1993) was hopefully minimised by the use of intermoult individuals.

Lack of induction of MT upon exposure to copper has been reported in laboratory studies (Lundebye and Depledge, 1998) and at field sites characterised by highly elevated sediment burdens of trace metals, including copper (Pedersen and Lundebye, 1996, Weddeburn *et al.*, 1998). Additionally, natural variability in MT concentrations due to salinity and season often conceals relationships with accumulated metal concentrations, as evidenced by a study on the crab *Pachygrapsus marmoratus* in the metal-rich Gironde

estuary, France (Mouneyrac *et al.*, 2001). Such studies illustrate the pitfalls associated with this biomarker. While it is accepted that copper is not as potent an inducer of MT as other metals (particularly cadmium), the decision to use it in the present experiments was based on its ubiquity in the environment and the extensive knowledge on its toxic effects in aquatic organisms (Hebel *et al.*, 1997, Viarengo, 1985).

It is also possible that the exposure period of 2 weeks was insufficient to push cellular metal pathways past capacity and induce MT synthesis to levels that would distinguish copper-exposed crabs from controls. Realistically, the nature of chemical stress in the environment is characterised by chronic, low level exposure over months or years (Handy *et al.*, 2003). This kind of continuous exposure is perhaps a requirement for detectable levels of MT in field studies. For example, *C.maenas* might only exhibit elevated copper and zinc MT concentrations in its gills when it is resident in a chronically metal-contaminated habitat, such as the Fal estuary, UK (Pedersen *et al.*, 1997). Similarly, excretion of exogenous copper may have kept pace with metal entry into the body over the exposure period. Since copper exposure here was *via* the dissolved route and therefore taken up by the gills, excess copper could have been successfully excreted directly back via the gill surfaces (Hebel *et al.*, 1997). Alternatively, copper excretion could have occurred via the gut (R. Uglow, personal communication). As a result, intracellular levels may not have been elevated sufficiently to trigger detoxification mechanisms i.e. MT synthesis (Hebel *et al.*, 1997).

Another possible explanation may be the inhibition of MT induction due to exposure to PAH (or pesticide) alongside copper. In fish, exposure to a metal (Cd) following exposure to a PAH (benzo(a)pyrene) has been shown to inhibit MT induction (Sandvik *et al.*, 1997). The same may be possible in crustaceans and suggests that the use of MT to indicate exposure to heavy metals might be compromised when other contaminants are present. The low MT concentration measured in crabs exposed to PAH

alone, may therefore be the result of depression of basal MT synthesis or turnover during the exposure period. Perhaps without the added stimulus of metal exposure to elevate MT back to control levels, PAH exposure keeps MT levels depressed. Klerks (1999) hypothesises that one contaminant might inhibit the detoxification of another and uses the example of a PAH induced reduction of lysosomal stability resulting in reduced metal detoxification. Lysosomes play an important role in metal detoxification and are a site of accumulation of metallothioneins (Viarengo, 1985). Since PAH can decrease lysosomal stability (Viarengo *et al.*, 1987) it is possible that exposure to a PAH can impair the MT response.

The results obtained for the urinary biomarker are important, as they demonstrate that this assay still provides meaningful results when contaminants other than PAHs are included in exposures. Previous chapters have shown the assay to respond in a concentration dependent manner and to be reliable, reproducible, comparable with more sophisticated techniques and applicable in field situations. However, the assay's response when applied to a multi contaminant system was still not determined. The above results prove that the assay is not invalidated by the presence of additional contaminants. Whilst it is true that exposure to multiple classes of compounds is inevitable at impacted field sites (chapters 3 and 4), it was still necessary to test the biomarker in the presence of other contaminants under controlled conditions to allow objective comparison. One interesting feature of the results is the significantly higher (KW $p=0.0038$) level of urinary equivalents in the PAH/metal/pesticide group after 14 days of the high concentration exposure trial. Exposure to another organic contaminant (i.e. chlorfenvinphos) has significantly elevated the level of pyrene equivalents excreted in the urine. The addition of copper alone did not cause such an increase. It is possible that the additional contaminant increased induction on the detoxification systems (cytochrome p450 enzymes in the hepatopancreas) of exposed individuals and resulted in a higher rate of conjugation and subsequent increase in eliminated metabolites.

The response of the carboxylesterase activity biomarker to OP pesticide exposure is also maintained in the presence of additional contaminants. Furthermore, despite suggestions that PAHs (Narbonne *et al.*, 1995) and metals (Elumalai *et al.*, 2002, Livingstone, 1993) can inhibit esterase activities, inhibition occurs only in pesticide exposed individuals, suggesting specificity for exposure to chlorfenvinphos in this study. The robustness of the CbE biomarker is important, as OP induced inhibiton of esterase activity in *C.maenas* correlates with cardiac irregularities and reduction in heart rate (Lundebye *et al.*, 1997). This causative link shows that detection of reduced esterase activity in wild populations indicates the potential for impacts on physiological processes and general health in this species (Lundebye *et al.*, 1997).

Haemolymph total protein level has been proposed as an physiological indicator of organismal health and pollutant exposure (Brown *et al.*, 2004) and has the potential to be used as a biomarker in its own right (as long as it is accompanied by other biomarkers in a "weight of evidence" appraisal of pollutant stress). A decrease in haemolymph total protein concentration has been reported for copper exposed *C.maenas* (Depledge *et al.*, 1995, Weeks *et al.*, 1993) and mysid shrimps (Lin and Chen, 2001) and this may be a useful indicator of contaminant stress in field studies. No statistically significant differences in haemolymph protein were found in the present study, although mean levels do decrease over the exposure period in exposed crabs ([high] trial), with this decrease being most marked in crabs exposed to all three contaminants (figure 5.11). Mean haemolymph total protein could be an indication of pollutant stress, although further work would be needed to substantiate this. Variability in haemolymph protein is also markedly greater in crabs exposed to the higher concentrations of the contaminants. It has been proposed that variability in biomarker response can itself be considered an indication of contaminant impacts (Depledge and Lundebye, 1996, Depledge, 1990). Whilst not reliable in isolation, significantly increased variability in haemolymph protein could be a useful indicator of exposure to chemical stressors.

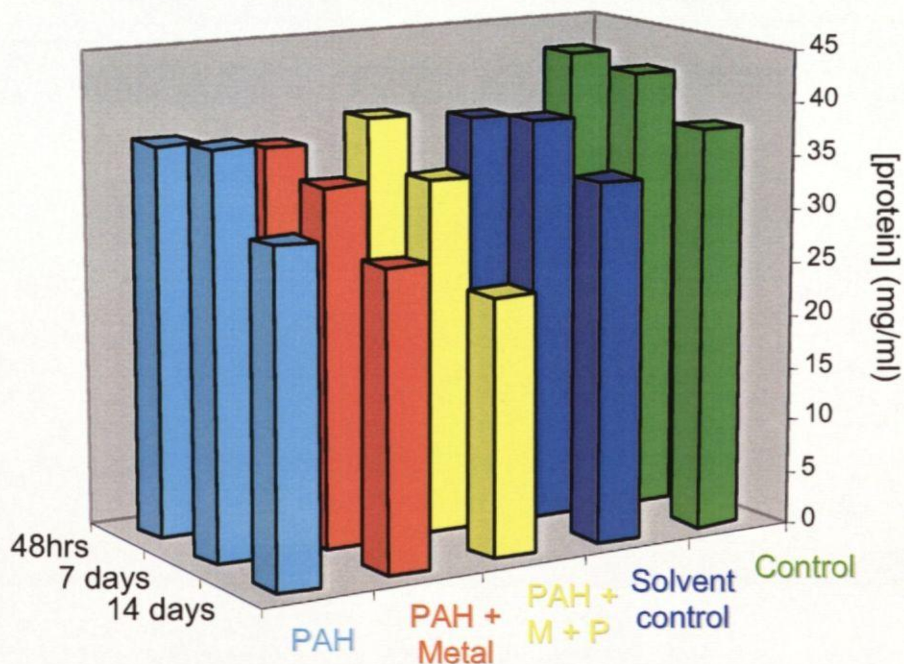
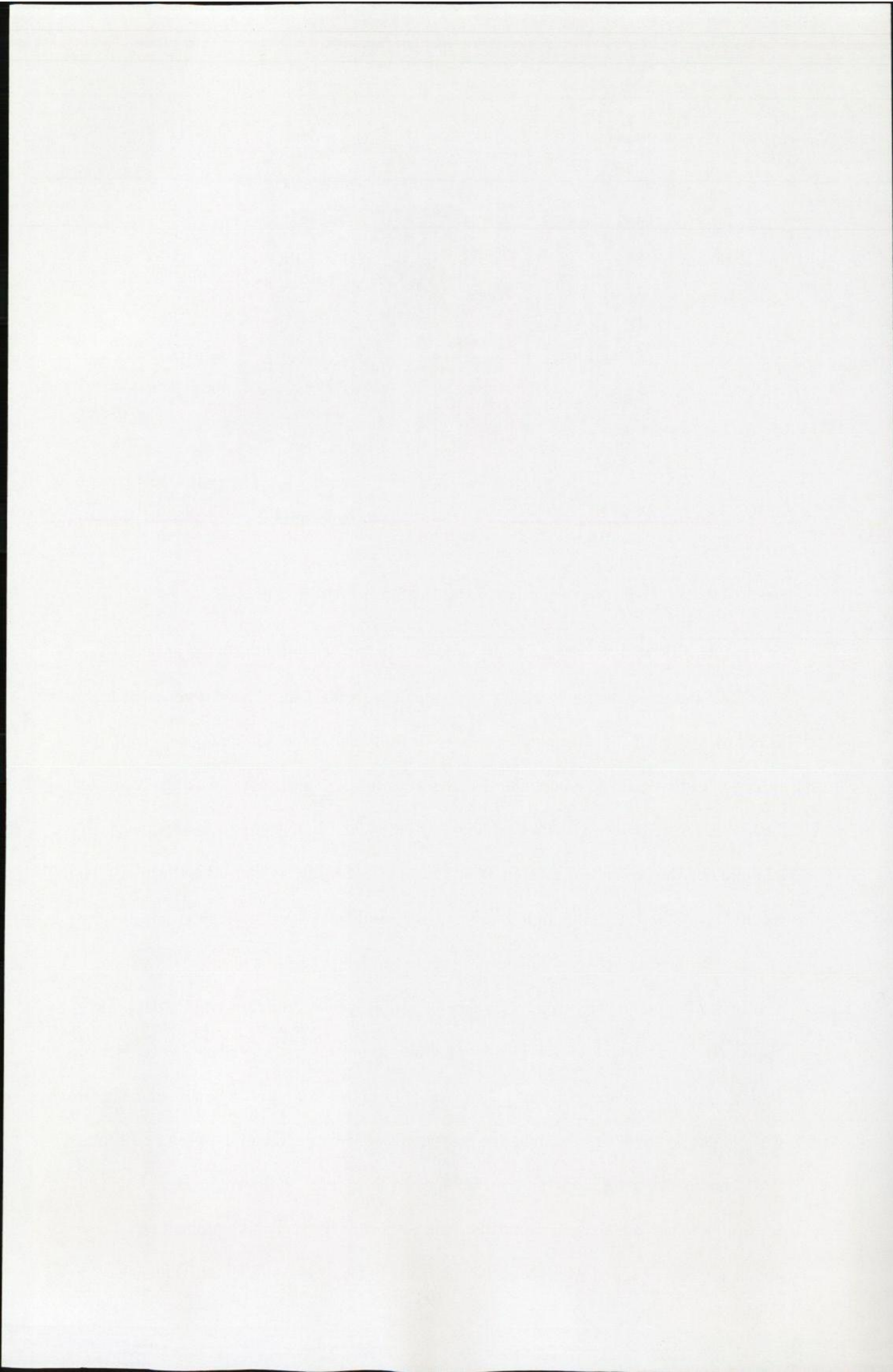


Figure 5.11 Mean total protein concentration (mg/ml) in the haemolymph of crabs exposed in the high trial.

PRIMER analysis revealed very little in terms of relationships between biomarker responses and no compelling trends were apparent in the data. Analysis revealed high levels of similarity between individuals in control and exposed treatments, with the exception of the pesticide group, after 14 days of exposure. This result is most likely due to the low carboxylesterase activities exhibited by individuals in this group, a consequence of inhibition by the pesticide. Levels in other groups were broadly similar. This illustrates the specificity of the CbE inhibition assay and the resultant values are responsible for the greater dissimilarity between individuals exposed to the pesticide and those not exposed. Similarly, in preliminary analyses the inclusion of the urinary PAH data gave disproportionate weight to PAH exposed crabs and steered the analysis towards two distinct, tightly clustered groupings. The lack of response in controls and solvent controls was not due to anything other than the fact that these individuals had not been exposed to PAH and so did not eliminate its metabolites in their urine. Following the omission of urinary data, individuals were compared with respect to the remaining biomarkers. These are endpoints associated with endogenous processes and as a result, all individuals have a



measurable response and can be compared. However, comparisons yielded few clear differences between the data sets.

5.5 Conclusions

The work in this chapter has shown that the response of selected biomarkers, developed to detect exposure and effects of specific single pollutants, changed little when crabs were exposed to more than one contaminant. Since biomarker endpoints are indicative of the organism's response to specific chemicals, it would appear that *C.maenas*' responses were largely unchanged following exposure to increasing degrees of toxic stress. Of the biomarkers investigated, MT, micronuclei and antioxidant status showed no significant differences between treatments, regardless of the number of contaminants. MT synthesis and formation of micronuclei were not induced and antioxidant levels were not increased. These biomarker responses might suggest that individuals of *C.maenas* were not subject to contaminant stress, illustrating the potential for contaminant impacts to be underestimated if only a limited number of biomarkers are employed. In the present work however, despite the lack of response in some of the biomarkers tested, contaminants were clearly interacting with biochemical pathways in the test organisms, as evidenced by clear responses in exposure (urine assay) and exposure/effect (CbE inhibition) biomarkers. The specificity of these assays was not compromised by the presence of additional contaminants. Levels of urinary equivalents in PAH exposed crabs were significantly elevated over controls but the presence of additional contaminants (copper and/or chlorfenvinphos) had no significant effect on these levels. Carboxylesterase activity was only reduced significantly following exposure to chlorfenvinphos. Multivariate analysis of all biomarker responses revealed very little and the measured responses could not clearly discriminate individuals from each other with respect to their treatments.

Results presented here highlight the importance of employing biomarkers at multiple levels of biological organisation. In field situations, restricting investigations to one level of organisation (biochemical, cellular, physiological), or a limited number of specific biomarkers may result in pollutant impacts being overlooked. This is central to the

suite of biomarkers and weight of evidence approaches and a philosophy advocated by many authors (Handy *et al.*, 2003, Fossi *et al.*, 2000, 1997, Astley *et al.*, 1999, Livingstone, 1993).

The extent of investigations into the interrelationships between biomarkers was limited by the lack of response in several of those tested. The integrity of the responding biomarkers was, however, maintained, upon exposure to the three contaminants suggesting that adverse interactions were minimal. An increase in the urinary levels of 1-OH pyrene equivalents following exposure to chlorfenvinphos is the only statistically significant difference due to an additional contaminant. Overall, biomarker results suggest that the responses of *C.maenas* following exposure to combinations of chemicals are not radically different from those following exposure to the same contaminants singly.

Chapter 6: Investigating the effects of exogenous chemicals on endocrine mediated processes in *C.maenas*: Possible endpoint biomarkers of endocrine disruption.

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6.1 Introduction

Through the development and field application of biomarkers, work described in previous chapters has sought to evaluate the responses of *C.maenas* to environmental contaminants. The use multiple biomarkers following exposure to combinations of contaminants also attempted to understand the responses of this species at various levels of biological organisation.. The lack of suitable endpoints that specifically indicate impacts at the level of the endocrine system has meant that measurements of endocrine disruption were not included (and are often omitted from environmental monitoring programs).

To address this deficiency, there is a similar requirement for the development of a reliable biomarker of endocrine disruption in decapod crustaceans. It is important, however, to first determine if exposure to exogenous chemicals causes any changes in endocrine-mediated processes in these organisms (i.e.- is disruption to endocrine processes a mechanism by which environmental chemicals can elicit toxic effects in crustaceans?).

A general introduction to the field of endocrine disruption can be found in the main introductory chapter of the thesis. Widely observed and well-documented in vertebrate species, endocrine disruption is still poorly understood in invertebrates and few examples exist for its occurrence in wild animal populations. Invertebrate research has often used vertebrate hormones and analogs that have been implicated in the induction of endocrine disruption (sewage effluent, nonylphenol, oestradiol etc.), but has revealed little evidence for endocrine disruption due to these compounds. It is reasonable to suggest that a greater risk is posed by chemicals that mimic or antagonise the action of specific invertebrate hormones e.g. ecdysteroids, arthropod juvenile hormone, methyl farnesoate (see below). Therefore, experiments in this final chapter aim to measure the responses of endocrine regulated processes in *C.maenas* (moulting, locomotor activity and vitellogenesis) following exposure to exogenous chemicals and assess the risks posed to these processes

by such exposure. It is hoped that these any observable changes to these processes will have the potential to be used as an indication of endocrine disruption.

In order to observe the responses of the endocrine system to exogenous chemicals, it is necessary to employ compounds that are most likely to interact with it. In doing so, "disruptable" processes might be identified with measurable endpoints that could be applied in the field as evidence of endocrine disruption. The hormone applied exogenously is the ecdysteroid moulting hormone 20-hydroxyecdysone (20-HE). This hormone is integral to the moulting process and has also been shown to be involved in several other endocrine-mediated processes in crustaceans, including reproduction (vitellogenesis). Previous research has also suggested it has a role to play in controlling behaviour and rhythmic locomotory processes (see below). Consequently, it is considered the most appropriate candidate for investigating perturbations in the endocrine system of *C.maenas*. Crabs are also exposed to the moult-inducing insecticide tebufenozide (RH-5992). Tebufenozide is a bisacylhydrazine ecdysteroid agonist whose mechanism of toxicity involves interaction with ecdysteroid receptor proteins (Dhadialla *et al.,* 1998) leading to precocious, incomplete moults and mortality (Smagghe *et al.,* 1999). Endocrinological similarities between insects and crustaceans (Chang, 1993), particularly with respect to ecdysteroids, means that crustacean endocrine systems are potentially at risk from this chemical and others like it in the environment. Its mode of action means that it also serves as a useful model for investigating how interactions of exogenous chemicals with endocrine pathways can manifest themselves in the processes they control.

6.1.1 Introduction to the endocrine system of crustaceans

A brief introduction to the endocrine system of crustaceans is appropriate here. What follows is a description of crustacean endocrinology most relevant to the work in this chapter, and introduces the endocrine mediated functions under investigation.

The endocrine system is responsible for the control of a multitude of vital functions in crustaceans, the most important being moulting, reproduction, glucose metabolism and water balance although integumentary colour, retinal pigment changes and locomotor rhythmicity are also endocrine controlled. Most of what is known about the endocrine systems of crustaceans has been elucidated from studies on decapods and what follows describes the generalised endocrinology of decapods with reference to examples from certain species. The main organs involved in the control of endocrine mediated processes are the sinus-gland/X-organ complex, the Y-organ, the mandibular organs, the pericardial organs, the androgenic gland in males and the ovaries in females.

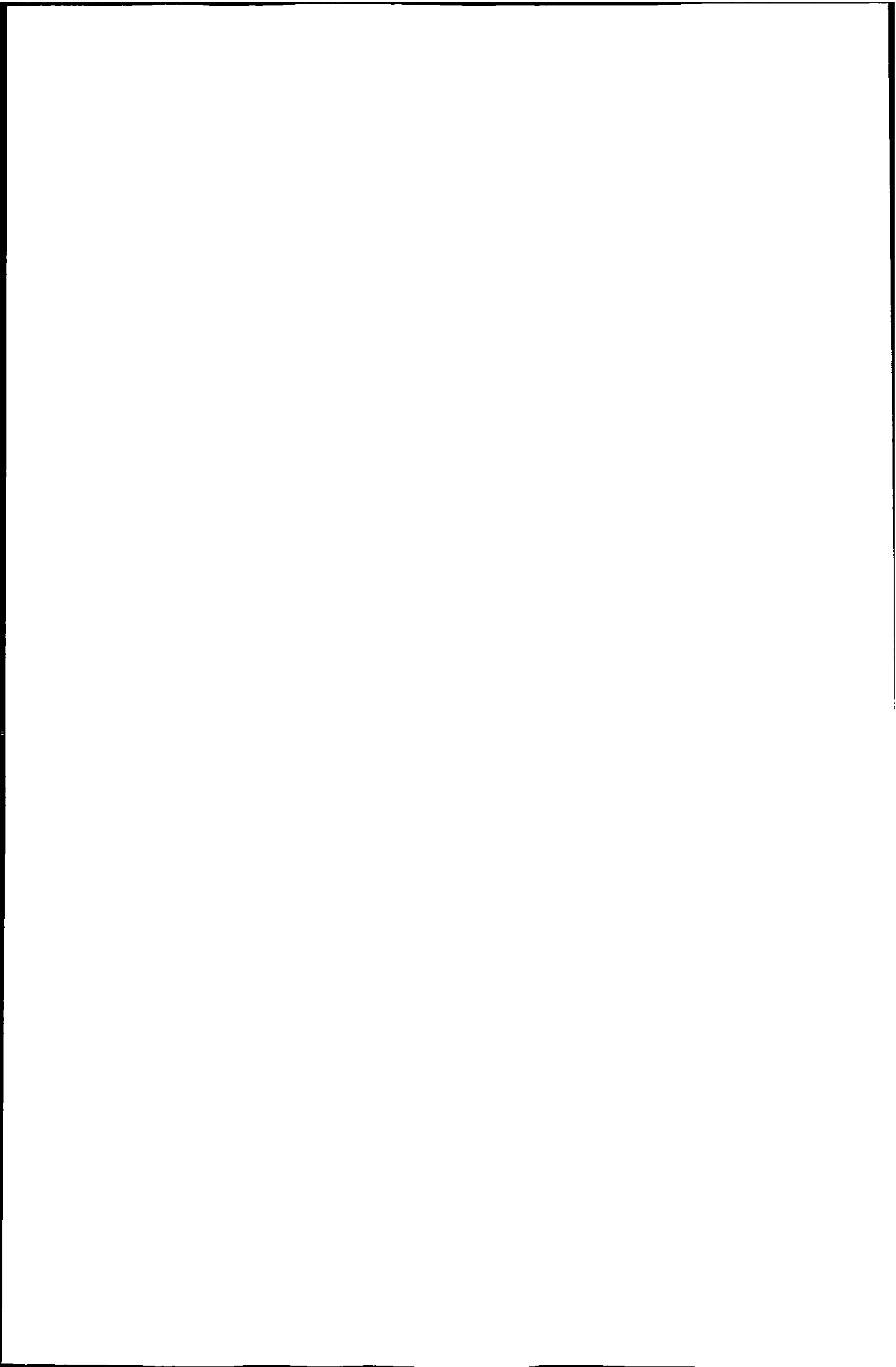
The sinus gland/X organ complex is the main endocrine control centre in decapods (Fingerman, 1987). The sinus gland is located in the eyestalk in most higher crustaceans (Fingerman, 1997) and is made up primarily of axonal terminals (associated with glial cells), 90% of which originate from cell bodies in a region of the medulla terminalis called the X-organ. The sinus gland secretes a moult inhibiting hormone (MIH), which exerts inhibitory control on hormonal moulting pathways, crustacean hyperglycemic hormone (CHH) involved in glucose metabolism and vitellogenesis inhibiting hormone (VIH) which acts on the ovary. These three neuropeptide hormones exhibit many similarities in amino acid sequence and function and are grouped together in the same peptide family (Fingerman, 1997). Two additional low molecular weight compounds are produced in the eyestalk complex, both of which display significant inhibitory activity on ecdysteroid synthesis in the Y organ. These are 3-hydroxy-L-kynurenine (3 OH-K) and xanthurenic

acid (XA) (Lachaise *et al.,* 1993, Chang, 1989). The sinus gland also secretes a peptide neurodepressing hormone (NDH), which depresses the responsiveness of motor and sensory neurons and decreases the spontaneous firing of motor neurons (Fingerman, 1987). It is thought to be involved in the modulation of circadian activity in the CNS (Williams, 1985, Arechiga *et al.,* 1974, 1979) and its release is rhythmic, reflecting the rhythmic activity patterns seen in crustaceans (Webb, 1983).

The Y organ is a paired epithelial (non-neural) endocrine gland located in the maxillary segment of decapods and is the source of the steroid moulting hormone ecdysone (Fingerman, 1987). The Y organ converts dietary cholesterol into ecdysone and secretes it into the haemolymph. Here it is transported to peripheral tissues which convert it to the bioactive haemolympatic form, 20-hydroxyecdysone (or β-ecdysone), titres of which increase just prior to moulting (Lachaise *et al.,* 1993). In addition to 20-hydroxy ecdysone, the Y organ also synthesises and secretes the ecdysteroids 25-hydroxyecdysone and 3 dehydroecdysone (Lachaise *et al.,* 1993). Ecdysteroids exert their effect by binding to an intracellular receptor protein within the target tissue. Synthesis of ecdysteroids by the Y organ is under inhibitory control from the eyestalk neuropeptide MIH, 3 OH-K and XA and under stimulatory control from another steroid hormone, the sesquiterpenoid methyl farnesoate (MF), produced by the mandibular organ (Tamone and Chang, 1993).

The pericardial organs are neurohaemal organs lying in the venous (pericardial) cavity surrounding the heart (Fingerman, 1987) and release several different cardioexcitatory amines (5-hydroxy tryptamine- 5-HT, dopamine and octopamine) and peptide hormones (proctolin and crustacean cardioactive peptide) (Pinder *et al.,* 1999). These cardioexcitatory substances increase the frequency and amplitude of the heartbeat (Fingerman, 1987).

The androgenic gland of males is a non-neural epithelial endocrine gland attached to both *vas deferens* in most species (Fingerman 1987, 1995). It produces a peptide



(androgenic gland hormone or AGH) that stimulates and coordinates the differentiation of the male reproductive system and the development of secondary sexual characteristics. The androgenic gland and the release of AGH are under stimulatory control from GSH produced by the brain and thoracic ganglia and under inhibitory control from GIH released by the sinus gland of the eyestalk (Fingerman, 1995, 1997, Pinder *et al.*, 1999). The testes are themselves not endocrinologically active (Pinder *et al.*, 1999).

In females the endocrine structures analogous to the androgenic gland are the ovaries, which produce ovarian hormone that induces the development of female secondary sexual characteristics (Fingerman, 1997) and stimulates the synthesis of yolk lipoproteins (vitellogenesis) in the ovaries and hepatopancreas. The ovarian hormone has been called "vitellogenin stimulating ovarian-hormone" or VSOH (Charniaux-Cotton and Payen, 1988). The eyestalk neuropeptide GIH (or VIH) inhibits vitellogenesis in the ovaries and synthesis of precursor lipoproteins in the hepatopancreas. Eyestalk ablation invariably results in rapid ovarian maturation and precocious vitellogenesis in decapods (Subramoniam, 1999). Ovarian growth and vitellogenesis are also under additional stimulatory control from GSH (or VSH) produced by the brain and thoracic ganglia (Charniaux-Cotton and Payen, 1988), which acts on the ovaries and influences vitellogenin synthesis in ovarian tissue and extraovarian vitellogenesis in the hepatopancreas (Pinder *et al.*, 1999). Conflicting evidence exists in the literature as to the extent of extraovarian vitellogenesis. It is widely thought that vitellogenin is the direct precursor of the yolk protein vitellin and is synthesised in the hepatopancreas and then transported via the haemolymph to developing oocytes to be incorporated into yolk globules (Lee *et al.*, 1996). However, it has been suggested that in some species eg: *Callinectes sapidus*, the ovary is the exclusive site of vitellin synthesis (Lee *et al.*, 1996).

6.1.2 Endocrine mediated processes investigated

In order to identify any hormonally related disturbances in the processes of moulting, vitellogenesis and locomotor activity in crustaceans, it is important to understand their normal functioning.

6.1.2.1 Moulting

Crustaceans must shed their exoskeleton (ecdysis) periodically for growth to occur, a process known as moulting. Connectives between their living tissues and extracellular cuticle must first be loosened, followed by the actual shedding of this old cuticle (exuviation or ecdysis). Water is then taken up to expand the new, flexible exoskeleton, which is then hardened to allow effective defence and locomotion (Chang, 1995). Ecdysis is in fact only a small part of the moult cycle, which can take up to a year or more and involves profound physiological and biochemical changes (Chang, 1995).

The moult cycle

The crustacean moult cycle can be divided and subdivided into several stages and substages. However, for the purposes of this work, a description of the four general stages (postmoult, intermoult, premoult and ecdysis - from Chang, 1989 and Skinner, 1985), will suffice.

Postmoult (*Metecdysis*): Immediately follows ecdysis (or exuviation) and is when the exoskeleton expands and haemolymph volume increases due to rapid uptake of water. Following expansion, the exoskeleton hardens until rigid.

Intermoult (*Anecdysis*): The exoskeleton is furthur hardened by the deposition of minerals, including calcium, and proteins. Storage of organic reserves (glycogen. lipids) in midgut gland, haemolymph and muscle.

Premoult (*Proecdysis*): In early premoult, secretion of the new cuticle is initiated and then maintained. Just before ecdysis, the old exoskeleton separates from the epidermal layer beneath it (apolysis). Minerals are resorbed from the old exoskeleton for the new and energy reserves in the midgut gland are mobilized.

Ecdysis: The old exoskeleton is split open (usually along the dorsal junction of the abdomen and thorax) and the animal quickly frees itself. Immediately following ecdysis the animal is extremely vulnerable to predation as it is soft, its mobility is restricted and it has limited use of its appendages for defence (Chang, 1995). Hence postmoult processes come into immediate effect.

It has been shown that ecdysis in *C.maenas* has an endogenous rhythmicity, with moulting being most frequent around the times of expected high tide in late premoult females (Abello *et al.,* 1997). It is suggested that this rhythmicity is adaptive for efficient moulting, predator avoidance, reduced cannibalism and reduced competition for shelter sites or to facilitate the copulatory process in paired males and females (Abello *et al.,* 1997). Clearly, any disruption to this process has implications for postmoult survival and reproductive success.

Endocrine control of moulting

During the moult cycle, circulating levels of ecdysteroids vary considerably. At postmoult, haemolymph titers of ecdysteroids are negligible and remain so throughout intermoult. Ecdysteroid levels increase dramatically in premoult and then drop steeply prior to ecdysis (Chang, 1989). During intermoult, production and secretion of ecdysteroids by the Y-organ is under inhibitory control from MIH, produced by the x-organ/sinus gland complex, keeping circulating titers low. High affinity binding of MIH to Y organ membrane bound receptors has been demonstrated in *C.maenas* (Webster, 1993). During early premoult, the Y-organ is freed from inhibition and ecdyteroid levels rise,

triggering the latter stages of premoult, before dropping prior to ecdysis. Postmoult sees ecdysteroid production inhibited and haemolymph titers returned to basal levels. Removal of Y-organ inhibition by eyestalk ablation leads to a rapid and dramatic increase in the levels of circulating ecdysteroids and therefore to precocious moulting (Chang, 1989, 1995, Skinner, 1985). This shortening of the moult cycle has been observed in many crustaceans (Skinner, 1985, Chang, 1989 and 1995).

Work in this chapter includes experiments designed to determine if exposure to exogenous chemicals i.e. 20-HE or its agonist, tebufenozide can trigger precocious moulting. Clearly this would be distinctly disadvantageous for field-exposed crustaceans as individuals might prematurely enter into stages of the moult cycle whilst being subject to unfavourable environmental conditions or an increased risk of predation.

6.1.2.2 Vitellogenesis

Female decapods develop a large number of heavily yolked eggs (oocytes) in the ovary. *C.maenas* for example, produces an average of 185,000 eggs in each reproductive cycle (Crothers, 1967). The yolk, containing proteins, lipids and carbohydrates provides nourishment for the developing embryos and nauplii which must subsist on it for up to several weeks following hatching (Tom *et al.,* 1992). The process of yolk synthesis and deposition is termed vitellogenesis (Subramoniam, 1999) and the major yolk protein that accumulates in the oocytes is vitellin (Vt) (Lee *et al.,* 1996). The primary translation product and precursor of vitellin is a high-density lipoprotein called vitellogenin (Vg), present in the haemolymph of vitellogenic females. Vg is immunologically identical to Vt (Lee *et al.,* 1996). Quantitative and semiquantitative correlations between haemolymph Vg and ovarian Vt concentrations in developing oocytes have been observed in many crustaceans (Adiyodi, 1985, Okumura and Aida, 2000). It is suggested that vitellogenin is synthesised in the ovary and also in extraovarian tissues (specifically the hepatopancreas) and transported in the haemolymph to the ovary, where it is converted to vitellin and

incorporated as yolk globules into developing oocytes (Lee *et al.,* 1996, Chen and Chen, 1994).

Endocrine control of vitellogenesis

Vitellogenesis is under the strict control of a number of antagonistic hormones. There are four main hormones involved in the control of vitellogenesis - the neuropeptide gonad inhibiting hormone (GIH, or VIH), neuropeptide gonad stimulating hormone (GSH), the sesquiterpenoid methyl farnesoate (MF), and ecdysteroid(s). This chapter is concerned primarily with the role of 20-HE in vitellogenesis and attempts to elucidate the effect of exogenous application of this hormone on Vg levels in female crabs.

There is evidence in the literature that in some crustaceans, ecdysteroids play a role in vitellogenesis. It has been suggested that high ecdysone titres related to premoult processes may promote the early stages of vitellogenesis (Skinner, 1985). Ecdysteroids (ecdysone, 20-hydroxy ecdysone and ecdysteroid conjugates) have been detected in follicles, oocytes and embryos of several species of shrimp, crab and amphipod (Chang, 1989), and the available evidence indicates that these ecdysteroids are sequestered into the ovary by binding to yolk precursor proteins (Subramoniam, 2000). The specific function of these ovarian ecdysteroids is, however, unclear. Increased ecdysteroid titres in the haemolymph have been correlated with the progression of vitellogenesis in certain species, including *C.maenas* (Lachaise *et al.*, 1981), the spider crab *Acanthonyx lunulatus* (Chaix and de Reggi, 1982) and the freshwater prawns *Macrobrachium nipponense* (Okumara *et al.,* 1992) and *M.rosenbergii* (Young *et al.,* 1993b). However, other studies have reported decreasing levels of haemolymph ecdysteroids during vitellogenesis e.g. *Penaeus monodon* (Young *et al.,* 1993a). Young *et al.,* (1993b) suggest the differences observed may indicate that the roles of ecdysteroids in vitellogenesis in these two species differ i.e. ecdysteroids stimulate vitellogenesis in *M.rosenbergii,* but are not directly involved in *P.monodon* vitellogenesis. Similar conflicting results are reported in studies where ecdysteroids are

administered *in vivo* or *in vitro*, with the effect being either inhibitory or stimulatory (Chang, 1993, Chang, 1989). Clearly further research is required to determine the roles of ecdysteroids in vitellogenesis, an area the present work might contribute to.

6.1.2.3 Locomotor activity and endogenous rhythmicity

In addition to the processes of moulting and reproduction, this chapter investigates locomotor activity in *C.maenas*. At present, information on the hormonal regulation of locomotor activity in crustaceans is limited. Presented below is a general overview of *C.maenas* locomotor activity patterns relevant to the research and a brief description of what is presently known about their hormonal regulation.

Locomotory activity patterns in C.maenas

The locomotory activity exhibited by *C. maenas* is regulated by interlinked endogenous circadian and circatidal rhythms. The resultant locomotor pattern consists of peaks of activity around times of high tide (circatidal) overlaid with periods of increased activity at times of nocturnal high tides as compared to diurnal high tides (circadian) (Williams, 1985). Essentially, the level of activity associated with a tidal peak is modulated by the circadian rhythm (Webb, 1983). This rhythmicity in locomotor activity allows *C. maenas* to undertake daily migrations into inter-tidal areas where food and mating sites can be found. Increased activity at the time of nocturnal high tides also affords crabs increased foraging opportunities whilst limiting predation risk. Cessation of activity at low tide allows crabs to seek shelter, avoiding desiccation and avian predation (Naylor, 1985).

The characteristic pattern of locomotor activity is maintained in the laboratory under constant conditions for 4-6 days before starting to break down (Williams, 1985) with crabs kept in normal light-dark, non-tidal conditions exhibiting a "daily rhythm" overlaid with a weak approximate tidal component (Webb, 1983). Crabs kept for a month or more

under constant conditions, whilst subject to constant illumination, showed a circadian rhythm with no tidal component (Webb, 1983).

Under natural conditions, circadian/tidal rhythms are continually entrained (synchronised with predictable patterns of environmental change) by a number of exogenous variables (salinity, hydrostatic pressure, temperature, immersion and wave action) to ensure the expressed behaviour is in phase with the environmental regime (Bolt and Naylor, 1986, Naylor, 1985, Reid and Naylor, 1985). Under constant laboratory conditions, in the absence of environmental cues, the accuracy of endogenous free running rhythms slowly decreases and rhythmic patterns of behaviour become imprecise (Naylor, 1985). However, tidal rhythms in *C. maenas* can be entrained in the laboratory by simulated tides with peaks of high salinity (Bolt and Naylor, 1986), and subtle changes in temperature and pressure associated with tides in the normal habitat (Williams and Naylor, 1969, in Naylor, 1985). The entrained rhythmicity is then maintained when the animal is returned to constant conditions.

Endocrine control of locomotor activity and rhythmicity

Little is presently known regarding the endocrine regulation of locomotor activity and endogenous rhythms in decapod crustaceans. Experiments involving eyestalk ablation identified the presence of an eyestalk factor responsible for the regulation of locomotor activity in crustaceans. Further research determined this factor to be a neuropeptide, termed neurodepressing hormone (NDH) (see above). It is thought to have a role in the modulation of circadian activity (Williams, 1985, Arechiga *et al.*, 1974, 1979) and its rhythmic release reflects the rhythmic activity patterns seen in crustaceans (Webb, 1983). The cyclical nature of its release also suggests it is under the partial control of a biological clock mechanism elsewhere in the CNS (Naylor, 1985). Removal of this inhibitory influence by eyestalk ablation results in prolonged heightened, arhythmic locomotory activity in *C. maenas* (Williams, 1985, Bolt and Naylor, 1986). Eyestalk ablation has also been shown to

abolish entrainability in *C.maenas*, consistent with the view that the eyestalk neurosecretory complex is the site of a possible component of the crab's physiological clock (Bolt and Naylor, 1986).

The biogenic amine 5-HT has been shown to stimulate release of NDH and produces hyperglycaemia in crayfish, presumably by stimulating release of CHH from the sinus gland (Fingerman, 1995). GABA (gamma amino butyric acid) has been shown to inhibit the release of NDH (Fingerman, 1995). The regulation of release of these two peptide hormones has implications for locomotor activity in crustaceans. Locomotor activity may also be affected by the cardioexcitatory influence of 5-HT, dopamine and octopamine, released from the pericardial organ (Pinder *et al.,* 1999), which increase the frequency and amplitude of the heartbeat (Fingerman, 1987).

Studies on the effect of exogenous hormones on locomotor activity are limited. Administration of exogenous 5-HT, to juvenile lobsters (*Homarus americanus*) has been shown to adversely affect substrate seeking locomotor activity and result in lobsters losing fights and not securing or retaining possession of shelters in laboratory aquaria (Peeke *et al.,* 2000). Movements of 5-HT injected lobsters were also less coordinated than controls, consistent with observations reported by McPhee and Wilkins (1989) on locomotor activity and 5-HT in *C. maenas*. These results suggest that if a chemical with a hormonal mechanism of action can gain access to the internal environment of the organism, then hormonally controlled processes might be affected. Cooper and Ruffner (1998) also report some interesting results on the depression of synaptic efficacy by 20-HE in crayfish, which has implications for locomotor activity and behaviour. These authors found that exposure of an isolated crayfish nerve muscle preparation to 20-HE markedly reduced the size of excitatory postsynaptic potentials (EPSPs) in the muscle, which in turn reduced its contraction. 20-HE appeared to act rapidly at the presynaptic site, resulting in fewer synaptic vesicles being released. The authors suggest that increased haemolymph titres of

20-HE therefore account for the quiescent behaviour seen in crustacean during premoult, which minimises injury to the soft new cuticle lying beneath the old. Whether such a reduction in locomotor activity is seen in intact crabs exposed *in vivo* to 20-HE, remains to be seen. Preliminary experiments conducted with *C.maenas* (S. Bamber, pers. comm.) have also suggested a possible role for ecdysteroid-mediated perturbations to locomotor activity. Exposure to waterborne 20-HE abolished the characteristic locomotor activity in several test animals measured in an actograph system under laboratory conditions. It is therefore possible that interaction with endocrine pathways might result in "behavioural toxicity", a hypothesis that the present work aims to investigate further using waterborne 20-HE exposures.

6.2 Materials and Methods

6.2.1 Development of quantitative ELISA for vitellogenin

In order to measure any changes in haemolymph vitellogenin (Vg) titres that might be caused by exogenous chemicals, it was necessary to develop a quantitative assay suitable for Vg. A semi-quantitative enzyme linked immunosorbent assay (ELISA) for Vg in haemolymph samples has been developed previously and used for establishing whether Vg is expressed in male crabs as a result of endocrine disruption in the field (Matthiessen *et al.*, 2002). This assay was adapted into a quantitative assay for use in this work, following several developmental steps.

The results of preliminary experiments showed that Vg preferentially binds to the plate wells. This conclusion was reached following several experiments investigating the binding characteristics of vitellin (Vt) standards. Vt is immunologically identical to Vg, the haemolymphatic form (Lee *et al.*, 1996). The results of each experiment led to the subsequent step in the development of the ELISA and are described in turn below.

Microplate well capacity for vitellin

Vitellin standards from 10ng/100µl to 1µg/100µl were run using a standard ELISA to determine the capacity of the plate wells for vitellin only. When absorbance is plotted against Vt concentration an absorbance response plateau at approximately 400ng/100µl can be seen; i.e. above 400ng/100µl all binding sites are occupied and concentrations in excess of this amount will therefore produce the same absorbance response. This experiment was repeated several times and found to be reproducible (figure 6.1).

Having determined the well capacity for vitellin to be approximately 400ng/100µl, it was necessary to determine if this behaviour was affected by the presence of other proteins competing for space on the well walls. To do this, vitellogenin-free haemolymph from male crabs was sampled and assayed to determine its total protein concentration. Solutions of male haemolymph in coating buffer were then spiked with known quantities of vitellin standard to yield a standard series of vitellin from 10ng/100µl to 400ng/100µl with an overall concentration of 400ng/100µl. For example 10ng Vt + 390ng male protein in 100µl, 20ng Vt + 380ng male protein in 100µl and so on. The presence of male haemolymph had no effect on the response seen, suggesting preferential binding of Vt to the well (figure 6.2).

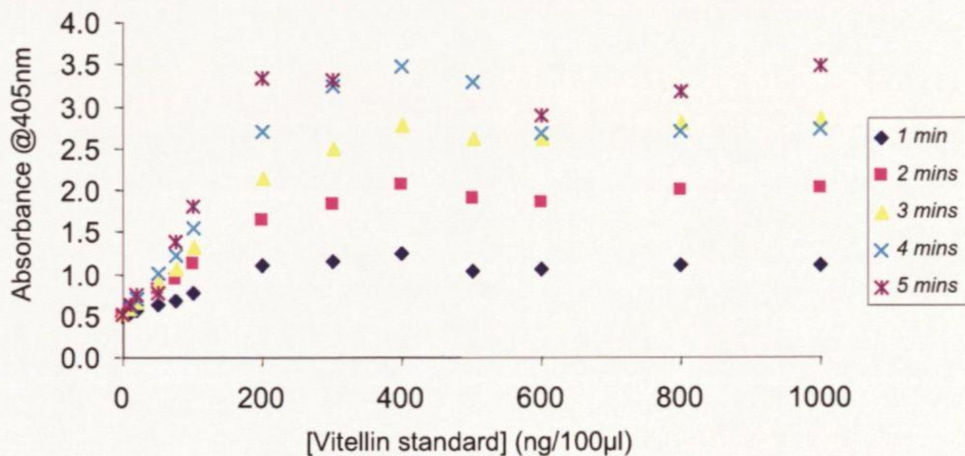
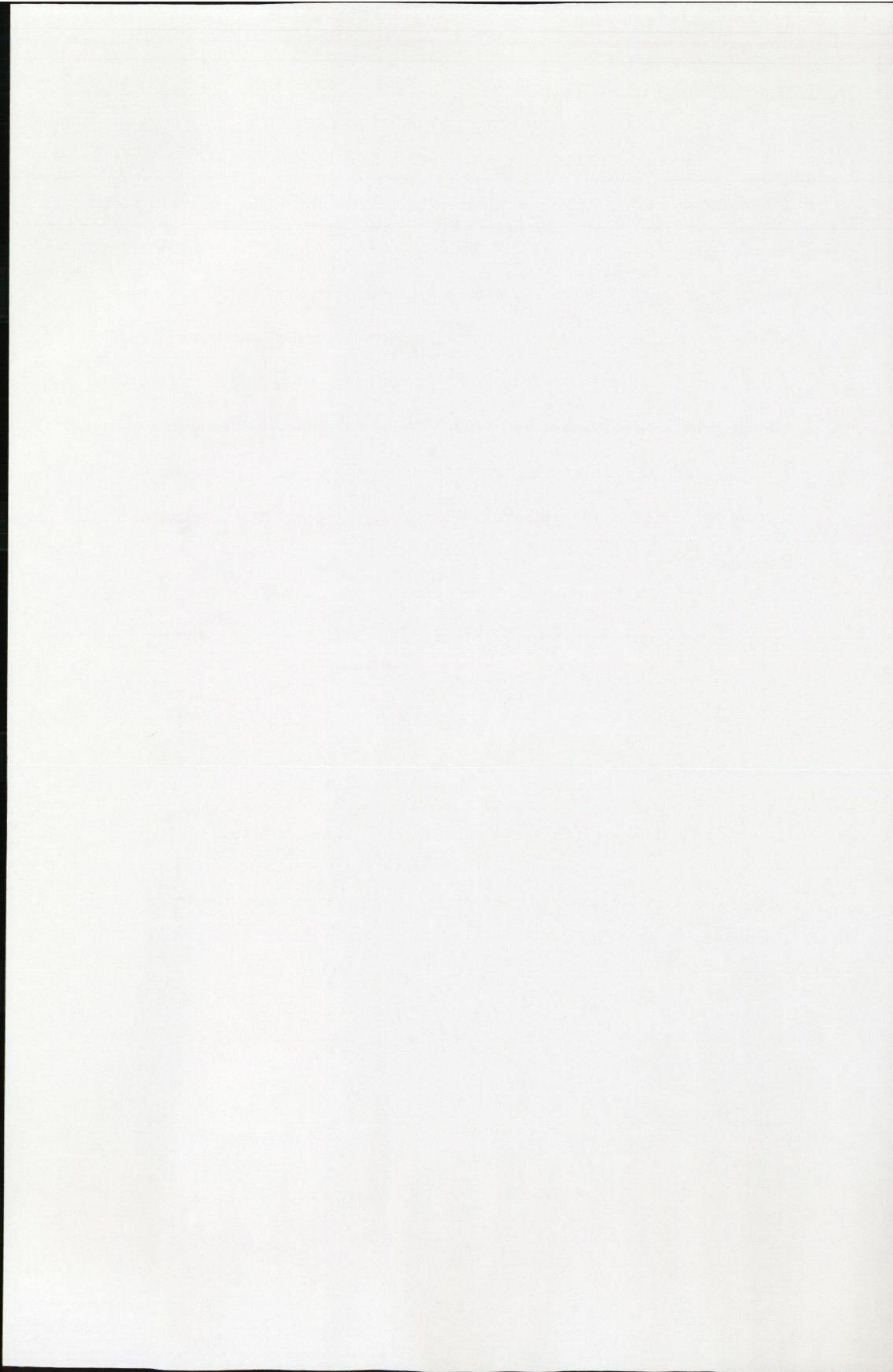


Figure 6.1 Absorbance measurement at minute intervals of vitellin standard series, illustrating the response plateau at approximately 400ng/100µl Vt concentration.



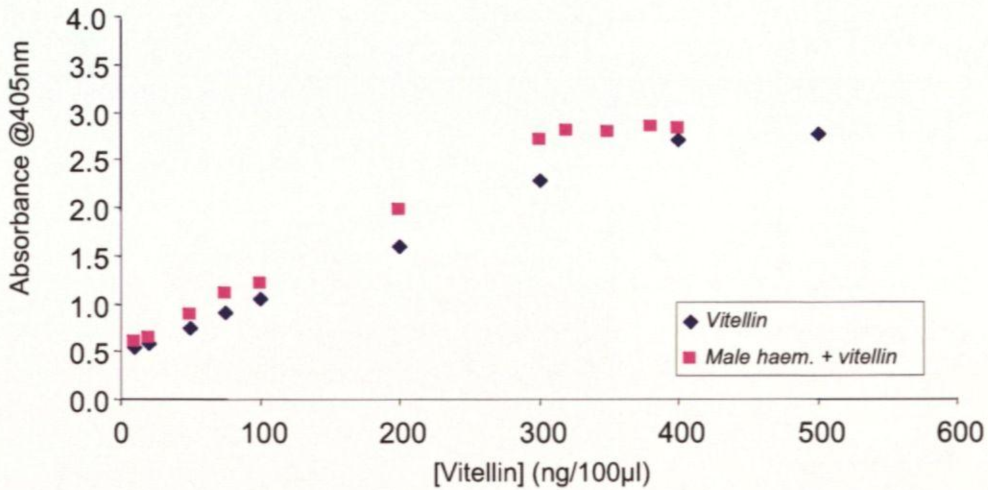


Figure 6.2 Absorbance measurements of series of male haemolymph samples spiked with increasing amounts of vitellin standard (10ng/100µl to 400ng/100µl).

To further validate this finding, four additional experiments were performed.

Increasing male haemolymph proteins vs fixed concentration of Vt

First, a series of standards, containing a fixed amount of vitellin (200ng/100µl) but increasing amounts of protein from male haemolymph (from zero up to 900ng/100µl) were added to wells in triplicate. Despite the presence of additional protein, the response produced was approximately the same in all standards (figure 6.3).

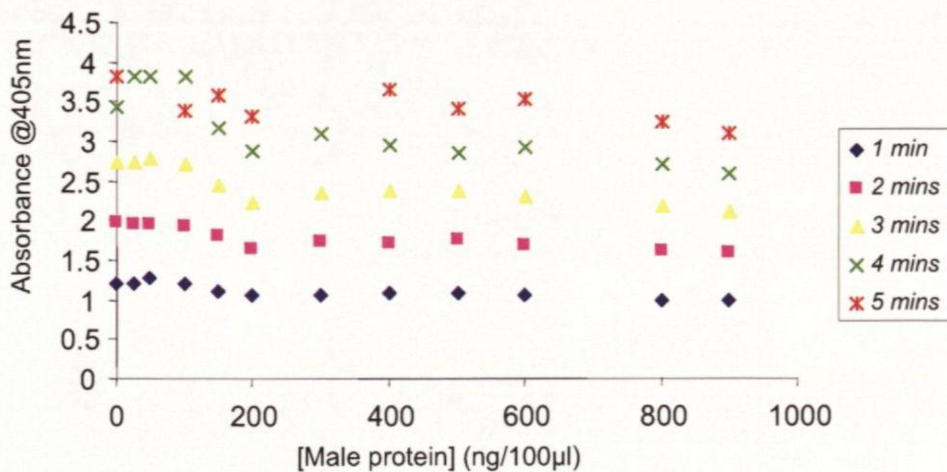
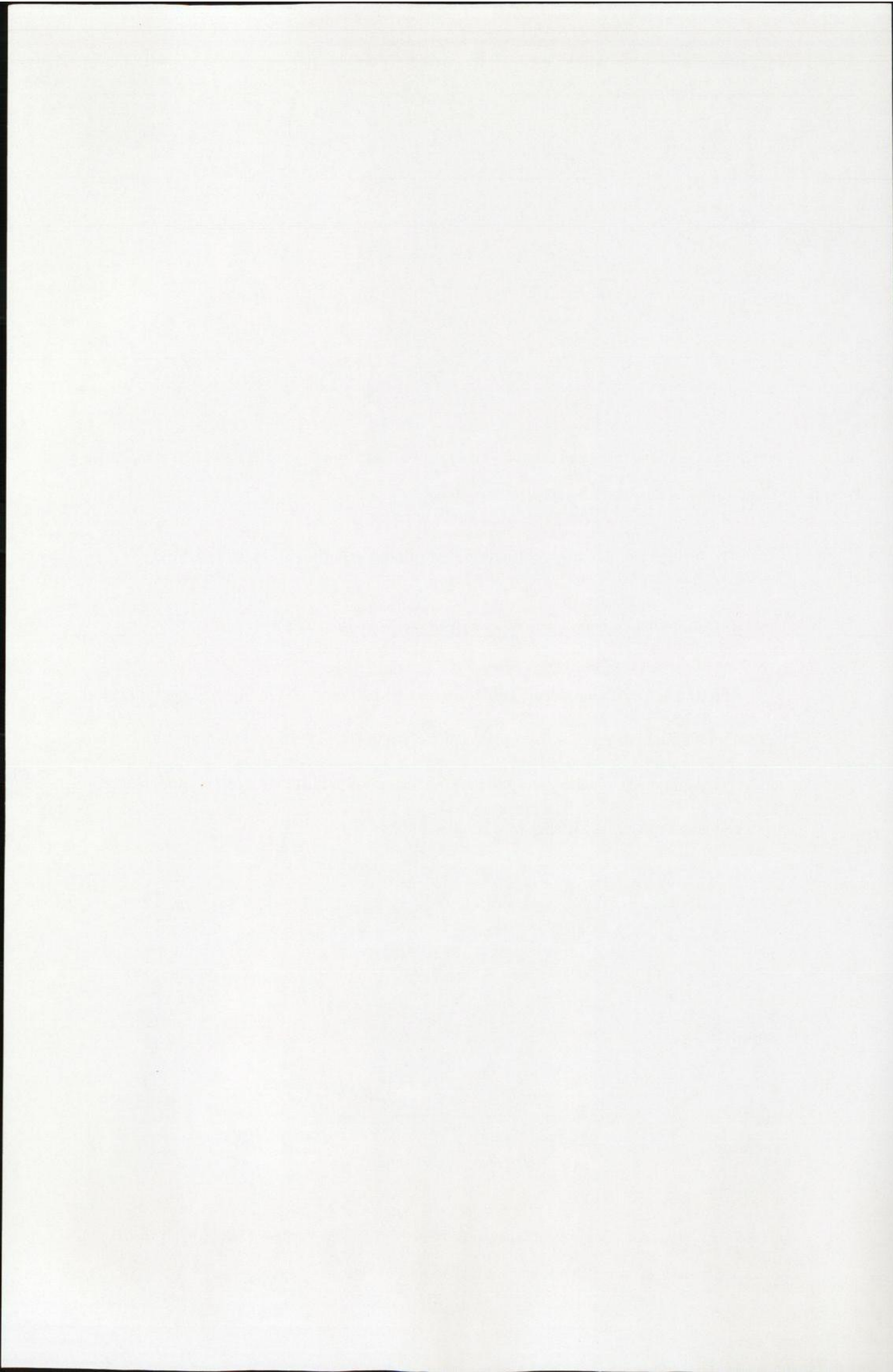


Figure 6.3 Absorbance measurements at minute intervals of vitellin standard (200ng/100µl) spiked with increasing amounts of male haemolymph protein (0 to 900ng/100µl).



50% vitellin and 50% male protein

Secondly, a series of standards, containing a fixed ratio of 50% vitellin and 50% male protein (from 10ng/100μl to 800 ng/100μl) were added to the wells in triplicate. Again, these standards behaved in the same way as pure vitellin standards, with a plateau seen at 400ng/100μl vitellin. Additional haemolymph proteins, even when their total concentration was equal to that of vitellin, did not compete with Vt for binding sites (figure 6.4).

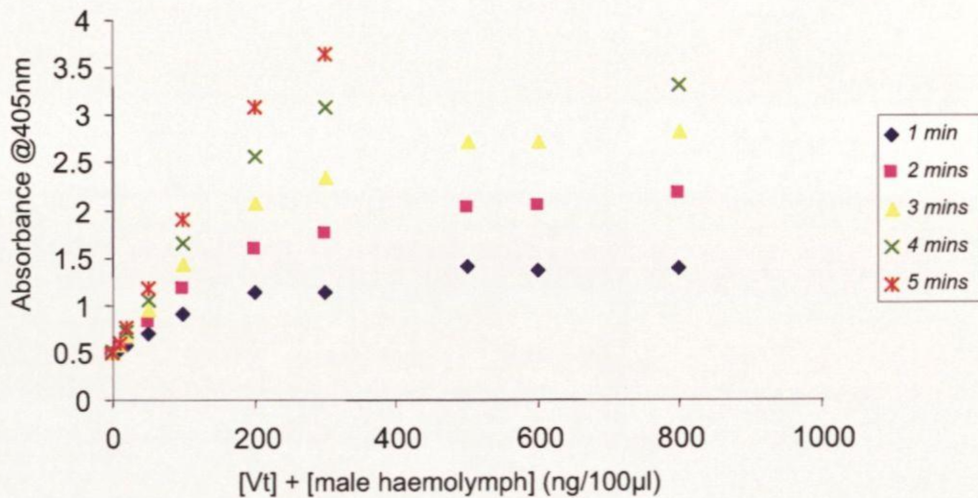
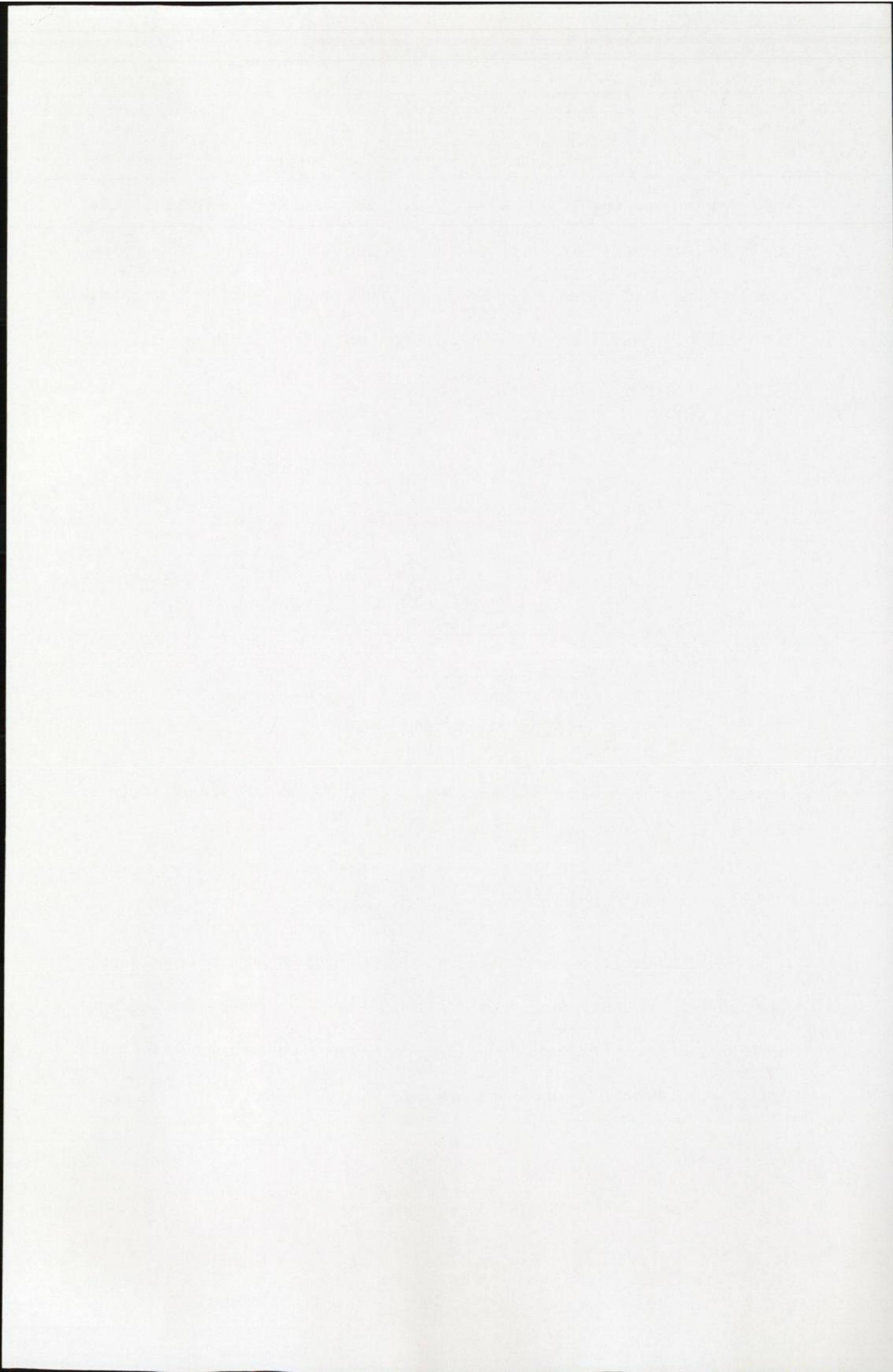


Figure 6.4 Absorbance measurements at minute intervals of a 50:50 vitellin/male haemolymph protein standard series from 10-800ng/100μl.

Increasing vitellin vs fixed concentration of male protein

A third series of standards, containing a fixed amount of male protein (400ng/100μl) and an increasing amount of vitellin (10 to 800 ng/100μl) were added to the wells in triplicate. The response was the same as the pure vitellin standard series, with a plateau seen at 400ng/100μl of vitellin (see figure 6.5).



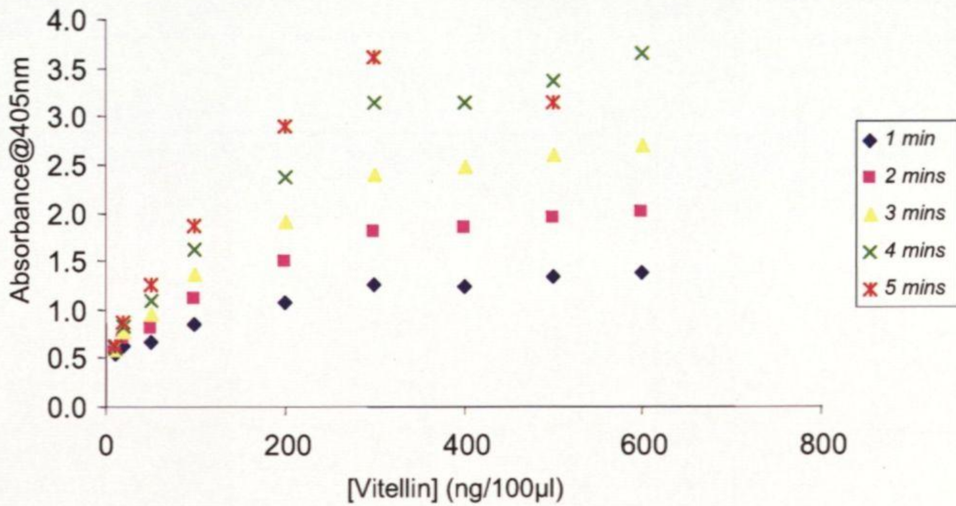


Figure 6.5 Absorbance measurements at minute intervals of a standard series consisting of a fixed concentration of male protein (400ng/100µl) spiked with increasing amounts of vitellin (10-600ng/100µl).

Non-vitellogenic female *haemolymph proteins vs vitellin.*

Lastly, the preferential binding theory was tested using non-vitellogenic *female* haemolymph proteins. This was to investigate whether female specific proteins influence vitellin binding. A series of solutions of haemolymph protein from non-vitellogenic green and red females were spiked with known quantities of Vt (in the same way as in the initial experiment with male haemolymph). It was found that non-vitellogenic female haemolymph proteins do not affect vitellin binding (figure 6.6).

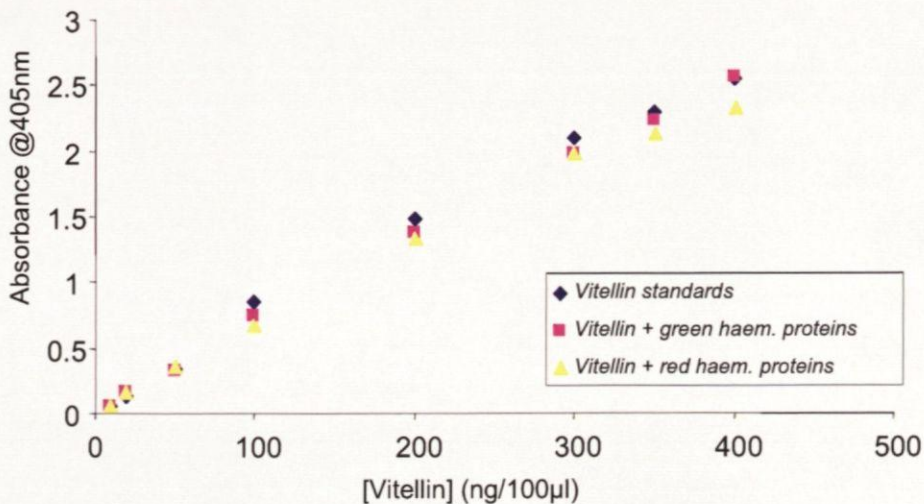
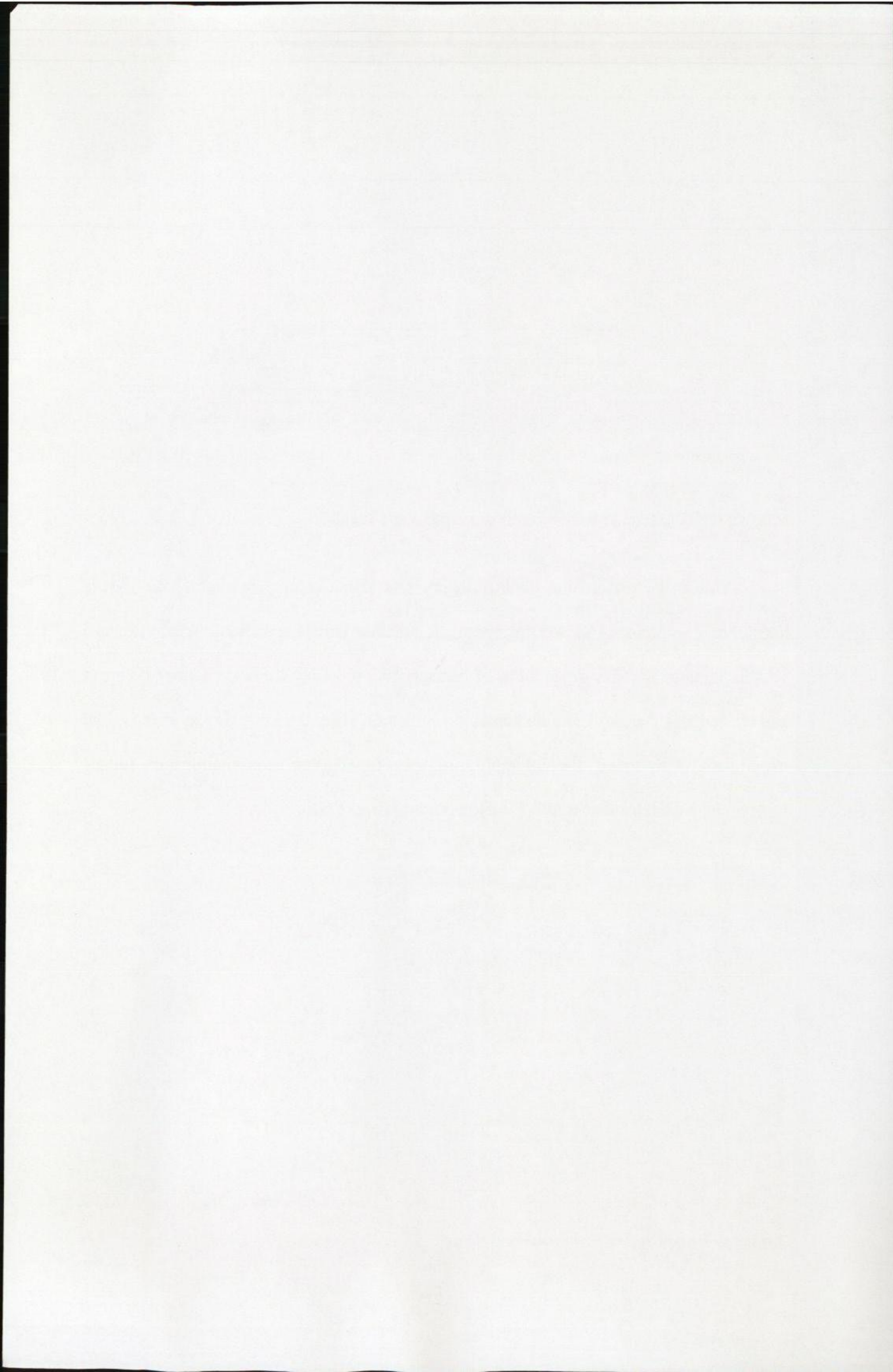


Figure 6.6 Absorbance measurements of a series of protein standards from non-vitellogenic green and red females spiked with increasing amounts of vitellin. (10-400ng/100µl).



These experiments suggest that vitellin is preferentially binding to the well and that other haemolymph proteins (male or female) do not compete for binding sites. As a result, it can be assumed that all the vitellogenin in any given sample will bind when added to a microplate well, provided its concentration is not greater than 400ng/100µl. Any change in total vitellogenin concentration (per 100µl of sample) can be therefore be measured using the present ELISA protocol. The ELISA can then be applied to confidently quantitate Vg in haemolymph samples, allowing the measurement of any fluctuations in Vg level associated with exposure to exogenous ecdysteroids.

6.2.2 Collection of experimental animals and laboratory conditions

For the vitellogenesis experiments, female *C.maenas* were collected on incoming tides using a baited drop-net from Jenkins Quay on the Avon estuary at Bantham, South Devon, England. On return to the laboratory, crabs were maintained for an acclimation period of 1 week in holding tanks containing natural, well-aerated 34ppt, 15+/-1°C seawater, under a 12h light: 12h dark regime. During exposure experiments laboratory conditions were unchanged from those of the acclimation period.

For the locomotor activity experiments, intermoult male crabs of carapace width (<65mm) were collected in the same way as above, but transferred immediately to the actograph test system on return to the laboratory. During the experimental period, crabs were kept in glass aquaria containing 8L of aerated, prepared seawater (Instant Ocean, salinity 34ppt), at 20°C under constant low-level light.

For the moulting experiments, juvenile crabs (of carapace width <5mm) were collected by hand at low tide from a rocky shoreline (Jennycliff Beach, Plymouth). On return to the laboratory they were size classed, transferred to the moulting "cages" described below (see moulting methods), and immersed in glass aquaria containing 10L of natural, aerated, 34ppt, 15+/-1°C seawater, under a 12h light: 12h dark regime.

6.2.3 Waterborne 20-hydroxyecdysone exposure

Waterborne exposures were of the semi-static renewal type. Water changes were immediately followed by redosing with the test chemical.

The ecdysteroid moulting hormone 20-hydroxyecdysone (20-HE) (Sigma cat no: H-5142) was dosed into seawater using methanol as a solvent vehicle (to increase its solubility). Firstly, a 10mg/ml stock solution of 20-HE in methanol was prepared. A dosing solution was then prepared by dissolving a 250μl aliquot of the stock solution in 24.75ml of filtered seawater. This avoided the addition of small volumes of stock solution into the large volume of seawater in each aquarium.

6.2.4 Vitellogenesis

Using the vitellogenin ELISA on haemolymph samples, the effects of exposure to exogenous 20-HE on vitellogenesis in females was investigated.

Pre-screening of female crabs for exposure experiments

Before exposures could begin, it was imperative that the vitellogenic status of the experimental animals be known. Knowledge of the level of haemolymph vitellogenin at time zero is needed so that each animal can be used as its own control. Changes, if any, in vitellogenin titres can then be placed in the context of the animals starting point and then compared to control animals whose vitellogenic history is also known.

Initially, it was hoped that all crabs used would be at the same stage of vitellogenesis. The best way to ensure this is to collect females at the same moult stage and at the beginning of the vitellogenic cycle. This would allow the comparison of like with like and measurement of the whole reproductive cycle. Green coloured (intermoult) females usually fit the above criteria as they are recently moulted, mated and ready to start

vitellogenesis. Sampling trips (to Jenkins Quay and additional locations), however, yielded far fewer green females than were needed. Red coloured (premoult) females are much further into vitellogenesis and show greater variability with respect to haemolymph Vg, but were much more plentiful at the time of sampling (June/July 2001).

Therefore, the decision was made to pre-screen all crabs for Vg and assign them to classes according to their approximate vitellogenic state. Each treatment group would then contain representatives from each class, whose Vg levels were known at the time of exposure. Future measurements of Vg would not be compared directly as a mean for each group but be presented as a value relative to the starting point of each crab. Each crab is essentially assigned a position on the vitellogenesis continuum and effects of exogenous hormone would be measured as deviations from the norm at each position (determined by the control group).

Before vitellogenin screening, all crabs were first numbered and labelled (with labels glued to the carapace) according to their approximate moult stage. Pre-moults were identified with the prefix "R" e.g. R2 and intermoults with "G" e.g. G11. These colour classifications can only be considered approximate since subtle differences in moult stage in similarly coloured crabs are common. Pre-screening of female crabs was then carried out to determine their vitellogenic status. Following the pre-screen, individuals were assigned to groups according to their vitellogenic status. Representatives from each group were then included in each treatment set and were identifiable by their colour-number for repeated sampling.

20-hydroxyecdysone exposure trial

Prior to 20-HE exposure, all crabs were kept under control conditions (as described above) for two weeks, and haemolymph sampled weekly. These two pre-exposure samples were then included in the sample set for each crab.

Pre-screened female crabs were then exposed to waterborne 20-HE at the nominal concentrations of 100μgl^{-1}, 5μgl^{-1}, 1μgl^{-1} and 100ngl^{-1} for a period of 16 weeks, in glass aquaria containing 10L of natural, filtered, aerated seawater. Ten females were exposed at each concentration, in two aquaria containing five crabs each. Two groups of pre-screened females were also included as solvent (n=8) and seawater controls (n=10). Haemolymph samples (100μl) were taken weekly from each crab, via the arthrodial membranes at the base of the walking legs. Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Crabs were fed every three days with irradiated whole cockle and their water changed and re-dosed within 12h of feeding. Moulted crabs were removed wherever possible to avoid cannibalism from other individuals. These crabs were then mated and their exoskeleton allowed to fully harden before being returned to the exposure trial. Dead crabs were also removed to avoid fouling of the exposure water.

Baseline data set for non-exposed crabs

In order to establish the normal pattern of haemolymph Vg levels in vitellogenic females over time, a baseline study was run alongside the exposure trials. 40 female pre-moult crabs, collected and maintained as above, were haemolymph sampled weekly over a period of 24 weeks. Samples were then quantitatively assayed for total protein and then Vg as described below.

Quantitative ELISA

Each weekly haemolymph sample was first assayed for determination of total protein by the BioRad Bradford method (as described previously in chapter 5) using bovine serum albumin (BSA) as standard (0.21-1.4mg/ml). This was required to calculate the volume needed to yield a concentration of 400ng/100μl for use in the quantitative Vt ELISA. All samples from each individual crab were run on the same microplate, so as to avoid any interindividual variation in binding affinity between plates.

For analysis of haemolymph vitellogenin levels, 400ng/100μl of haemolymph protein was added to triplicate wells of a 96 well microplate, alongside a triplicate series of Vt standards (from 2ng/100μl to 100ng/100μl). Coating buffer was then used as the diluent for both standards and samples. As before, all samples from each individual crab were run on the same microplate.

The plate, containing standards and diluted samples in triplicate was then incubated overnight at 4°C or for two hours at room temperature. Following incubation the plate was washed three times with TPBS to remove any unbound samples/standards. The remaining sites on the well walls were then blocked using 200μl of a bulk protein mixture (dried skimmed milk powder dissolved in PBS) which was left to incubate at room temperature for 1 hour.

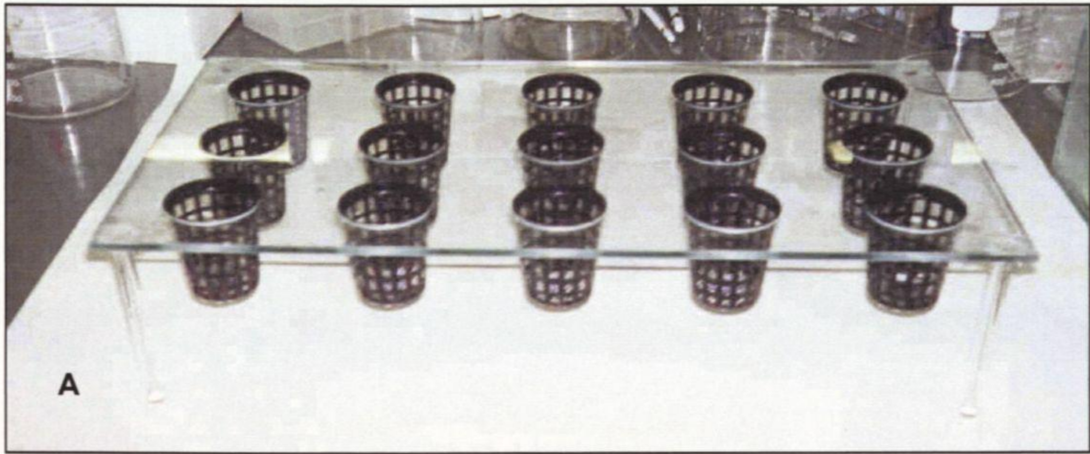
Following incubation with blocking buffer, the plate was again washed three times and 100μl of primary antibody (conjugated with milk powder dissolved in PBS) was added to each well. This primary antibody was raised against vitellin and binds to any Vg bound to the well walls. The plate was then incubated at 37°C for 1 hour, washed 3 times with TPBS and then 100μl of secondary antibody (conjugated with milk powder in PBS), was then added to each well. This secondary antibody binds to the primary antibody. The secondary antibody was left to incubate for 1 hour at room temperature. The plate was then

washed 4 times with TPBS and 100µl of developer solution added to each well. The absorbance of each well of the plate was then read at 405nm at minute intervals using a microplate reader. Concentrations of Vt in the samples were then interpolated from the standard curve.

6.2.5 Moulting

The effect of exogenous hormones and mimics on the process of moulting can be studied by observing moulting frequency of *C.maenas* in control and exposed individuals. Juveniles of the species (carapace width of <12mm) are used since the frequency of moulting at this life stage is highest. To preclude cannibalism of conspecifics (a common occurrence following moult in *C.maenas*) and to allow identification of moulted individuals, crabs were kept separately in sealed cages suspended from glass frames, immersed in glass aquaria of aerated seawater (figure 6.7). In this way, up to 15 individuals per tank were exposed to the test chemical simultaneously and under identical conditions of constant temperature, light:dark cycle and salinity, without loss of newly moulted individuals.

Waterborne exposures were carried out at the nominal concentrations of 100µgl^{-1}, 5µgl^{-1} and 1µgl^{-1} 20-HE (n=15) with solvent and seawater controls (n=9) over a period of 16 weeks. Crabs were fed every 2 days and water changed and redosed within 12h of feeding. Tanks were checked daily and exuviae were counted, removed and measured following moult. Any mortalities were also recorded. The interval (days) between moults and size increase following moult was calculated for all crabs with more than one recorded moult within the exposure period.



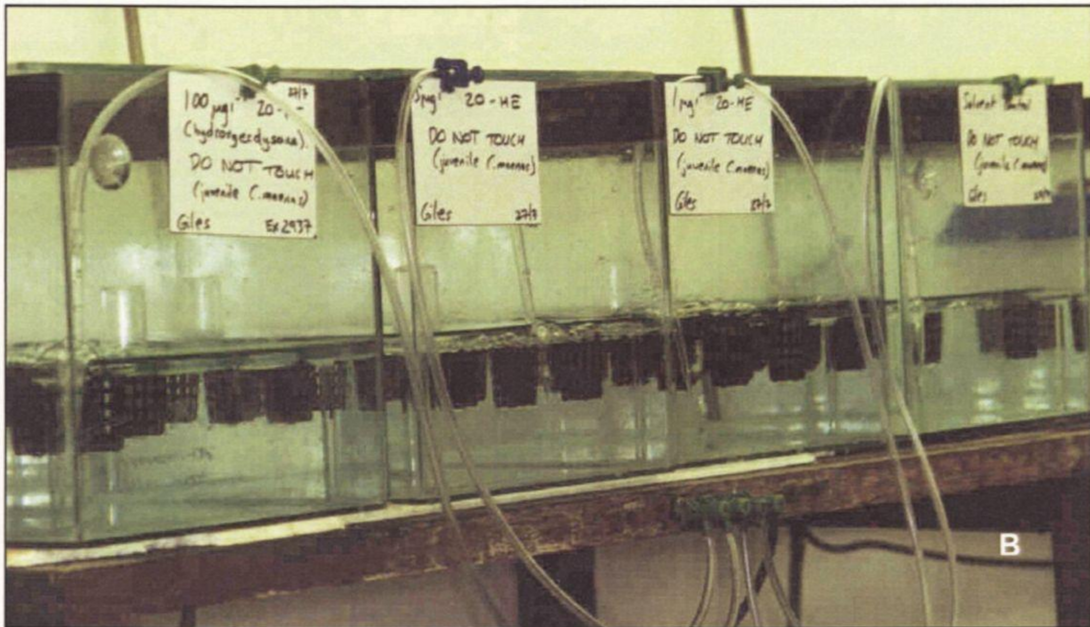
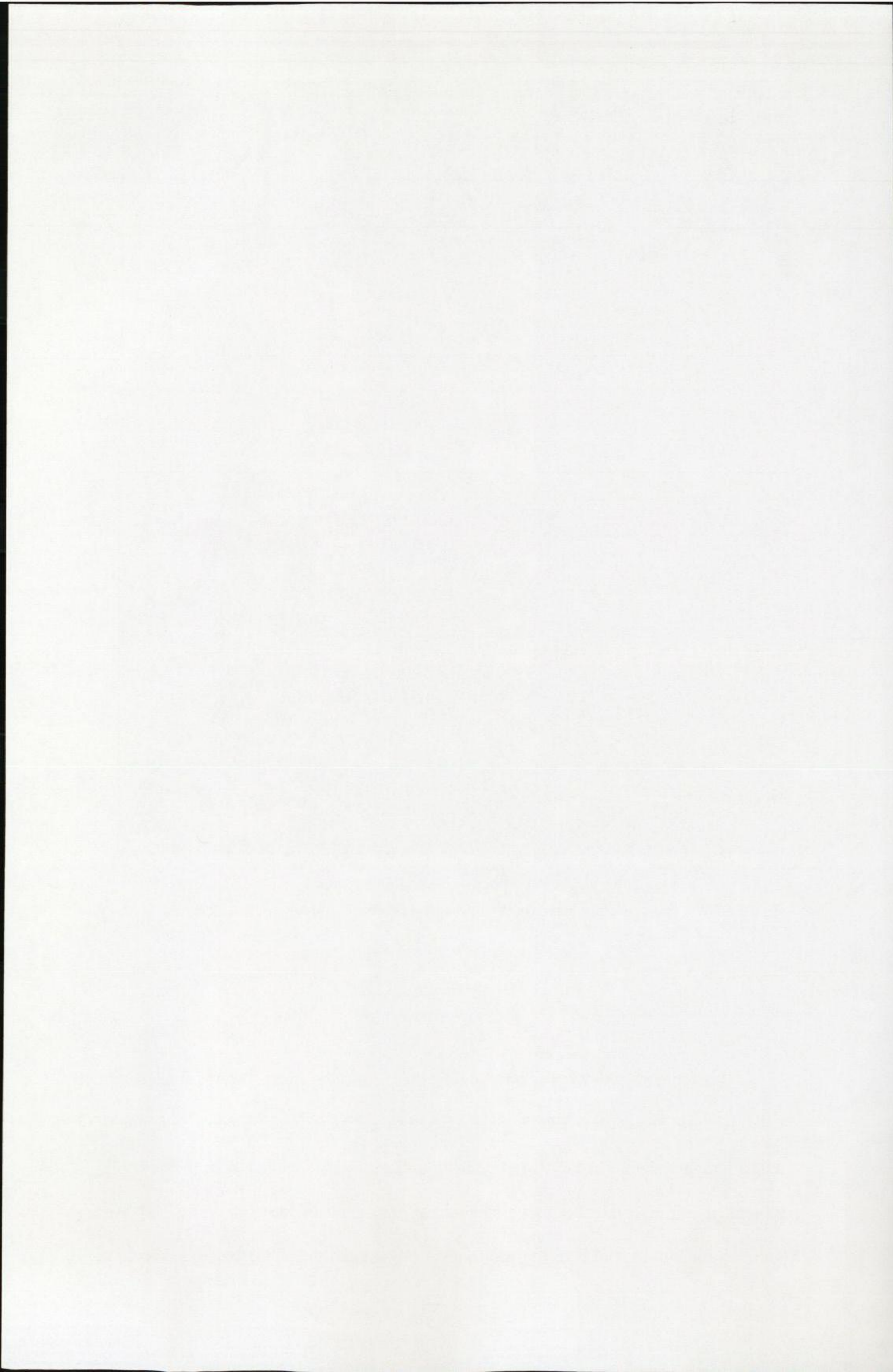


Figure 6.7 A - Glass moulting trays with individual containers. B - Moulting trays deployed in exposure tanks.

6.2.6 Locomotor activity

Locomotor activity rhythms of freshly collected crabs were studied under conditions of constant low level light, temperature and salinity using tanks equipped with an actograph system. The activity of crabs in individual glass tanks was monitored using a system of infrared light beams passing across the width of the tank. As a crab passes between the emitter and detector, the beam is broken and this is logged as a "locomotory



event" by an attached computer (figure 6.8). Beam breaks over a period of 4-5 days were plotted against time to produce characteristic peaks of activity coincident with times of expected high tide and darkness. Such a system is ideally suited to observe the effect of exogenous chemicals and hormones on this predictable expression of locomotory behaviour in *C.maenas*.

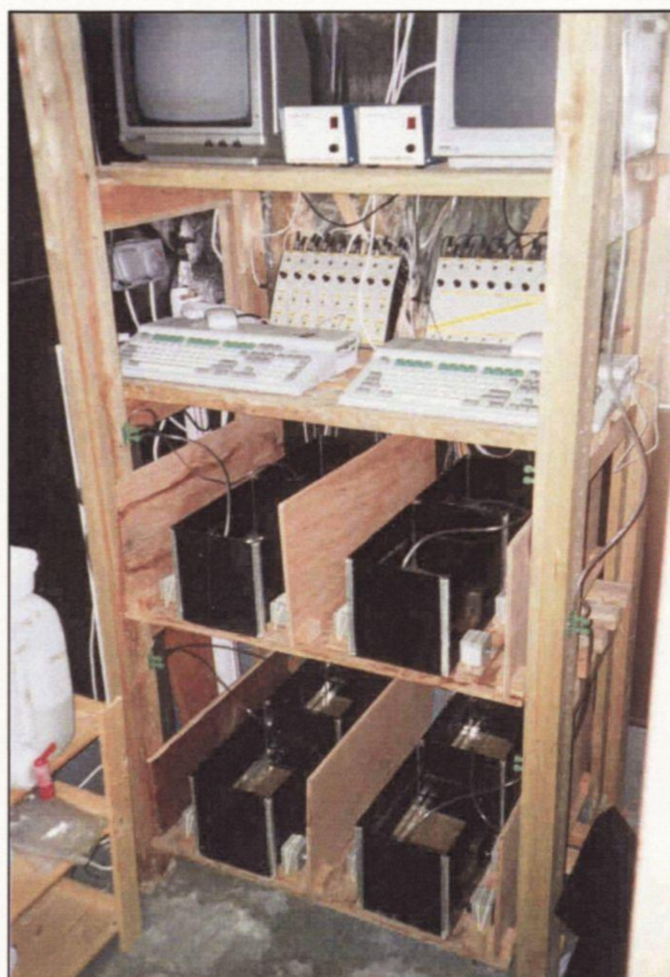


Figure 6.8 Actograph system showing tanks, infrared emitters/detectors and control computers.

Before any exposure experiments were performed, the activity of 8 crabs (kept in control conditions) was monitored in the actograph system over a period of 6 days. This was to provide a seasonal measure of expected activity in crabs collected in winter. In each of two separate exposure trials, 4 crabs were exposed to 100μgl^{-1} 20-HE, alongside 4 solvent controls, for a period of 6 days. Crabs were kept in glass aquaria containing 8L of

aerated, prepared seawater (Instant Ocean, salinity 34ppt), at 20± 3°C under constant low-level light.

6.2.7 Tebufenozide exposure trial

The effect of the moulting hormone agonist tebufenozide on moulting and locomotory activity was investigated using waterborne exposures. The experimental system was the same as described previously for investigating both parameters. Vitellogenin titre following exposure to tebufenozide were not investigated due to time constraints and seasonal unsuitability (the only available time for such an exposure trial was late winter (February), when vitellogenesis is typically quiescent).

Waterborne tebufenozide exposure

Tebufenozide (purity 99%, catalogue number PS-2188) was obtained from Greyhound Chromatography and Allied Chemicals (Birkenhead, England) and dosed into seawater using acetone as a solvent vehicle at the nominal concentrations of $100\mu gl^{-1}$ and $10\mu gl^{-1}$. Firstly, a 10mg/ml stock solution of tebufenozide in acetone was prepared. A dosing solution was then prepared by dissolving a 100µl aliquot of the stock solution in 4.9ml of acetone (for $100\mu gl^{-1}$) and 10µl in 4.99ml of acetone (for $10\mu gl^{-1}$) and dissolving this in 1L of seawater. This dosing solution was then mixed thoroughly for 1 hour using a magnetic stirrer and then immediately added to 9L of filtered seawater in 20L aquaria to yield the appropriate nominal concentration.

Juveniles (carapace width of <12mm) were exposed at the nominal concentrations of $100\mu gl^{-1}$ and $10\mu gl^{-1}$ tebufenozide (n=27) with solvent and seawater controls (n=15) over a period of 4 weeks. Crabs were fed every 2 days, water changed and redosed within 12h of feeding and checked daily for mortality and moults. Locomotor activity rhythms of freshly collected crabs exposed to tebufenozide were studied using the actograph system

described above. In each of two separate exposure trials, two crabs were exposed to $100\mu gl^{-1}$ tebufenozide, alongside two solvent controls, for a period of six days (n=4 for each treatment).

6.3 Results

6.3.1 Moulting

Figure 6.9 shows cumulative mortality over the length of the exposure period for each treatment. Mortality for the two highest exposure groups (100 and 5μgl^{-1}) was clearly elevated over the remaining three treatments. The majority of these mortalities were amongst the smallest size class of crabs (5-6mm carapace width).

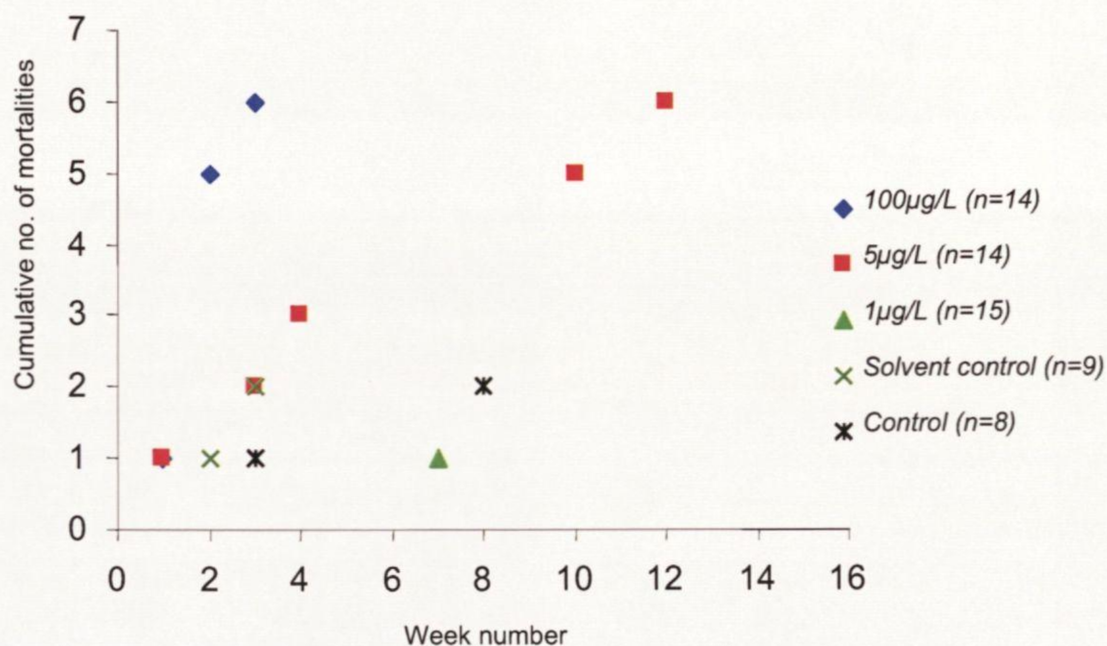
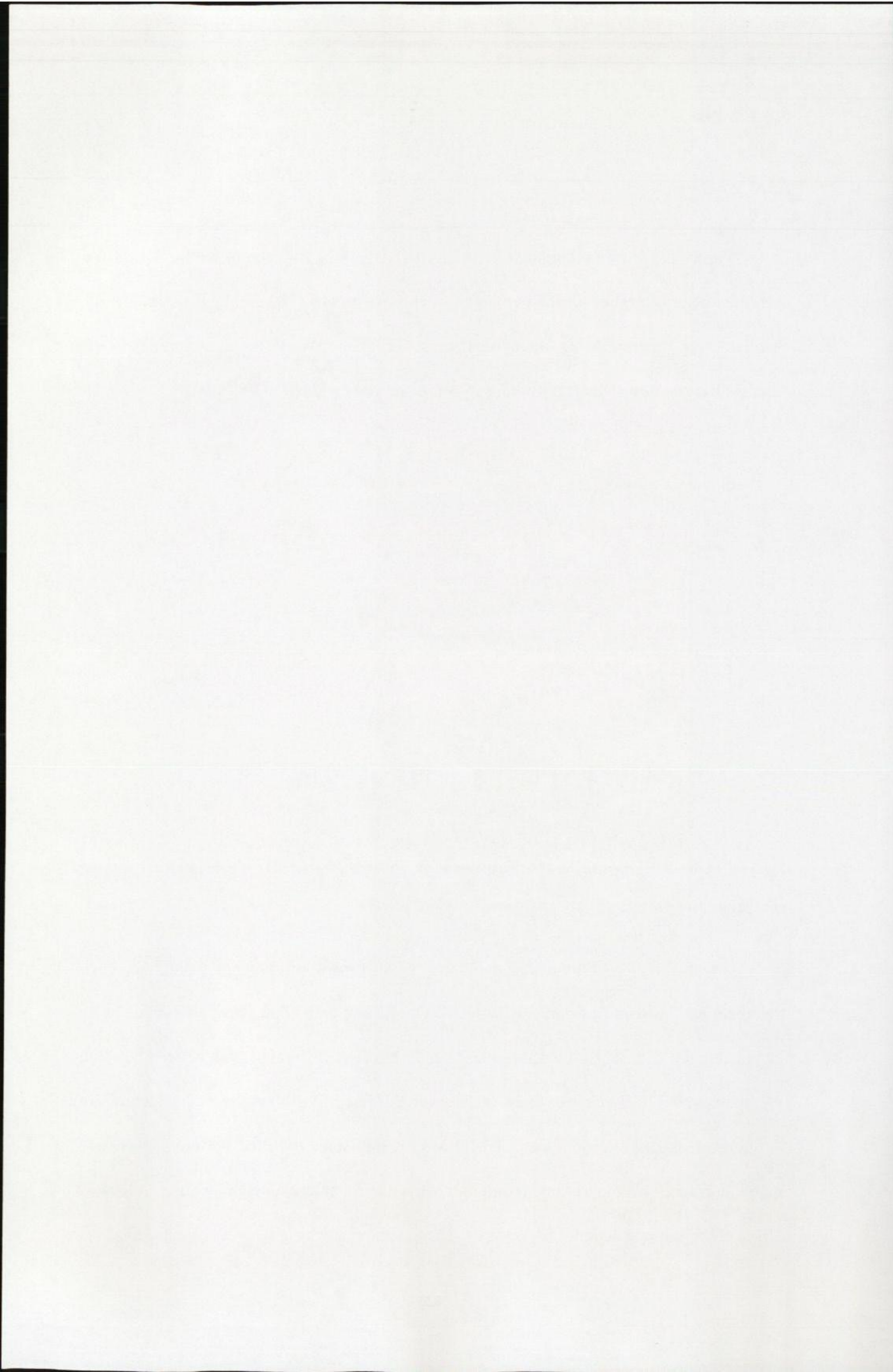


Figure 6.9 Cumulative mortality in 20-HE exposed and control crabs (n= number of individuals at beginning of exposure period).

Mean number of moults and number of successful subsequent moults for each treatment can be seen in figures 6.10 - 6.14. Only those crabs that survived a moult were included in the data set for the subsequent moult. No clear differences can be seen between the treatments. The number of crabs undergoing third and fourth moults was reduced from the number undergoing their first and second moults, since the time between successive moults increases with increasing moult number. This is a natural phenomenon and is not an effect of the test chemical.



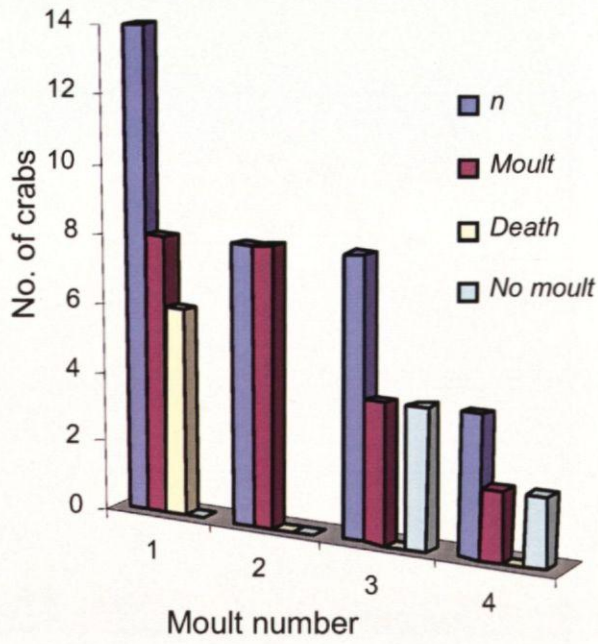


Figure 6.10 Number of moults, deaths and non-moults in the 100μg/l treatment group.

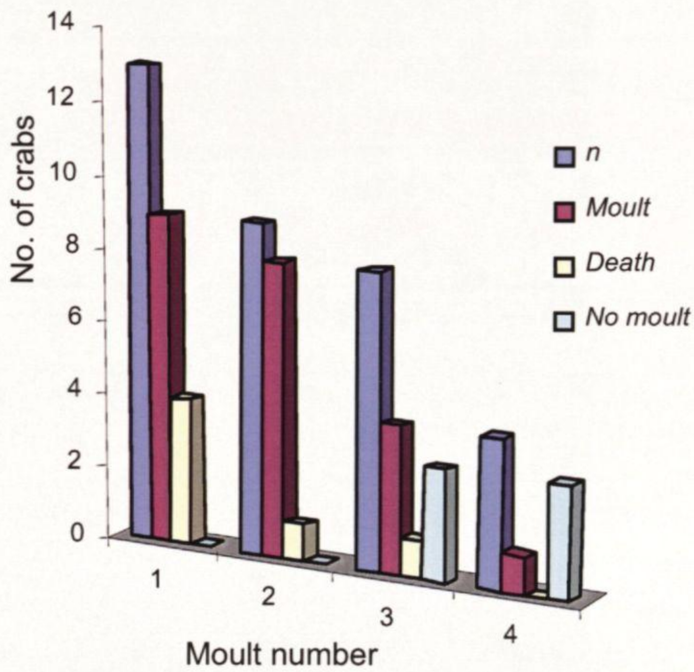
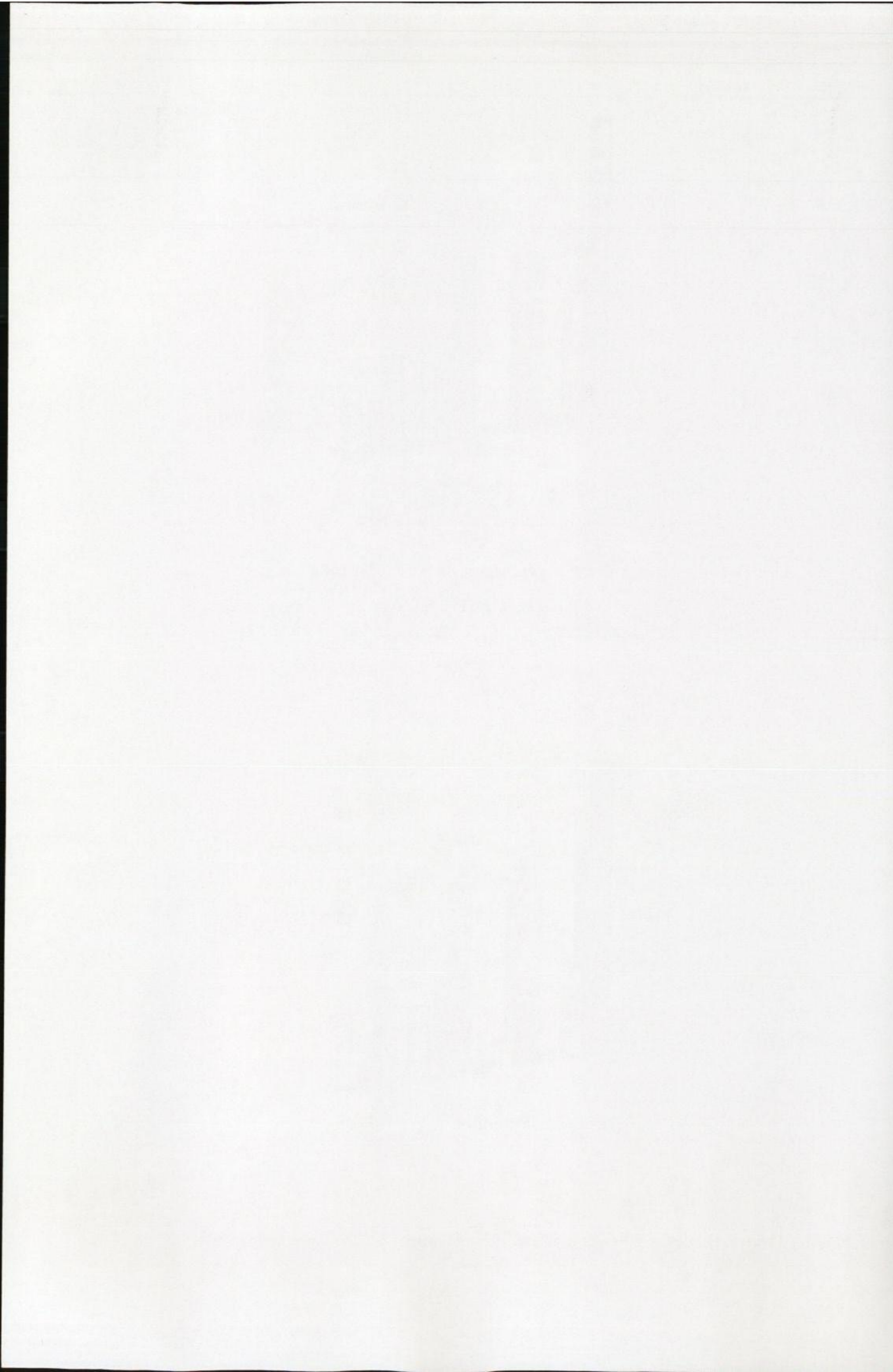


Figure 6.11 Number of moults, deaths and non-moults in the 5μg/l treatment group.



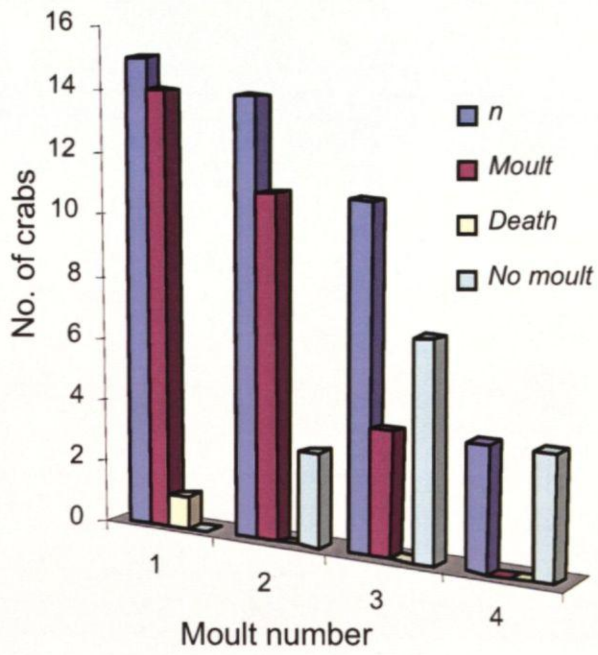


Figure 6.12 Number of moults, deaths and non-moults in the 1μg/l treatment group.

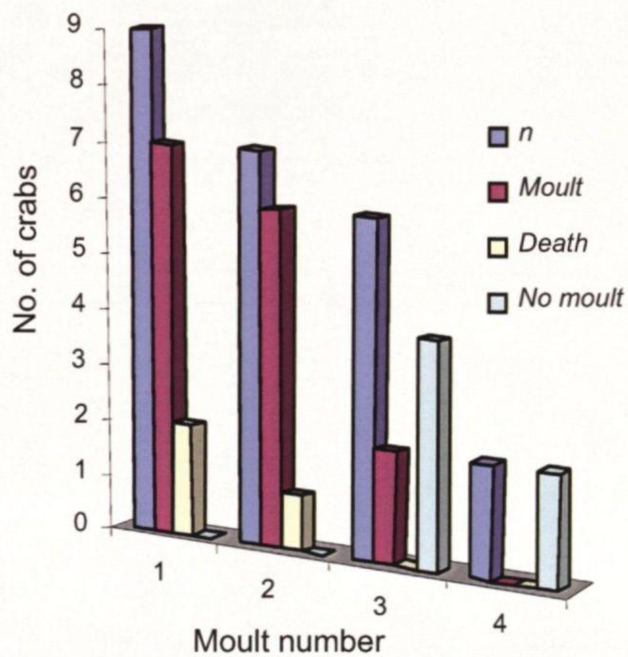
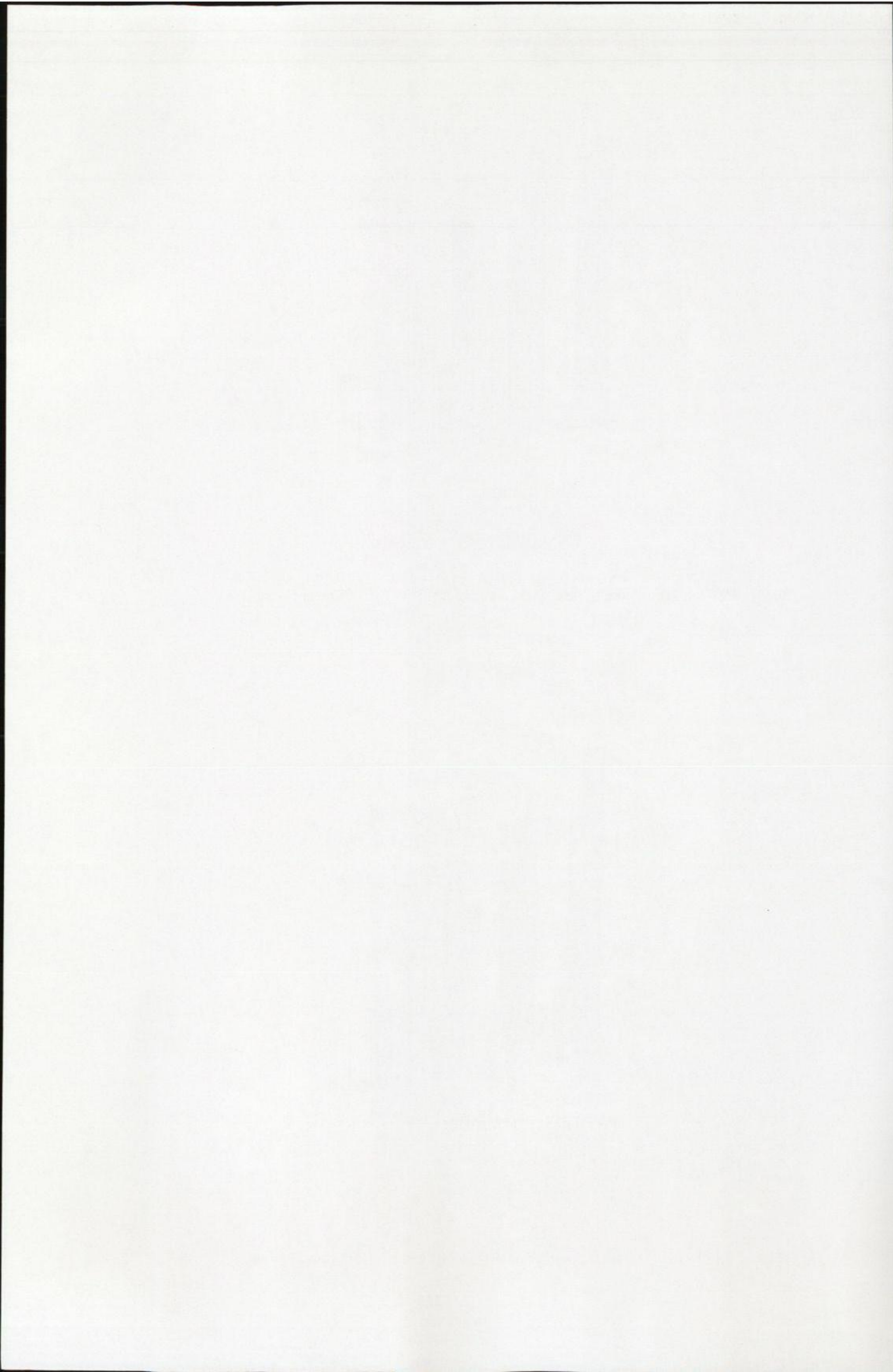


Figure 6.13 Number of moults, deaths and non-moults in the solvent control group.



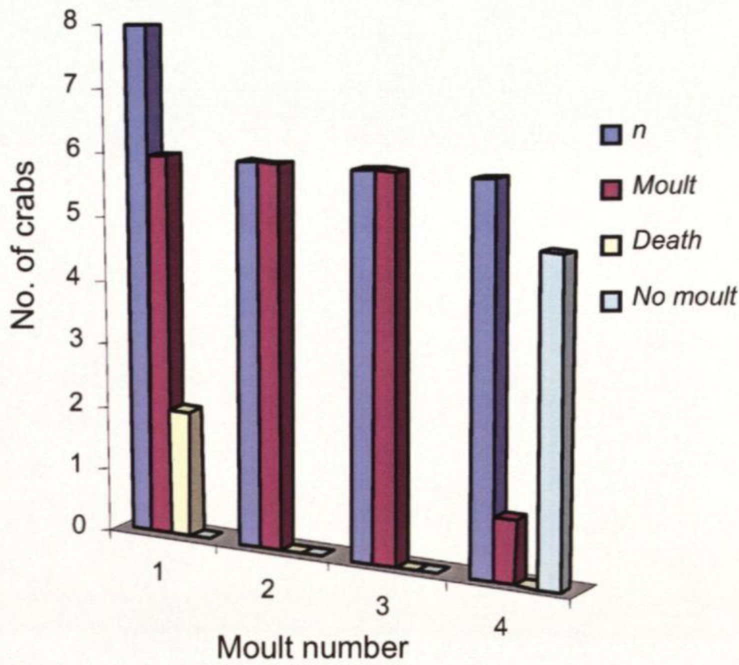
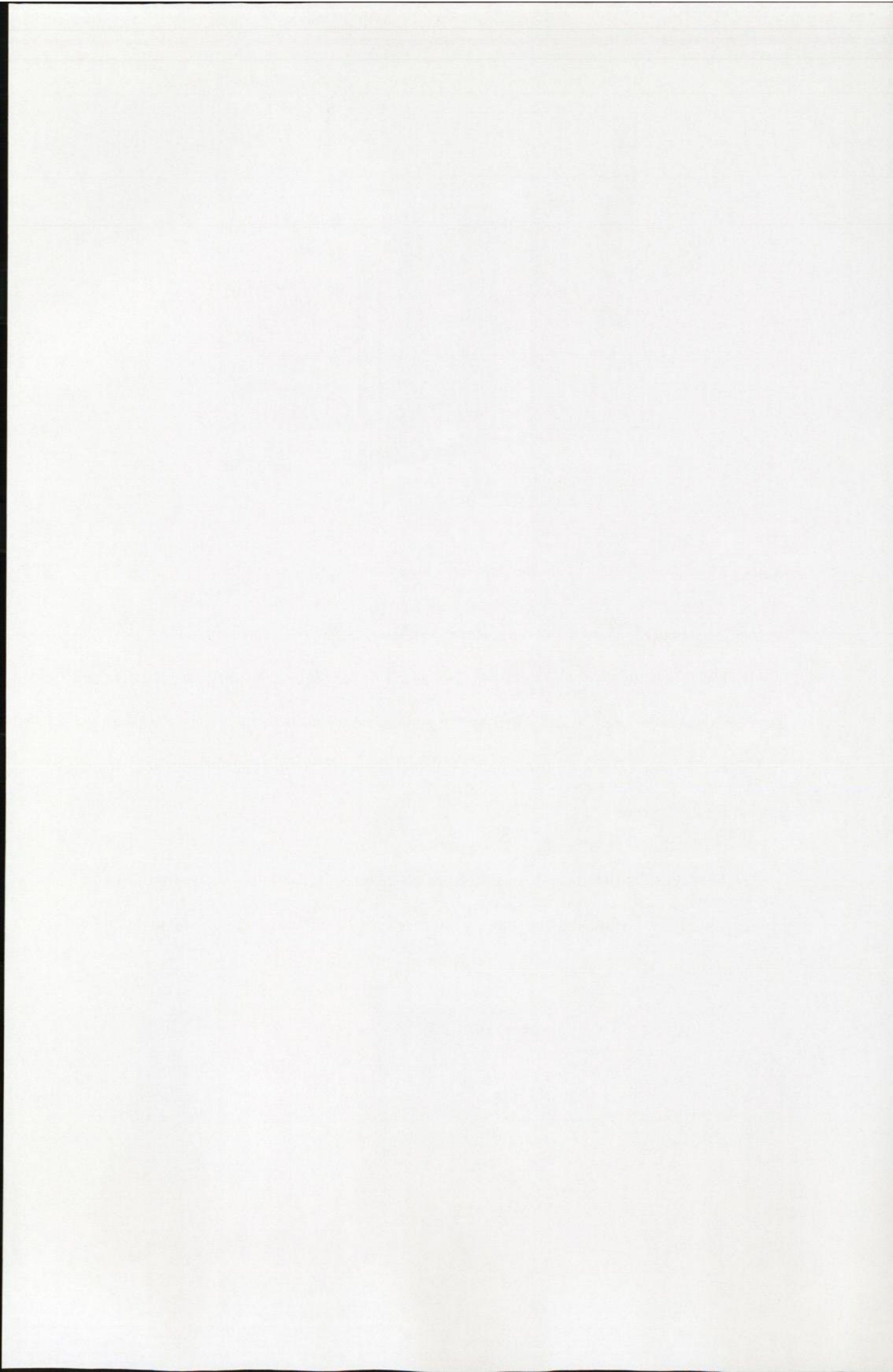


Figure 6.14 Number of moults, deaths and non-moults in the control group.

Figures 6.15 - 6.20 show the mean moult interval and mean size increase following moult at each treatment, for moults 1-2, 2-3 and 3-4. No significant differences between the treatments were seen, as indicated by the standard deviations of the mean. Exposure to 20-HE at the concentrations used had no discernible effect on moult frequency or on growth following moult.

These results illustrate that aside from an increase in mortality in the smallest size classes, 20-HE exposure did not have a gross effect on moulting processes in juvenile *C.maenas*.



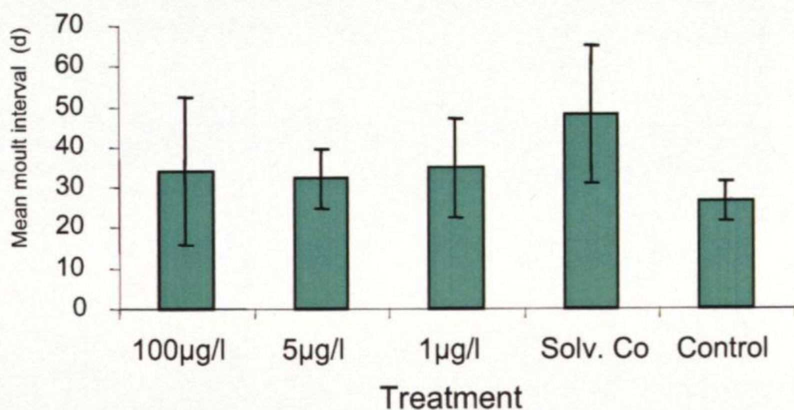


Figure 6.15 Mean (±SD) moult interval (1st to 2nd moult) for all treatment groups.

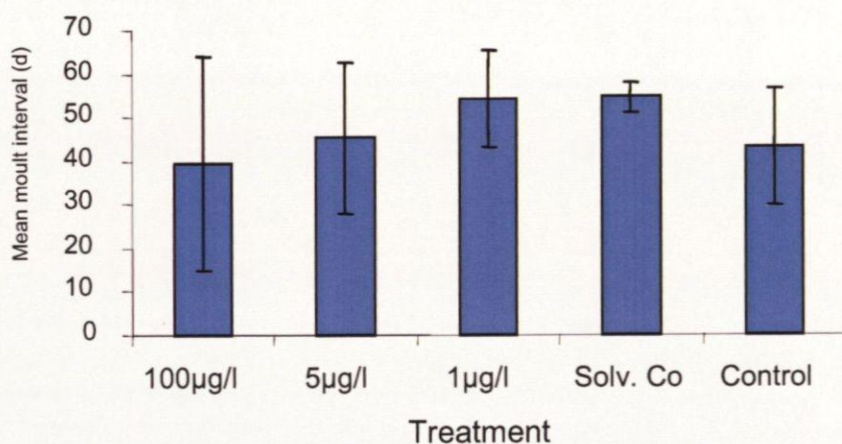


Figure 6.16 Mean (±SD) moult interval (2nd to 3rd moult) for all treatment groups.

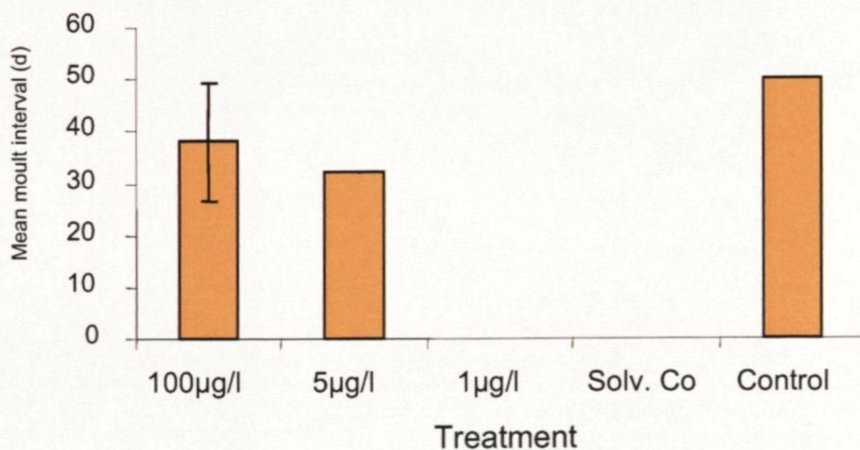
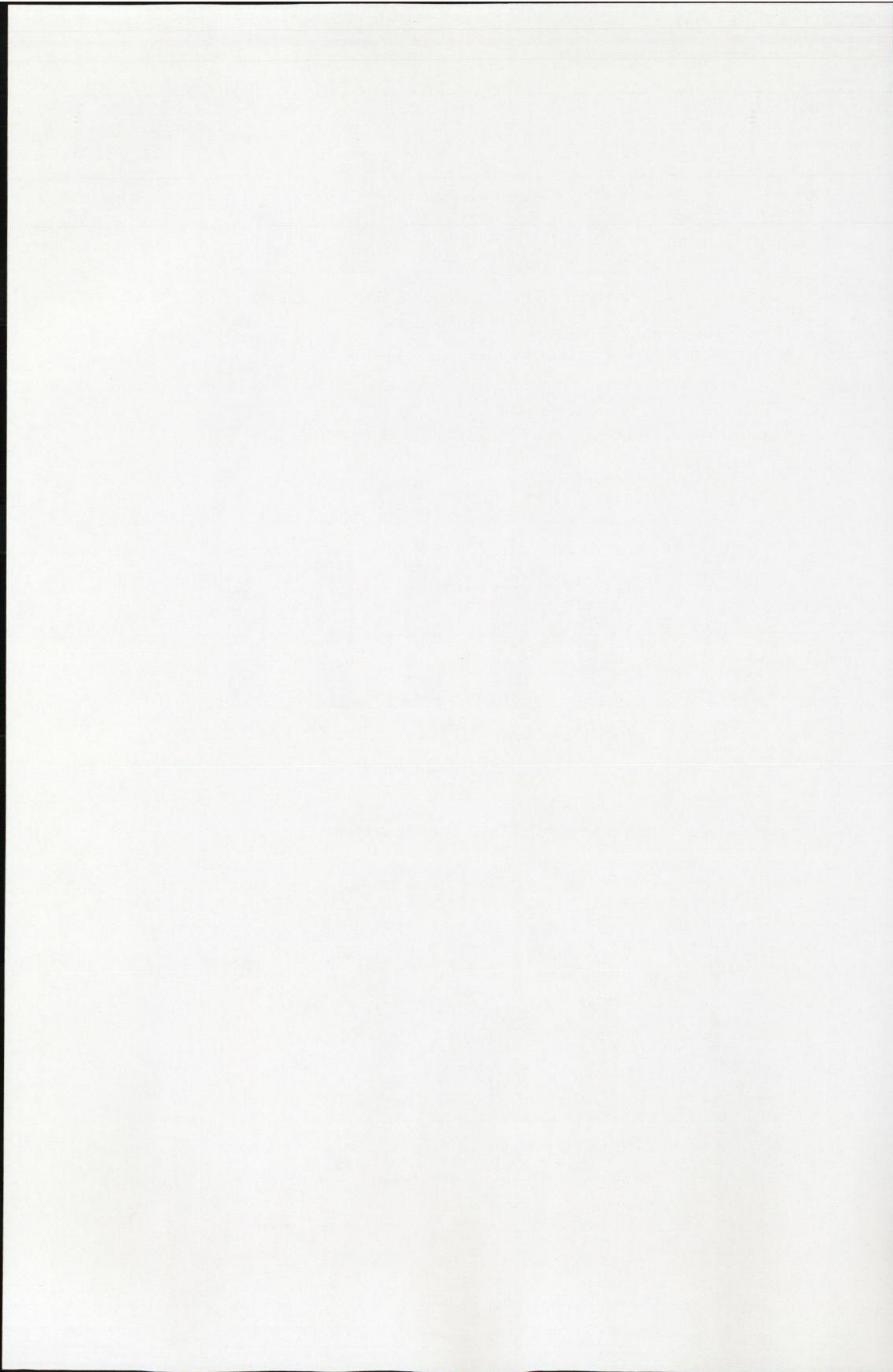


Figure 6.17 Mean (±SD) moult interval (3rd to 4th moult) for all treatment groups.



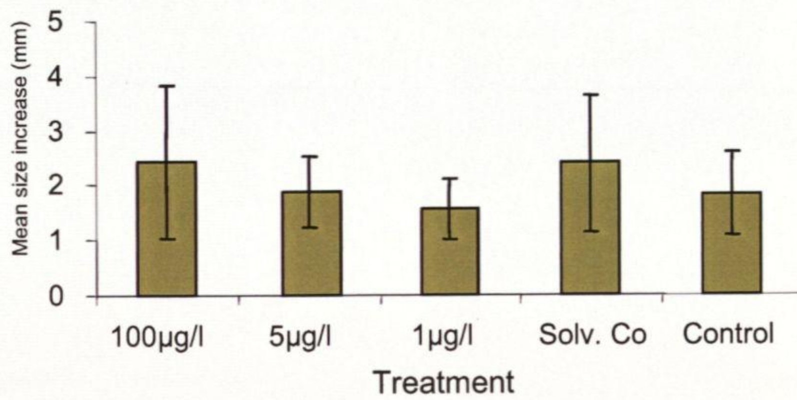


Figure 6.18 Mean (±SD) size increase following moult (1st- 2nd) in all treatment groups.

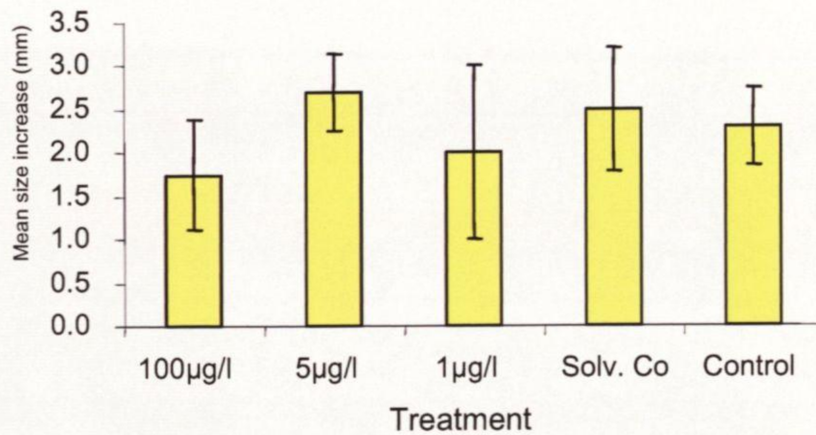


Figure 6.19 Mean (±SD) size increase following moult (2nd-3rd) in all treatment groups.

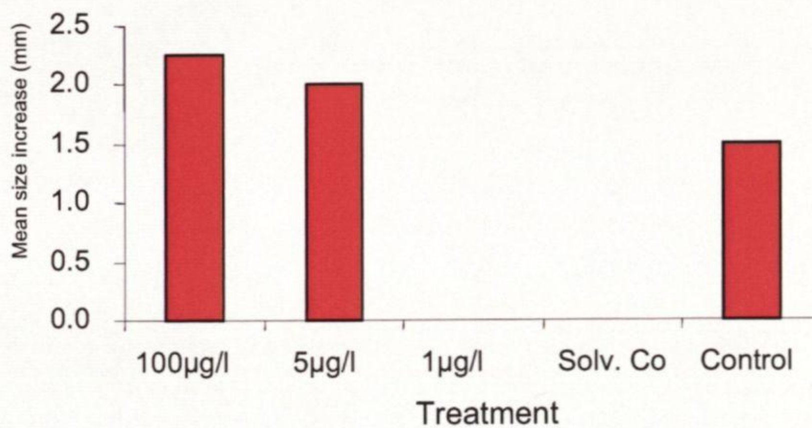
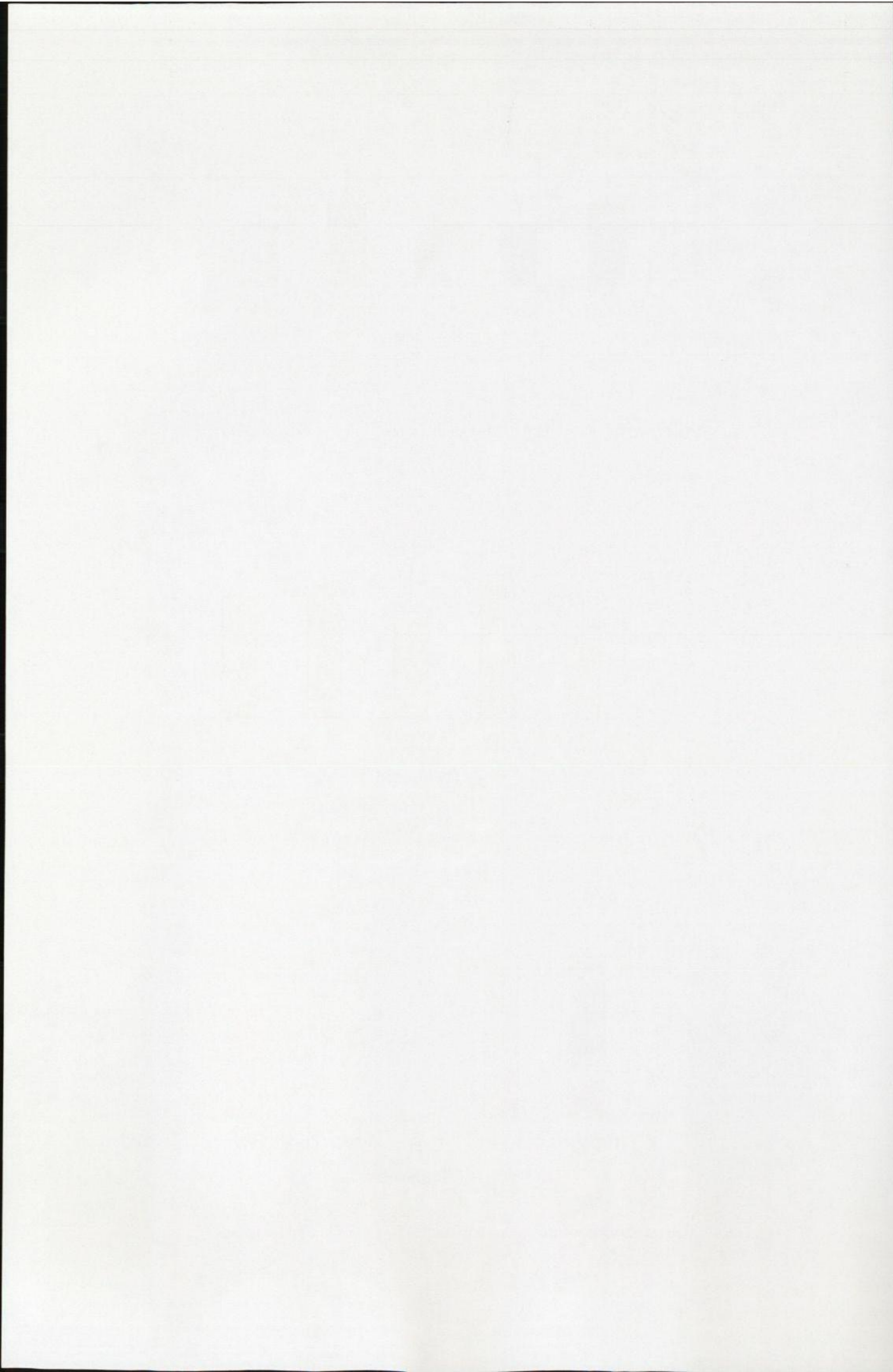


Figure 6.20 Mean (±SD) size increase following moult (3rd- 4th) in all treatment groups.



6.3.2 Locomotor activity

A typical mean locomotor activity trace for 8 crabs kept under control conditions can be seen in figure 6.21. Times of high water and approximate light and dark periods are shown. Peaks of activity can be seen around the time of expected high tide, with the amplitude of these peaks greatest at times of nocturnal high tides. Over time, in the absence of environmental cues, the rhythmicity of the activity pattern slowly breaks down and amplitude is slightly decreased.

Locomotor activity of both the 20-HE exposed and control crabs in the first exposure trial can be seen in figures 6.22 and 6.23. Mean activity of both treatment groups is shown in figure 6.24. No gross deviations from the expected pattern were observed in the exposed group. No discernible loss of rhythmicity was seen in the earlier stages of the exposure period and amplitudes of activity are broadly similar for both exposed and control groups. Breakdown of the characteristic pattern of rhythmic behaviour was consistent with the results of the initial control trial. The amplitude of mean activity increased towards the end of the experimental period in control crabs. This can be attributed to the increased locomotor activity of two crabs in particular (see figure 6.23).

Locomotor activity of 20-HE exposed and control crabs in the second exposure trial can be seen in figures 6.25 and 6.26. Mean activity of both treatment groups is shown in figure 6.27. No gross deviations from the expected pattern were observed in the exposed group. No discernible loss of rhythmicity was seen in the earlier stages of the exposure period and amplitudes of activity were broadly similar for both exposed and control groups. Breakdown of the characteristic pattern of rhythmic behaviour occurred earlier than expected in both treatment groups, and in control crabs, activity levels were greatly decreased after 48h. The mean activity pattern for exposed crabs (figure 6.25) is largely attributable to the contribution of one particularly active crab in the treatment group, thus creating the impression that locomotor activity was greatly increased above control levels

by exposure to 20-HE. A closer look at the results would reveal this not to be the case, as without the contribution of the aforementioned individual, control and exposed crabs were again broadly similar.

It would therefore appear that 20-HE did not cause gross perturbations to rhythmic locomotor activity patterns in *C.maenas* at the time of year the present study was undertaken.

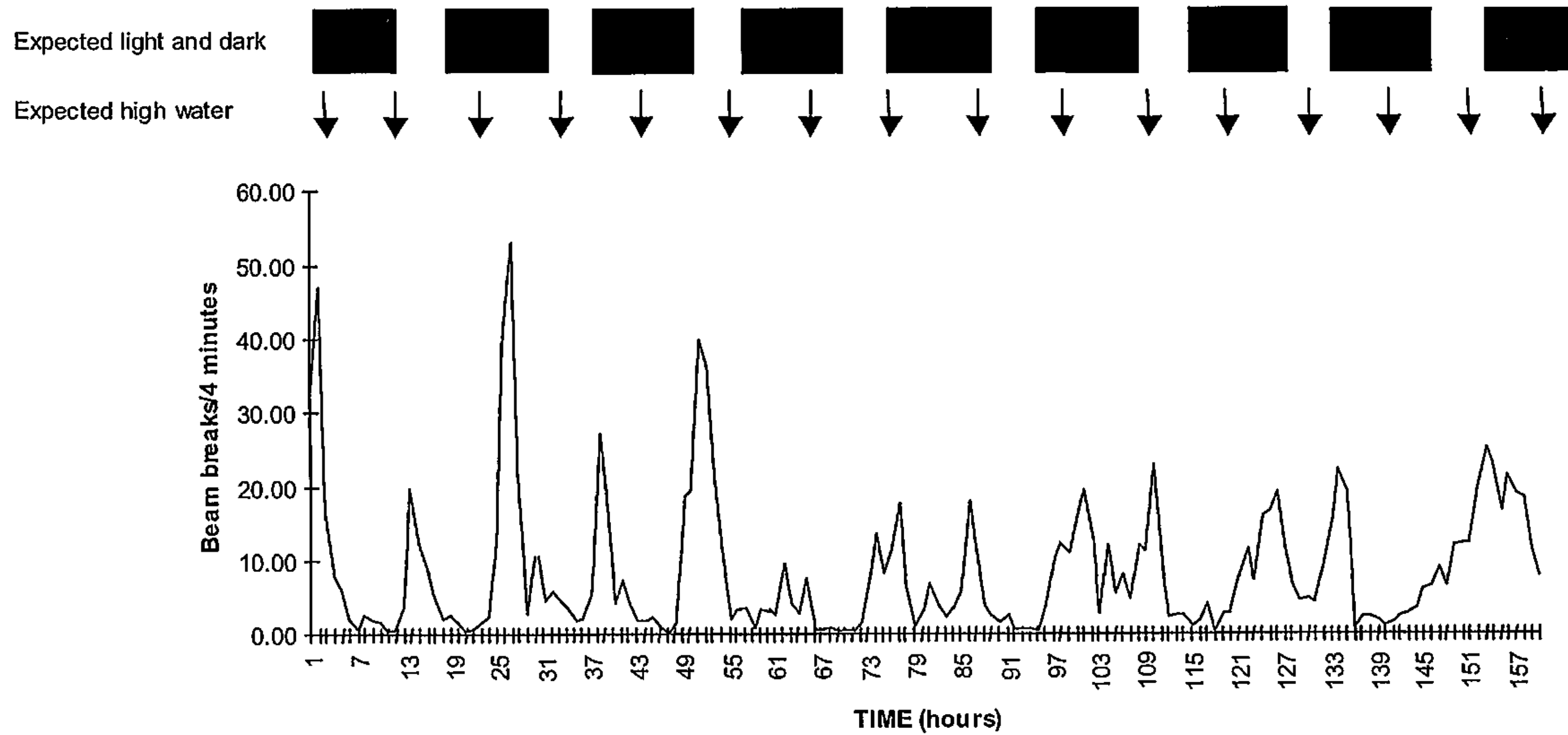


Figure 6.21: Mean locomotor activity for 8 crabs kept under controlled laboratory conditions.

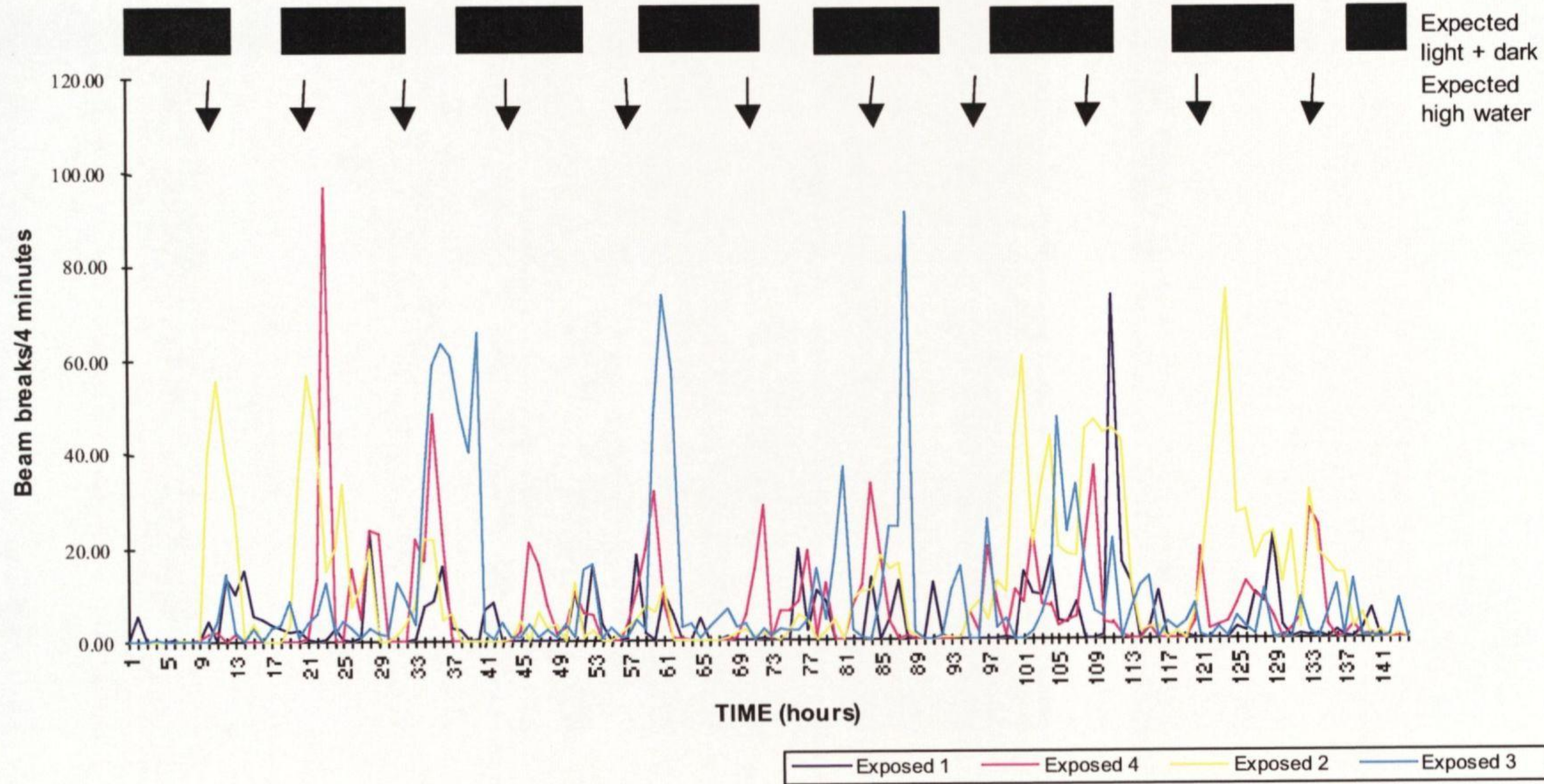
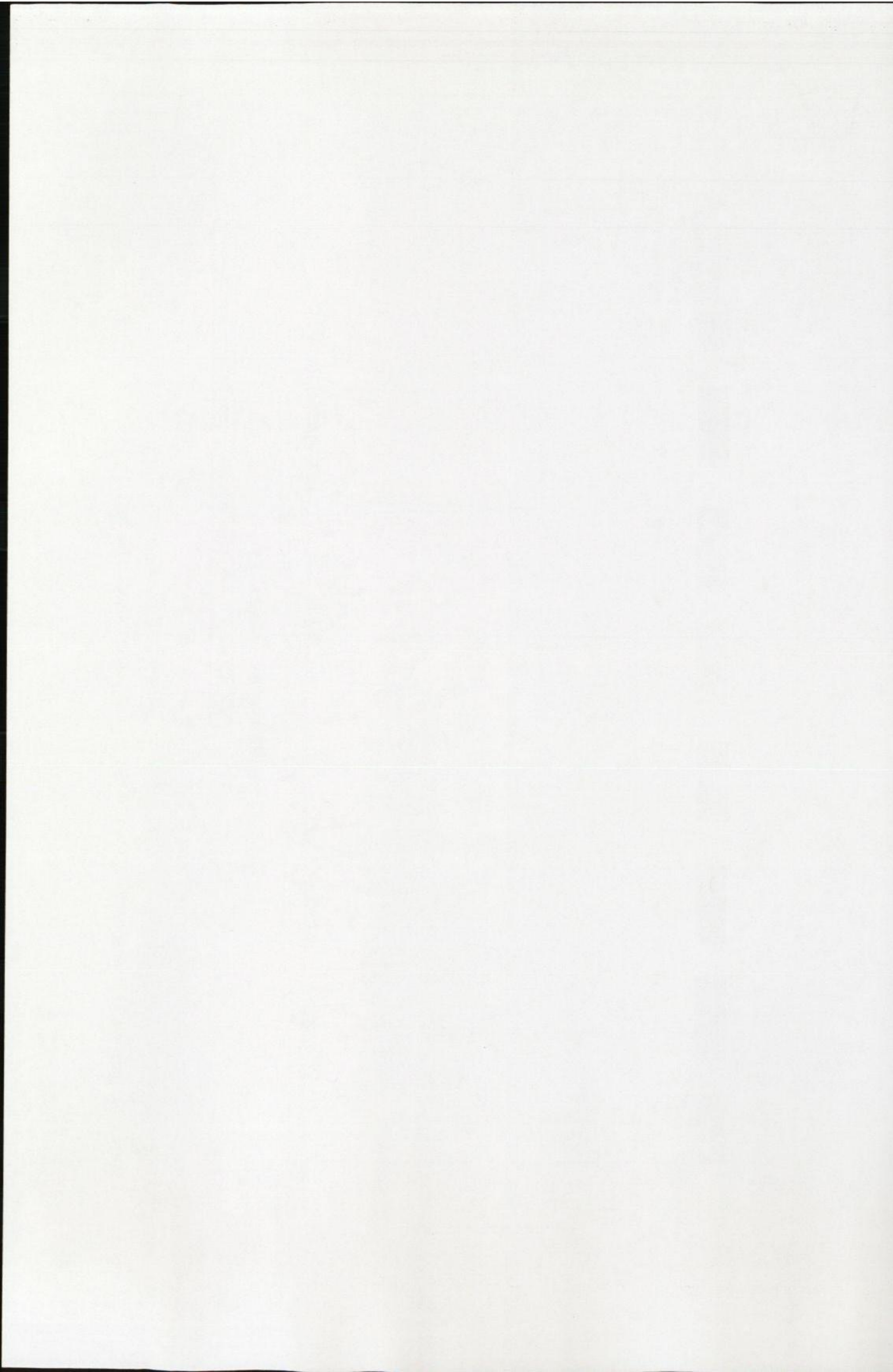


Figure 6.22: Individual locomotor activity for 4 20-hydroxyecdysone exposed crabs (trial 1).



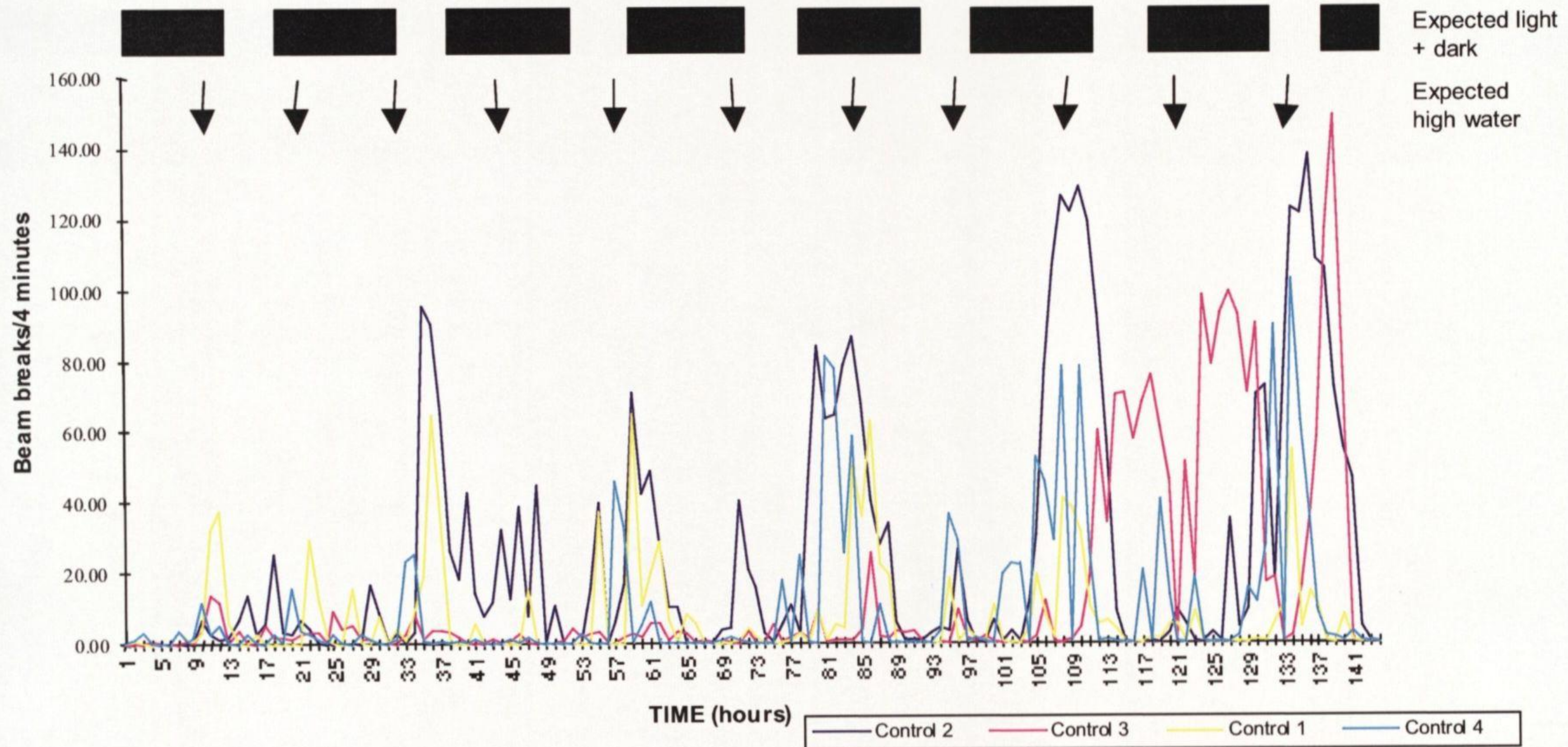
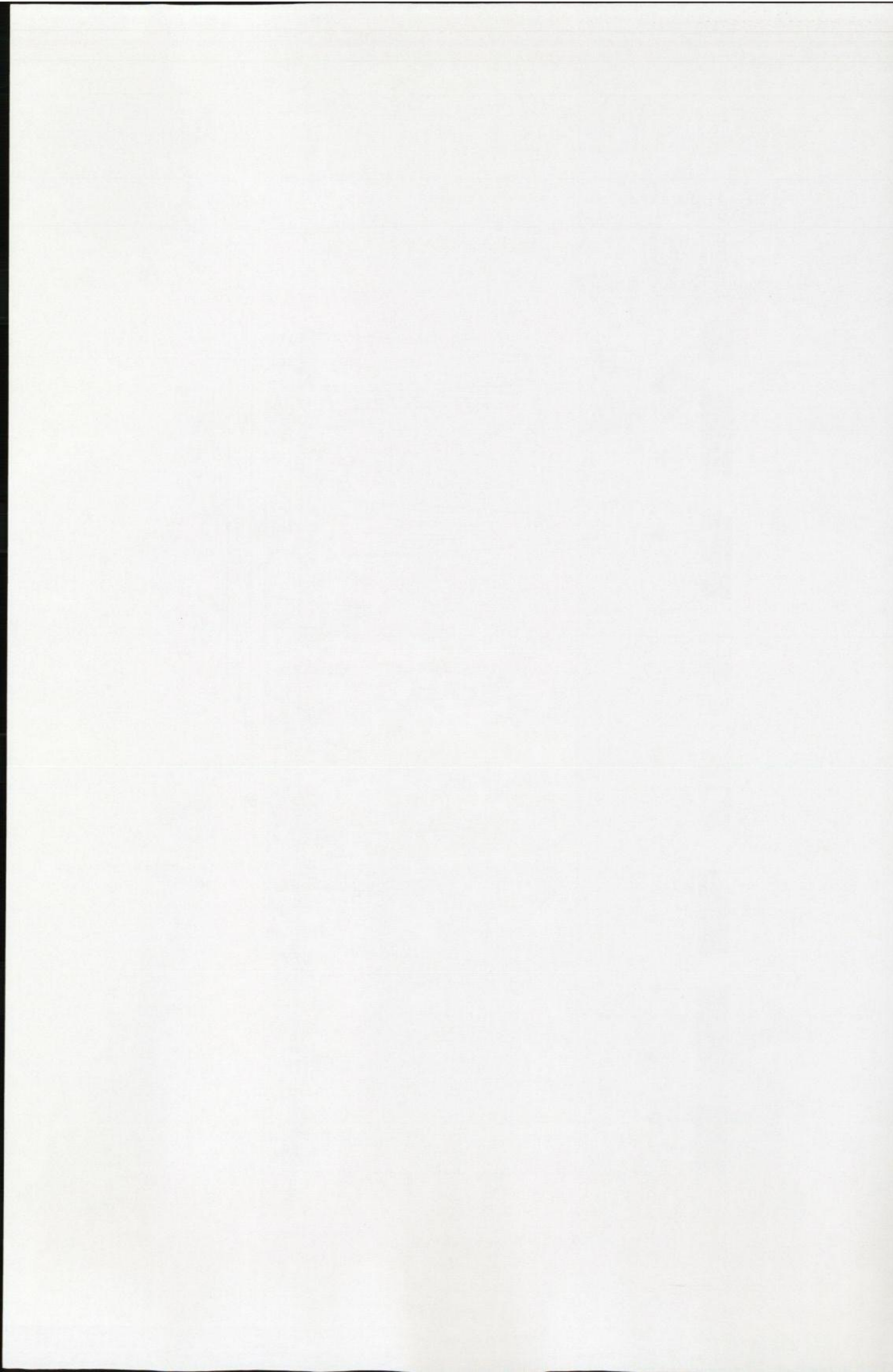


Figure 6.23: Individual locomotor activity for 4 control crabs (trial 1).



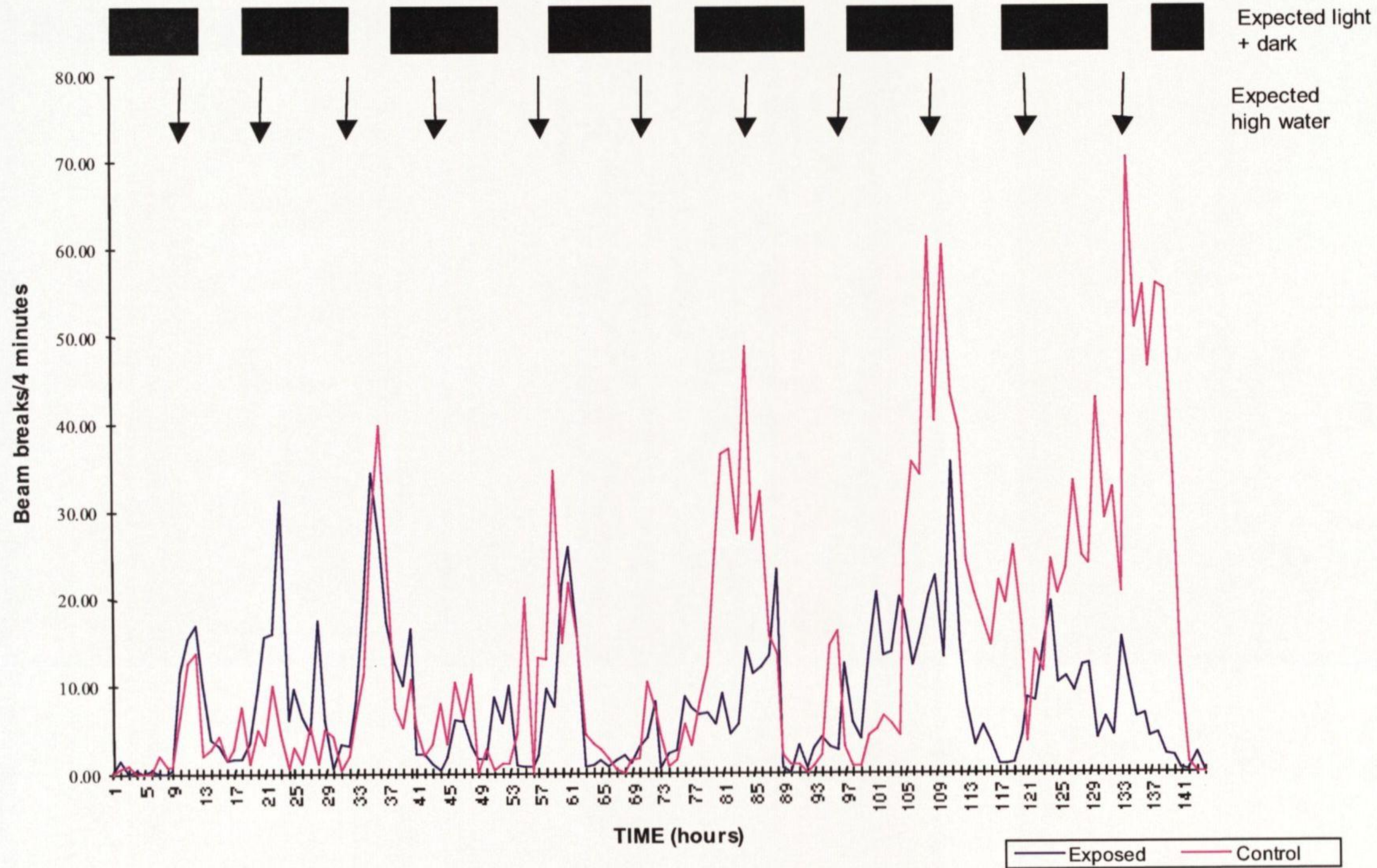
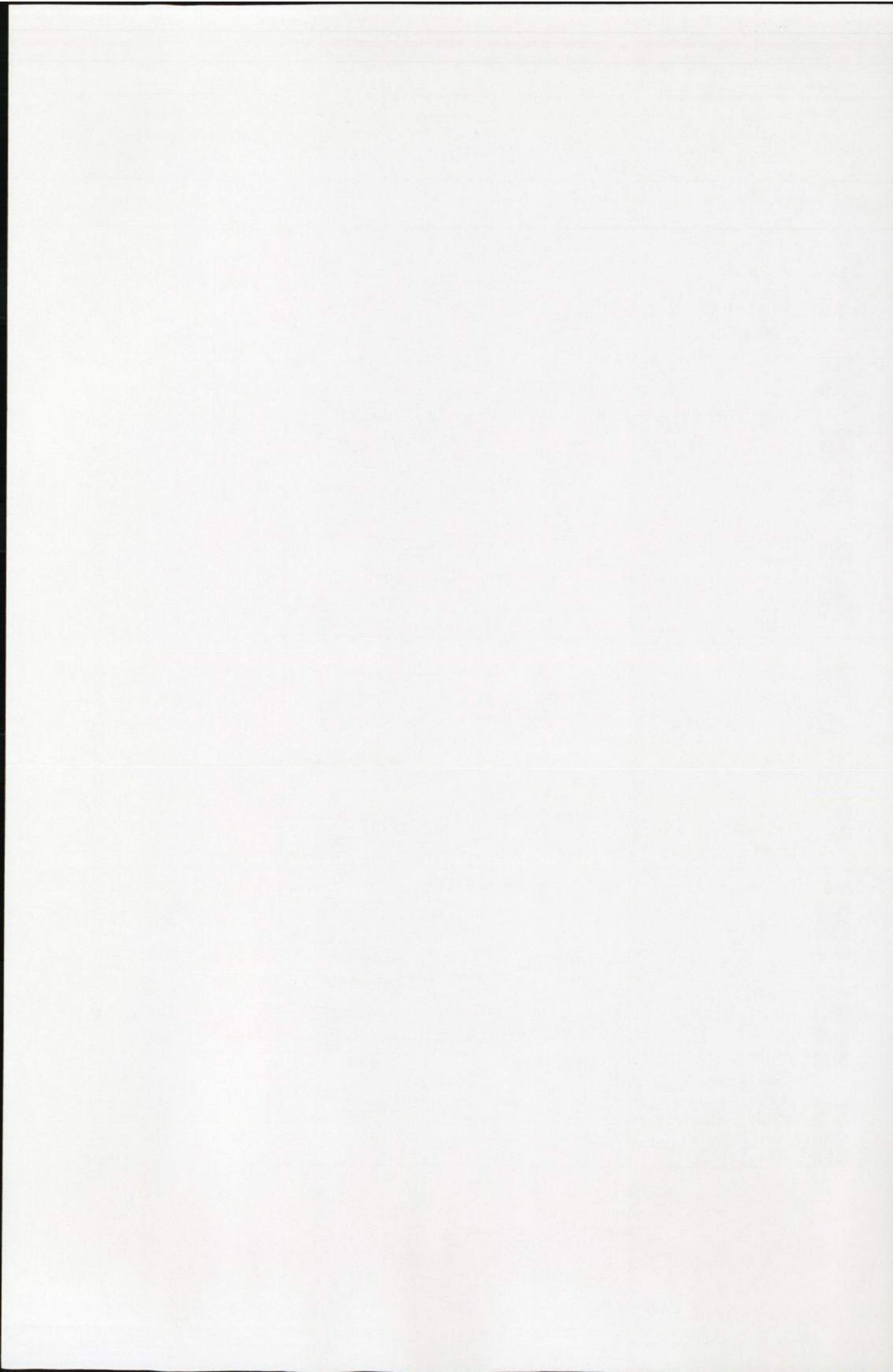


Figure 6.24: Mean locomotor activity for 20-hydroxyecdysone exposed and control crabs (trial 1).



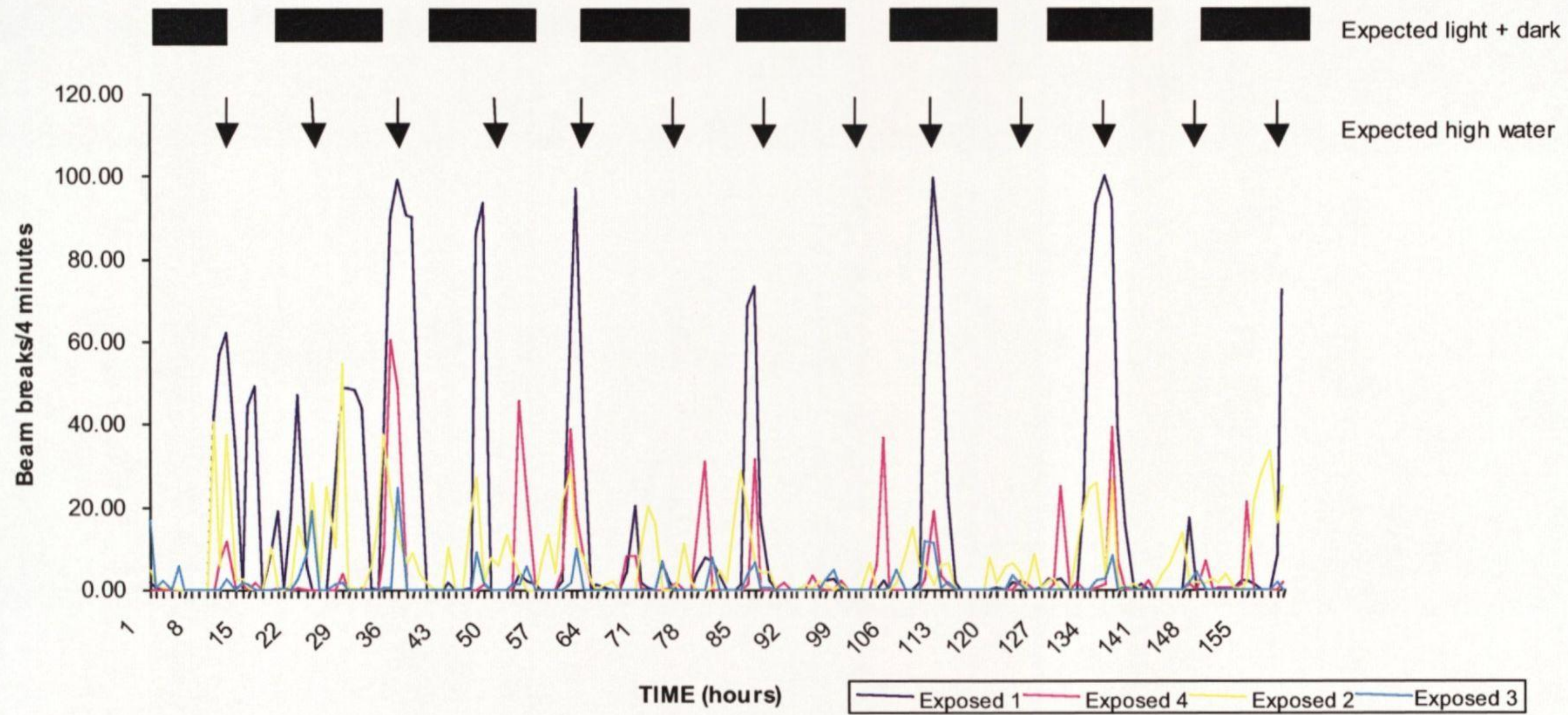
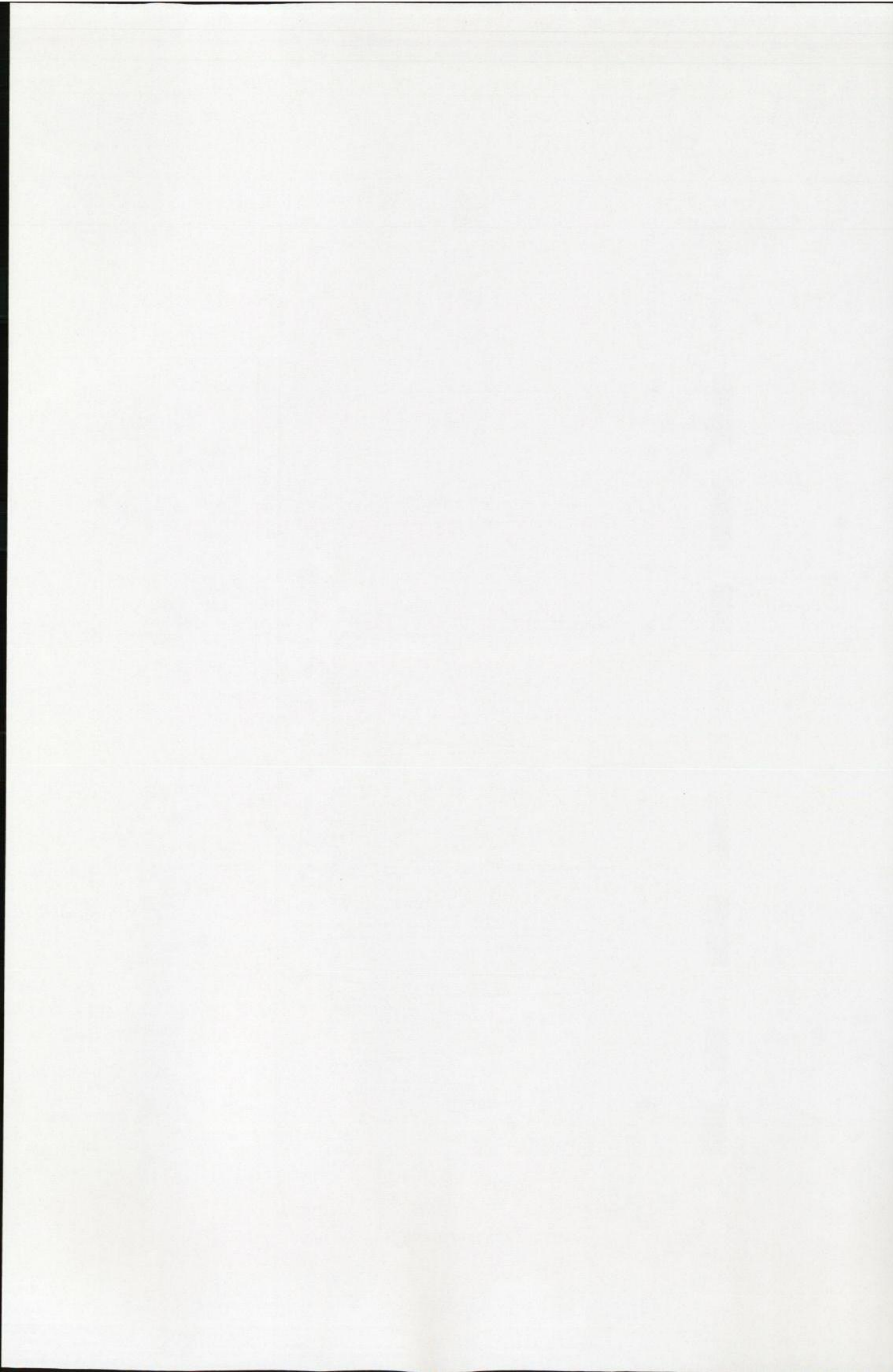


Figure 6.25: Individual locomotor activity for 4 20-hydroxyecdysone exposed crabs (trial 2).



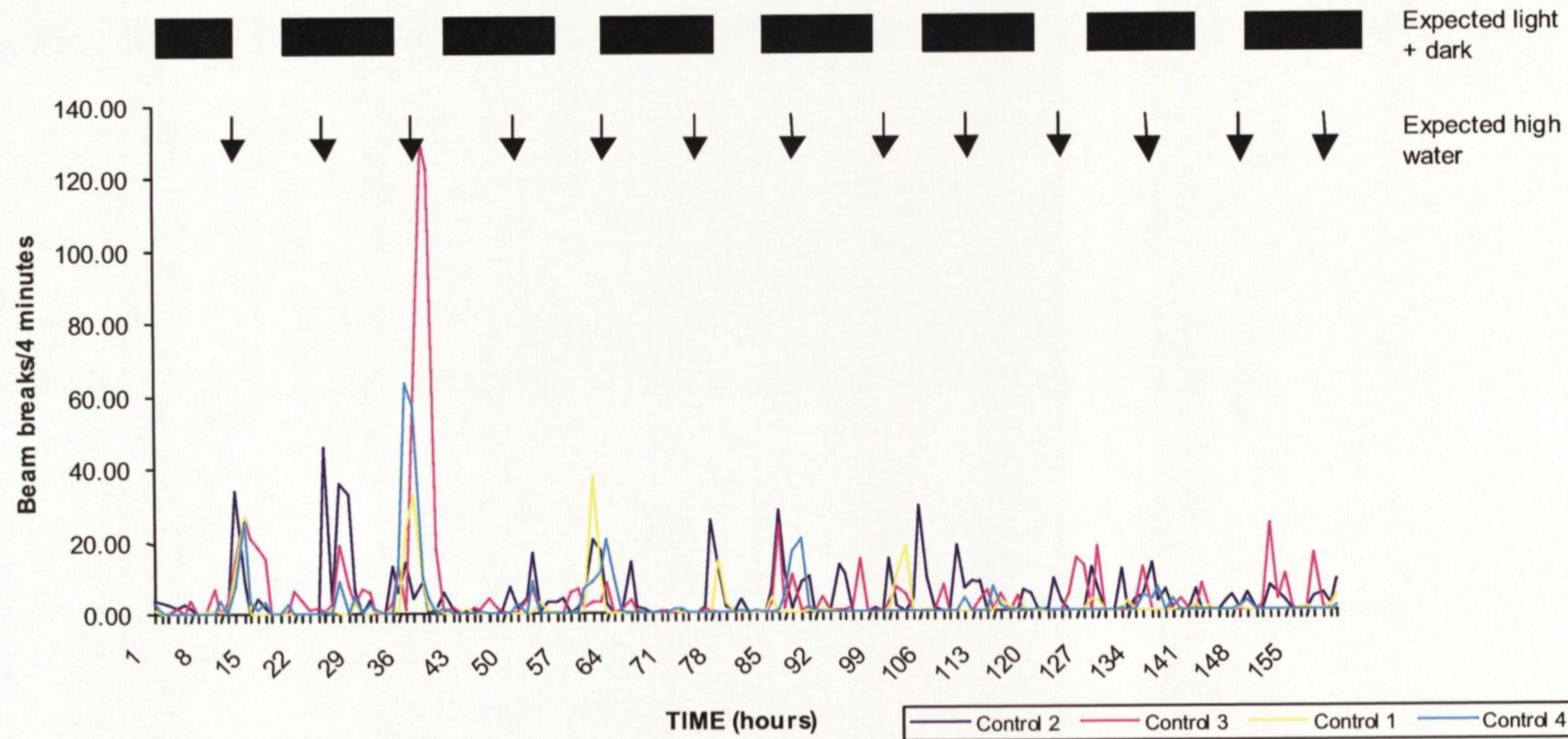
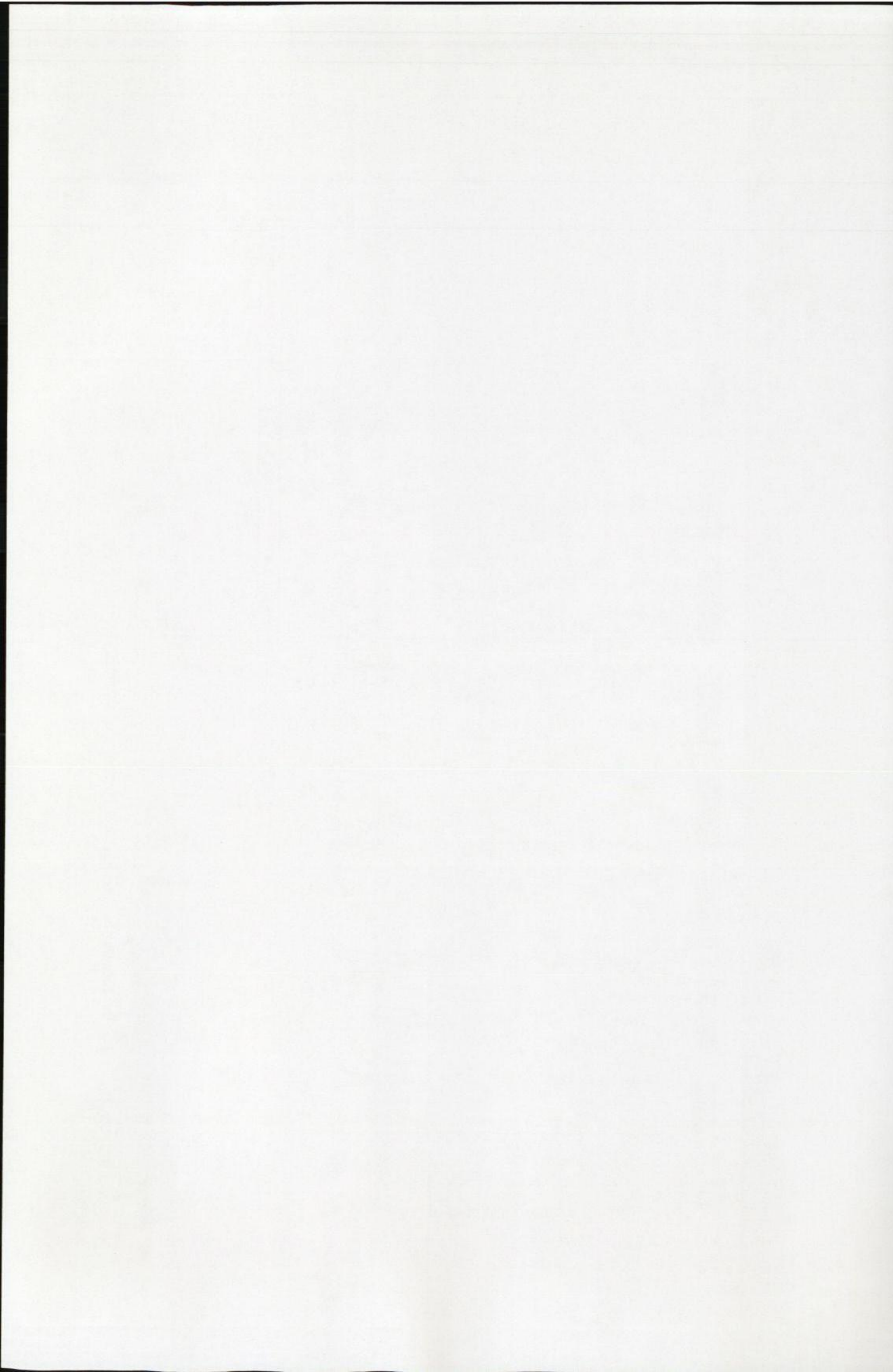


Figure 6.26: Individual locomotor activity for 4 control crabs (trial 2).



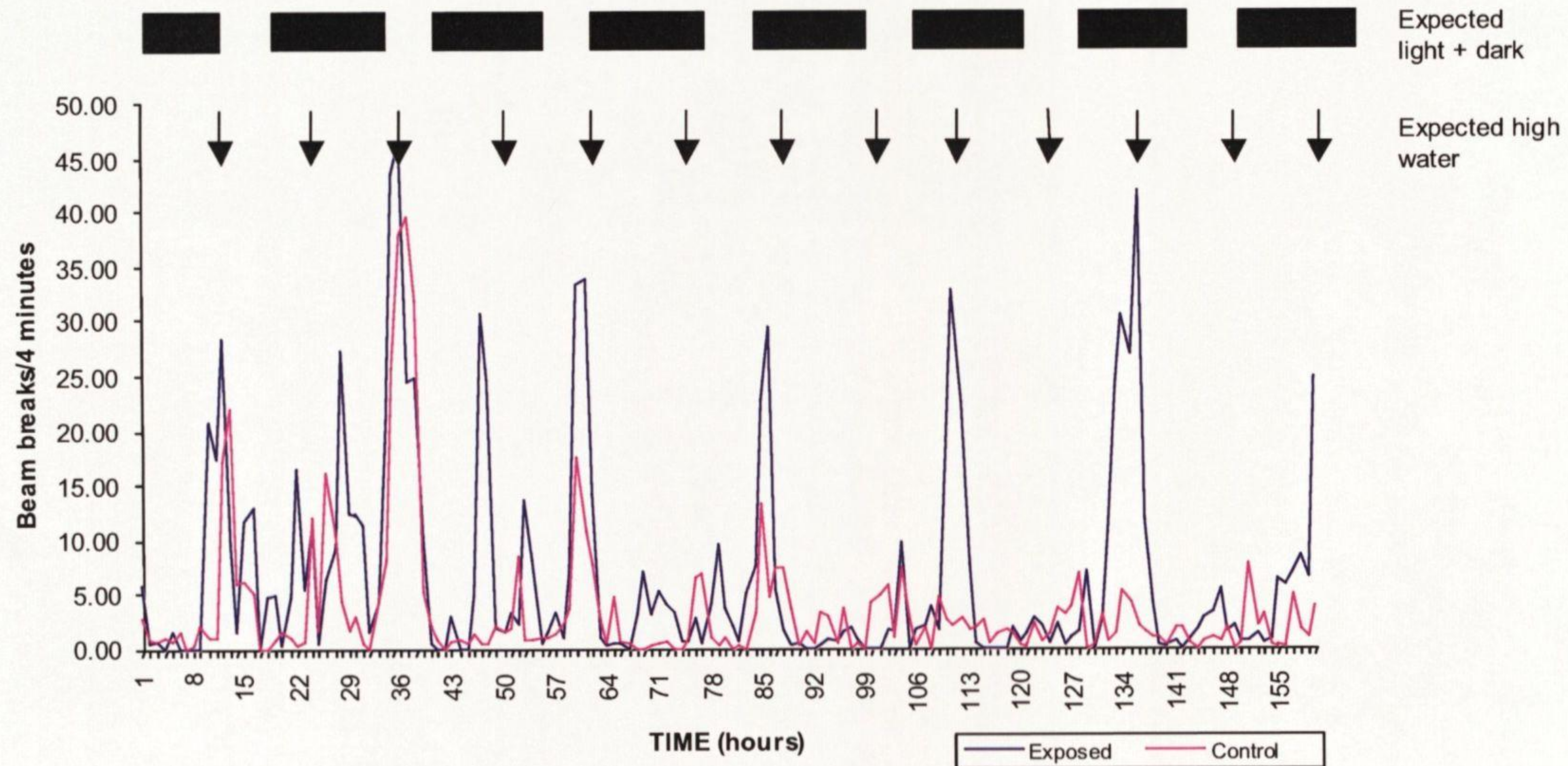
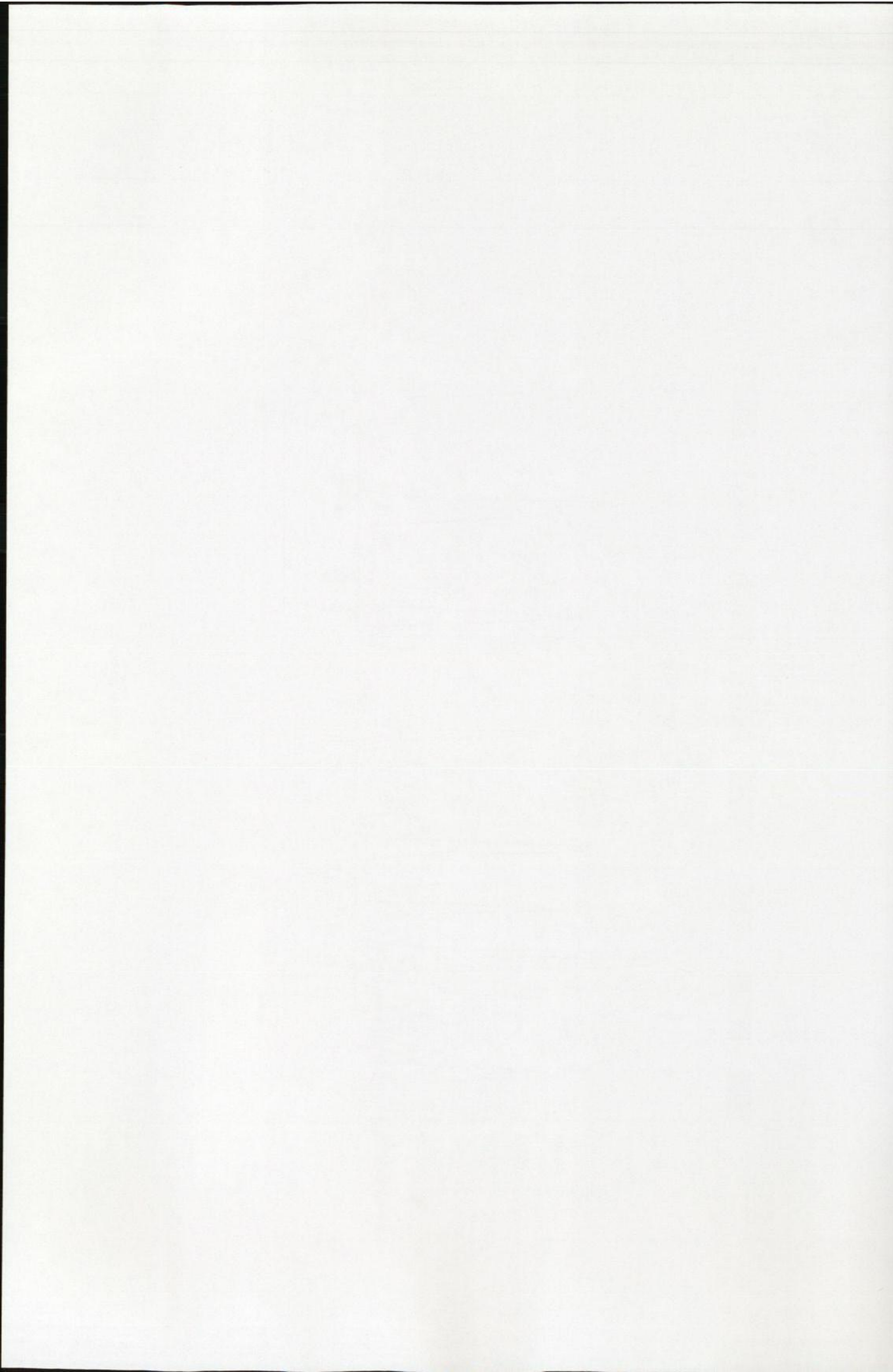


Figure 6.27: Mean locomotor activity for 20-hydroxyecdysone exposed and control crabs (trial 2).



6.2.3 Vitellogenesis

Development of quantitative ELISA for vitellogenin

Results from the various steps in the development of the quantitative ELISA can be found in the methods section.

Baseline vitellogenesis study

The haemolymph vitellogenin (ngVg/100µl) levels of selected baseline individuals are presented in figure 6.28. Data sets that come to an end before the completion of the observation period were the result of mortality or moulting and subsequent mortality (caused by cannibalism by conspecifics).

A clear pattern of changing vitellogenin titre can be seen in certain individuals, with a sharp increase culminating in an initial large peak at the beginning of the baseline sampling period and a smaller, secondary peak towards the end. The presence of these twin peaks was verified by repeat analysis of the same samples and was reproducible. This general pattern had a few exceptions. For example, crab 32 exhibited a very large secondary peak of Vg, similar in shape and amplitude to the initial peak. Individuals such as crab 6, 20 and 35 had elevated levels of Vg in their haemolymph that were maintained following the initial increase. Other baseline crabs (9, 40) showed a very low level of Vg throughout the sampling period (for the purposes of the present study, a vitellogenin concentration of less than 10ng/100µl was considered a basal or background level).

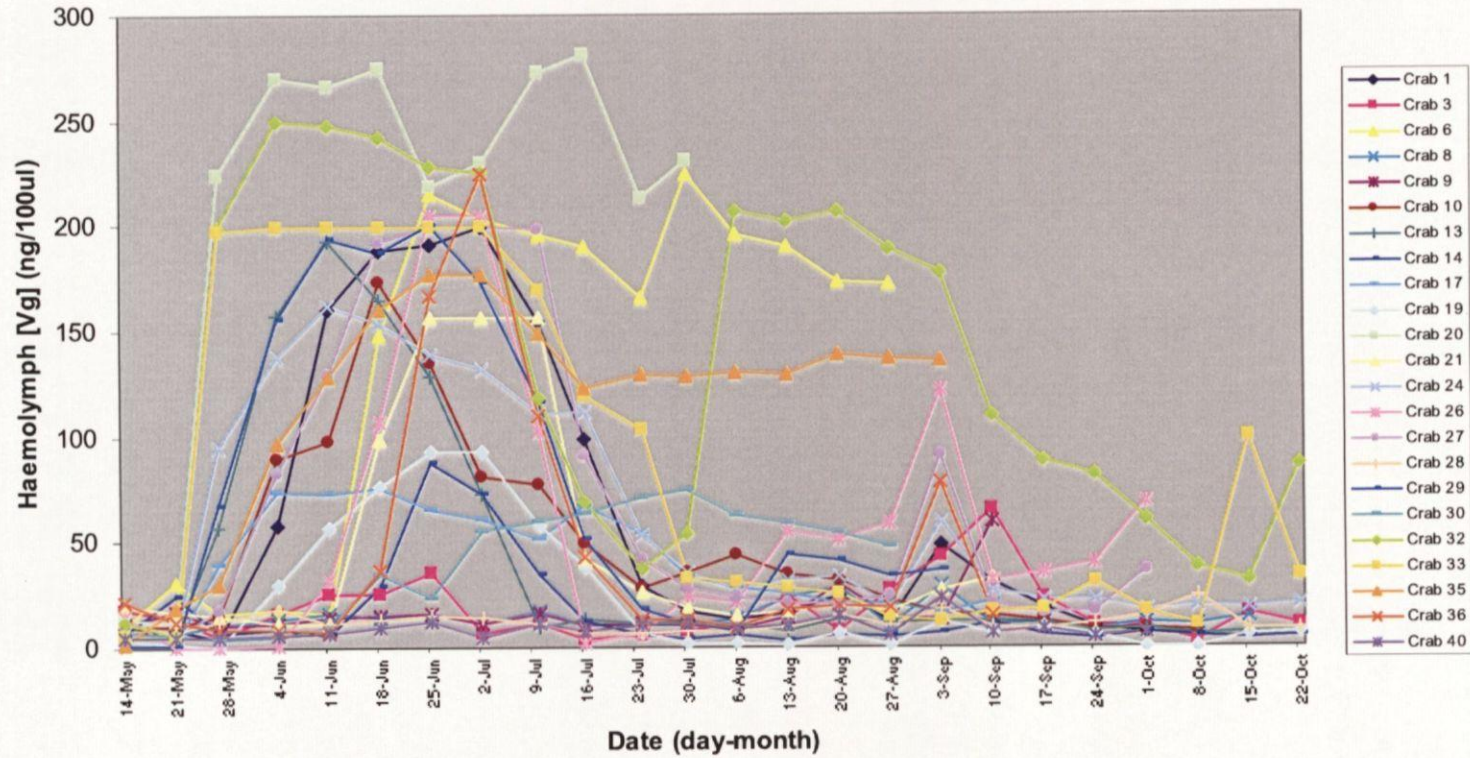
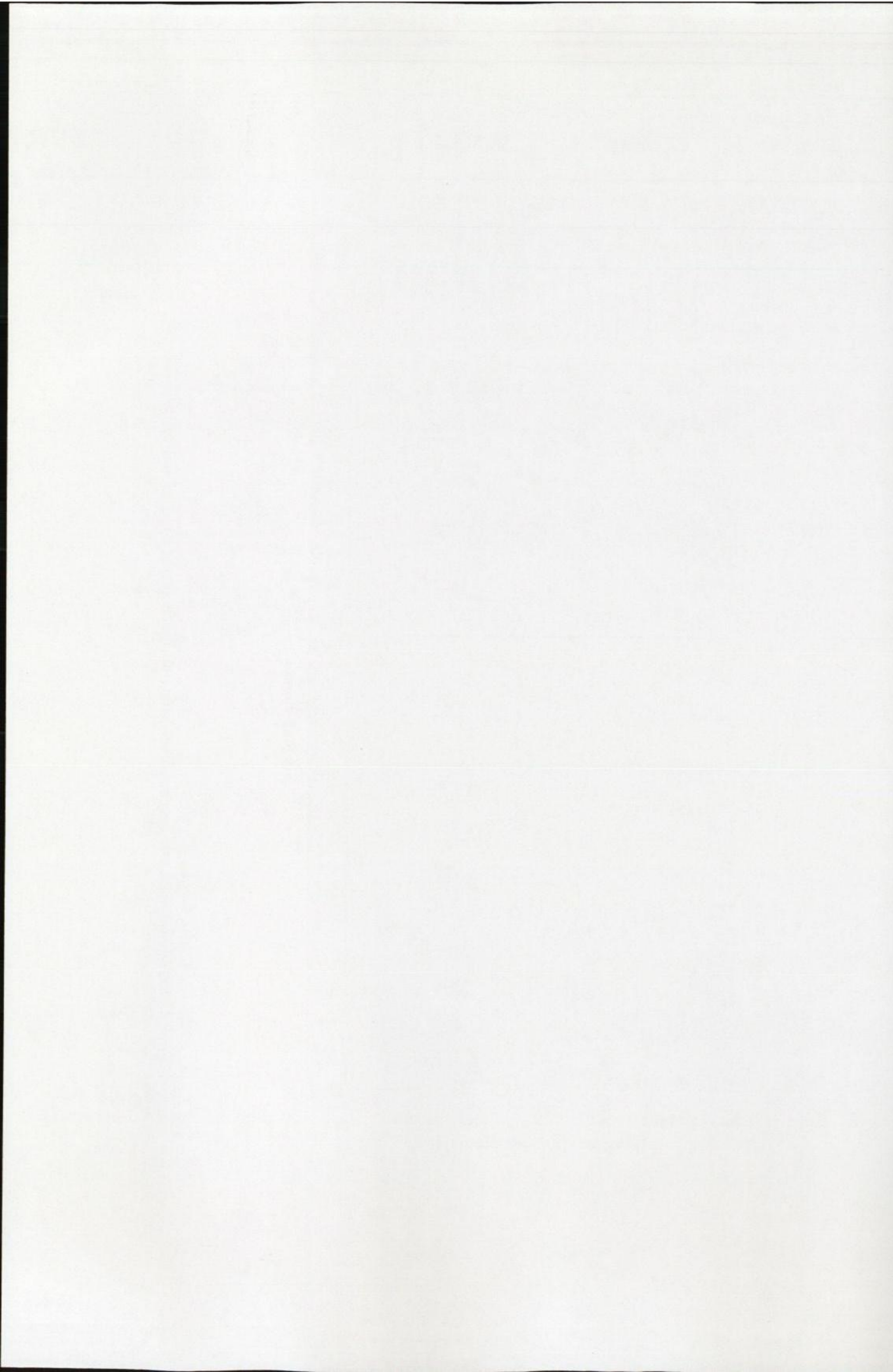


Figure 6.28: Haemolymph vitellogenin levels (ng/100µl) of selected baseline individuals.



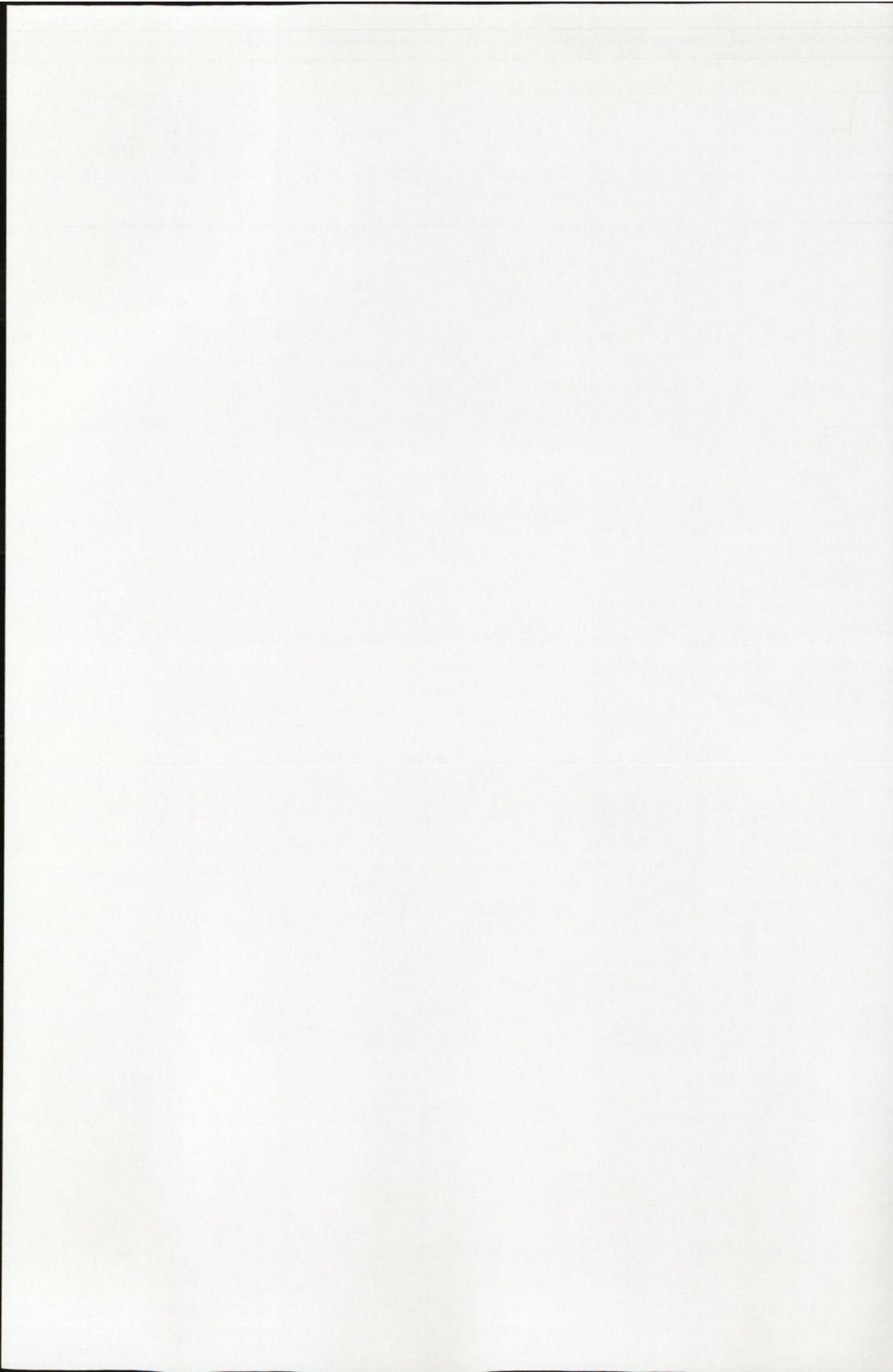
20-hydroxyecdysone exposure trial

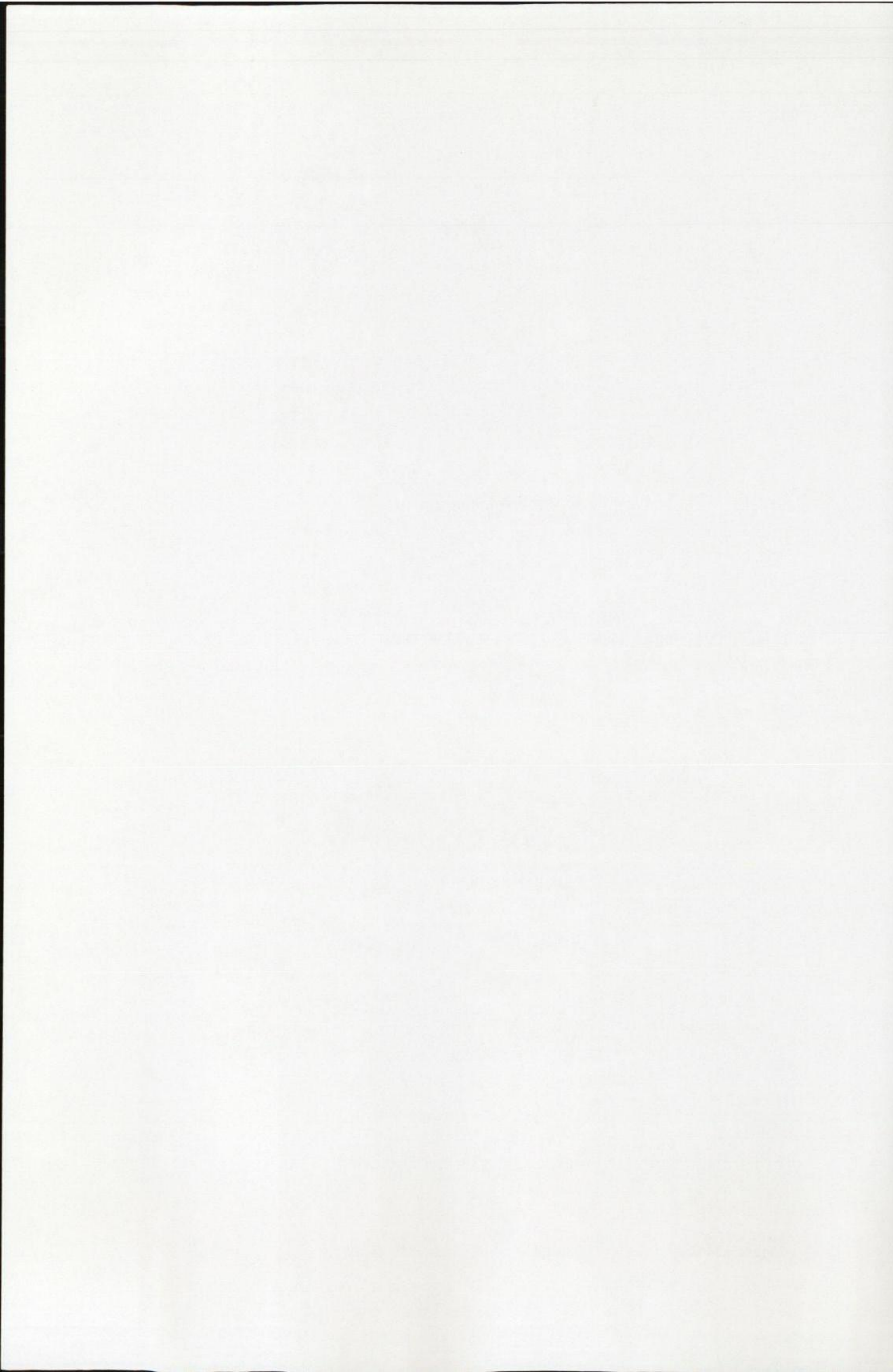
The haemolymph vitellogenin levels of 20-HE exposed and control individuals can be seen presented in figures 6.29-6.34. Data sets that come to an end before the completion of the exposure period were the result of mortality or moulting and subsequent mortality (due to cannibalism by individuals in the same tank). Data sets that end prematurely and then resume later in the exposure period were from moulted individuals that were successfully removed from the trial, mated and returned when suitably hardened. Missing data points are the result of loss or exhaustion of samples.

Haemolymph Vg titres of the majority of individuals included in the exposure trial remained at a basal level throughout the exposure period and their vitellogenin profile lacked the large peaks seen in the baseline. Certain individuals exhibited the large peaks similar to those seen in the baseline crabs (for example R2 and G11 seen in the 100μgl^{-1} and 5μgl^{-1} treatment groups respectively). Other crabs also show less marked peaks (e.g. G21 and R27 from the 100ngl^{-1} exposure group) whilst others exhibit subtle increases over longer time scales e.g. G30 (solvent control group). Overall, however, there seems to be little discernible pattern to the haemolymph Vg levels in the majority of the crabs in the exposure trial. There are also no clear differences between the treatment groups, with the majority of individuals in all groups exhibiting background levels of haemolymph Vg throughout (with the exception of those mentioned above). Therefore, despite the inclusion of the Vg pre-screen step, it would appear the bulk of crabs assayed did not subsequently undergo the events of vitellogenesis so clearly evidenced in the baseline study.

6.3.4 Tebufenozide exposure trials

The moulting hormone agonist tebufenozide had no gross observable effects on moulting and locomotory activity (data not presented).





6.4 Discussion

No significant changes was observed in any of the processes under investigation, suggesting that exposure to the chemicals used in this study does not affect the endocrine systems and pathways of *C.maenas.*

The moulting trial produced little conclusive evidence of disruption to this process caused by exogenous 20-HE. Increased mortality in the two highest exposure groups (100 and 5μgl^{-1}) was the only result to indicate any disruption due to exposure. The majority of these mortalities were amongst the smallest size class of crabs (5-6mm carapace width), which is to be expected since moult frequency is highest in crabs of this size (Crothers, 1967) and the risk from moulted related complications is therefore increased. However, exposure to 20-HE at the concentrations used has had no discernible effect on moult frequency or on growth following moult. A recent study produced a similar result in *Daphnia magna* (Baldwin *et al.,* 2001).

The lack of gross perturbations to moult processes might be due to the behaviour of 20-HE in water. Without appropriate chemical analyses, the persistence of 20-HE in the exposure system is not known. It is possible that it is rapidly degraded and therefore is only active and able to exert an effect for a relatively short period of time. In contrast, moult inhibiting insecticides such as tebufenozide are by their nature more persistent, in order to maximise the chance of exerting a toxic effect. However, experiments carried out to investigate the effect of tebufenozide on *C.maenas* (a potential non-target invertebrate) showed that moult processes were unaffected. It is possible that tebufenozide is only effective when applied as a formulation, which might explain the lack of effects seen following waterborne exposure to the pure chemical.

Despite the lack of effects in juvenile *C.maenas,* it is possible that moult processes might be disruptable by other contaminants, in other species of crustacean or at earlier life

stages. For example, PAH and constituents of crude oil have been implicated in disrupting moult processes. After 48h of exposure to 0.32ml/L crude oil, Tanner crabs (*Chionectes bairdi*) showed reduced moulting success compared to controls (60% to 85% respectively) (Karinen and Rice, 1974). Wang and Stickle (1987) also report that exposure of juvenile *C.sapidus* to the water soluble fraction of crude oil inhibits moulting and growth in this species. Growth (percentage size increase following moult) was significantly reduced in crabs exposed to 1.5 and 2.5mg/L aromatic hydrocarbons. Intermoult period was also prolonged in exposed crabs. Exposure to pyrene also caused a significant delay in moulting in male grass shrimp (*Palaemontes pugio*) (Oberdorster *et al.*, 2000).

Karinen and Rice (1974) noted that the closer *Chionectes bairdi* was to moulting, the less sensitive it was to crude oil's toxic effects and that moulting success actually increased. The work of Oberdorster *et al.* (1999) provides a possible explanation for this result and suggestions for the mechanism of this interaction. They report that four PAHs (benzo(a)pyrene, benzo(a)fluoranthene, pyrene and chrysene) can enhance the effect of ecdysteroids, suggesting they are capable of interacting *in vitro* with the ecdysone receptor (in conjunction with ecdysteroids) to enhance moulting. This may explain the increased moulting success seen in crustaceans exposed chronically to crude oil whilst close to moult, when ecdysteroid titres in the haemolymph are reaching their maximum. Precocious moulting may however be as disadvantageous as inhibited moulting, since an individual may moult at an inappropriate time, when protective shelter is in short supply and predator stress is high.

These examples illustrate the potential for ubiquitous environmental contaminants to interact with endocrine mediated pathways, but it must be noted that the concentrations used were very high and environmentally unrealistic.

The locomotor activity experiments were tentatively included in the experimental program, due to the lack of available information on the hormonal control of rhythmicity.

No obvious link with 20-HE exposure was found, although other behaviours could have indirectly been influenced by disruption elsewhere (e.g. Bolingbroke and Kass-Simon, 2001). Investigation into such behaviours was beyond the scope of the present study, due to the simplicity of the actograph system. Only a crude level of activity over time could be measured using this system. Research into more complex behavioural patterns could be the focus of future work, utilising new high-tech behavioural observation systems.

Preliminary exposure studies suggested the abolition of activity by 20-HE, but the present work did not support this. The preliminary study was undertaken during the summer (Bamber 2000, unpublished results) when large proportions of the population are actively migrating with the tide. During winter however, the number of migrants declines (McGaw and Naylor, 1992). As a result, it is possible that the present study tested crabs with more persistent locomotor activity not so easily overridden by exogenous influences.

Hunter and Naylor (1993) report differences in locomotor activity between "colour-forms", with green (early intermoult) male crabs being more active and making more extensive intertidal migrations than red (late intermoult) crabs. Green intermoult males were chosen for the present exposure trial and their characteristically active locomotory habits seemed to have prevailed despite 20-HE exposure. The hormone could potentially have a more profound effect on different colour forms whose locomotory activity patterns are not quite as robust. Exposure to tebufenozide also had no gross effects on locomotor activity patterns, suggesting this chemical poses a limited risk to locomotor behaviour in *C.maenas*. However, solubility and availability for uptake may have been influential, due to the use of the pure chemical, not a formulation (see above).

As mentioned previously, there is some evidence reported in the literature for the involvement of ecdysone in vitellogenic events although its role is still poorly understood. It has been suggested that ecdysteroids may stimulate vitellogenesis (Fingerman, 1997) and ovarian maturation and protein synthesis (Chan, 1995, Oberdorster and Cheek, 2001).

Conversely, in some species, administration of ecdysteroids has inhibited vitellogenesis (Chang, 1989). It appears that the ability of ecdysteroids to promote vitellogenesis in the hepatopancreas is species dependent (Loeb, 1993) as is the need for ecdysteroids for the completion of vitellogenesis (Pinder *et al.,* 1999). Correlations between haemolymph ecdysteroid titres and vitellogenesis have been reported (Chang, 1993, Okumura *et al.,* 1992, Young *et al.,* 1993a,b). However, it is still unclear as to whether ecdysteroids directly influence vitellogenesis, or that their levels during vitellogenesis are simply indicative of the corresponding stage of the moult cycle.

The results of the present study have unfortunately provided little further clarification of this situation. No obvious changes in vitellogenic events were observed in exposed individuals and consequently no real conclusive evidence of disruption by exposure to 20-HE could be found. Whilst it appears that vitellogenesis is not disrupted by 20-HE, it may still be disruptable by any number of exogenous chemicals in the field. For example, PAHs and crude oil hydrocarbons have been shown to impact reproductive processes in crustaceans.

Naphthalenes, major constituents of the water-soluble fraction of crude oil, have been implicated in significant effects on ovarian development in crayfish and prawns. The ovaries of red swamp crayfish (*Procambarus clarkii*) exposed to 10ppm naphthalene were smaller (as determined by a decreased ovarian index) than controls kept in clean seawater (Sarojini *et al.,* 1994, 1995). This decrease was due to intense ovarian atresia and yolk resorption, with degeneration of previtellogenic and vitellogenic oocytes. Ovaries of control crayfish developed into ripe gonads. The authors suggest that this atresia in the ovary is a result of naphthalene inhibiting release of gonad-stimulating hormone (GSH) from neuroendocrine cells in the brain and thoracic ganglia (Sarojini *et al.,* 1994). This is supported by the observation that immature oocytes reappeared in crayfish returned to clean seawater for depuration following exposure (Sarojini *et al.,* 1995). This can be

considered as evidence of endocrine disruption in this species, distinct from acute toxicity. Similar effects are seen in freshwater prawns (*Macrobrachium lamerrii*) chronically exposed to diesel. Effects included decreased ovarian index, degeneration of oogonial cells and presence of avitellogenic oocytes (Rao *et al.,* 1990). In the grass shrimp *Palaemontes pugio,* vitellin levels were elevated in the ovary by exposure to pyrene, perhaps due to this PAH up-regulating vitellin synthesis (through enhancing ecdysone-dependent gene expression) (Oberdorster *et al.,* 2000). Again, interaction with endocrine pathways appears to be the route by which effects were triggered.

Once our understanding of vitellogenesis in *C.maenas* is more thorough, the newly optimised quantitative assay provides a valuable tool to investigate changes in Vg titre, caused by chemicals or other environmental factors. It is clear from this work that the fundamental baseline data on vitellogenesis needs to be more comprehensive and our understanding of this process must be improved before any fluctuations can be attributed to chemical endocrine disruption. A year round picture of the vitellogenin profile correlated with a clear measure of reproductive status (ovarian development) and moult/colour stage is required. In this way, a vitellogenic continuum could be combined with a moult/reproductive component so that deviations from the norm might be identified.

For example, females could be collected regularly throughout the year and moult staged by a specific measurement of shell thickness or colour. The reproductive status could also be ascertained by investigation of ovarian development. These parameters could then be compared with an ELISA derived haemolymph Vg titre for each individual. A clearer picture could then be composed of the interplay between these processes over an entire year or perhaps more. Mean data on haemolymph Vg level and ovarian development would then exist for females of a certain size and colour/moult stage in any month, or even week of the year. Females sampled from a population could then be compared to the established baseline data set. If the Vg level does not correlate with the expected level of

an individual sharing the same physiological characteristics at the same time of year, then there could be a case for a level of disruption to vitellogenic events caused by exogenous influences. It is important to note here that, due to regional differences in the timing and duration of various physiological events in crustacean populations, allowances would need to be made to account for variations between these populations.

On a similar note, this work has illustrated that a simple measure of Vg level at the time of collection tells us little about the true stage of vitellogenesis an individual has reached. The pre-screen provides us with limited information since it does not tell us which side of the large vitellogenic baseline peak the individual is. Additionally, crude classification of crabs into green or red groups is no guarantee that their respective positions in the vitellogenic continuum are before or after the large peak seen in the baseline.

The absence of high vitellogenin titres in exposed crabs may be a simple issue of the timing of the trial. The exposure trial was started later in the summer and after the time period encompassing the vitellogenic peak in the majority of the baseline samples (from mid May to late July). A possible explanation for the lack of increased Vg titres could be that the period of increased vitellogenin synthesis had already occurred in the exposure trial individuals. This increase in synthesis and circulation of Vg is clearly the crucial stage for our investigation and alterations in Vg level would be most easily detected during this time. During the initial planning of experiments, however, this scenario was considered unlikely, as it is reported that vitellogenesis can occur over a wide time period and well into late summer/early autumn (Crothers, 1967). The results of this work now cast doubt on this assumption, at least in the population studied. It is possible that the population sampled at Bantham is characterised by a compressed temporal period for the events of vitellogenesis. For example, where vitellogenesis might occur throughout some populations from early May to October, with a maximum in June, crabs from Bantham

may be characterised by vitellogenesis occurring from late May to early August only. Later in August and through September into October, the proportion of the population exhibiting the increased Vg titres would therefore be smaller. This would explain the small number of crabs in the exposure trial that showed any kind of vitellogenic peak. The need to avoid repetition of this situation further illustrates the need for a fully comprehensive baseline data set.

The majority of crabs in the exposure trial and a small number in the baseline group were characterised by a Vg titre that stayed at basal levels (<10ng/100μl) throughout the exposure period. If still in the intermoult or early premoult stages such individuals are most probably non-vitellogenic (Vafopoulou and Steel, 1995) and may lie to the left of the peak on the vitellogenic continuum. Without an accurate measure of moult stage however, this remains conjectural. Alternatively, females may have previously undergone vitellogenesis i.e. are now situated to the right of the vitellogenic peak on the continuum. These are individuals whose ovaries have sequestered the vitellogenin previously circulating in their haemolymph where it is converted to the ovarian form vitellin (Lee *et al.*, 1996, Chen and Chen, 1994). Recirculation into the haemolymph and/or basal levels of protein synthesis/turnover might account for the small amounts found in samples throughout the exposure period (Vafopoulou and Steel, 1995). A measure of ovarian development might confirm this, but this was not undertaken due to time constraints. Again, comparison to a well-established and comprehensive baseline would clarify the situation.

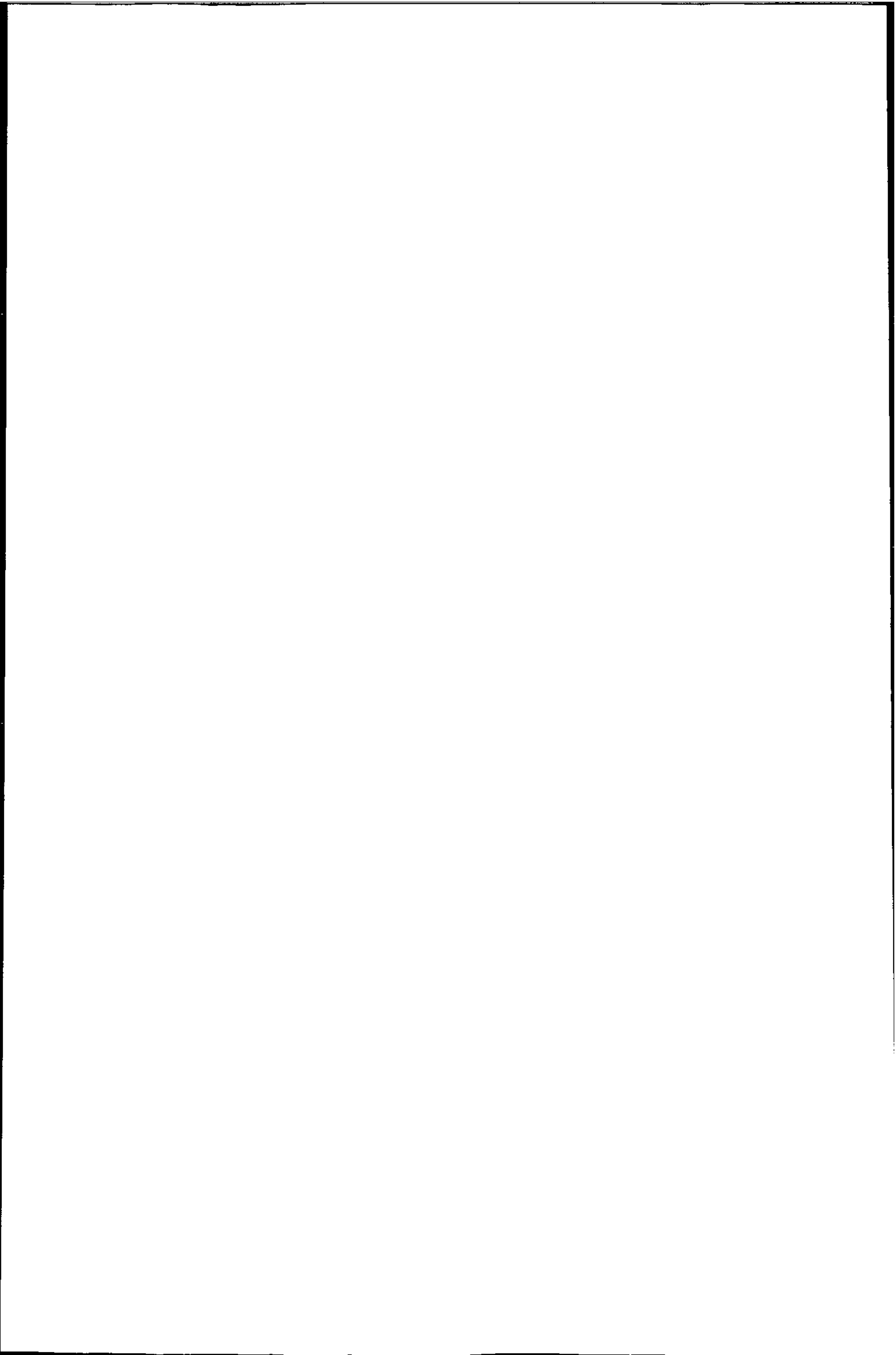
The disparity between the amplitude of the vitellogenic peaks of the haemolymph of baseline and exposure crabs may be partially explained by the activity of the Vt standard used to run the ELISA. The baseline haemolymph samples were analysed using aliquots of a concentrated stock of Vt. These aliquots were stored at -20°C for several months and thawed individually when required. Inevitably, all may have undergone partial thawing and re-freezing when individual aliquots were removed for use in the ELISA. As a result, the

binding affinity of the standard may have decreased over time. In contrast, the Vt standard used in the analysis of the exposure samples was aliquotted from the concentrated stock (kept at -80°C) just prior to analysis and kept at -20°C for only a few weeks whilst running the ELISA. The absorbance values produced by the fresher standards were therefore higher despite their concentrations being identical. As a result, interpolation from the steeper curve produced lower values. This effect can be minimised by regular normalisation of standards against an initial fresh curve and is important now that the Vg ELISA is fully quantitative.

As mentioned briefly above in reference to the moulting experiments, chemical analysis would have been a valuable addition to this work. It is not presently known whether waterborne exposure to 20-hydroxysone is effective in elevating endogenous ecdysone levels in *C.maenas*. An unfortunate disadvantage of the semi-static renewal system of dosing experiments is the potential for degradation and/or loss of the test chemical from the exposure system. Monitoring of waterborne levels over time would offer insight into the degree of these processes and provide information on the required frequency of redosing. Nevertheless, the present study minimised potential degradation and loss by a regime of regular water changes and redosing.

6.4.1 A biomarker of endocrine disruption?

While it is relevant to address the potential risks posed by contaminants, realistically, the endpoints investigated in this chapter have certain limitations that preclude them from being used as biomarkers of endocrine disruption in the field. Detecting disruption to moulting processes caused by exposure to chemicals in the laboratory may inform us of the risk posed by these chemicals should the organism encounter them, but this cannot be employed in the field. For example, contaminant induced premature moulting could not be distinguished from that caused by other factors, unless the precocious moult resulted in a visibly altered morphology. Unless individuals were caged at different impacted and reference sites, it would also be impossible to measure moult frequency and interval in the field, as the shed exoskeletons could not be recovered or assigned to specific individuals. Similarly, locomotor activity may be disrupted by exogenous hormones or xenobiotics in the laboratory, but this would only tell us of the potential adverse effects which may result from exposure in the field. Observing locomotory activity in a dynamic field environment would be far too difficult and complex as to be prohibitive. The only parameter with the potential to be used as a biomarker of exposure to endocrine disrupting chemicals is vitellogenin titre in the haemolymph of female crabs. However, a more comprehensive knowledge of the fundamentals of vitellogenesis in *C.maenas,* and a complete baseline data-set of year round vitellogenin levels is needed before this marker can be used to assess endocrine disruption in the field. It is this author's opinion however, in light of the complexity of the vitellogenic cycle and large differences between crab populations and the environments and conditions they are adapted to throughout Europe, that this assay will not be applicable in the future. Only with comparison to comprehensive data sets for year round normal levels of vitellogenin in crab populations in every distinct part of the UK and Europe will the assay by usable for biomonitoring.



The original aim of this work was to measure the responses of endocrine regulated processes in *C.maenas* following exposure to exogenous chemicals. It would appear that these processes are unaffected by exposure i.e. endocrine processes in *C.maenas* are not readily disruptable, at least by the chemicals investigated here. Despite exposure to two chemicals that are arguably most likely to cause endocrine disruption, the endocrine system of *C.maenas* has proved itself to be robust. This coupled with the fact that evidence of endocrine disruption in this species has not been reported in wild UK populations, it would seem as though endocrine disruption is not a phenomenon that is capable of occurring to the extent of that seen in vertebrate species such as riverine fish. Nevertheless, while the present work has provided limited evidence for endocrine disruption, crustacean studies on the effects of crude oil and PAHs suggest endocrine mediated processes are indeed disruptable. This highlights the potential for adverse effects on moulting and reproduction and could stimulate future work on the effects of PAH on these processes. Furthermore, should evidence of PAH mediated endocrine disruption be confirmed in *C.maenas*, the exposure biomarker described in earlier chapters could then be applied to identify crab populations whose endocrine systems might be at risk.

6.5 Conclusions

No gross effects on moulting processes were observed, aside from increased mortality in the two highest exposure groups, and there were no discernible effects on moult frequency or growth. Moulting processes were also unaffected by tebufenozide, which is good for non-target invertebrates such as *C.maenas* and supports findings for other aquatic invertebrates (Kreutzweiser *et al.*, 1994). Despite the lack of gross effects on the life stage of *Carcinus* tested, it is still unclear whether other life stages (e.g. larval forms) and species of crustacean are affected. These questions highlight the need for further studies, but such work was outside the scope of the present thesis. No obvious effects of 20-HE exposure on locomotory activity were observed. Research into more complex behavioural patterns, utilising modern behavioural observation systems, might reveal subtle changes that our system missed. No obvious changes in vitellogenic events were observed in 20-HE exposed individuals. However, the optimisation of the Vg ELISA provides us with a powerful tool for future exploration of this complex process. Importantly, the present work has highlighted the considerable gaps in knowledge of vitellogenesis and the need for a comprehensive baseline data set.

The lack of endocrine disruption observed in the field suggests endocrine systems in crustaceans are not easily disrupted by environmental levels of exogenous chemicals. The results presented here suggest laboratory exposures cannot cause significant alterations to endocrine processes either, although other authors have had more success using PAHs and crude oil. Furthermore, the endocrine mediated processes investigated in this chapter did not offer suitable endpoints for use as biomarkers of endocrine disruption in *C.maenas*. Disruption of vitellogenic events has the greatest potential as a biomarker of endocrine mediated toxicity, but this would require the timing and nature of vitellogenesis to be elucidated, to allow any changes due to xenobiotic chemicals to be placed in context.

Chapter 7: General discussion and conclusions

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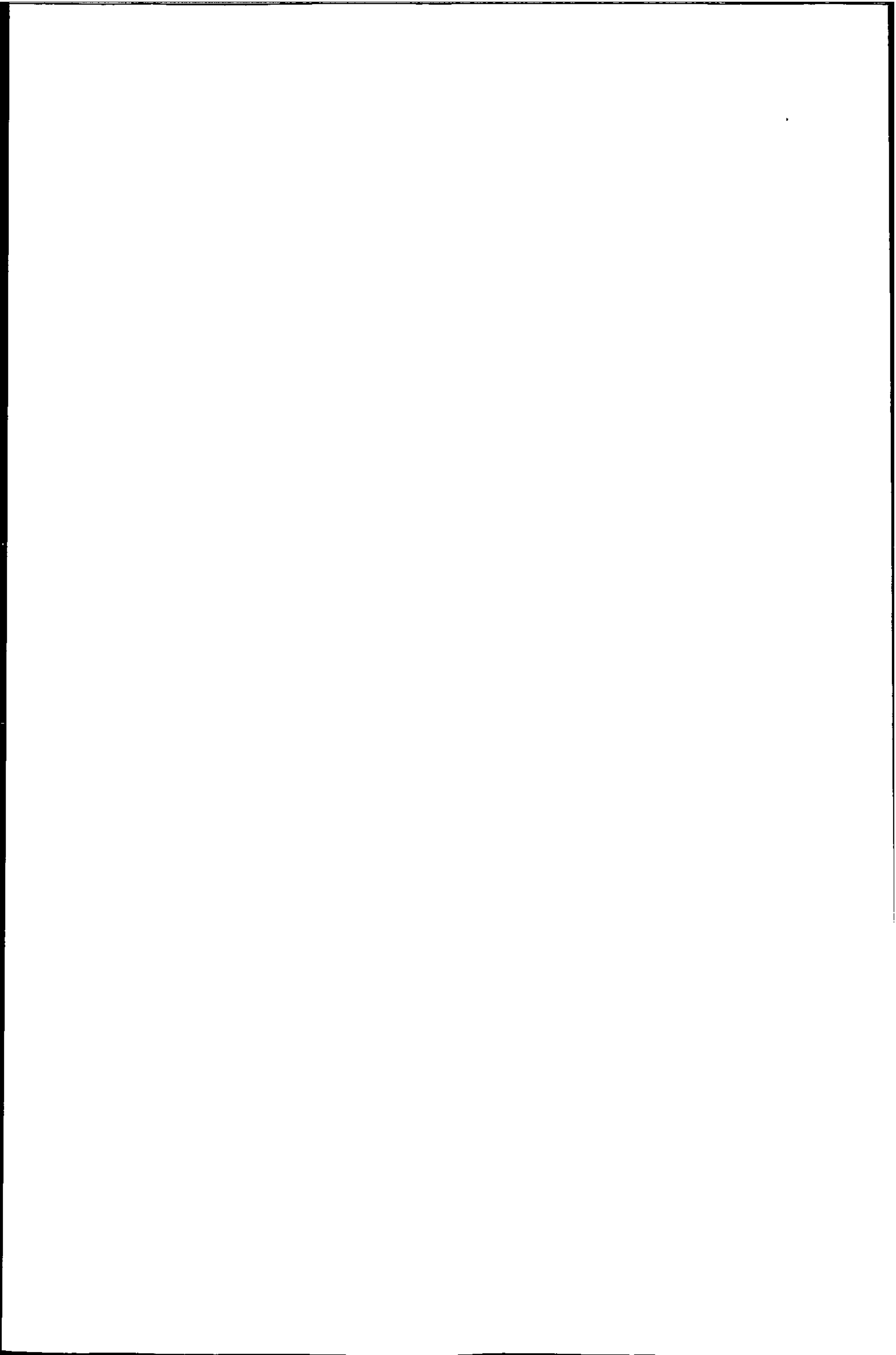
7.1 General discussion

While the discussion of specific experimental results can be found in their respective chapters, it was considered pertinent here to briefly discuss two recurrent themes that have been a feature of the work in this thesis.

The present work has consistently illustrated a very important aspect of biomarker responses and the use of biomarkers for environmental monitoring - that of inter-individual variability. Wherever biomarkers or biological parameters have been observed, there has always been a large degree of variability in organismal response over a large range. This is, however, nothing new and is an almost universal feature of biomarker studies (Fossi *et al.*, 2000, Fossi *et al.*, 1997, Lundebye *et al.*, 1997, Pedersen *et al.*, 1997, Lin *et al.*, 1994, Krahn *et al.*, 1986). In natural ecosystems, it is likely the result of a population not being homogenously exposed to the contaminant or due to differences in the morphology and biochemical/physiological status of exposed organisms (Depledge and Fossi, 1994). A common misconception is that this variability effectively invalidates biomarkers and renders them insensitive compared to traditional chemical analyses, but recent reviews have addressed this issue (Handy *et al.*, 2003, Depledge, 1994). Handy *et al.* (2003) suggest various approaches to minimise variability when applying biomarkers. These include careful selection of reference and contaminated sites (similar in their hydrology and geochemistry), normalisation of biomarker responses against critical chemical variables, introduction of sentinel organisms at reference sites to calibrate temporal changes in response, and increased replication.

It has been proposed that variability in biomarker response is an indication of contaminant impacts itself (Depledge and Lundebye, 1996, Depledge, 1990). Wide variability illustrates that contaminant stress has discriminated between those organisms capable of mounting a response at a certain level and those incapable of doing so. This



suggests that the toxic effects of the contaminant have already incapacitated a portion of the population and that the overall health of that population is under threat. This should be viewed as a clear signal that remedial action is required (Depledge and Fossi, 1994). When considered alongside a suite of biomarkers, variability in response may strengthen multivariate analyses with respect to diagnosis of impacts (Handy *et al.*, 2003).

Secondly, there is also a need for a more thorough understanding of temporal, spatial and individual fluctuations in organismal response when using biomarkers for environmental assessment.

> "To be confident in assessments based on biomarker measurements, it is absolutely vital that...the range of inherent variability in the biomarker measurements in healthy organisms is well known". (Depledge, 1994)

Without an intimate knowledge of the way an organisms response, measured using biomarkers, naturally changes with temperature, photoperiod, food availability, salinity, reproductive state, sex of the organism and numerous other biotic and abiotic factors, it is not possible to put biomarker responses into context. A thorough understanding of background levels or baseline behaviour of biomarkers is needed before we can attribute any changes to contaminant exposure or "pollutant stress". This is particularly important for functional biomarkers such as MT or antioxidant systems. Concerns over the use of MT in evaluating trace metal exposure have been discussed in chapter 4, since natural variability can obscure changes due to pollutant impacts. It has long been argued that MT levels in crustaceans are too heavily influenced by endogenous factors to be reliable indicators of metal contamination in the field (George and Olsson, 1994). Equally, the temporal progression of vitellogenesis in *C.maenas* must be more thoroughly understood before the interaction of environmental chemicals with endocrine pathways can be held responsible for any observed anomalies.

7.2 Conclusions and fulfilment of specific aims

While it was not possible within the scope of this PhD to answer all of the additional questions generated by the investigations, the findings presented here have fulfilled, in part, the original aims of this work (see introduction).

Waterborne exposures have provided an insight into the disposition of PAH in *C.maenas* and allowed the response of this organism to these compounds to be measured. Using a simplistic, rapid and inexpensive analytical technique (direct fluorimetry), *C.maenas* has been shown to eliminate PAH metabolites in its urine (and circulate them in its haemolymph) following waterborne exposure to the parent. PAH metabolism has been shown to be comprehensive *via* the urinary route, with levels of metabolites in the urine being dose and time dependent. HPLC/F identification of the metabolic products has revealed that conjugation is extensive in this species. Validation experiments have shown that results obtained by direct fluorescence correlate well with other analytical techniques (ELISA, HPLC/F). Levels of pyrene metabolites in the haemolymph were also exposure concentration dependent, but fluorimetric results did not correlate well with the ELISA, due to sample matrix interference. Application of the fluorimetric urinary assay to both pyrogenic and petrogenic PAH contamination situations in the field has illustrated its suitability as an indicator of exposure in wild populations. The net result is a rapid, inexpensive, non-destructive and sensitive technique, using a ubiquitous, ecologically important invertebrate species for environmental PAH monitoring.

The responses of *C.maenas* following exposure to contaminant mixtures were also studied, with the aim of improving our knowledge on the mechanisms of toxicity of environmental chemicals and how aquatic organisms handle, detoxify and eliminate them. Investigations at the biochemical, cellular and physiological level in this species have demonstrated it to be remarkably robust when faced with xenobiotic challenge. Of the responses investigated, the majority remained unchanged following exposure to

increasingly numerous combinations of contaminants at both high and low concentrations. It would appear that *C.maenas* is capable of handling increasing degrees of sub-lethal pollutant stress with minimal alteration to its normal biochemical and physiological processes (concentration of metal binding proteins, activity of neuronal enzymes, DNA repair mechanisms, efficacy of detoxification enzymes, total protein levels). This is perhaps of no surprise, since contamination in the field is characterised by the presence of numerous anthropogenic chemicals and yet this species remains highly abundant. Importantly, the responses of individual biomarkers were not abolished when exposed to chemical mixtures and still provided accurate information about the health of the organism and its endogenous processes.

Further experiments investigated the potential for endocrine disruption in this species, by observing the effects of chemical exposure on endocrine mediated processes. Exposure to exogenous chemicals did not have measurable effects on endocrine mediated processes in *C.maenas*. As a consequence, it was not possible to evaluate the mechanisms of toxicity of chemicals on these processes or investigate the response of *C.maenas* following exposure. Nor was it possible to develop a reliable biomarker for endocrine disruption in this species, as the endpoints investigated were not altered by exposure. The quantitative Vg ELISA holds promise as a tool to monitor potential disruption to vitellogenesis in *C.maenas*, but only after the temporal progression of this very complex process is understood in this species. The results suggest that endocrine processes are not easily disruptable in *C.maenas* and that exposure to the chemicals used in this work does not pose a significant threat to its endocrine pathways. A much greater understanding of the vulnerability of endocrine processes to disruption by environmental chemicals is also required before their associated endpoints can be used as biological indicators.

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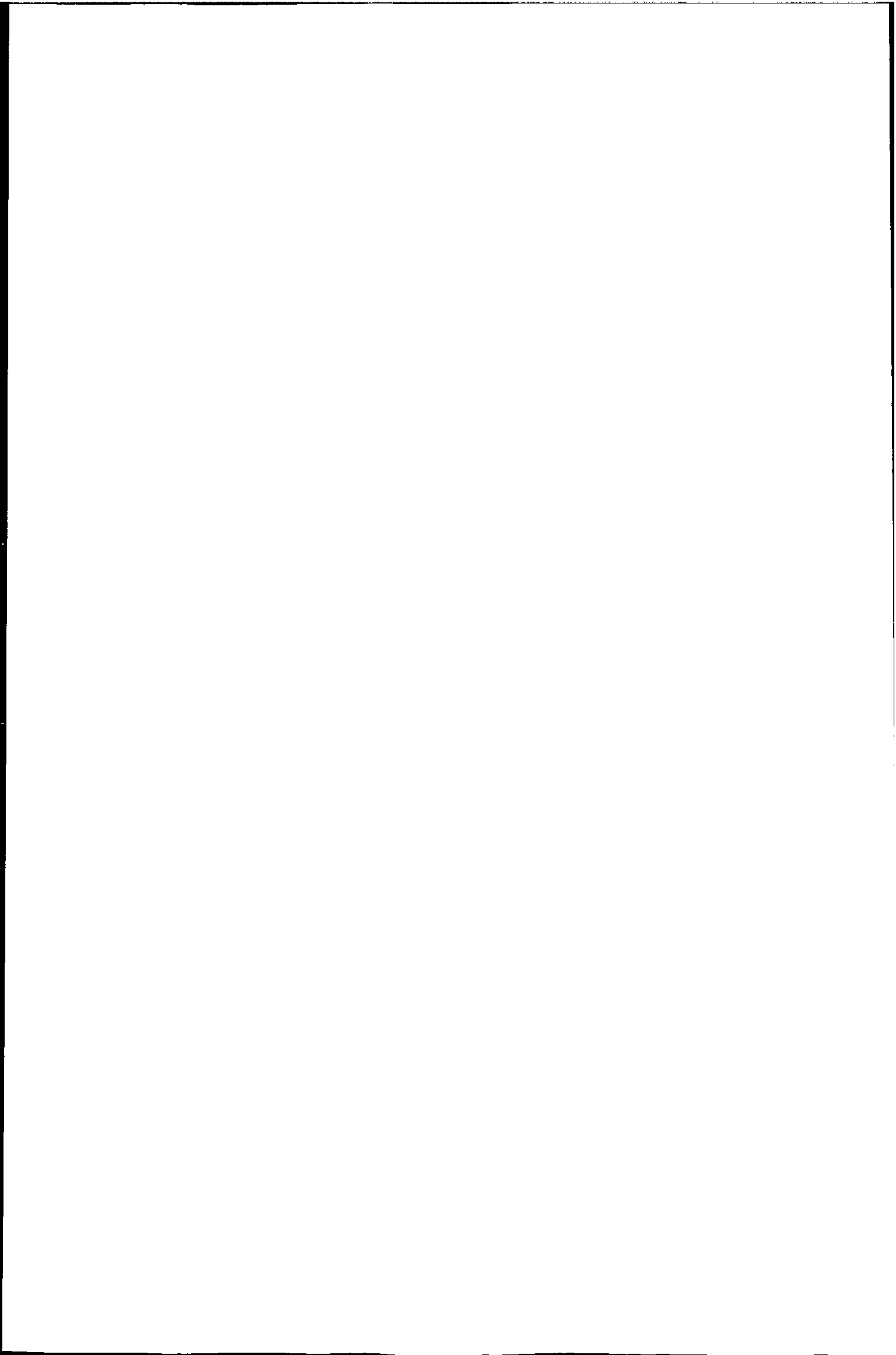

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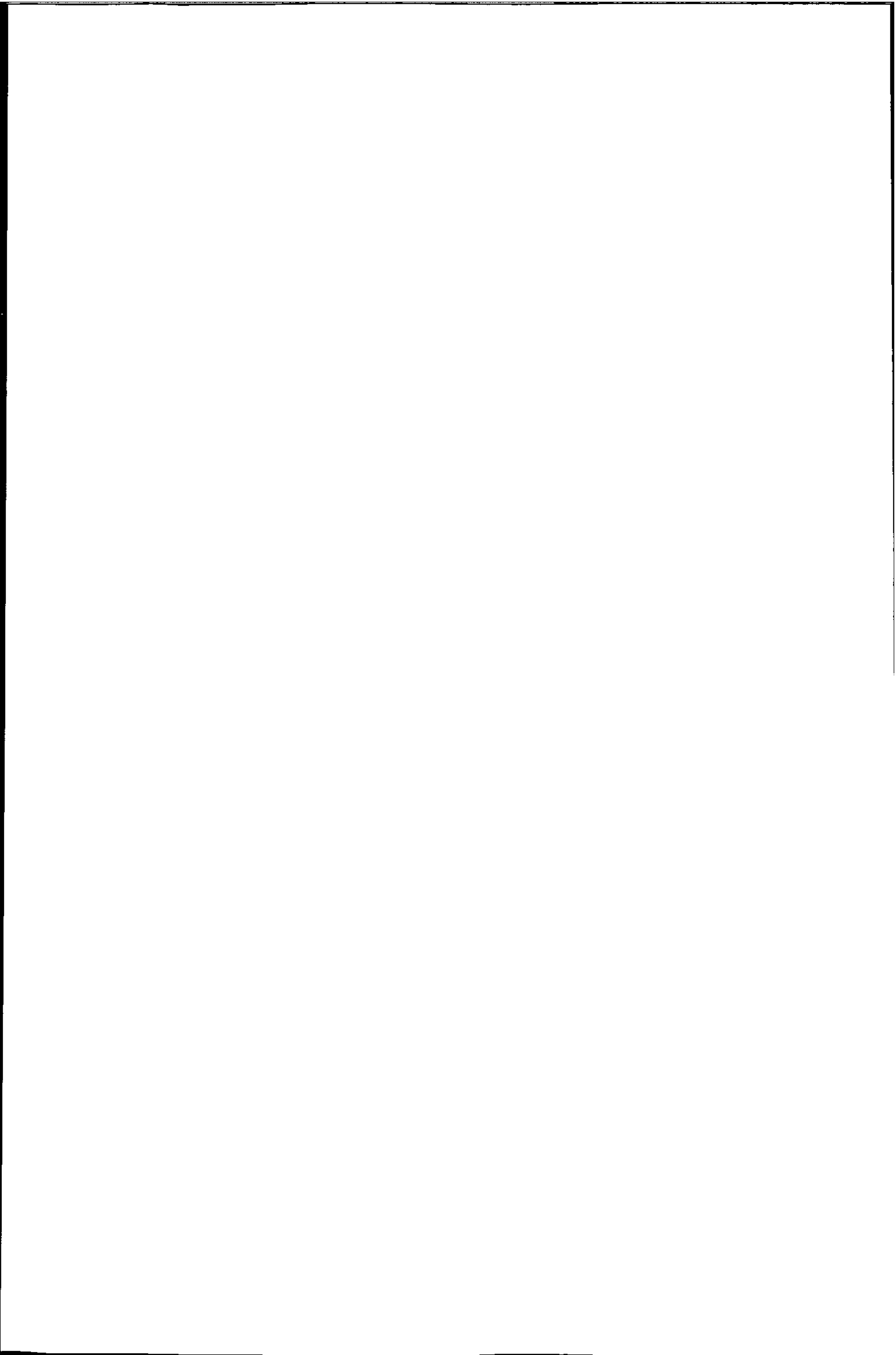

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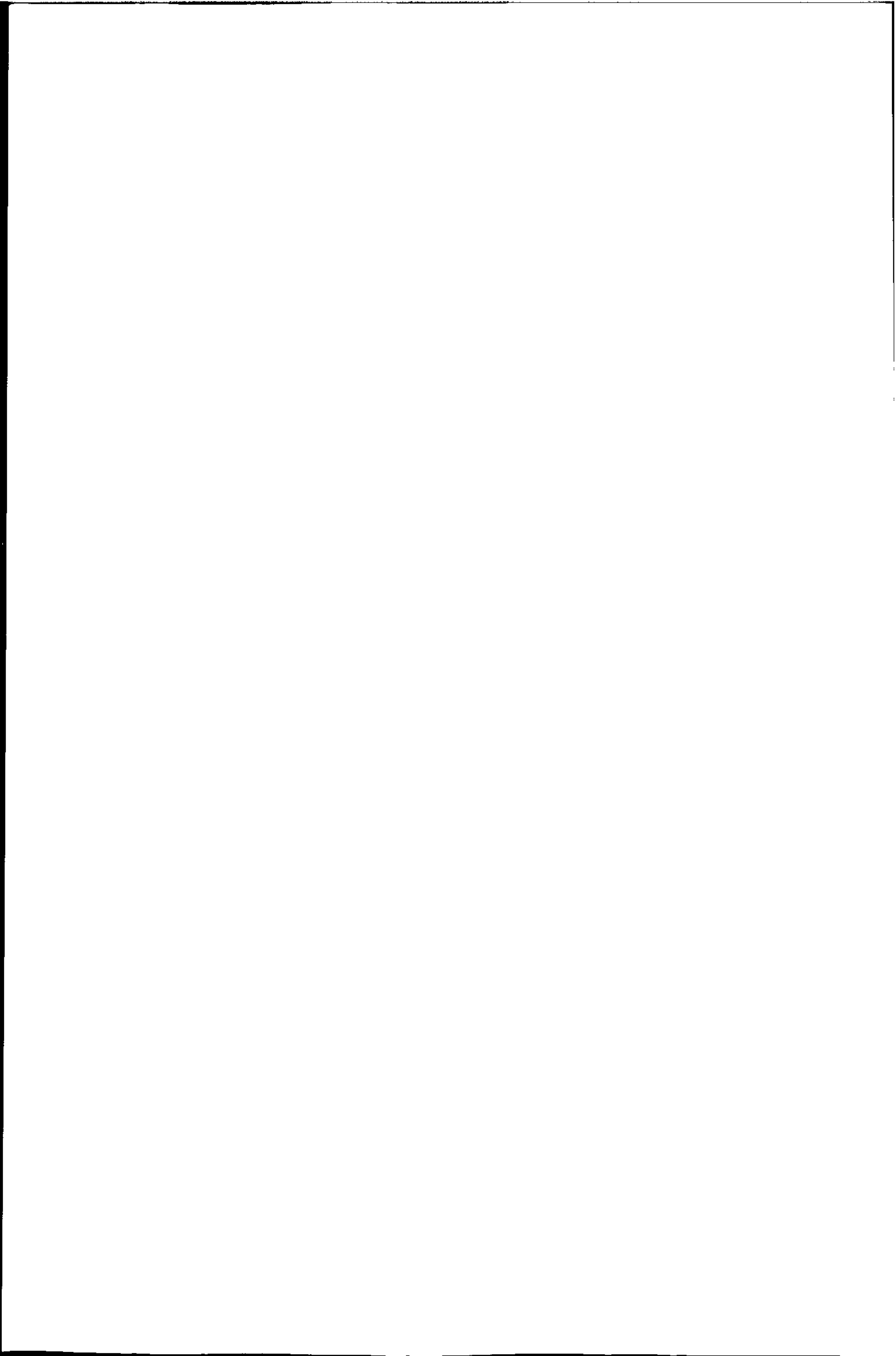

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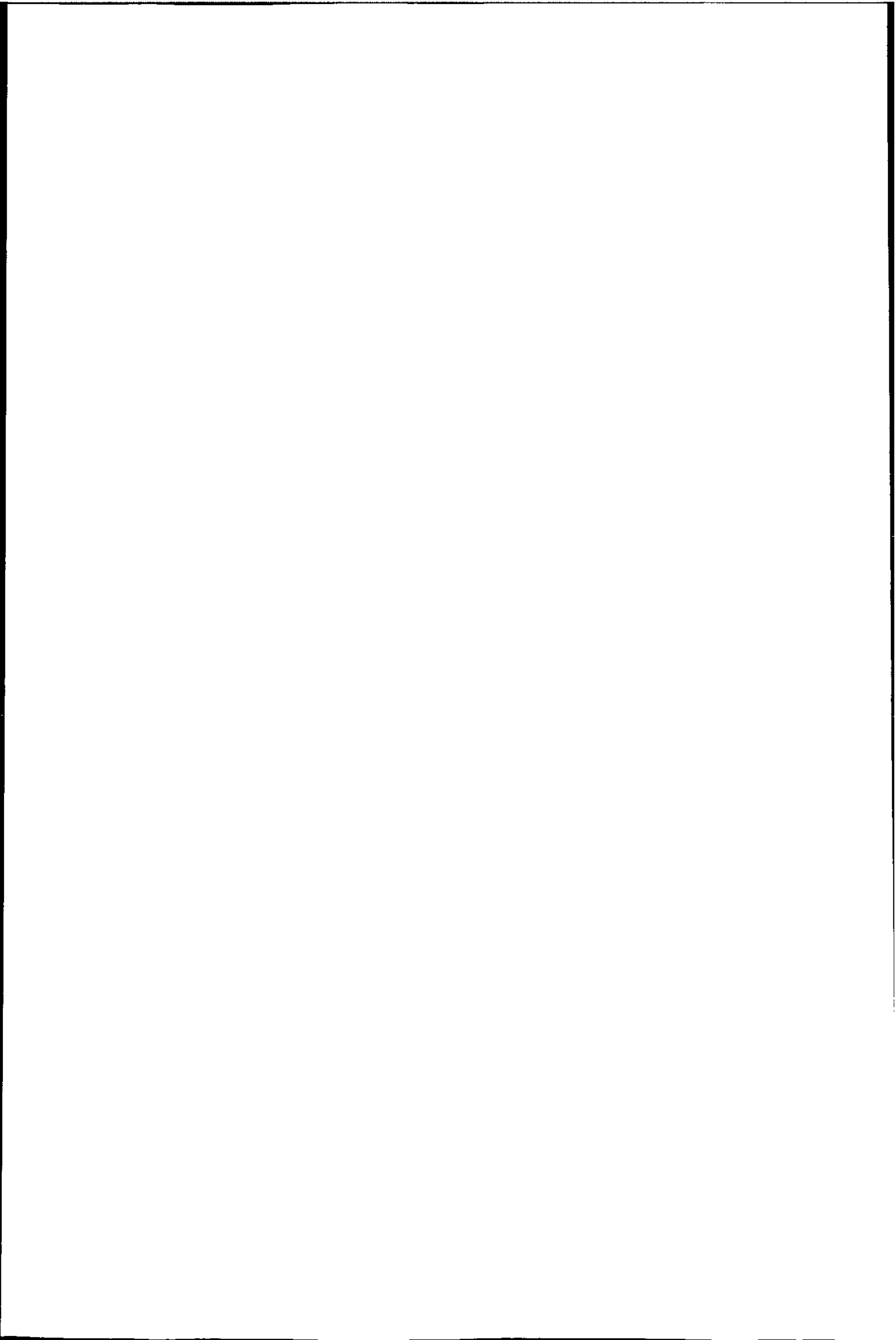

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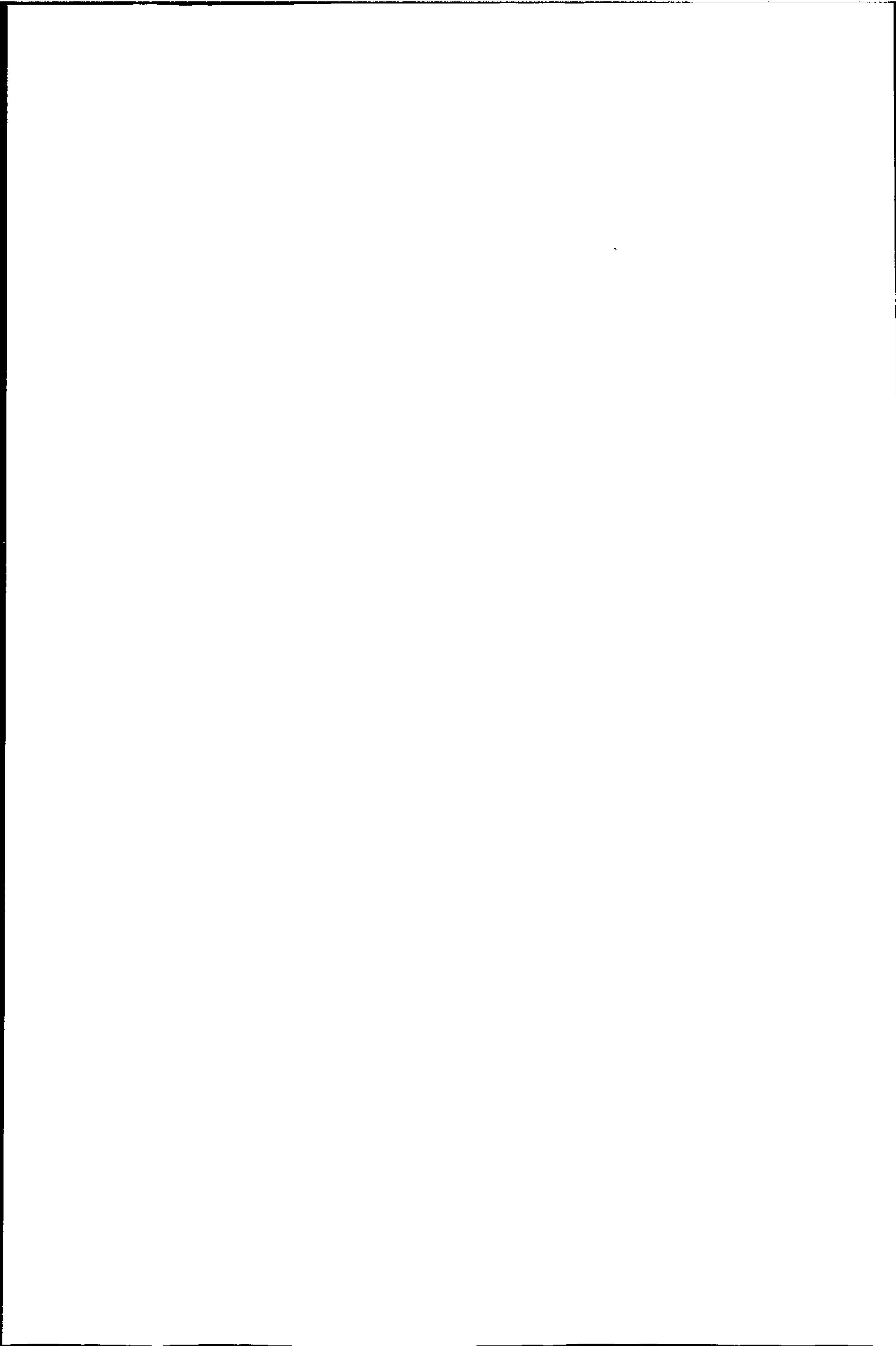

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APPENDIX

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Marine Environmental Research 54 (2002) 823–828

MARINE ENVIRONMENTAL RESEARCH

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A non-destructive assessment of the exposure of crabs to PAH using ELISA analyses of their urine and haemolymph

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Abstract

Urine and haemolymph can be repeatedly sampled from crabs with no (or limited) damage to the organism. Their analysis offers a measure of the animals' exposure to biologically available contaminants. Shore crabs (*Carcinus maenas*) were exposed to the PAHs phenanthrene and pyrene at concentrations ranging from 20 to 200 μg l^{-1}. After 48 h, urine and haemolymph samples were taken and analysed using ELISA and UV-fluorescence spectrophotometry. High correlations were recorded between the two sets of results from the urine analyses ($r^2=0.83$ for phenanthrene and $r^2=0.88$ for pyrene). Contaminant concentrations were much lower in haemolymph than in the urine. Analyses of urine taken from crabs collected from clean and contaminated sites confirm the suitability of these analyses for environmentally exposed organisms. Again, a good correlation was recorded between the ELISA and spectrofluorimetric analysis ($r^2=0.83$). In this instance, difficulties were experienced with haemolymph analyses owing to a lack of sensitivity. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: PAHs; Urine; Haemolymph; Crabs; ELISA; PAH metabolites; Immunoassay

Immunoassays of pesticides and contaminants have been extensively applied to evaluate environmental contamination (Aga, 1997; Sherry, 1997). Although

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PII: S0141-1136(02)00109-5

extensively used in medicine (Knopp, Schedl, Achatz, Kettrup, & Niessner, 1999; MacKenzie, Strilley, Biagini, Stettler, & Hines, 2000; Mastin et al., 1998), to date, we are not aware of other studies using immunoassays of urine and haemolymph from aquatic organisms. Biological samples are important because pollutants such as PAHs and their metabolites become concentrated in tissues, body fluids and excreta thereby offering a measure of exposure to biologically available contaminants. Such analyses can provide a non-destructive tool for monitoring exposure to PAH.

The goal of the present study was to determine the effectiveness of immunoassay methods in measuring PAH/PAH metabolite levels in the urine and haemolymph of exposed invertebrates (shore crab—*Carcinus maenas*). A commercially available immunoassay kit (PAH RaPID Assay®, SDI, Newtown, PA), which was designed to measure PAHs in water, was selected for adaptation to biological sample monitoring. Validation for both experimental and environmental exposure was considered necessary.

The toxicity test (exposure system) consisted of a static test (Rand, 1995). Parental PAH (either pyrene or phenanthrene) was added to the pre-filtered seawater (34 PSU) to produce six nominal concentrations [200, 100, 50, 25, 10 and 0 (control) μg l^{-1}]. Concentrations were verified using solid phase extraction (SPE) C18 cartridges (IST, Hengoed, UK) followed by GC/MS analysis. Measured concentrations confirmed that the nominal (spiked) values were within $\pm 12\%$. The experiment was maintained under a 12 h light/12 h dark regime at 15 ± 1 °C. Crabs were not fed during the exposure period and were sampled for urine and haemolymph after 48 h. Urine and haemolymph samples were taken from each crab using the techniques described by Bamber and Naylor (1997). Briefly, crabs were removed from their aquaria and placed in a bucket containing clean seawater. After being drained, the third maxillipeds were moved aside and kept apart. Under a dissecting microscope ($\times 10$), the operculum of each antennal gland bladder was lifted and urine (20–400 μl per crab) was collected. Haemolymph samples (100–200 μl) were taken with a fine capillary with plunger from an arthrodial membrane at the base of a walking leg. Samples were split (volumetrically) for analysis by immunoassay and spectrofluorimetry and were stored at −80 °C until analysis. After the experiment, the crabs were transferred to clean seawater to depurate (for 3 weeks) before being returned to the environment.

The PAH RaPID Assay® ELISA was used according to the manufacturer's recommendations. Urine and haemolymph samples (50-μl aliquots) were diluted with 50% v/v methanol/buffered aqueous solution with stabilisers (SDI diluent) and were assayed in triplicate together with four standards. Appropriate amounts of samples (or standards), antibody-coated onto paramagnetic particles and enzyme conjugate (PAH-horseradish peroxidase) were mixed and incubated. After washing twice with kit buffer using a magnetic rack to retain the antibodies, substrate (hydrogen peroxide) and chromogen (3,3′,5,5′-tetramethylbenzidine) were added (and incubated). Stop solution (2 M sulphuric acid) was added and the colour produced was measured at 450 nm using an Optimax microplate reader (Molecular Devices, Menlo Park, CA). Sample concentrations were calculated using a log–logit standard curve and multiplying results by the appropriate dilution factor.

Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer following an adapted method of Ariese et al. (1993) and Aas, Beyers, and Goksoyr (2000). Standards (pyrene, 1-OH-pyrene, phenanthrene, and 9-OH-phenanthrene), blanks and samples (urine—1:100; haemolymph—1:20) were diluted with 50% v/v ethanol/Milli-Q water. Fixed excitation wavelength fluorescence (FF) and synchronous excitation/emission fluorescence spectrometry (SFS) measurements were carried out with excitation and emission slit widths of 2.5 nm. The assigned wavelength pairs were Ex345/Em382 nm (FF) for pyrene and Ex252/Em357 nm (FF) for phenanthrene, and $\Delta\lambda$ of 37 and 54 nm, respectively, for SFS analyses.

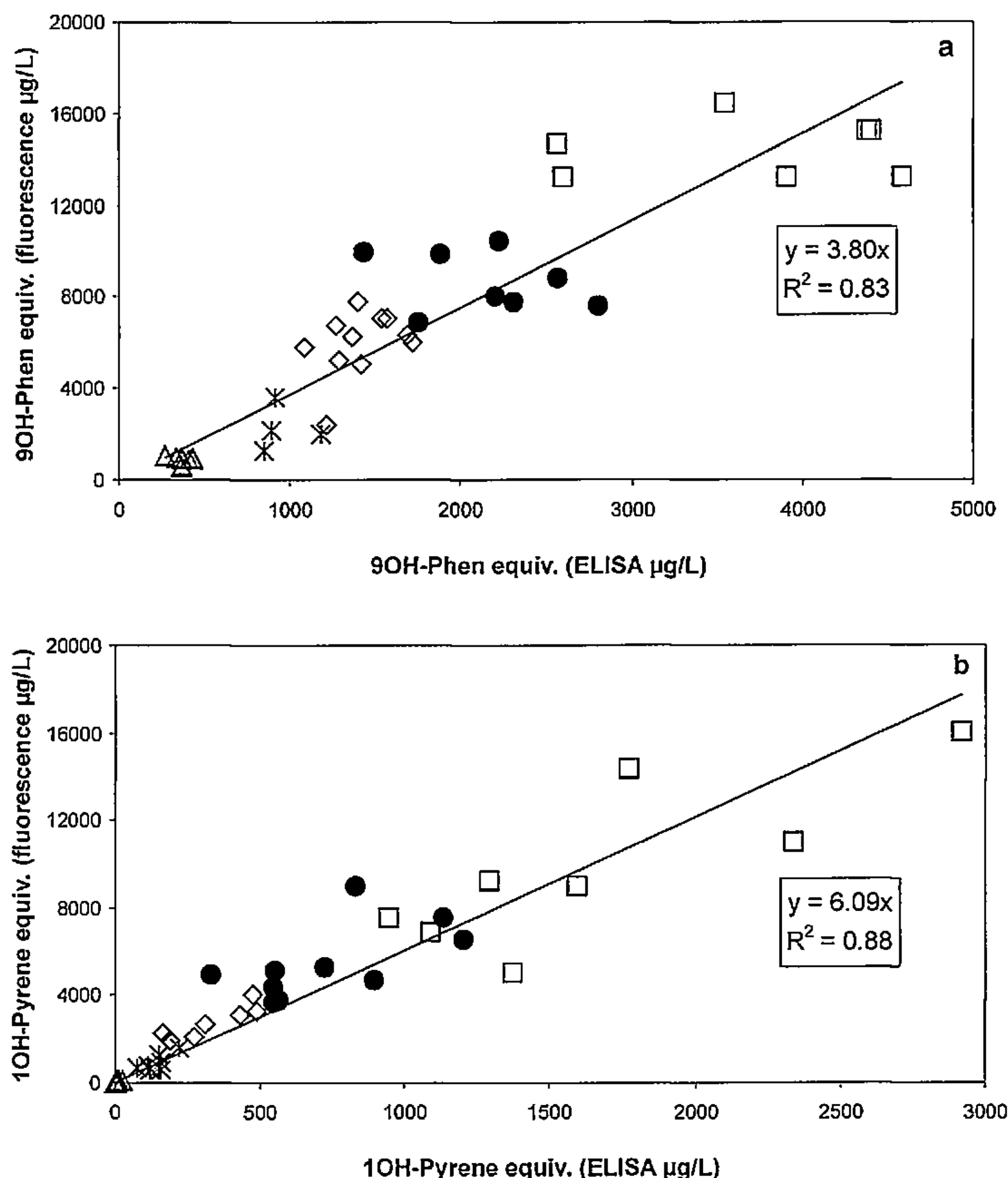

Fig. 1. Plot of results from PAH RaPID Assay® ELISA and spectrofluorimetry analyses. (a) Phenanthrene metabolites in crab urine ($FF_{252/357}$); (b) pyrene metabolites in crab urine ($FF_{345/382}$). Exposure concentrations: (□) 200 µg l^{-1}, (●) 100 µg l^{-1}, (◇) 50 µg l^{-1}, (⋇) 25 µg l^{-1}, (△) 0 µg l^{-1}.

Samples were quantified against 1-OH-pyrene and 9-OH-phenanthrene standards (200, 100, 75, 50, 25, 10, 5 μg l^{-1} for urine samples and 10, 5, 2, 1, 0.5 μg l^{-1} for haemolymph samples). Results are reported in terms of 1-OH-pyrene or 9-OH-phenanthrene "equivalents".

Results of the immunoassay and spectrofluorimetric urine analyses from the exposure experiments are shown in Fig. 1a and b. An exposure response relationship

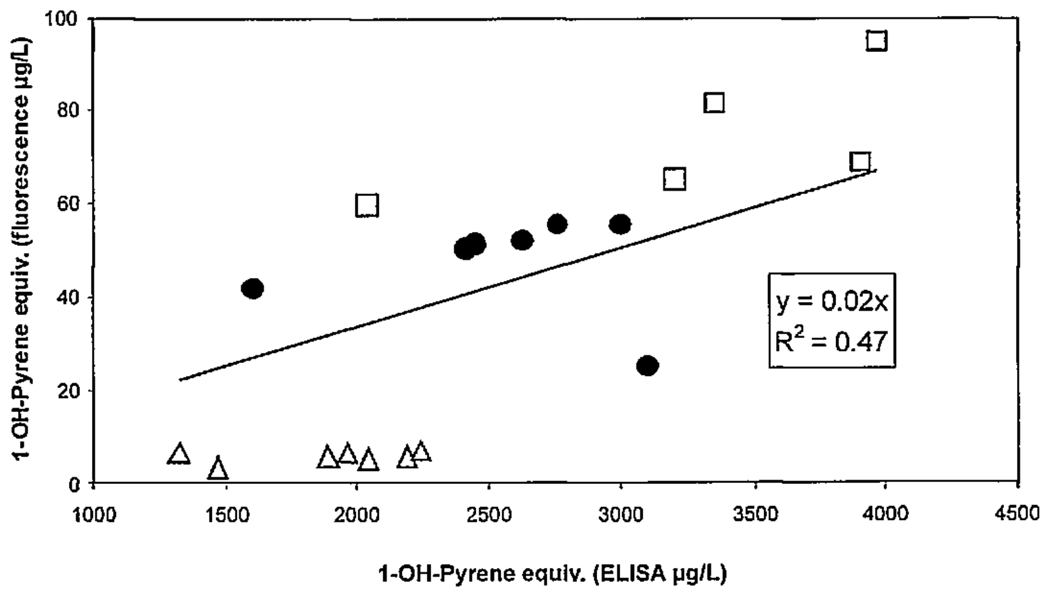

Fig. 2. Plot of results from PAH RaPID Assay® ELISA and spectrofluorimetry ($FF_{345/382}$) analyses for pyrene metabolites in crab haemolymph. The poor correlation indicates matrix interferences associated with the ELISA. Exposure concentrations: (□) 200 μg l^{-1}, (●) 100 μg l^{-1}, (Δ) 0 μg l^{-1}.

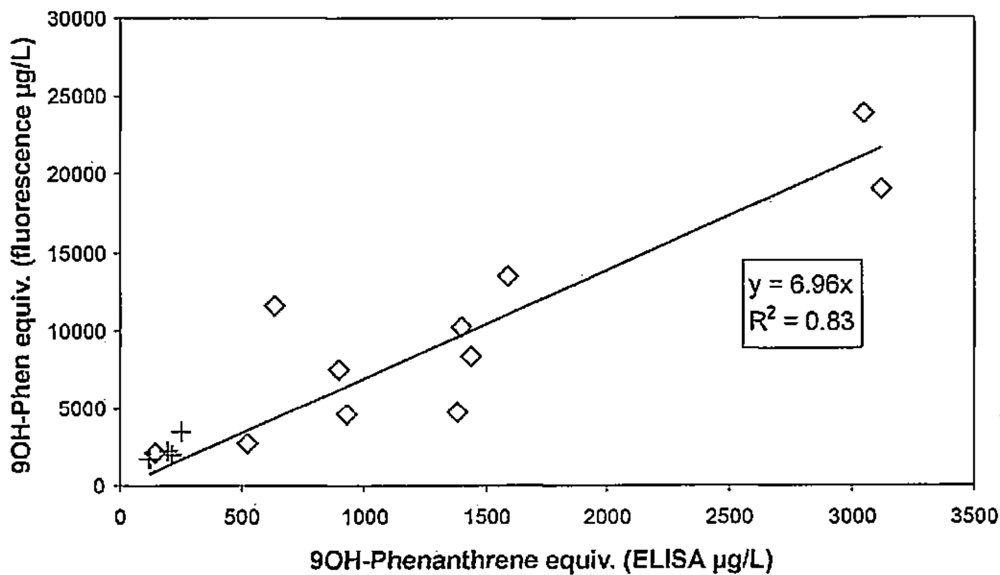

Fig. 3. Plot of results from PAH RaPID Assay® ELISA and SFS (42 nm diff.) analyses of PAH metabolites in environmental samples of crab urine. (+) Bantham (clean control site) and (◇) Sutton Harbour (polluted)—Southwest England.

and high correlations between the ELISA and spectrofluorimetric analyses are demonstrated ($r^2=0.83$ for phenanthrene and $r^2=0.88$ for pyrene). PAH and metabolite concentrations were much lower in the haemolymph, although FF and SFS spectra appeared similar to those seen in the urine samples. Difficulties were experienced with haemolymph analyses owing to a lack of sensitivity and matrix interactions (Fig. 2). Matrix effects with urine analyses were overcome by dilution (20-fold) of the samples. However, even after 80-fold dilution of the haemolymph matrix interactions were still apparent (Fig. 2).

Urine and haemolymph samples from crabs living at clean and contaminated sites were also analysed using the immunoassay and UV-fluorescence. Both techniques detected PAH (mainly petrogenic) contamination in urine. The agreement between techniques was good ($r^2=0.83$) (Fig. 3). The haemolymph intensities, however, were much lower than those in urine and the variability of the data much greater. As noted before, more dilution was needed to avoid matrix effects in the haemolymph immunoassay, rendering detection limits a problem.

There is a clear need for simple, cost-effective, and non-invasive methods in environmental studies to measure organism exposure levels, and ELISA is therefore a good option. Our results demonstrate the suitability of the immunoassay technique described in measuring exposure of crabs to PAH. Urine analyses are shown to be more sensitive than those of haemolymph. Further research is required to investigate causes underlying variability in the data.

Acknowledgements

G. Fillmann is sponsored by CAPES (BEX1536/96–6) and WWF-Brazil/USAID (CSR-151–00). This research is partially funded by the Department for International Development (DFID-UK).

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Aquatic Toxicology 67 (2004) 127–142

AQUATIC TOXICOLOGY

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Rapid assessment of polycyclic aromatic hydrocarbon (PAH) exposure in decapod crustaceans by fluorimetric analysis of urine and haemolymph

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Received 19 July 2002; received in revised form 3 November 2003; accepted 17 November 2003

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and potentially harmful contaminants of the coastal and marine environment. Studies of their bioavailability, disposition and metabolism in marine organisms are therefore important for environmental monitoring purposes. Detecting PAH compounds in the biological fluids of marine organisms provides a measure of their environmental exposure to PAHs. In the present study, the shore crab *Carcinus maenas* was exposed to waterborne pyrene for 48 h. Urine and haemolymph samples were analysed by direct fluorimetry utilising both fixed wavelength (FF) and synchronous scanning fluorescence (SFS) techniques. Samples from exposed crabs exhibited fluorescence due to 1-OH pyrene equivalents, whilst samples from control crabs did not. Levels of equivalents were exposure dependent. Urine was shown to be a more suitable medium for the analysis of PAH equivalents. In a separate experiment, depuration of pyrene equivalents in urine was monitored over time. Urinary levels reached a maximum 2–4 days after initial exposure and decreased steadily thereafter. No unchanged parent pyrene was detected in samples from exposed crabs. While fluorimetric techniques could discriminate between 1-OH pyrene equivalents and parent pyrene, identification of specific metabolites was only possible with HPLC/F analysis. This revealed crabs had bio-transformed pyrene into 3 major conjugates of 1-OH pyrene, which were excreted in the urine. While such biotransformation of PAH is well documented in fish and several crustaceans, this is the first study to use direct fluorimetry to detect PAH equivalents in exposed crustacean urine. Fluorimetric results correlated well with those obtained by HPLC/F and ELISA techniques. The technique has great potential as a rapid, inexpensive and non-destructive technique for field biomonitoring of PAH exposure in crustaceans.
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Keywords: PAH; Shore crab; Urine; Haemolymph; Non-destructive biomarker; Biotransformation; Metabolites; HPLC/F; ELISA

1. Introduction

Marine waters and sediments, particularly those receiving anthropogenic inputs, contain a multitude of

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0166-445X/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2003.11.006

chemical contaminants that are potentially toxic to aquatic organisms (Krahn et al., 1984). One group of particular concern are the polycyclic aromatic hydrocarbons (PAHs). These are highly lipophilic chemicals, ubiquitous in soils, sediments, air and water (Lin et al., 1994). PAHs in the aquatic environment are derived largely from inputs of petroleum and its products, from sewage effluents, runoff and atmospheric deposition from the incomplete combustion of organic matter (Law et al., 1994). Industrial activities such as metal smelting (Naes et al., 1995; Beyer et al., 1996) and the electrolytic production of aluminium (Beyer et al., 1998) also release PAHs into rivers, estuaries and inshore waters. Coastal waters additionally receive substantial amounts of PAHs from products such as creosote, coal tar and coal tar pitch which are used as preservatives and antifouling agents (Uthe and Musial, 1986).

The conventional assessment of impacts involves measurements of contaminant concentrations in water and sediments. PAHs are sparingly soluble however, and their concentrations in water are very low (Lin et al., 1994). Consequently, they are difficult to detect. Large water volumes are needed for analysis, and their hydrophobicity/lipophilicity results in preferential partitioning into sediments (Lin et al., 1994). Analysis of sediments is equally laborious and time-consuming, involving numerous clean up and extraction steps. Furthermore, water/sediment-based measurements provide little information about the potential for contaminants to reach organisms and cause deleterious effects (i.e. bioavailability and exposure are not addressed). To assess exposure to PAHs (and other contaminants) more accurately, it is pertinent to detect them in the tissues and biological fluids of aquatic organisms.

Tissues such as liver, hepatopancreas, kidney, muscle and gills, and body fluids (blood/haemolymph, bile and urine) have been analysed previously to determine the concentrations and types of PAHs that enter biota. However, differences in the ability of aquatic species to metabolize contaminants affects their suitability as biological indicators. In organisms capable of metabolizing PAHs, measuring metabolites instead of simply quantifying parent compounds avoids underestimation of true uptake (McElroy et al., 1989). Measurement of parent PAH residues is more suited to species such as bivalve molluscs, which have limited ability for biotransformation of PAHs and tend to accumulate these compounds in their tissues (Hufnagle et al., 1999; McElroy et al., 1989). Determination of PAH metabolites is particularly relevant in fish (Stegeman and Lech, 1991) as it is the biotransformed, active metabolites that exert significant toxic, mutagenic and carcinogenic effects (James, 1989; Stroomberg et al., 1996).

The development of easy-to-use, accurate and cost-effective techniques for the measurement of PAHs and their metabolites (and other contaminants) in the various matrices of aquatic biota is becoming increasingly relevant, as it provides useful information regarding contaminant exposure and adverse effects in populations in situ (Depledge, 2000; Wells et al., 2001). This information can then be used in the rapid assessment of the pollution status of the ecosystem and of the potential risk to humans who consume PAH contaminated animals. For example, the rapid assessment of marine pollution (RAMP) approach utilises a wide array of simple, inexpensive techniques to provide an indication of the extent of exposure of marine organisms to contaminants as well as evidence of effects (Depledge, 2000; Wells et al., 2001; Galloway et al., 2002). This information can then be used in setting priorities for more detailed and costly studies utilising more sophisticated techniques.

In the present study, a simple technique for the spectrophotometric analysis of urine and haemolymph from shore crabs has been developed and evaluated as a means of assessing exposure to polyaromatic hydrocarbons.

2. Materials and methods

2.1. *Collection of experimental animals and laboratory conditions*

Green, male, intermoult shore crabs, *Carcinus maenas* (carapace width 50–72 mm) were collected on incoming tides from Jenkins Quay on the Avon estuary at Bantham, South Devon, UK on three occasions between the months of April and July 2000. On return to the laboratory, they were maintained in holding tanks containing filtered (10 μm carbon filtered), well aerated 34 ppt, 15 ± 1 °C seawater,

under a 12 h light: 12 h dark regime for 1 week to permit acclimation. Crabs were fed twice weekly with irradiated cockles. Water was changed within 12 h of feeding.

2.2. Chemicals

Pyrene (98%, cat. no. 18, 551-5) and 1-OH pyrene (98%, cat. no. 36, 151-8) were purchased from Sigma–Aldrich (The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK). Pyrene-d_{10} was acquired from Promochem (Welwyn Garden, UK). Ethanol (GPR™, 96%, v/v) and acetone (GPR™) were obtained from BDH Laboratory Supplies (Poole, BH15 1TD, UK).

2.3. Exposure–response exposure experiment

Crabs were transferred to glass aquaria containing 10 l of filtered (10 μm carbon filtered), well-aerated seawater (34 ppt, 15 ± 1 °C), under a 12 h: 12 h light: dark regime. After measurement of carapace width, the animals were assigned to one of 14 groups of 4 in separate aquaria, with groups of 8 crabs exposed to one of seven treatments, for 48 h. For exposed crabs, pyrene was added to the water in an acetone carrier (at a ratio of 1:1, w/v, pyrene/acetone) to increase solubility. Pyrene exposure was at five nominal concentrations (200, 100, 50, 25 and 10 $\mu g\,l^{-1}$). Acetone controls were exposed to acetone only and eight crabs were held in seawater alone (seawater controls). Animals were not fed during the exposure period. The crabs remained in the aquaria for the duration of the exposure, whereupon they were transferred to clean seawater to depurate for up to 3 weeks, during which time feeding was resumed.

2.4. Time response exposure experiment

In a separate experiment, groups of eight crabs were exposed, under the conditions described above, to waterborne pyrene at the nominal concentrations of 400 and 200 $\mu g\,l^{-1}$. An acetone control group and a seawater control group, each comprising eight individuals, were also included as described above. Individuals were exposed and kept in their respective aquaria for a period of 10 days. After this time, they were removed and held in clean aerated seawater. For repeat sampling, individuals were identified by labels super-glued onto the dorsal surface of the carapace.

2.5. Urine and haemolymph sampling

In the exposure–response experiment, urine and haemolymph samples were taken from each crab after 48 h exposure using the technique described by Bamber and Naylor (1997). Briefly, crabs were removed from their aquaria and placed in a bucket containing clean seawater. After being drained of residual seawater, they were restrained with the ventral surface uppermost on a plastic board using rubber bands. The third maxillipeds were moved aside and kept apart by inserting absorbant paper between the base of the appendage and top of the sternum. The epistome was dried (to prevent seawater contaminating the urine) and the crab placed under a dissecting microscope (10×). The operculum of each antennal gland bladder was lifted using a hooked seeker, causing urine to flow from the bladder, through the opercula, where it was collected using a 200 μl Gilson© pipette fitted with a flexible flat tip. Samples (20–400 μl per crab) were then transferred to siliconized microcentrifuge tubes, snap frozen in liquid nitrogen and stored at −80 °C until analysis.

With the crab still restrained, the absorbant paper holding the maxillipeds was removed and a haemolymph sample was taken from a suitable arthrodial membrane at the base of a walking leg. The membrane was first gently punctured using a fine bore needle and then a fine capillary, with plunger, was used to draw out 200 μl of haemolymph. Samples were then expelled into siliconized microcentrifuge tubes, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Test animals were returned to their respective aquaria immediately after sampling,

Urine and haemolymph samples were taken in this way wherever possible, but occasionally urine could not be obtained since the urinary bladders of some crabs were empty. It was necessary therefore, to repeat the above exposure and sampling procedure three times, to obtain sufficient replicates ($n > 10$) of urine samples. For the time–response experiment, urine samples were taken from all crabs (as described above) after 12, 24, 48 and 96 h, and 8 and 10 days.

2.6. Direct fluorescence spectrophotometry (fluorimetry)

2.6.1. Analysis of standards and urine samples

Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer, coupled to a software package operated by Windows 3.1. Fifty percent ethanol was the solvent used for samples, standards and blanks. Measurements were carried out in a 3.5 ml quartz fluorescence cuvette. All FF and SFS measurements were performed with excitation and emission slit widths of 2.5 nm. Resulting FF and SFS spectra were smoothed and fluorescence intensity measured at a fixed excitation and emission wavelength pair for solvent blanks, standards and samples alike.

2.6.2. Determination of wavelength pairs

Various authors have reported differing wavelength pairs for the detection of pyrenes in fish bile, including 341/383 nm (Aas et al., 1998; Camus et al., 1998; Beyer et al., 1998), 345/395 nm (Ariese et al., 1993; Escartin and Porte, 1999) and 349/391 nm (Sundt and Goksoyr, 1998). For synchronous fluorescence analyses of fish bile, wavelength differences of 34 nm (Lin et al., 1994) 37 nm (Ariese et al., 1993; Lin et al., 1994) and 42 nm (Aas and Klungsoyr, 1998; Aas et al., 2000a,b) have been used.

The optimum wavelength pairs for the detection of "pyrenes" (the parent compound and its metabolites) in the present study were selected after first analysing a subset of samples and several pyrene and 1-OH pyrene standards using a range of different excitation and emission wavelengths.

2.6.3. Preliminary analysis of standards and samples

Various concentrations (200, 100, 75, 50, 25, 10, 5 $\mu g\,l^{-1}$) of pyrene and 1-OH pyrene were prepared in 50% ethanol and analysed to determine the optimal wavelength pair for these compounds. The standards were analysed using fixed fluorescence (FF) at a range of excitation wavelengths (including 341,345 and 349 nm, according to the papers reported above), with emission spectra taken from 330 to 500 nm. For synchronous fluorescence (SFS) analysis, $\Delta\lambda$'s of 34, 37 and 42 nm were tested, with excitation/emission spectra taken from 240 to 500 nm.

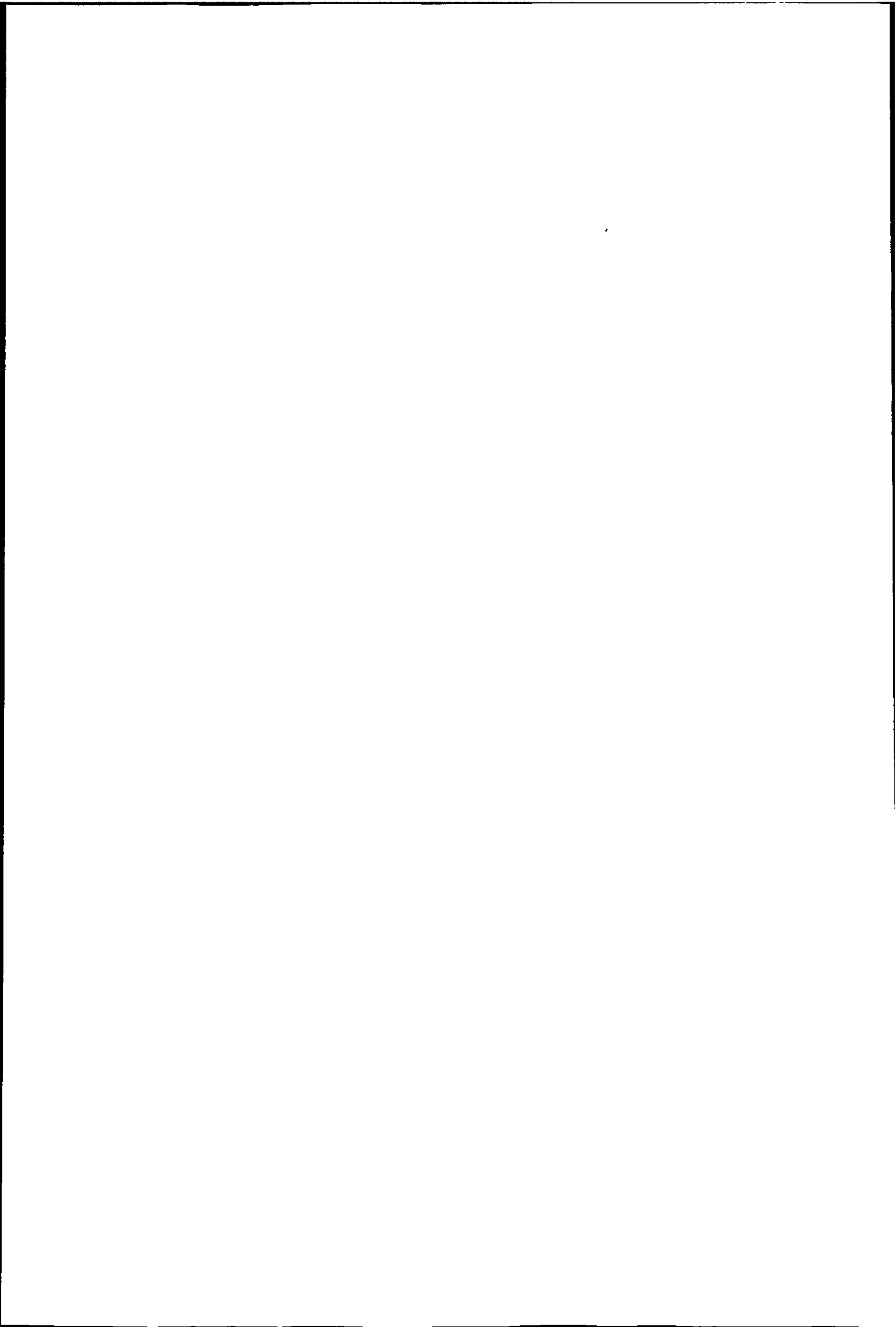
Several representative urine and haemolymph samples from each treatment were also analysed. A 20/50 µl aliquot of urine/haemolymph was dissolved in 1980/950 µl of 50% ethanol in the cuvette, to yield a 1:100/1:20 dilution, respectively. Analyses were performed using the above wavelength regimes for detection of pyrenes.

FF at Ex345 nm produced the largest and most clearly resolved peaks for standards and samples. For synchronous fluorescence spectrometry (SFS) analysis, 37 nm was the optimal $\Delta\lambda$. The predominant urinary peaks more closely approximated those produced by the 1-OH pyrene standards than the parent standards. Therefore, analysis of samples and 1-OH pyrene standards was undertaken with a fixed excitation wavelength of 345 nm and synchronous analysis was performed with a $\Delta\lambda$ of 37 nm.

For operational purposes, the fluorescence value assigned to individual standards and samples was the intensity measured at a fixed wavelength pair rather than at the maximum peak height of the dominant peak on the emission spectra. In all cases this value was within 5% of the peak maxima. The fixed wavelength pair was determined by calculating the mean emission wavelength of the dominant peak on both standard and sample spectra. For analyses, this was Ex345/Em387 nm (standards) and Ex345/Em382 nm (samples). For SFS analyses, wavelengths were Em387 nm (standards) and Em381.4 nm (samples).

2.6.4. Quantification of sample peaks

To take into account instrumental drift over the lifetime of the experiment and to allow all samples to be compared despite fluctuations in instrumental response, sample peaks were quantified with respect to a series of 1-OH pyrene standards (200, 100, 75, 50, 25, 10, 5 $\mu g\,l^{-1}$ for urine samples and 10, 5, 2, 1, 0.5 $\mu g\,l^{-1}$ for haemolymph samples), analysed at the fixed wavelength pair of Ex345/Em387 nm (FF) and Em387 nm (SFS). The use of 1-OH pyrene was favoured over the parent compound as it more closely approximates the peaks seen in the samples. 1-OH pyrene does not serve to quantify sample peaks in terms of $\mu g\,l^{-1}$ of 1-OH pyrene as the peaks seen in samples are blue shifted by 5 nm and are unlikely to be the pure standard. Results are therefore reported in terms of $\mu g\,l^{-1}$ of 1-OH pyrene "equivalents".



2.6.5. Urine and haemolymph samples

Urine (diluted 1:100) and haemolymph (diluted 1:20) samples from exposed and control crabs were analysed for pyrenes as described above. Fluorescence intensity was measured at the assigned wavelength pair of Ex345/Em382 nm (FF) and Em381.4 nm (SFS) and expressed as $\mu g\,l^{-1}$ of 1-OH pyrene equivalents following comparison to standards. Before analysis of either standards or samples, a blank of 50% ethanol was scanned by FF and SFS. This provided a value for the background fluorescence contribution of the solvent, for comparison with samples and standards.

2.6.6. Statistical analysis

Data were analysed using the software package Statgraphics Plus 5.1 and are presented using notched box and whisker plots. The rectangular part of the plot ("box") extends from the lower quartile to the upper quartile, covering the middle 50% of data values (interquartile range). The centre lines within each box show the position of the medians, whilst the plus signs show the position of the mean. The "whiskers" extend from the upper and lower quartiles to maximum and minimum values lying within 1.5 times the interquartile range. Outliers are shown as individual points. Also included are notches covering a distance either side of the median line, representing the 95% confidence interval for the median. Where notches for any pair of medians overlap, there is no statistically significant difference between medians at the 95% confidence level. Where no overlap occurs, the difference is significant at the 95% confidence level.

All urine and haemolymph data were variance checked for homogeneity of variance between groups and found to be not normally distributed (a statistically significant difference amongst the S.D., $P \leq 0.05$). This large variability in the data (more than a 3 to 1 difference between the smallest S.D. and the largest) invalidates an analysis of variance, which assumes that S.D. at all treatments is equal. All urinary data were therefore log-transformed for comparison of means by one-way ANOVA, using Fisher's least significant difference (LSD) procedure to discriminate between means at the 95% confidence level. Haemolymph data, when variance checked, were found to be not normally distributed, even following log transformation. Data were therefore subject to a Kruskal–Wallis test to compare medians at the 95% confidence level.

2.6.7. GC/MS analysis of water samples

To investigate the uptake of waterborne pyrene by exposed crabs, a series of tanks were set up in duplicate (one containing crabs, the other without). Pyrene was added to the water at the same nominal concentrations as for the exposure experiment and water samples taken at 0, 2, 6, 12, 24, 48 and 96 h. Samples were analysed for parent PAH using GC/MS analyses (Hewlett-Packard Model 5890 II Plus GC and a 5972 mass selective detector (MSD) (Palo Alto, CA)). Internal standard spiked (pyrene-d_{10}) water samples (100–500 ml) were concentrated using C_{18} cartridges (IST, Hengoed, UK), which were subsequently eluted (three times) using 3 ml of ethyl acetate. The eluent was then concentrated down to 1 ml before analyses by GC/MS.

2.6.8. Inner filter effects and matrix effects

Provided the total absorbance of a sample is low, a linear relationship exists between the intensity of fluorescence emitted by a compound within the sample, and its concentration. If the sample absorbs more than 5% of the incident light, fluorescence intensity will be reduced by any compound in the sample which absorbs either the excitation or emission light. This is known as "inner filter effect" (UNEP/IOC/IAEA, 1992). To test for inner filter effects, representative urine samples from each treatment were serially diluted in 50% ethanol (1:100, 1:50, 1:25, 1:10, 1:5) and analysed as above (FF Ex345/Em382 nm). Fluorescence intensity results were then plotted against the relative concentration of the sample to establish the relationship between dilution and fluorescence signal.

To determine whether the 5 nm difference between the standard peak and the urinary peak was not due to a urinary matrix effect, an additional experiment was carried out. Urine from control crabs was spiked with known amounts of 1-OH pyrene standard (yielding final concentrations of 25, 50, 100 and 200 $\mu g\,l^{-1}$). These spiked samples were then analysed as described above and the spectral position of the resulting peak was compared to the peak seen in urine from pyrene exposed crabs.

2.6.9. HPLC/F identification of urinary metabolites

HPLC/F analysis on selected urine samples from control and exposed crabs was kindly carried out by Dr. Mike Howsam, Vrije Universiteit, The Netherlands. Methodology and results are reported in full in Fillmann et al. (2003).

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

Samples were diluted 1:30 with ethanol (modified with 5 mg ml^{-1} ascorbic acid) and stored at −20 °C. Peaks were identified according to their retention times (Stroomberg et al., 1999), and confirmed for the system used in this study (Howsam, personal communication). Quantification of 1-OH pyrene was performed using a series of 1-OH pyrene external standards, while conjugates were quantified using their relative fluorescence efficiencies compared to 1-OH pyrene; pyrene-1-glucoside = 2.0 ± 0.31, pyrene-1-sulphate = 1.23 ± 0.09, pyrene-1-'conjugate' = 1.75 ± 0.18 (Stroomberg et al., 2003).

2.6.10. Comparison of fluorescence results with other analytical techniques

To validate the direct fluorimetric technique, levels of urinary equivalents in selected samples were analysed using HPLC/F and an immunoassay technique and then compared to fluorescence results. Samples were analysed with a PAH-competitive tube format ELISA kit (PAH RaPID Assay®, SDI Europe, Alton, UK) shown to be highly suitable for detecting PAH in biological samples (Fillmann et al., 2002). The ELISA was calibrated using 1-OH-pyrene.

3. Results

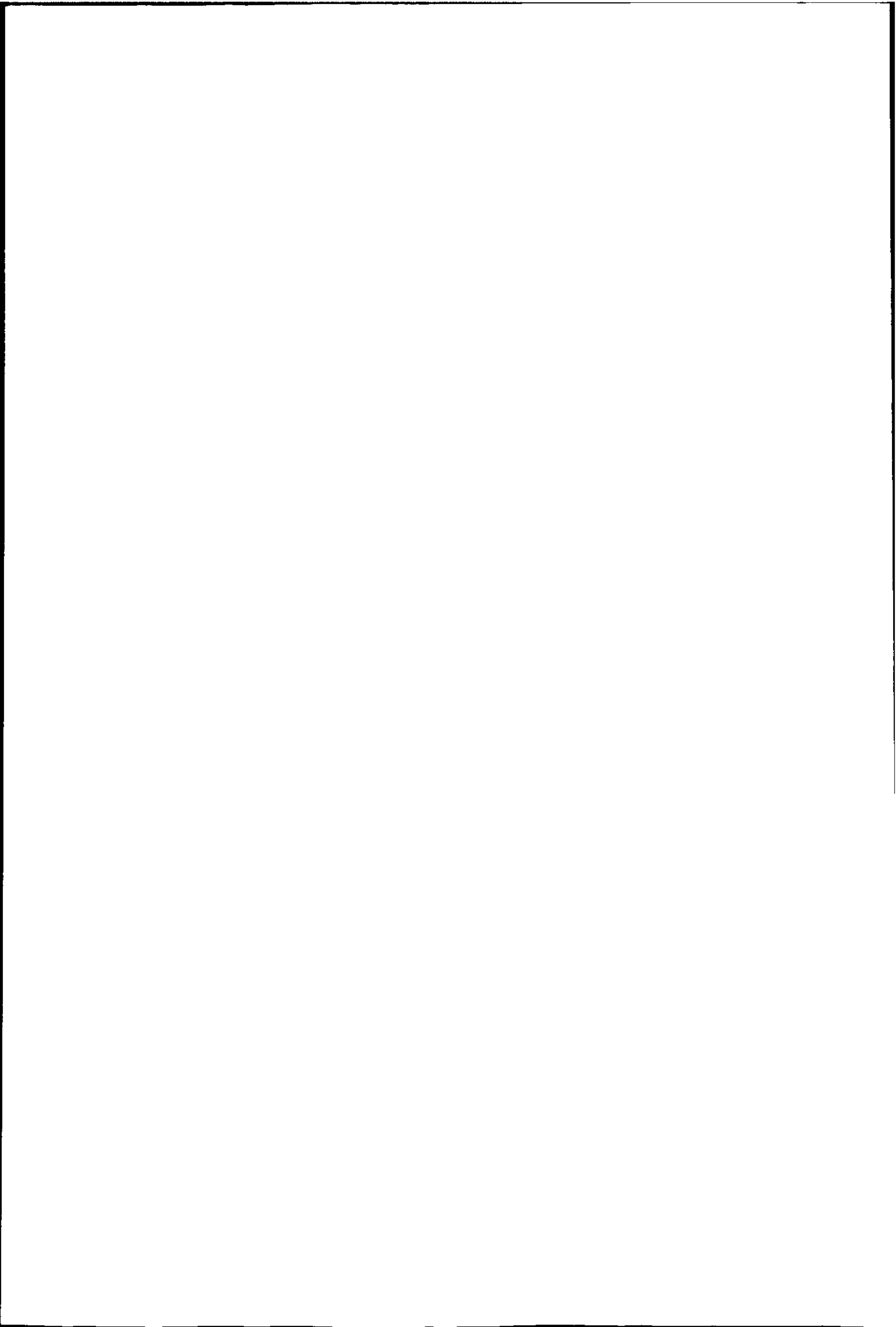
3.1. Pyrene and 1-OH pyrene standards

FF analysis of pyrene standards with an excitation wavelength of 243 nm produced a predominant peak at Em372–373 nm, with an additional smaller peak at Em392 nm. Ex345 nm produced no peaks. SFS with a $\Delta\lambda$ of 37 nm yielded a sharp peak at Em 372 nm (Fig. 1c). FF analysis of 1-OH pyrene standards yielded three peaks, with the largest and most clearly resolved at 386–387 nm emission (see Fig. 1a). SFS ($\Delta\lambda$37 nm) of the same standards yielded a large sharp peak at the wavelength pair of Ex349–350/Em386–387 nm (Fig. 1c).

3.2. Urine samples

FF analysis of urine samples from exposed crabs (from both dose response and time response experiments) produced three clear peaks on the emission spectrum, with the largest of these being at approximately 381–383 nm (see Fig. 1a). These peaks were conspicuously absent from control samples. SFS reduced these peaks to a single emission band, with a large sharp peak around Em381–383 nm in "exposed" samples (see Fig. 1c), with controls lacking this peak. The position of the dominant emission peaks seen in FF and SFS is shifted approximately 5 nm from that of the largest peak seen in the hydroxylated standards (~Em387 nm). This has important implications for quantitation and metabolite identification, which will be discussed below. The position of the SFS peak also strongly suggests that the fluorescence signal is produced almost exclusively by pyrenes other than the parent compound.

Results are reported in terms of μg l^{-1} of 1-OH pyrene equivalents, determined by interpolation of sample fluoresence intensities (at FF Ex345/Em382 nm and SFS Em381.4 nm) from a 1-OH pyrene standard curve (using intensities at FF/SFS Em387 nm). No significant differences ($P > 0.05$) in fluorescence signal were seen between acetone control and seawater control groups, so these data were combined and are presented simply as "controls".



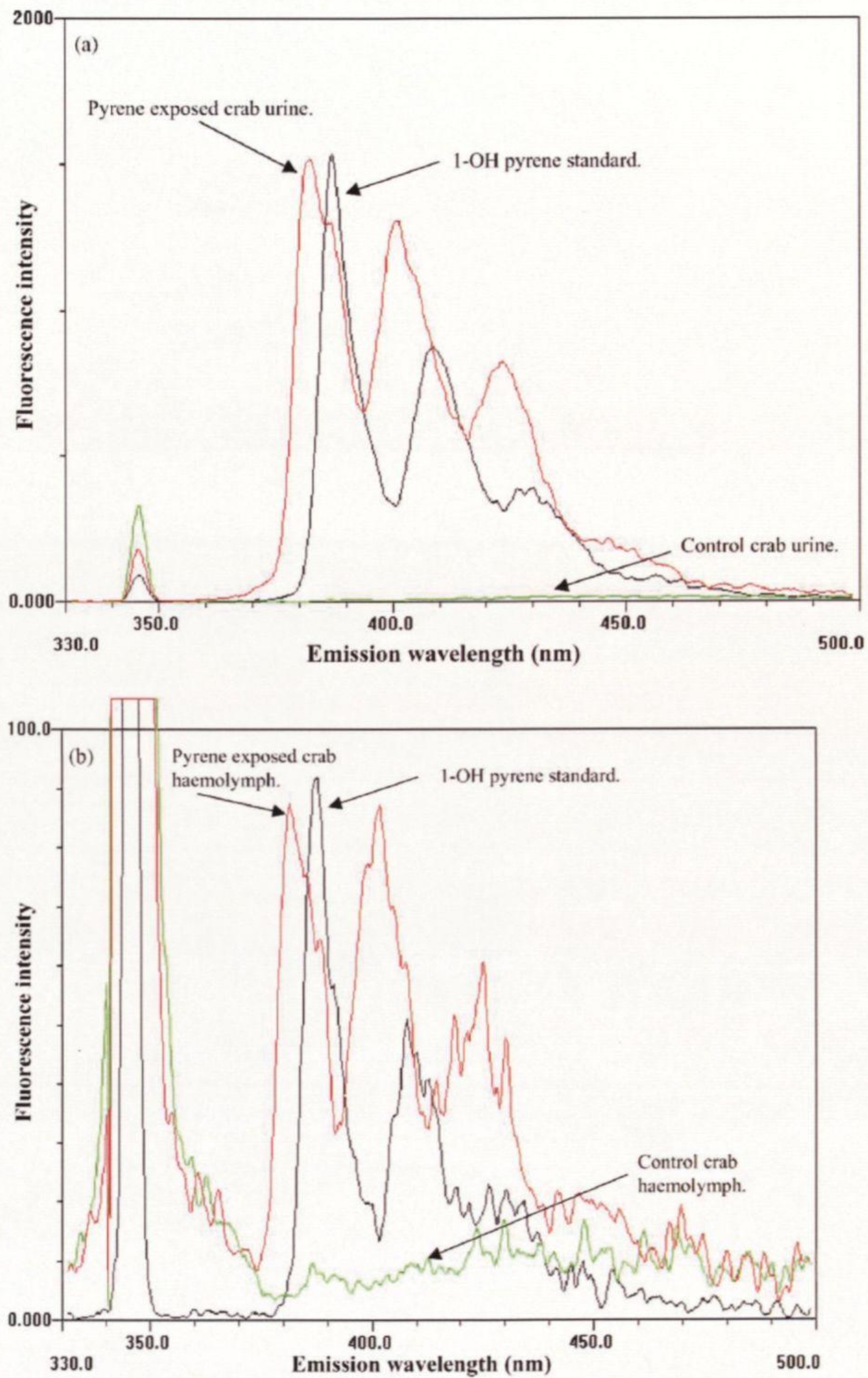
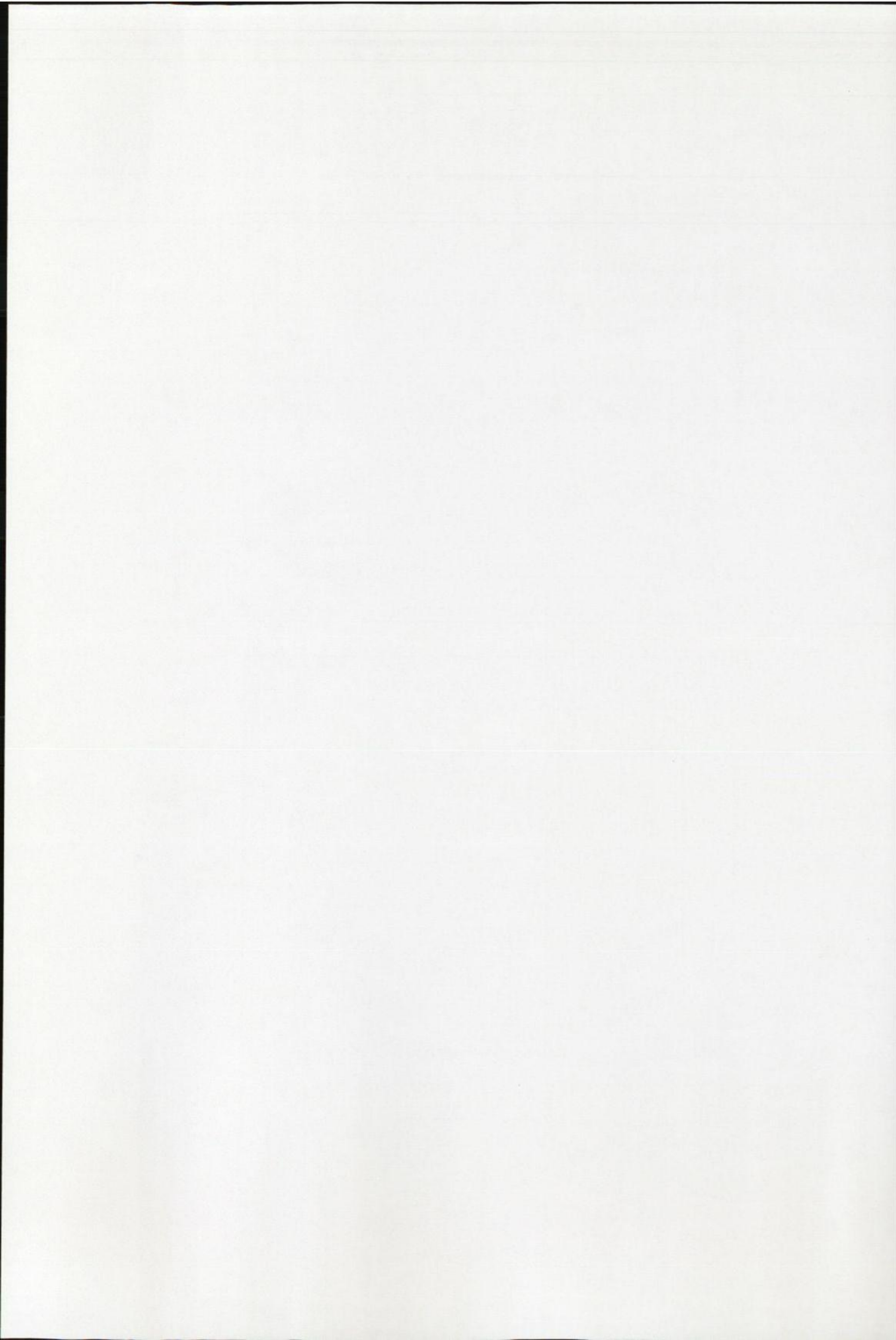


Fig. 1. (a) Fluorescence (FF, Ex345 nm) spectra from 200 $\mu g\,l^{-1}$ pyrene exposed crab urine, control crab urine and 100 $\mu g\,l^{-1}$ 1-OH pyrene standard. Urine samples diluted 1:100. (b) Fluorescence (FF, Ex345 nm) spectra from 200 $\mu g\,l^{-1}$ pyrene exposed crab haemolymph, control crab haemolymph and 5 $\mu g\,l^{-1}$ 1-OH pyrene standard. Haemolymph samples diluted 1:20.



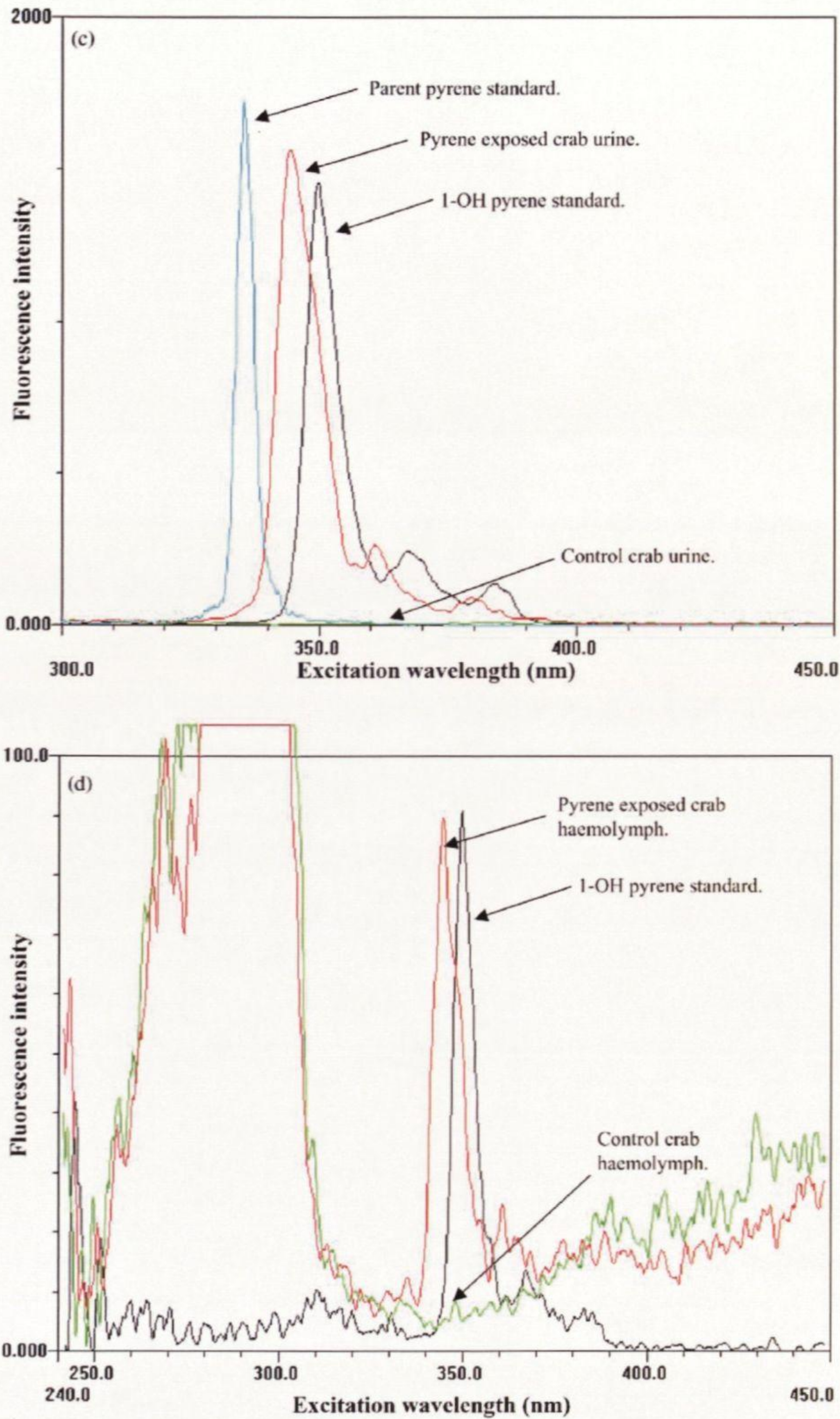
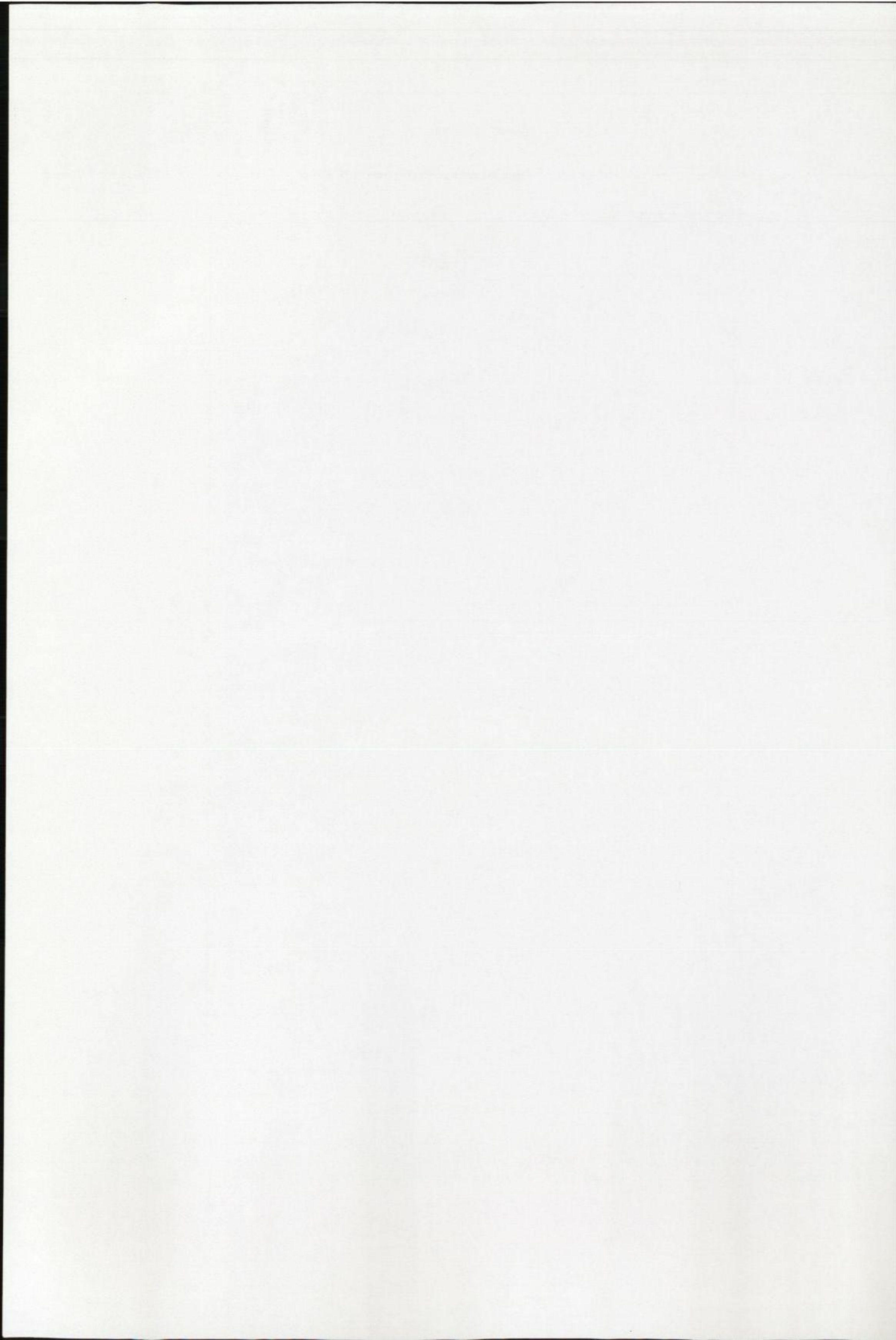


Fig. 1(c) Fluorescence (SFS, $\Delta\lambda$ 37 nm) spectra from 200 $\mu g\,l^{-1}$ pyrene exposed crab urine, control crab urine, 100 $\mu g\,l^{-1}$ 1-OH pyrene and 100 $\mu g\,l^{-1}$ pyrene standards. Urine samples diluted 1:100. (d) Fluorescence (SFS, $\Delta\lambda$ 37 nm) spectra from 200 $\mu g\,l^{-1}$ pyrene exposed crab haemolymph, control crab haemolymph and 5 $\mu g\,l^{-1}$ 1-OH pyrene standard. Haemolymph samples diluted 1:20.



3.3. Exposure–response experiment

Untransformed data are presented in Fig. 2a and b; Table 1. A moderately strong exposure–response relationship can be seen with median and mean levels of urinary 1-OH pyrene equivalents increasing with waterborne pyrene concentration (FF, $r^2 = 68.23\%$, correlation coefficient $= 0.826$, SFS, $r^2 = 66.12$, cc $= 0.813$) (Fig. 2a and b). Mean values determined by SFS are marginally higher than those determined by FF. There was a statistically significant difference (KW, $P < 0.05$) between the medians at each treatment, whether determined by FF or SFS, with the notable exception of the medians from the 100 and 200 $\mu g\,l^{-1}$ treatment groups (SFS). Analysis of variance of log-transformed data revealed statistically significant differences in mean fluorescence between treatments at the 95% confidence level ($^{***}P = 0.00$).

Baseline fluorescence at Ex345/Em382 nm and Em381.4 nm is reported for controls (as these samples lack a peak at the wavelengths), and was very low compared to exposed samples. The control level of fluorescence at these wavelengths was comparable to that seen in the solvent blank (data not presented).

The data from both methods showed considerable inter-individual variability. Variability increased with exposure and was most marked in the 100 and 200 $\mu g\,l^{-1}$ treatment groups.

3.4. Haemolymph samples

Results are reported in terms of $\mu g\,l^{-1}$ of 1-OH pyrene equivalents, determined as above. There were no significant differences (KW, $P > 0.05$) in fluorescence signal between acetone control and seawater control groups, so these data were combined and are presented simply as "controls".

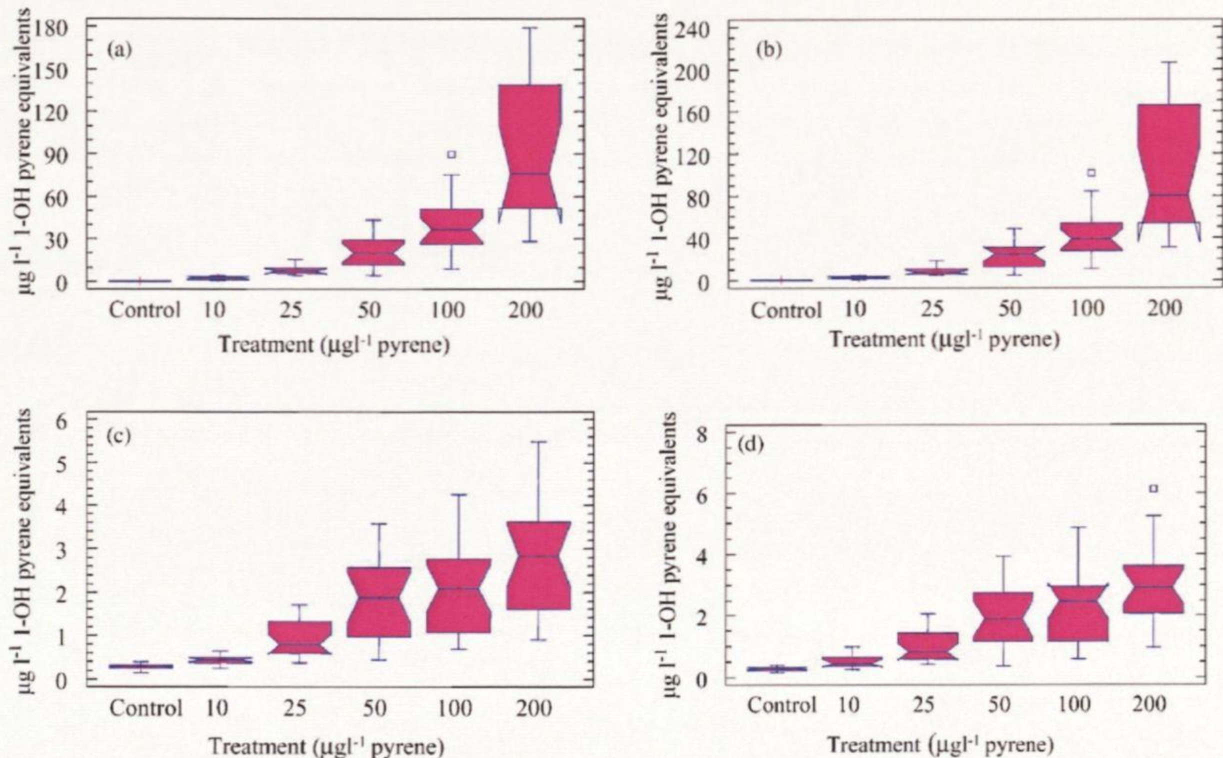


Fig. 2. (a) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C. maenas*, determined by FF. (b) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C. maenas*, determined by SFS. (c) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C. maenas*, determined by FF. (d) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C. maenas*, determined by SFS.

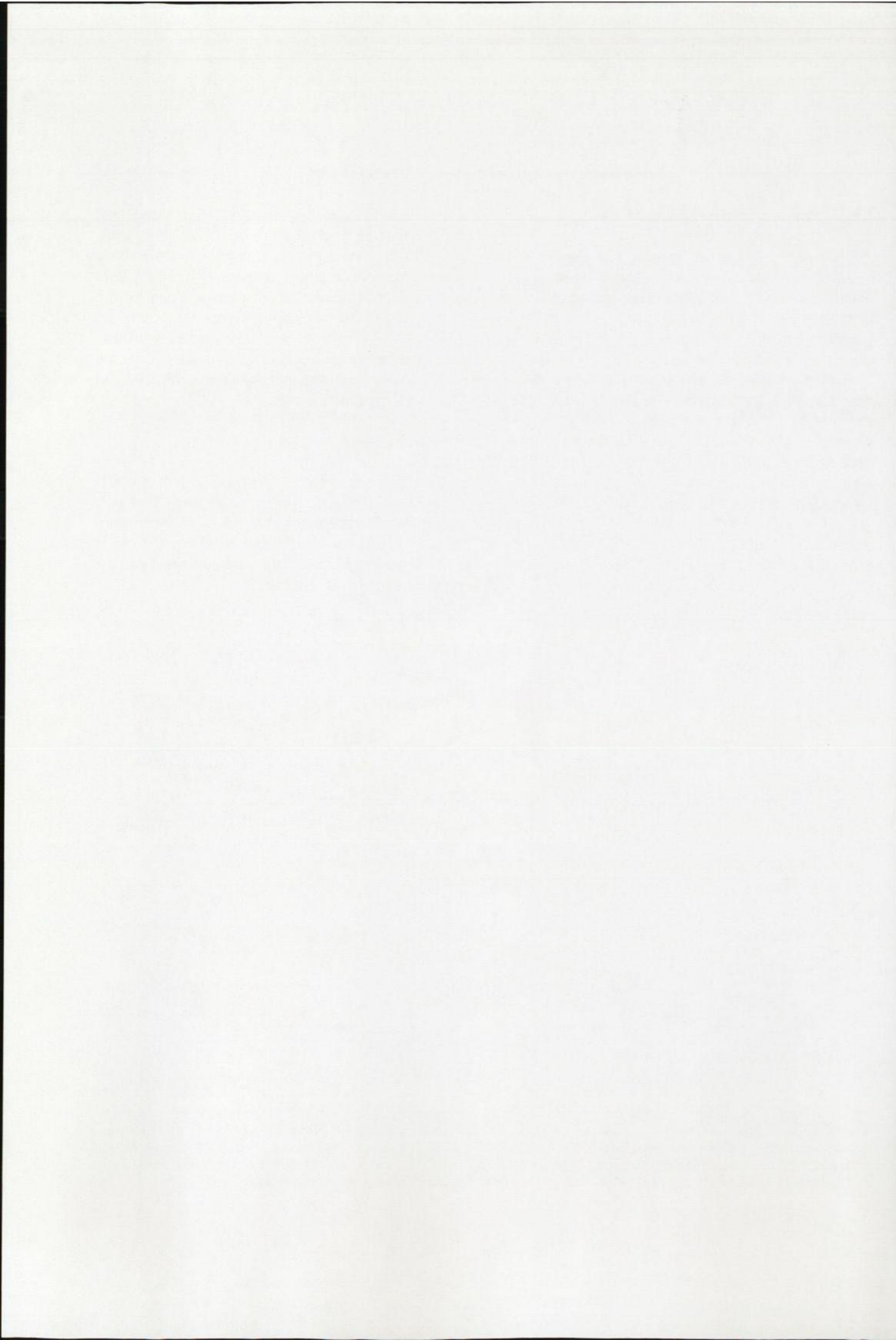


Table 1
Mean concentrations of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C. maenas*, determined by FF and SFS

Treatment ($\mu g\,l^{-1}$ pyrene)	$\mu g\,l^{-1}$ 1-OH pyrene equivalents (FF @ Ex345/Em382 nm)	$\mu g\,l^{-1}$ 1-OH pyrene equivalents (SFS @ Em381.4 nm)
200	89.77 ± 47.40 (15)	100.44 ± 55.69 (15)
100	40.58 ± 20.70 (19)	43.75 ± 23.49 (19)
50	21.50 ± 11.66 (16)	24.27 ± 13.15 (16)
25	8.10 ± 3.21 (15)	8.93 ± 4.01 (15)
10	2.59 ± 1.00 (12)	2.92 ± 1.22 (12)
Controls	0.38 ± 0.21 (20)	0.44 ± 0.26 (20)

Data are mean ± S.D. Numbers in parentheses indicate the number of samples analysed.

3.5. Exposure–response experiment

Untransformed data are presented in Fig. 2c and d; Table 2. FF and SFS analysis of haemolymph samples produced peaks in the same positions as those seen in the urine samples (Fig. 1b and d) but with greatly reduced intensities (see Table 2). Median and mean levels of 1-OH pyrene equivalents, determined by both FF and SFS, increase in a concentration dependent manner with waterborne pyrene concentration (FF, $r^2 = 49.79\%$, cc = 0.706 and SFS, $r^2 = 48.64$, cc = 0.697) (Fig. 2c and d). Mean values determined by SFS were marginally higher than those determined by FF.

FF and SFS data showed large variability, which increased with exposure concentration and was most pronounced in the 50, 100 and 200 $\mu g\,l^{-1}$ groups. This variability was greater than that seen in the urine samples from the same crabs. Despite log-transformation, the standard deviations of the FF and SFS data at each treatment differed significantly at the 95% confidence level ($^*P < 0.05$), excluding an analysis of variance. Kruskal–Wallis analysis of FF and SFS data revealed a statistically significant difference ($^{***}P = 0.00$) amongst the medians at each treatment. Medians from the control, 10 and 25 $\mu g\,l^{-1}$ groups differed significantly from each other and from the medians of the remaining treatment groups. The box and whisker plots show however, that there was no statistically significant difference between the 50, 100 and 200 $\mu g\,l^{-1}$ groups ($P > 0.05$).

3.6. Time response experiment

The time response data determined by both FF and SFS (Fig. 3a and b) showed a clear pattern of uptake and elimination of pyrene in *C. maenas*. Levels of equivalents in the urine of 200 and 400 $\mu g\,l^{-1}$ exposed crabs increased to reach a maximum after 2 and 3–4 days, respectively, and dropped steadily over the remainder of the exposure period. The maximum mean level seen in the 400 $\mu g\,l^{-1}$ exposed crab urine was approximately twice the level in the 200 $\mu g\,l^{-1}$ exposed crabs. Fluorescence at 1-OH pyrene equivalent wavelengths in control crab urine was not detectable above the background baseline for the entire experimental period. After 10 days, levels of equivalents in exposed crabs had decreased to levels similar to those after 12 h exposure, but was still significantly different to controls (KW, $^*P < 0.05$).

Table 2
Mean concentrations of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C. maenas*, determined by FF and SFS

Treatment ($\mu g\,l^{-1}$ pyrene)	$\mu g\,l^{-1}$ 1-OH pyrene equivalents (FF @ Ex345/Em382 nm)	$\mu g\,l^{-1}$ 1-OH pyrene equivalents (SFS @ Em381.4 nm)
200	2.79 ± 1.25 (19)	2.93 ± 1.32 (19)
100	2.08 ± 1.02 (23)	2.24 ± 1.15 (23)
50	1.93 ± 0.99 (17)	2.00 ± 1.01 (17)
25	0.97 ± 0.43 (24)	1.95 ± 0.52 (24)
10	0.44 ± 0.11 (13)	0.51 ± 0.21 (13)
Controls	0.29 ± 0.06 (26)	0.29 ± 0.06 (26)

Data are mean ± S.D. Numbers in parentheses indicate the number of samples analysed.

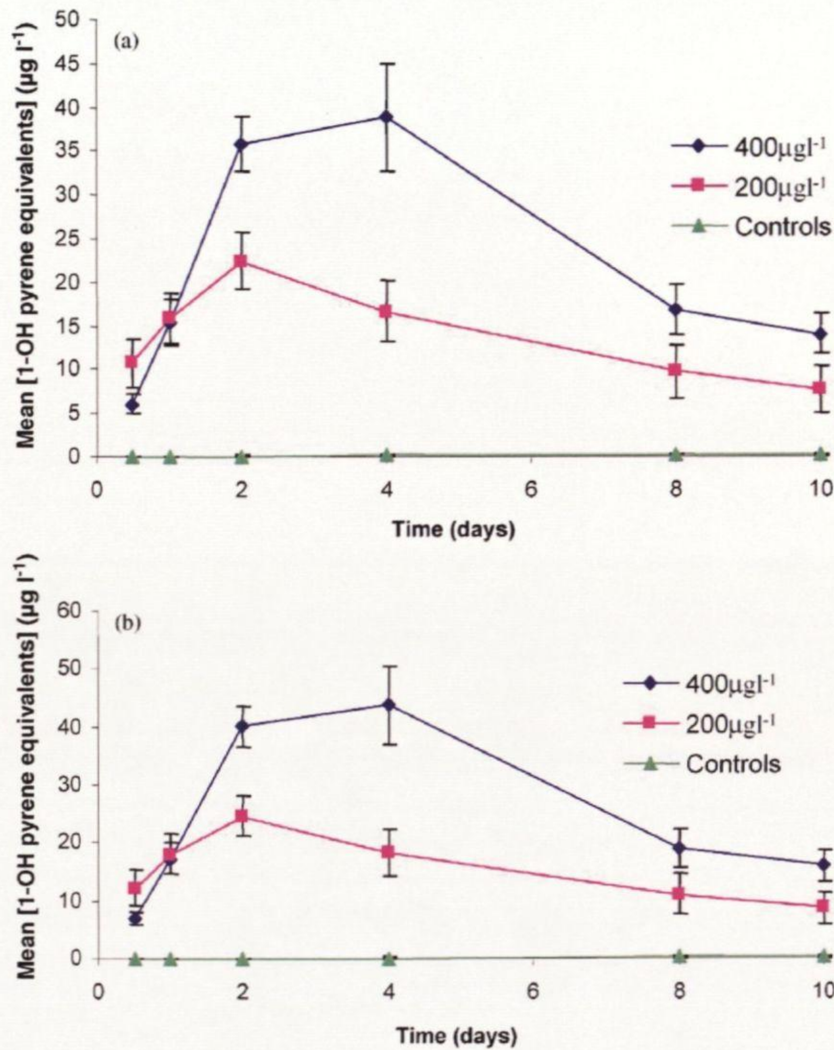


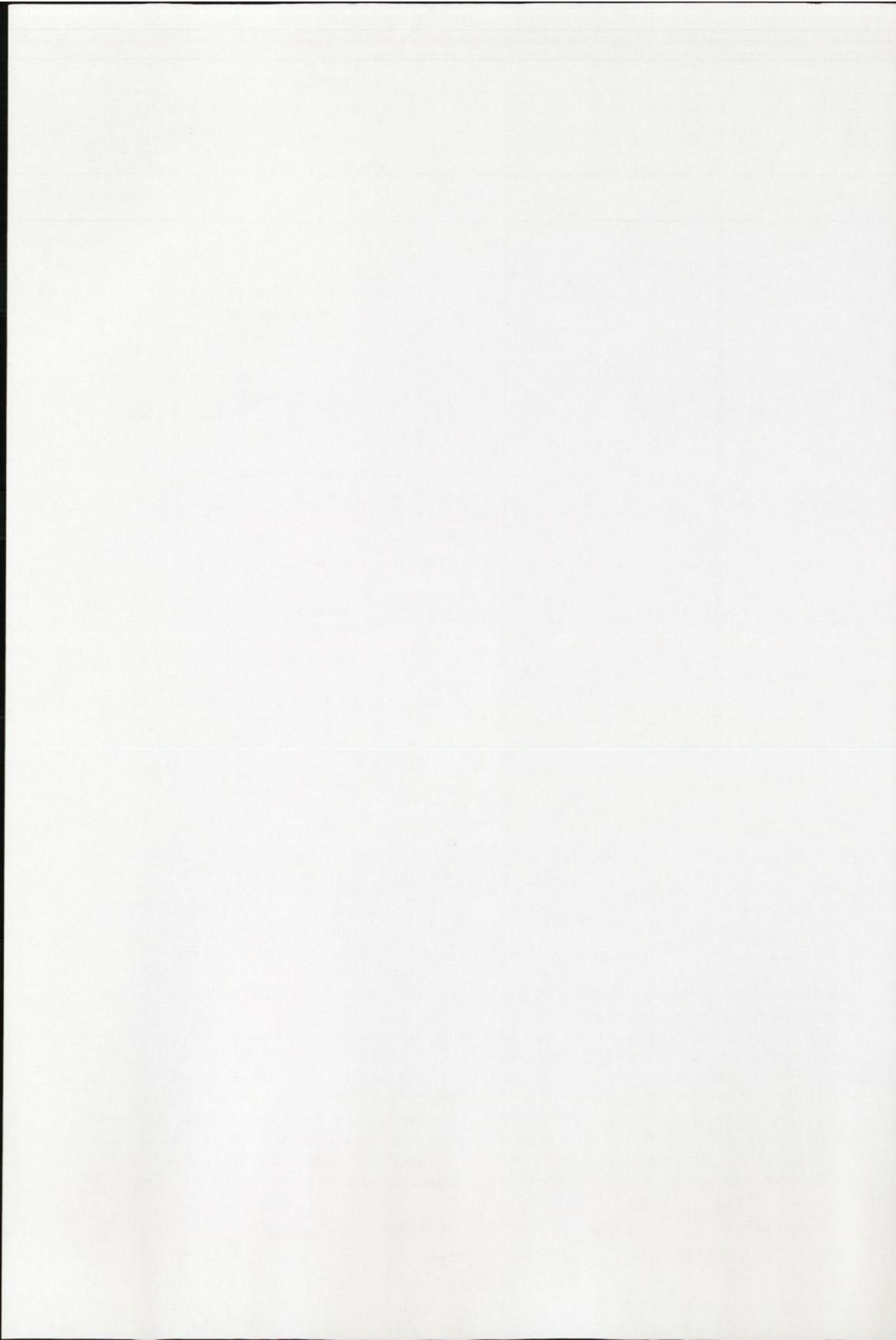
Fig. 3. (a) Temporal response of 1-OH pyrene equivalent levels in the urine (diluted 1:100) of pyrene exposed *C. maenas*, determined by FF. Values are mean ± S.E. 400 $\mu g\,l^{-1}$, $n = 7$; 200 $\mu g\,l^{-1}$, $n = 6$–7; controls $n = 3$–8. (b) Temporal response of 1-OH pyrene equivalent levels in the urine (diluted 1:100) of pyrene exposed *C. maenas*, determined by SFS. Values are mean ± S.E. 400 $\mu g\,l^{-1}$, $n = 7$; 200 $\mu g\,l^{-1}$, $n = 6$–7; controls $n = 3$–8.

3.7. GC/MS analysis of water samples

Recoveries were $95 \pm 3\%$ ($n = 18$). Measured concentrations confirmed that nominal values were within ±12%. Results from tanks with and without crabs are presented in Fig. 4, revealing that whilst pyrene was lost from water without crabs (most likely through photo-oxidation and adsorption processes), its levels decreased much more rapidly in the water from tanks with crabs. For example, after 2 h, an average of 8% of the nominal concentrations of parent pyrene was lost from seawater alone, whilst 34% was lost from seawater being filtered over the gills of crabs. After 6 h of exposure, these losses increased to 37 and 52% of the initial concentration, respectively.

3.8. Inner filter effects and matrix effects

A linear relationship between fluorescence intensity and sample concentration (dilution factor) indicates there is no significant inner filter effect, permitting any measurements to be used for quantification (UNEP/IOC/IAEA, 1992). This result also signified that fluorescence from the compounds of



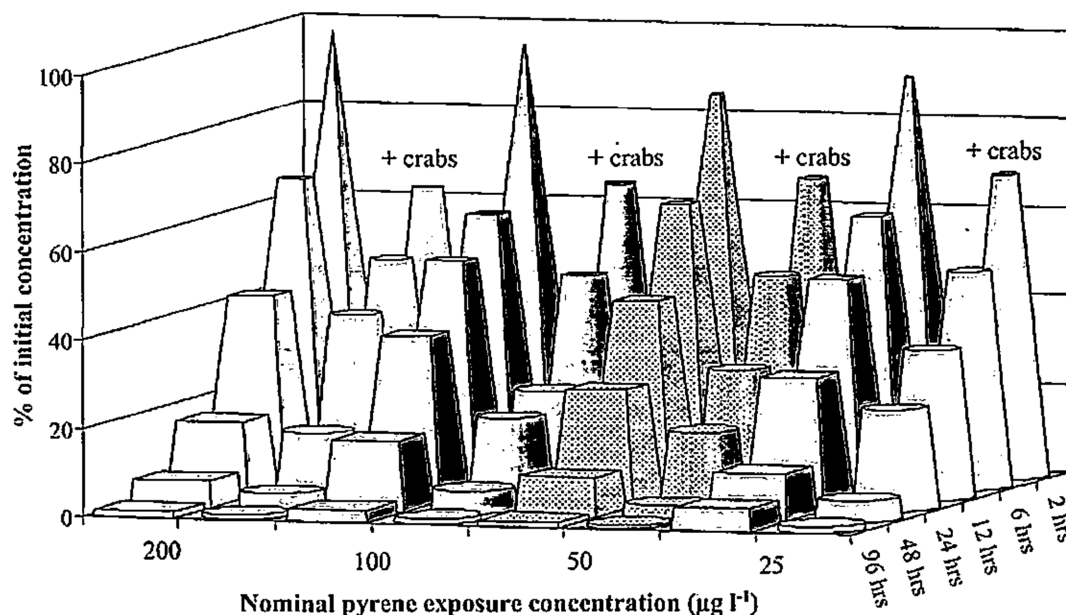


Fig. 4. Levels of pyrene in exposure water over the experimental period, as a percentage of the initial concentration (y-axis). Circular and square columns indicate levels of pyrene in tanks with and without crabs, respectively.

interest increases in a linear fashion with their concentration, allowing values for undiluted samples to be extrapolated from diluted values.

In each spiked sample, the peak observed was that of the 1-OH standard (~Em387 nm), with no shift to ~Em382 nm. The urinary matrix therefore does not alter the fluorescence characteristics of 1-OH pyrene. This suggests the peak seen in exposed crab urine is not the free-hydroxylated metabolite but is due to a compound or compounds with subtly different fluoresecent characteristics.

3.9. HPLC/F identification of urinary metabolites

HPLC/F analysis revealed conjugation of 1-hydroxypyrene into three major metabolites. Two of the conjugates were identified as pyrene-1-glucoside (elution time 28.0 min) and pyrene-1-sulphate (26.3 min). The relative distributions of conjugates show that pyrene-1-glucoside and an as yet unidentified pyrene-1-"conjugate" (25.3 min) dominated. Comparing the relative amounts of 1-OH-pyrene and its conjugates, it was found that an average of 38.9% is present as pyrene-1-glucoside, 9.7% as pyrene-1-sulphate, 47.7% as the unknown conjugate and 3.7% as non-conjugated 1-hydroxy-pyrene (39.1 min). The unknown pyrene-1-"conjugate" is not a glucuronide conjugate nor (derived from) a glutathione conjugate (M. Howsam, personal communication). Parent pyrene (47.7 min) was shown to be negligible in exposed samples. Results can be found in full in Fillmann et al. (2003).

3.10. Comparison of fluorescence results with other analytical techniques

Comparison of urinary equivalent levels determined by HPLC/F with those of direct fluorescence showed a strong correlation ($r^2 = 0.91$) (Fillmann et al., 2003). Results obtained from the ELISA also correlated well with fluorescence results ($r^2 = 0.88$) (Fillmann et al., 2002).

4. Discussion

The aim of the present study was to develop and evaluate a rapid, simple and inexpensive technique to measure PAH exposure in decapod crustacea. Results have shown that direct fluorometric detection of PAH metabolites in biological fluids of the shore crab (*C. maenas*) is a reliable means of doing so. Compelling

evidence is presented that exposure of crabs to pyrene can be assessed by measuring pyrene "equivalents" in their urine and to a lesser extent, their haemolymph. Urine and haemolymph from control crabs was conspicuously free of peaks at Em381–383 nm. Parent pyrene was not detected in the urine with the present method, providing evidence that biotransformation of pyrene has occurred. Fluorescence spectrophotometry has proven to be a valuable method for establishing proof of exposure and metabolism, and has potential for use in environmental assessments of hydrocarbon impacts. Comparison of fluorometric data with that from HPLC/F and immunochemical analysis of the same samples shows good agreement, reinforcing the use of the fluorometric screen as a biomonitoring tool. Immunoassay techniques can also be applied in conjunction with fluorimetry to rapidly screen crustaceans for PAH exposure (Fillmann et al., 2002). The presence of urinary metabolites not only provides evidence of exposure but also highlights the potential for deleterious effects, as biotransformation products are often more toxic and bioactive than their parent compounds.

Urinary levels of PAH equivalents were exposure concentration-dependent, a finding which may help in prioritising contaminated sites according to PAH exposure and for detecting gradients of pollution in estuaries and coastal ecosystems. In a similar study on fish bile, Aas et al. (2000a) state that "a prerequisite for application of the FF technique for monitoring purposes is the establishment of a reasonable dose–response relationship". A simple regression analysis of mean urinary levels over the range of exposure concentrations produces a correlation coefficient of 0.83, indicating a moderately strong relationship. The data presented here therefore fulfil the above requirement. A field exposure level equivalent to the $10\,\mu g\,l^{-1}$ exposure concentration in the present study should be considered the minimum detectable by this method, since pyrene equivalent peaks become obscured by the baseline fluorescence below this level. Levels of pyrene equivalents in the haemolymph are also exposure-dependent, although this relationship is not as strong as in urine. The haemolymph fluorescence intensities are, on average, much lower than those in urine and the variability of the data much greater, with the three highest treatment groups showing no significant differences ($P > 0.05$). Additionally, as a result of ultrafiltration in the antennal gland, urine is free from high molecular weight compounds which may interfere with the analysis. It needs no extraction or clean-up before analysis and the only preparation required is dissolution in the appropriate solvent (ethanol). Urine is therefore a much more suitable biological medium to analyse than haemolymph, when available.

The time response data showed urinary levels of pyrene equivalents reaching a maximum after 48–96 h in exposed crabs and steadily declining for a further 6 days to levels similar to those at 12 h. Similar kinetics are seen in fish (Aas et al., 2000a; Camus et al., 1998) and various crustaceans (James et al., 1995; Solbakken et al., 1980; Lee et al., 1976; Sundt and Goksoyr, 1998). Time dependent changes in urinary levels of PAH equivalents may prove useful for monitoring episodic pollution incidents, e.g. oil spills (Johnston and Baumann, 1989), in the same way that concentrations of PAH equivalents in fish bile indicate levels of exposure at the time of sampling.

Several authors have identified metabolites of various PAHs in the biological fluids of aquatic species (fish, crustaceans) following exposure, and of the phase I biotransformation products, the phenolic (monohydroxy) and dihydrodiol derivatives appear to be the dominant class of metabolites identified (Lee et al., 1976; Solbakken et al., 1980; Pritchard and Bend, 1991; Law et al., 1994; Sundt and Goksoyr, 1998). Phase II conjugation of PAH in aquatic species is also extensive (James, 1987). The majority of metabolites detected in the bile and urine of fish exposed to PAH are found in their conjugated forms (Ariese et al., 1993; Aas and Klungsoyr, 1998; Aas et al., 1998) with sulphates and glucuronides being the most common type (Law et al., 1994; Solbakken et al., 1980). Despite occurring at a slower rate and to a lesser extent than in fish species, conjugation of PAH metabolites does occur in a wide variety of crustaceans including the edible crab, *Cancer pagurus* (Sundt and Goksoyr, 1998), the lobster *Homarus americanus* (Li and James, 1993) and *C. maenas* (McElroy and Colarusso, 1987).

Other authors report that conjugation of 1-OH pyrene is highly likely in *C. maenas* and that glucosides or sulphates are the most likely candidates (M.O. James, personal communication). Ariese et al. (1993) report the emission spectrum of conjugated 1-OH pyrene is blue shifted by 5 nm and more

intense, which accounts for the 5 nm peak shift seen in exposed urine samples and supports the above assumptions.

The spiking experiment revealed that the urinary matrix is not responsible for any peak shift, suggesting that the peak seen in urinary spectra is distinct from the free hydroxylated peak. Final confirmation of this was provided by the HPLC analysis, which showed that *C. maenas* is capable of conjugating the intermediate phase 1 metabolite 1-hydroxypyrene into three major metabolites (pyrene 1-glucoside, pyrene 1-sulphate and an "unknown"). These findings concur with suggestions made above regarding phase II biotransformation of the 1-OH pyrene intermediate. It is now clear that these compounds contribute the bulk of the observed fluorescence at ~Em382 nm on the urinary spectra. Since non-conjugated 1-OH pyrene only accounts for 3.7% of the equivalents eliminated in the urine, it is clear that the conjugation rate is high, at least in this excretory pathway and accounts for the lack of a distinct peak at Em387 nm. The ratio of glucoside to sulphate conjugate illustrates that glucosidation is more extensive than sulphation. The metabolite profile suggests glucuronidation is not occurring to any significant degree. Whilst glucuronides are common phase II metabolites in fish (Ariese et al., 1993; Aas et al., 1998; Law et al., 1994; Solbakken et al., 1980), they are seemingly absent in crustaceans. Concurrent with fluorometric results, levels of parent pyrene are negligible and suggest complete biotransformation of pyrene through this excretory route.

It should be noted however, that identification of specific metabolites, while useful, is not a necessary requirement for PAH exposure monitoring. Direct fluorometric (FF and SFS) techniques are perfectly adequate for discriminating between organisms exposed to PAHs and those that are not exposed, as demonstrated by the present study and many others using fish bile. Certain limitations associated with these techniques must be considered, however. As both FF and SFS lack any kind of separation, it possible that the fluorescent signals observed at specific wavelength pairs or $\Delta\lambda$'s are made up of contributions from a range of PAHs, even if these wavelengths are not optimal for the individual compound (Aas et al., 2000a; Camus et al., 1998; Lin et al., 1996; Vahakangas et al., 1985). Also, direct fluorimetry cannot accurately discriminate between compounds with overlapping excitation and emission spectra (Lin et al., 1994). Whilst this phenomenon is not problematic for single contaminant exposures, it is potentially important in field trials, where organisms are exposed to a multitude of contaminants, including numerous PAHs. It is also possible that certain compounds, produced by normal metabolic processes and excreted in the urine, might also fluoresce at the wavelengths used. Hellou and Upshall (1995) suggest that biogenic compounds such as cholesterol, progesterone and tesosterone can contribute fluorescence which can interfere with the PAH signal. However, the likelihood of such compounds doing so in crustaceans is small.

For the purposes of rapid environmental screening, direct fluorimetry analyses of crustacean urine can determine PAH exposure with sufficient resolution to aid decision-making. The technique can categorise crab populations in terms of their level of exposure to certain classes of PAH. Validation of fluorometric data using HPLC/F and ELISA, in the present work and in accompanying studies (Fillmann et al., 2002, 2003), greatly strengthens the case for this rapid technique to be used for assessing PAH exposure in crustacea.

It is worth noting here that the present study has dealt strictly with exposure following an acute dose of waterborne pyrene. Whilst analysis of urine has established proof of exposure via this route, waterborne levels of PAH from even severely impacted sites are often very low, given their affinity to bind to sediments or suspended organic material (Onuska, 1989; Burns et al., 1990; Lin et al., 1994; Woodhead et al., 1999). Ingestion of sediments and suspended particles containing adsorbed PAH, or of PAH contaminated prey, can result in PAH being absorbed from the stomach and intestine (James et al., 1995). This route is especially important in epi-benthic omnivores such as lobsters and crabs. Therefore, determination of urinary/haemolymph PAH equivalents following dietary and/or sediment exposure to parent compounds is desirable.

The urine and haemolymph data from the dose response experiment showed considerable inter-individual variability, particularly at the higher exposure concentrations. Variations in the rate and degree to which individual crabs take up, metabolise and excrete waterborne pyrene may explain this. Variation in rate and degree of metabolism will be largely due to the level of induction and efficacy of

the appropriate p450 enzymes and subtle differences in moult stage between crabs. Large inter-individual variation in PAH metabolite levels has been observed in field populations of fish, as observed by Krahn et al. (1986) and Lin et al. (1994).

Whilst the present technique can determine exposure to PAHs such as pyrene, it tells us nothing about the potential for deleterious effects such exposure might have. Biotransformation of a parent compound, such as in the present study, can result in its metabolites becoming more toxic and bioactive. Therefore, to fully understand the risks posed to organisms whose biological fluids contain detectable levels of PAH metabolites, it is necessary to combine the present technique with suitable measures of physiological, histopathological and biochemical parameters. Parameters commonly used in fish, which correlate with elevated PAH metabolites in the bile, include incidence of hepatic neoplasia (Krahn et al., 1984, 1986) and CYP1A induction (Beyer et al., 1996; Miller et al., 1999).

The primary aim of the present technique is application to field situations. To identify impacted sites by analysis of the urinary PAH profile of its crustacean populations is a step towards this method being employed as a robust technique for widespread coastal and estuarine monitoring. It is the present author's intent to employ this technique in several impacted and reference field sites to fully test its effectiveness for exposure screening.

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Environ. Sci. Technol. **2004,** *38,* 2649–2656

Urinary PAH Metabolites as Biomarkers of Exposure in Aquatic Environments

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While immunoassays have been extensively applied to evaluate environmental contamination, to date they have rarely been used for the analysis of biological fluids outside of human medicine. These media are important because pollutants such as polycyclic aromatic hydrocarbons (PAHs) and their metabolites become concentrated in tissues, body fluids, and excreta, thereby offering a measure of exposure to biologically available contaminants. Such analyses also provide a nondestructive tool for monitoring exposure. Crabs (*Carcinus maenas*) were exposed to phenanthrene and pyrene (separately) at concentrations ranging from 0 to 200 μg L^{-1}. After 48 h, urine samples were taken and analyzed by immunoassay and UV-fluorescence spectrophotometry. Urinary levels (calibrated against hydroxylated metabolites) proved to be dose dependent for both compounds, and good agreement was demonstrated between the immunoassay and the fluorescence techniques. The cross reactivity of the immunochemical technique (ELISA) for pyrene and hydroxy-metabolites was lower than for phenanthrene. HPLC analyses demonstrated that urine from the crabs exposed to pyrene contained mainly conjugate PAH metabolites whose concentrations (the sum of the four main pyrene metabolites/conjugates) showed very good agreement with the ELISA (r^2 > 0.94) and fluorescence (r^2 > 0.91) data. Environmental samples were also analyzed by ELISA and UV-fluorescence, and both techniques detected PAH (mainly petrogenic) contamination in the urine samples from a polluted harbor. These data demonstrate the potential of urine analyses by ELISA and UV-fluorescence to measure exposure of crabs to PAH.

Introduction

Biological monitoring data are often essential for exposure assessments. Polycyclic aromatic hydrocarbons (PAHs) occur ubiquitously as complex mixtures in the environment, and there is very strong evidence that some of them are carcinogenic to organisms (*1–3*). Generally, environmental exposure of organisms is assessed by monitoring their environment (sediment/soil, water, and air). Biomonitoring, however, can provide an assessment of the integrated uptake through all exposure routes. This is important since only the bioavailable chemical is likely to be assimilated/concentrated in tissues, body fluids, and excreta. Owing to the complexity of biological samples, however, intricate analytical protocols are traditionally used for analyses. Conventional biomonitoring methods are often time-consuming, labor intensive, and expensive.

Biomarkers of exposure can measure either the concentration of contaminants in body fluids or the tissues of exposed individuals (*4*). Since PAHs comprise a mixture of compounds, biomarkers can either address the whole group of PAHs or a specific/single PAH (*4*). A variety of biomarker techniques has been used to investigate PAH exposure. Specific hydroxy-PAHs (e.g., 1-OH-pyrene) in urine (*5–8*) and immunoassays using monoclonal antibodies to detect a specific DNA-adduct (*9, 10*) provide single PAH markers. ^{32}P-post-labeling assays can detect total aromatic DNA adducts, giving a measure of exposure to total PAH (*4, 11, 12*). Quantification of various PAH in urine has been suggested to be a useful biomarker, either after reversed metabolism (enzymatic hydrolysis) to parent PAH (*13*) or by determining a variety of hydroxylated PAH (*14–16*).

Immunochemical methods, such as enzyme-linked immunosorbent assay (ELISA), have been routinely used for human monitoring of exposure (*17–22*). Only recently have ELISA techniques been tested on body fluids and excreta of aquatic organisms (*23*). ELISA methods can provide a total PAH measure without the need for enzymatic hydrolysis (*23*). This allows contributions from both pyrolytic and petrogenic sources to be assessed. Compared to conventional chromatographic methods, ELISA is rapid, easier, and less expensive to use, can afford comparable limits of detection, and is easily adapted for use in the field (*19*). By integrating ELISA with urine analyses, an elegant (nondestructive) measure of exposure is afforded. Cross reactivity and kinetics of metabolism and excretion, however, require consideration and testing.

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

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10.1021/es0350839 CCC: $27.50 © 2004 American Chemical Society
Published on Web 03/30/2004

itoring (PAH RaPID Assay, Strategic Diagnostics Inc., Newtown, PA).

Experimental Procedures

Collection of Experimental Crabs and Laboratory Conditions. Green, male, intermoult crabs (carapace width 50–72 mm) of the species *Carcinus maenas* were collected on incoming tides from Jenkins Quay on the Avon estuary at Bantham (South Devon, U.K.) on three separate occasions between April and July, 2000. On return to the laboratory, they were kept in holding tanks containing aerated seawater (34 PSU, 15 ± 1 °C), under a 12 h light/12 h dark regime for a period of 1 week in order for them to depurate and acclimatize. During this period, crabs were fed twice a week with irradiated cockle, and their water was changed within 12 h of feeding. To test the technique on environmentally exposed organisms, crabs were collected from Bantham (South Devon, U.K.) and Sutton Harbor (Plymouth, U.K.). On return to the laboratory, urine was immediately sampled (see details next).

Dose–Response Exposure Experiment. The exposure experiment consisted of a static toxicity test (*24*). Parent PAH (either pyrene or phenanthrene) was added to the prefiltered (5 μm) seawater (34 PSU) to produce six nominal concentrations (200, 100, 50, 25, 10, and 0 (control) μg L^{-1}). The experiment was kept under 12 h light/12 h dark regime at 15 ± 1 °C. Crabs were not fed during the exposure period. After the experiment, crabs were transferred to clean seawater to depurate (for 3 weeks) before being returned to the environment. The experiment was run in triplicate for each parent PAH.

GC/MS Analyses of Water Samples. Concentrations of parent PAHs in the tanks were monitored using GC/MS analyses (Hewlett-Packard Model 5890 II Plus GC and a 5972 mass selective detector (MSD) (Palo Alto, CA)). Briefly, water samples (100–500 mL) were spiked with internal standards (pyrene-d_{10} and phenanthrene-d_{10}) and then concentrated using C_{18} cartridges (IST, Hengoed, U.K.), which were subsequently eluted (3 times) using 3 mL of ethyl acetate. The eluent was then concentrated down to 1 mL before analyses by GC/MS. Recoveries were 95 ± 3% (n = 18) for pyrene-d_{10} and 93 ± 5% (n = 18) for phenanthrene-d_{10}. Measured concentrations confirmed that the nominal (spiked) values were within ±12%.

Urine Sampling. Urine samples were taken from each crab after 48 h of exposure using the technique described by Bamber and Naylor (*25*). Briefly, crabs were removed from their aquaria and placed in a bucket containing clean seawater. After being drained, the third maxillipeds were moved aside and kept apart. Under a dissecting microscope (×10), the operculum of each antennal gland bladder was lifted, and urine (20–400 μL per crab) was collected. Samples were stored at −80 °C in siliconized microcentrifuge tubes until analysis. Crabs were returned to their respective aquaria immediately after sampling.

Fluorescence Analyses. Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer following an adapted method of Ariese et al. (*26*) and Aas et al. (*27*). Standards (pyrene (98%), 1-OH-pyrene (98%), phenanthrene (98%), and 9-OH-phenanthrene (96%) were obtained from Sigma-Aldrich Chemical Co, U.K.), blanks, and urine samples were diluted (20–100-fold) with 50% v/v ethanol/Milli-Q water. Fixed excitation wavelength fluorescence (FF) and synchronous excitation/emission fluorescence spectrometry (SFS) measurements were carried out with excitation and emission slit widths of 2.5 nm (pyrene) and 5.0 nm (phenanthrene). The assigned wavelength pairs were $\lambda_{ex/em}$ = 345/382 (FF) for pyrene and $\lambda_{ex/em}$ = 252/357 (FF) for phenanthrene. A $\Delta\lambda$ of 37 nm (pyrene) and 54 nm (phenanthrene) were selected for the SFS analyses. Samples were quantified against 1-OH-pyrene and 9-OH-phenanthrene standards (200, 100, 75, 50, 25, 10, and 5 μg L^{-1}). Results are reported in terms of 1-OH-pyrene or 9-OH-phenanthrene equivalents.

Immunoassay Procedure. PAH RaPID Assay (SDI, Newtown, PA) is a PAH-competitive tube format ELISA employing paramagnetic particles that are covalently coated with anti-PAH antibodies. Kits were supplied by SDI Europe (Alton, U.K.) and were used according to the manufacturers recommendations. Briefly, urine samples (50 μL aliquots) were diluted with 50% v/v methanol/ buffered solution (SDI diluent). Analyses were undertaken in triplicate with concurrent standard calibrations and blanks. Appropriate amounts of samples (or standards), antibody-coated onto paramagnetic particles, and enzyme conjugate (PAH-horseradish peroxidase) were mixed and incubated (to allowed competitive binding to the antibody). After washing twice with kit buffer (using a magnetic rack to retain the antibodies), substrate (hydrogen peroxide) and chromogen (3,3′,5,5′-tetramethylbenzidine) were added (and incubated). Stop solution (2 M sulfuric acid) was added, and the color produced was measured at 450 nm using an Optimax microplate reader (Molecular Devices, Menlo Park, CA). Sample absorbance was compared to a linear regression equation using a logarithm of the concentration versus logit B/B_o standard curve to calculate the final concentration of PAH (where B/B_o is the absorbance observed for a sample or standard divided by the absorbance at the zero standard). Sample concentrations were then corrected for dilution factors. The ELISA was calibrated using 1-OH-pyrene and 9-OH-phenanthrene for pyrene and phenanthrene exposed samples, respectively.

HPLC/Fluorescence. High-performance liquid chromatography was performed using an ion pair elution system under acidic conditions on a reversed phase C_{18} analytical column (Vydac 201TP54 column (250 × 4.6 mm), Hesperia, CA). The column temperature was maintained at 30 °C in a column oven. A gradient elution of acetonitrile (CH_3CN) and aqueous buffer (10 mM ammonium acetate, adjusted to ~pH5 with ~0.5–1.0 mL acetic acid/L buffer) was used at a flow rate of 0.5 mL min^{-1} (t = 0 min, 5% CH_3CN; t = 40 min, 90% CH_3CN; isocratic at 90% CH_3CN for 10 min). The instrument consisted of two Spectroflow 400 pumps (Applied Biosystems), a Spark-Holland PROMIS II autosampler (20 μL injection loop), a GT-103 in-line degasser (Separations, The Netherlands), and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo). Fluorescence detection was carried out at $\lambda_{ex/em}$ = 346/384 nm for pyrene metabolites (slit widths $\lambda_{ex/em}$ = 18/40 nm) [modified from ref *28*].

Samples were diluted 30-fold with ethanol (modified with 5 mg mL^{-1} ascorbic acid) and stored at −20 °C. Further dilutions were also made when necessary. Peaks were identified according to their retention times, building on the work by Stroomberg et al. (*28*), and confirmed for the system used in this study (Howsam, personal communication). Quantification of 1-OH-pyrene was performed using a dilution series of 1-OH-pyrene external standard, while the conjugates were quantified using their relative fluorescence efficiencies as compared to 1-OH-pyrene; pyrene-1-glucoside = 2.0 ± 0.31, pyrene-1-sulfate = 1.23 ± 0.09, and pyrene-1-conjugate = 1.75 ± 0.18. All conjugates are expressed as 1-OH-pyrene equivalents. The identity of the pyrene-1-conjugate is still unknown, but it is not a glucuronide conjugate nor (derived from) a glutathione conjugate (G. Stroomberg, AquaSense, Amsterdam, The Netherlands, unpublished data).

Results and Discussion

Analytical Performance. Fluorescence Spectrometry. The fluorescence spectrophotometry was calibrated against the major metabolites 1-OH-pyrene and 9-OH-phenanthrene.

TABLE 1. Specificity of Parent PAHs and Metabolites in the ELISA ($n = 3$)

	MDL[a] ($\mu g\ L^{-1}$)	50% B/B_0[b] ($\mu g\ L^{-1}$)	cross reactivity (%)
phenanthrene	0.92 ± 0.08	28.7	100
9-OH-phenanthrene	24.5 ± 2.7	655.2	4.4
pyrene	9.01 ± 0.76	280.6	10.2
1-OH-pyrene	8.82 ± 0.96	290.9	9.9

[a] MDL: method detection limit (90% B/B_0). [b] 50% B/B_0 is the concentration required to inhibit one-half of the color produced by the negative control.

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

Immunoassay. The linearity of standard calibration curves of pyrene and phenanthrene analyzed by ELISA was >0.97. The method detection limit (MDL) for the PAH RaPID Assay defined as the concentration giving 90% B/B_0 was 0.92 ± 0.08 $\mu g\ L^{-1}$ (phenanthrene), 24.5 ± 2.7 $\mu g\ L^{-1}$ (9-OH-phenanthrene), 9.01 ± 0.76 $\mu g\ L^{-1}$ (pyrene), and 8.82 ± 0.96 $\mu g\ L^{-1}$ (1-OH-pyrene) (Table 1). The coefficient of variation (%CV) for repeated analyses of a single sample was 12 ± 5% ($n = 8$), similar to variations with conventional analytical techniques. A 1:20 dilution was found to be required to minimize matrix effects (see next); thus, practical environmental MDLs are 20-fold higher than the concentrations quoted for the standards.

Table 1 shows the cross reactivity and 50% B/B_0 (IC_{50}) values (concentration required to inhibit one-half of the color produced by the negative control) determined for phenanthrene, 9-OH-phenanthrene, pyrene, and 1-OH-pyrene using PAH RaPID Assay. The ELISA proved to be highly sensitive to phenanthrene (indicated by low 50% B/B_0 values) but less so for pyrene and monohydroxy metabolites. The better performance for phenanthrene is explained because the immunoassay antibodies were raised against phenanthrene (*29*). These results indicate that the ELISA is likely to detect other PAHs (albeit with differing sensitivities) potentially affording an effective monitor of exposure to environmental PAHs.

Undiluted human urine interferes with ELISA to seriously hinder its sensitivity and dynamic range (*19, 21, 22*). To investigate matrix effects for the PAH ELISA assay, aliquots of diluted control urine (1:5 to 1:80) were analyzed by the ELISA. A representative plot of the urine samples is shown in Figure 1. Even though differences in absorbances (or B/B_0) were evident in undiluted or slightly diluted urine samples, at dilutions equal to or greater than 1:20 matrix-induced differences were minimal (Figure 1).

HPLC. Calibration linearity was good ($r^2 > 0.95$) for pyrene and 1-OH-pyrene analyzed by HPLC/fluorescence. Detection limits for all metabolites expressed as 1-OH-pyrene equivalents were 0.1 $\mu g\ L^{-1}$. Identification of the individual pyrene metabolite peaks is described in depth later in this section. To achieve results within the standard calibration range, dose−response experiment samples required dilutions of at least 1:30.

Performance of ELISA. Laboratory Exposure Experiments. Response to Exposure. To evaluate whether crabs are useful in biomonitoring PAH exposure, it is necessary to investigate the dose−response relationship. It is also important to understand the kinetics of metabolism and excretion. Time− and dose−response experiments for *C. maenas* demonstrate that urinary levels of pyrene metabolites reached a maximum after 48 h (at exposed levels of 200 $\mu g\ L^{-1}$) (Figure 2). Urinary levels proved to be dose-dependent for both phenanthrene and pyrene (*23*).

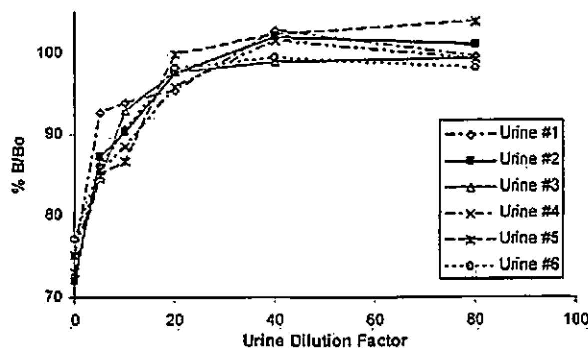


FIGURE 1. Matrix effects of crab urine on the PAH RaPID assay ELISA (B/B_0 is the absorbance observed for a sample or standard divided by the absorbance at the zero standard).

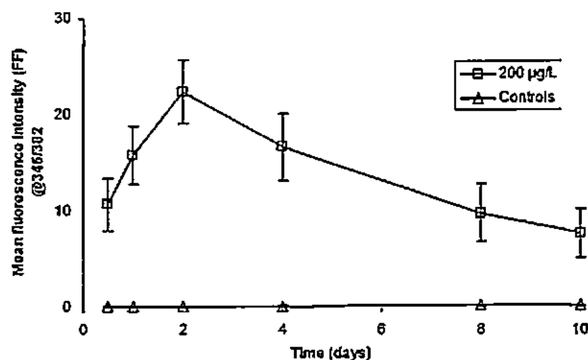
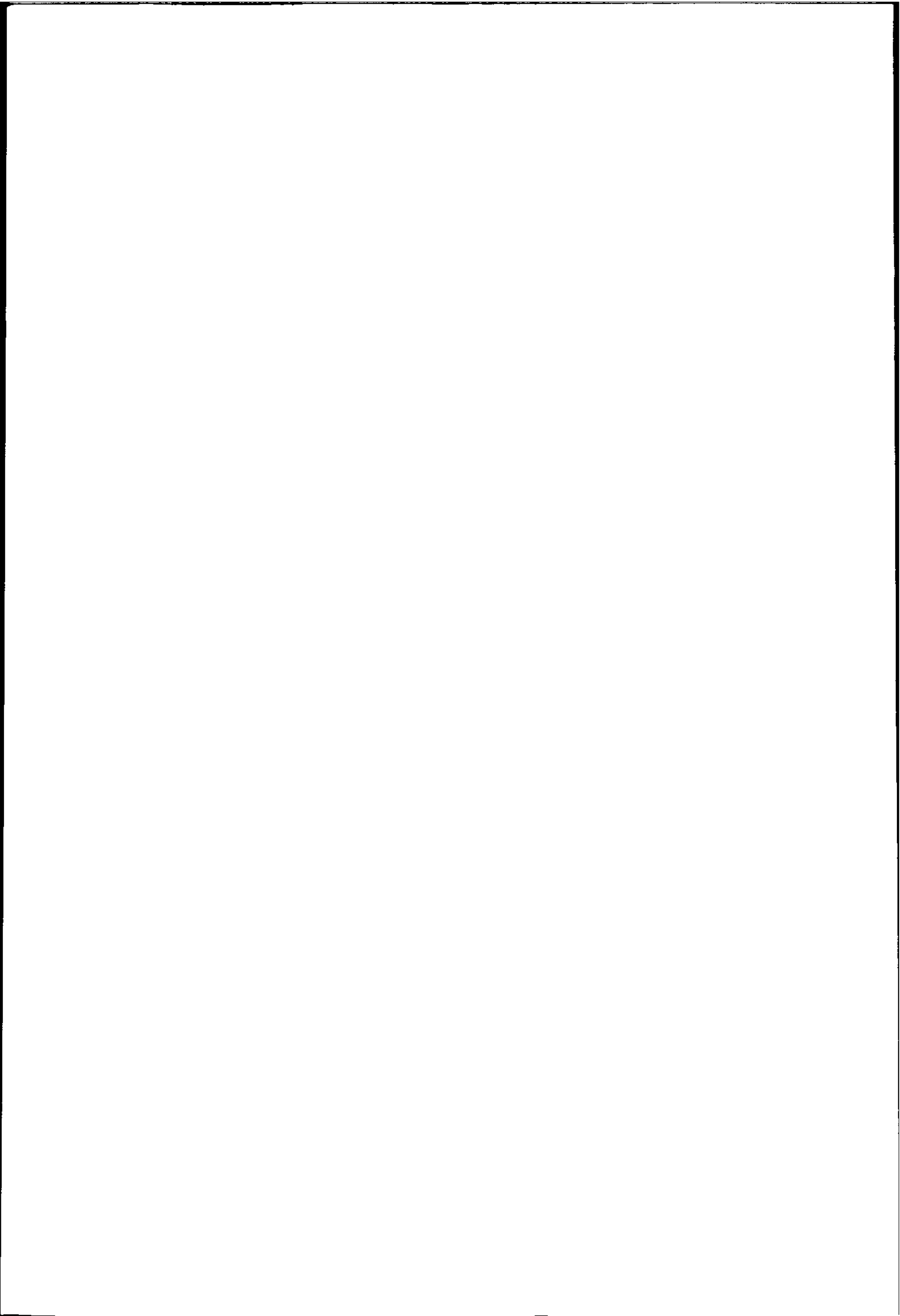


FIGURE 2. Fluorescence intensity (FF) measured in the urine of shore crabs exposed to pyrene for up to 10 days. Peak concentrations are reached between 2 and 4 days.

Fluorescence Spectra. Fixed excitation wavelength fluorescence (FF) analyses of urine samples from pyrene exposed crabs produced three distinct peaks on the emission spectrum, with the largest at approximately 382 nm (Figure 3a). In phenanthrene exposed crabs, urine analyses produced a broad fluorescence in the emission range of 350−400 nm (Figure 3b). These peaks are conspicuously absent from control samples (Figure 3a;b). Synchronous excitation/emission fluorescence spectrometry (SFS) reduced these peaks to a single emission band, with a large sharp peak around Ex340/Em382 nm in pyrene exposed samples and around Ex300/Em354 nm in phenanthrene exposed samples (not shown). Once again, controls lacked these peaks. The position of the dominant emission peaks seen in FF and SFS for pyrene exposed samples is shifted approximately 5 nm from that of the largest peak seen in the 1-OH-pyrene standard spectra (~Em387 nm) (Figure 3a). The unresolved shoulder to the main peak (exposed sample) is interesting since it indicates (together with the main peak) the presence of compounds with potentially similar properties to 1-OH-pyrene, possibly pyrene conjugates. Parent pyrene is almost certainly absent from the urine since its characteristic synchronous peak at 372 nm (wavelength difference of 37 nm) is not present in the urinary spectra of exposed crabs. Phenanthrene shows a similar pattern (Figure 3b). According to Ariese et al. (*26*), the emission spectrum of conjugated 1-OH-pyrene (e.g., pyrene-1-glucuronide) is blue shifted by



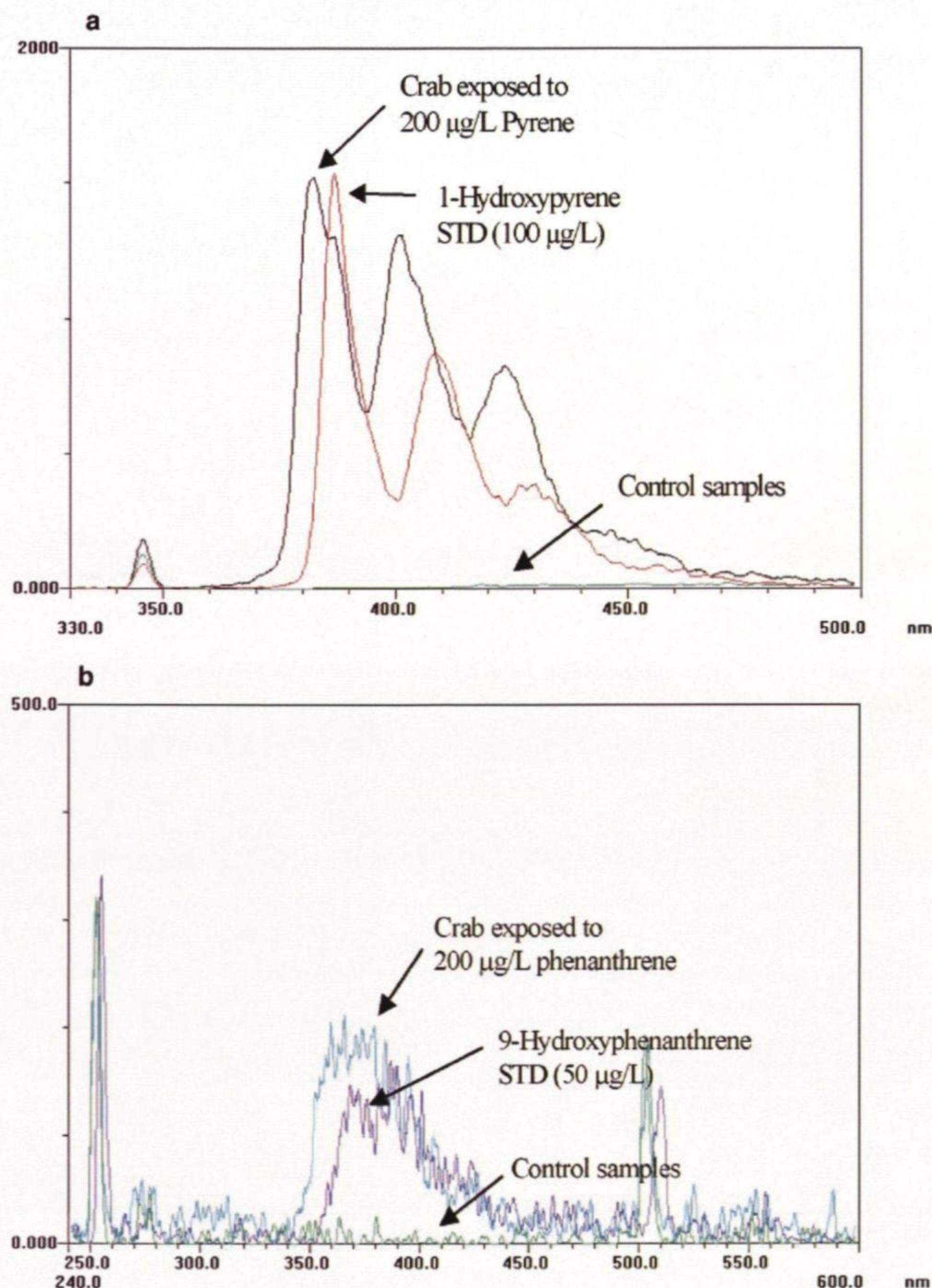
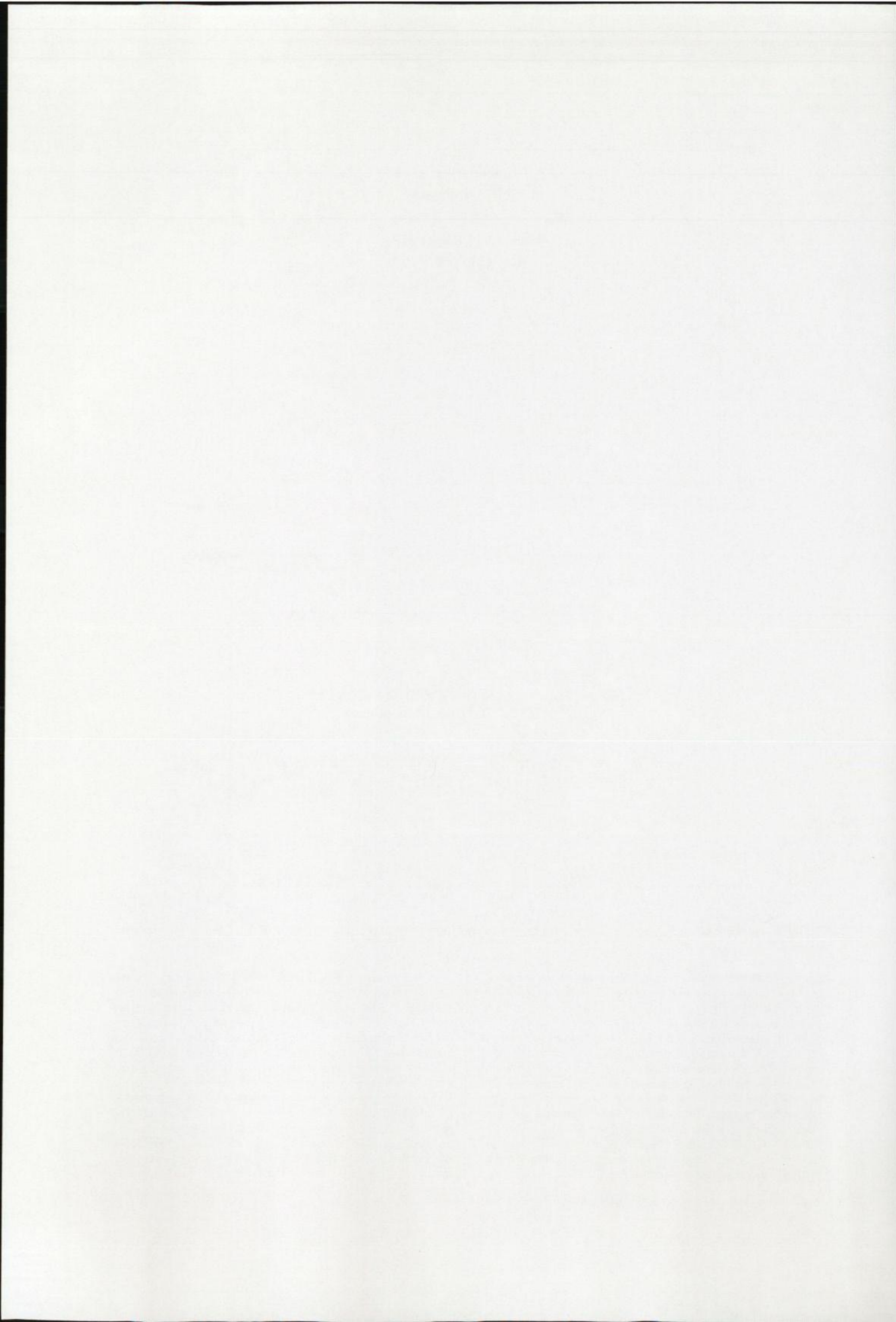


FIGURE 3. Fixed excitation wavelength fluorescence spectra of urine from crabs exposed to (a) pyrene ($FF_{345/382}$) and (b) phenanthrene ($FF_{252/357}$).

5 nm. The previous results might therefore indicate that C. maenas can transform pyrene and phenanthrene following exposure and excrete them in the urine as more water-soluble metabolites/conjugates.

From previous studies, it is known that aquatic organisms are capable of metabolising PAH. Primary and conjugated metabolites of pyrene, phenanthrene, and benzo[a]pyrene have been identified in bile and urine of vertebrates and invertebrates (26, 30–36). Marine crustacean studies have shown that hepatopancreatic and other organs microsomes contained cytochrome P450 (35, 36), which can metabolize PAH. PAH metabolism studies in shore crab (C. maenas) have shown that benzo[a]pyrene is rapidly metabolized to primary and conjugated metabolites (37, 38).

HPLC Analysis. The fluorescence data (described previously) strongly indicate that the pyrene is being metabolized. For this reason, HPLC analyses of urine samples were undertaken to investigate the metabolites. From the literature, the primary metabolite is thought to be 1-OH-pyrene, which is subsequently conjugated into phase II metabolites (28, 39, 40). Our results concur with this and indicate conjugation into three major metabolites. A typical chromatogram is shown in Figure 4. Two of the conjugates were identified as pyrene-1-glucoside and pyrene-1-sulfate (Figure 4). The identity of one other conjugate is still unknown, but Stroomberg and co-workers, who have found the same metabolite in isopods, have, using electrospray mass spectrometry, established that it has an m/z ratio of 467. Evidence



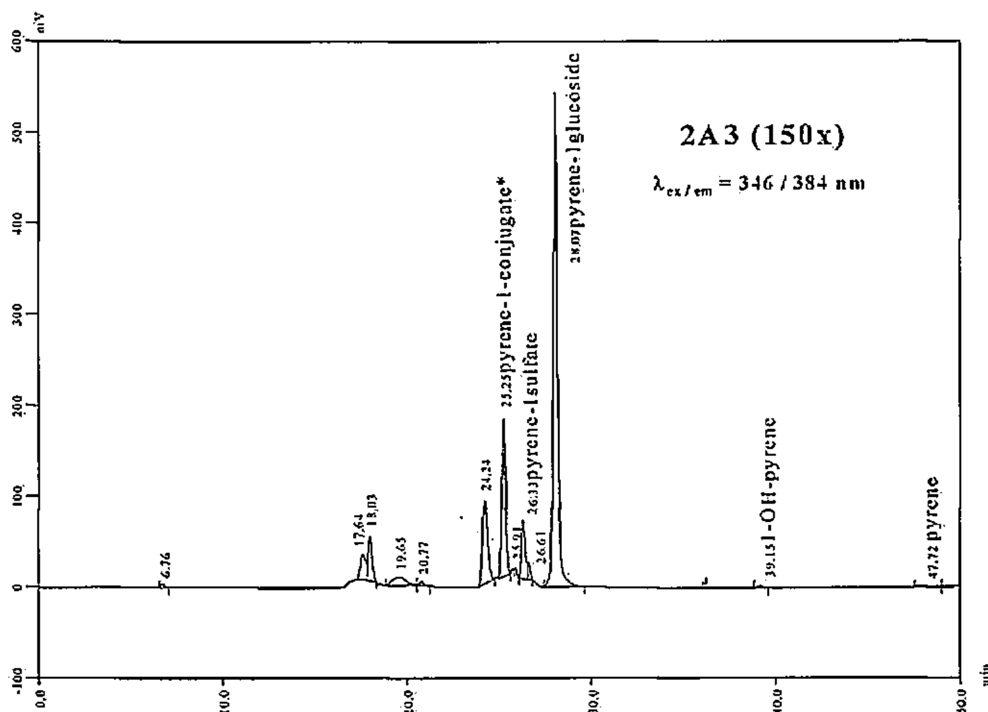


FIGURE 4. HPLC-fluorescence chromatogram ($\lambda_{ex/em}$ = 346/384 nm) of urine from crabs exposed to 200 μg L^{-1} pyrene. *Pyrene-1-conjugate has not been identified.

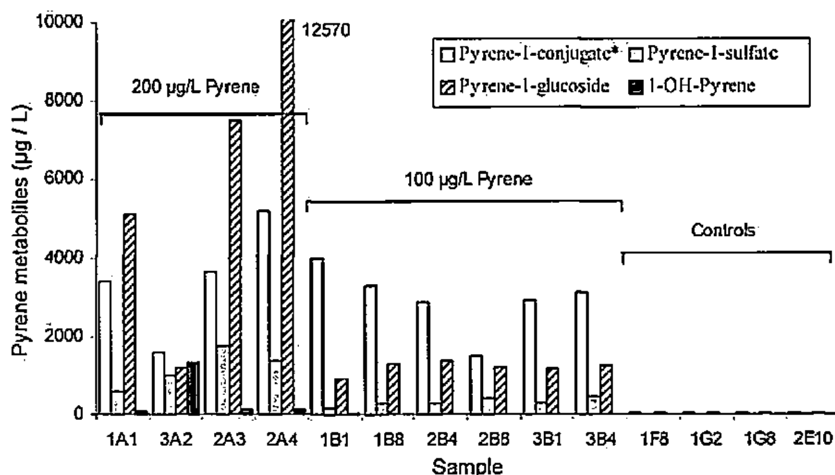


FIGURE 5. Concentrations of 1-OH-pyrene and conjugates (μg L^{-1}) in urine from crabs exposed to pyrene (200 and 100 μg L^{-1}) and control. *Pyrene-1-conjugate has not been formally identified.

of a malonate and glucoside functional groups on a pyrene moiety have also been obtained (G. Stroomberg, AquaSense, Amsterdam, The Netherlands, unpublished data). The other two peaks present in the chromatogram were not identified (~17.6/18 and 24.2 min, Figure 4), but from their fluorescence properties they are likely to include pyrene or pyrene-like structures. None of the metabolites observed coeluted with pyrene-1-glucuronide, although the peaks at 17.6/18 min were close. In vertebrates, the preferred carbohydrate co-substrate is UDP-glucuronic acid, giving rise to glucuronides. In crustaceans, however, the preferred cosubstrate is UDP-glucose, forming the glucosides (35, 41, 42). Finally, the last peak in the chromatograms was parent pyrene. Although not quantified, the pyrene peak was always negligible (Figure 4).

The relative distributions of conjugates show that pyrene-1-glucoside and pyrene-1-conjugate dominated (Figure 5). Comparing the relative amounts of 1-OH-pyrene and its conjugates, it was found that an average of 38.9% is present as pyrene-1-glucoside, 9.7% as pyrene-1-sulfate, 47.7% as the unknown conjugate, and 3.7% as nonconjugated 1-OH-pyrene. The fact that the amount of 1-OH-pyrene is rather small in comparison to the pyrene conjugates indicates a high conjugation rate of 1-OH-pyrene (28, 43). These ratios also indicate that glucosidation is more efficient than sulfation.

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

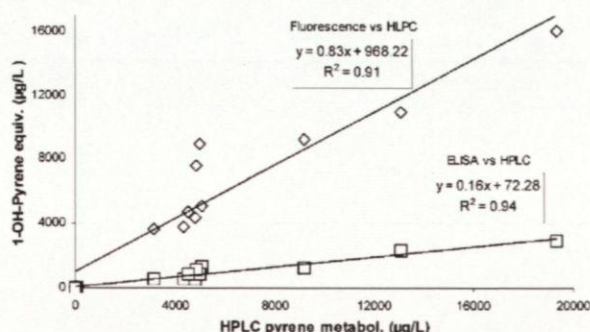


FIGURE 6. Correlations between PAH RaPID assay ELISA and HPLC results (□) ($n = 14$) and $FF_{345/382}$ and HPLC results (◇) ($n = 14$) of pyrene metabolites in crab urine.

of metabolites are different. The relative abundance of each metabolite of pyrene is not fully explained and requires further research. However, as compared to PAH metabolism studies on benzo[a]pyrene in shore crab (C. maenas) (38), the number of metabolites formed following pyrene exposure is limited to one major intermediate (1-OH-pyrene), which restricts the number of final conjugates.

Comparison of ELISA, Fluorescence, and HPLC Results. Comparison of results from the immunoassay and fluorescence techniques shows a high correlation for urine of crabs exposed to phenanthrene ($r^2 = 0.83$; n = 40) and pyrene ($r^2 = 0.88$; n = 42) (23). ELISA results, however, were consistently lower than those obtained by fluorescence by a factor of 0.26 (9-OH-phenanthrene equivalents) and 0.16 (1-OH-pyrene equivalents) (23). With polyclonal antibodies, reactivities differ between chemical structures such as the diverse metabolites and conjugates discussed in the previous section. It is therefore feasible that the PAH conjugates present in the crab urine samples are less reactive than the parent PAH used to raise the antibody.

PAH levels derived from HPLC results (sum of the four main pyrene metabolites/conjugates) show very good agreement with the ELISA ($r^2 > 0.94$) and fluorescence ($r^2 > 0.91$) data (Figure 6). This affords strong validation for both of the rapid assessment techniques. In addition, the ELISA and fluorescence methods developed appear fairly insensitive to urine matrix effects, and a simple dilution (≥1:20 or 1:20–1:40, respectively) is the only sample preparation required. With these methods, 100 samples can be run in duplicate (along with standards) per day.

The previous results provide evidence that exposure of crabs to PAH can be usefully assessed by analyses of their urine. While absolute levels may be difficult to determine, both ELISA and fluorescence appear to provide measures of exposure that are rapid and adequately sensitive to metabolites. However, it is important to extend the assessment from experimental systems to the field.

Biomonitoring is important because pollutants such as polycyclic aromatic hydrocarbons (PAHs) and their metabolites become concentrated in tissues, body fluids, and excreta, thereby offering a measure of exposure to biologically available contaminants. In this case, the use of these crustaceans will afford greater sensitivity to determine the direct effect of PAHs on the crustaceans (and consequently, other aquatic organisms).

Environmental Samples. To test the applicability of the urinary analyses to assess exposure of crabs to PAH under field conditions, crabs from clean and contaminated environments were investigated. Results demonstrate that urine analyses can clearly identify crabs from the differing environments (23). Variations in concentrations from Sutton Harbor (the contaminated site) are, however, large.

Comparison of the ELISA and UV-fluorescence results shows that both techniques detected PAH (mainly petrogenic) contamination in urine with good agreement ($r^2 = 0.83$; n = 15) (23). From the fluorescence results, however, mainly two to three ring PAHs were shown to dominate the environmental PAH (Figure 7) with only very small concentrations of four-ring PAHs such as pyrene. Samples taken from the clean site contained no such peaks (Figure 7). To further characterize the PAH mixture, extracts were also subjected to synchronous fluorescence spectrometry (SFS). HPLC/fluorescence analyses were run on selected extracts to identify and quantify any pyrene metabolites/conjugates present in the environmental samples. Results from both Bantham (clean) and Sutton Harbor (contaminated) showed very low concentration of pyrene metabolites (close to the limits of detection of the method). Indeed, concentrations of PAH metabolites/conjugates approached the levels in laboratory control samples.

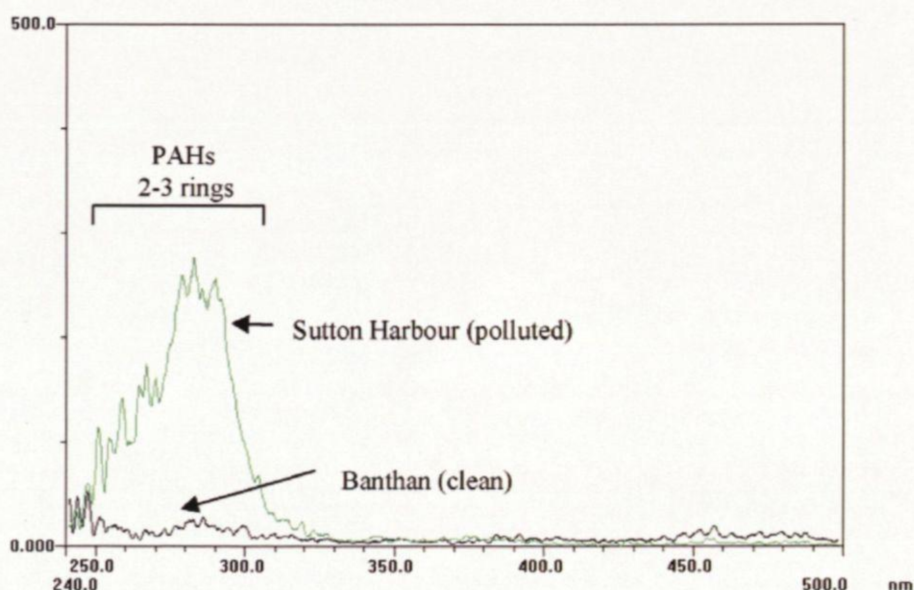
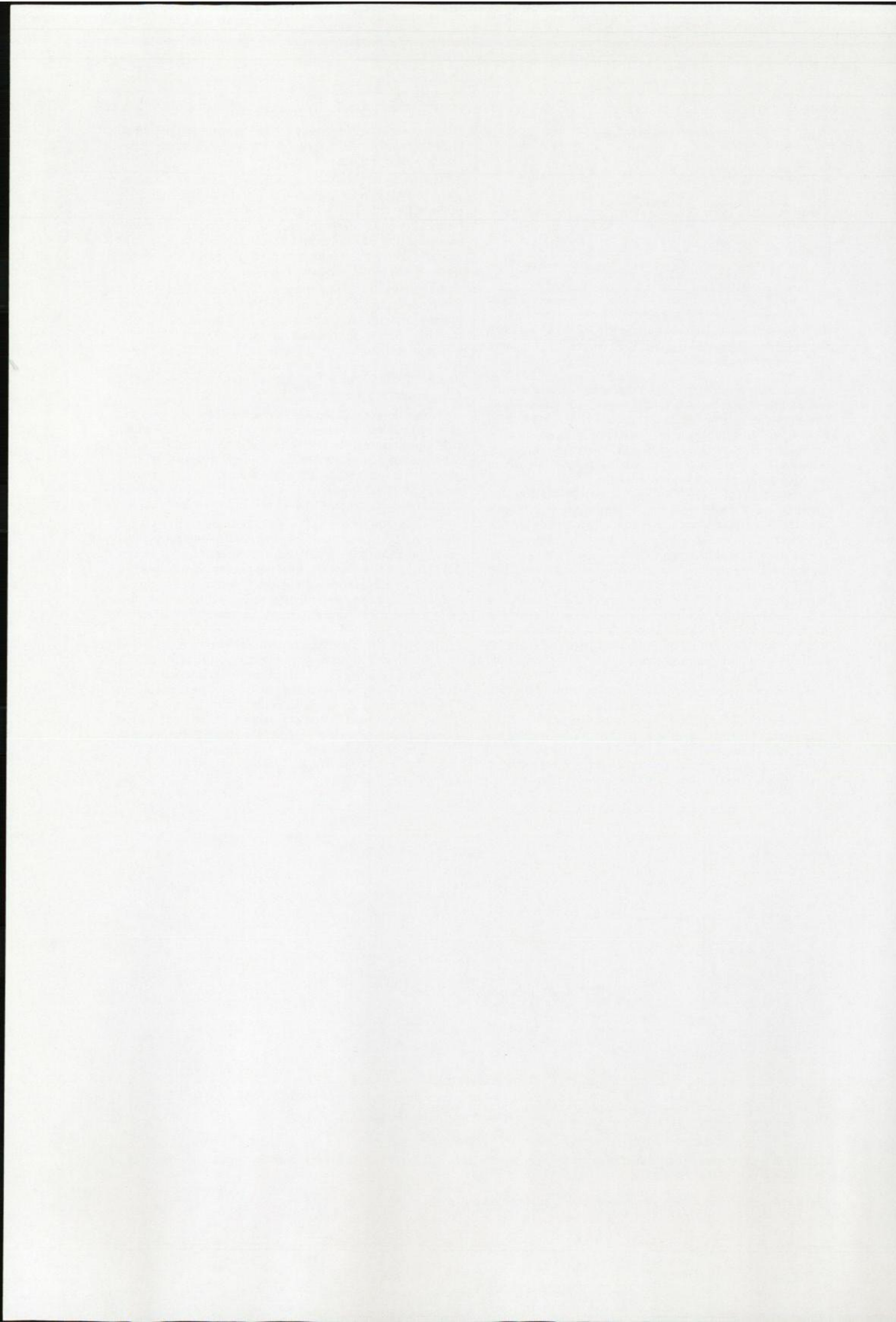


FIGURE 7. Synchronous excitation/emission fluorescence spectra ($SFS_{\Delta\lambda\ 42\ nm}$) of crab urine taken from environmentally exposed (Sutton Harbor) and control (Bantham) sites.



This preliminary study demonstrates significant differences in the extent and composition of PAH and their metabolites between crab populations sampled from contaminated (Sutton Harbor) and control sites.

Interindividual Variability of Urinary PAH Metabolites. Urinary PAH data showed considerable interindividual variability within experimentally and environmentally exposed populations. In some cases, levels of urinary PAHs were 3–4 times higher in some individuals as compared to others, despite them being exposed to the same concentrations of parent PAH. Interindividual variations most likely relate to the volume of urine/dilution and timing of urination but also to biological differences that are difficult to assess. While this might be easier to explain with field populations, where variations in dietary, preexposure to xenobiotics, and contaminant concentrations within sites is inevitable, it is more difficult to explain in a seemingly homogeneous group of organisms (similar size, sex, and moulting stage) kept under experimentally controlled conditions. This might partly be explained by interindividual variations in absorption, metabolism, and/or excretion of PAHs. Variations in the rate and degree of metabolism will also be influenced by the level of induction and efficacy of the appropriate P450 enzymes, possible preexposure to xenobiotics (44), and subtle differences in moult stage between crabs (45). Concerning field populations, interindividual variations in PAH metabolite levels in aquatic organisms (fish bile) have been previously reported (46, 47).

It is feasible that the variability in data might be better characterized with larger sample numbers (48). In the absence of previously published data concerning variability in crabs exposed to contaminants, the present design was considered optimal for a preliminary investigation. Other scientists have promoted normalization of urinary concentrations to other components to allow for fluctuations related to dilution (49). These studies, however, relate to human investigations, and while theoretically very appealing, no current research has identified potential candidate compounds in crustaceans. Conversely, some authors believe this normalization procedure has little effect on the results (5, 27, 50, 51).

Future Research. With recent developments into new antibodies, it is now possible to selectively detect major metabolites (e.g., the phenolic glucuronides (52)). This will afford greater sensitivities in future research and will also enable focusing on the most relevant metabolites.

Because metabolic oxidation of PAHs is responsible for the formation of toxic and carcinogenic intermediates (53), the determination of the level of metabolism by an organism can improve the assessment of risk. While the present technique offers a most useful extension to monitoring parent/metabolite compound levels in organisms, it still retains some constraints regarding the limit between levels and effects (54). Concurrent ecotoxicological investigation can bridge this gap.

Acknowledgments

G.F. was sponsored by CAPES (BEX1536/96-6) and WWF-Brazil/USAID (CSR-151-00). M. Howsam received a visiting postdoctoral fellowship from The Netherlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). This research is part of the Rapid Assessment of Marine Pollution (RAMP) program funded by a DFID-UK (Department for International Development) grant to M.H.D. and J.W.R.

Supporting Information Available

One figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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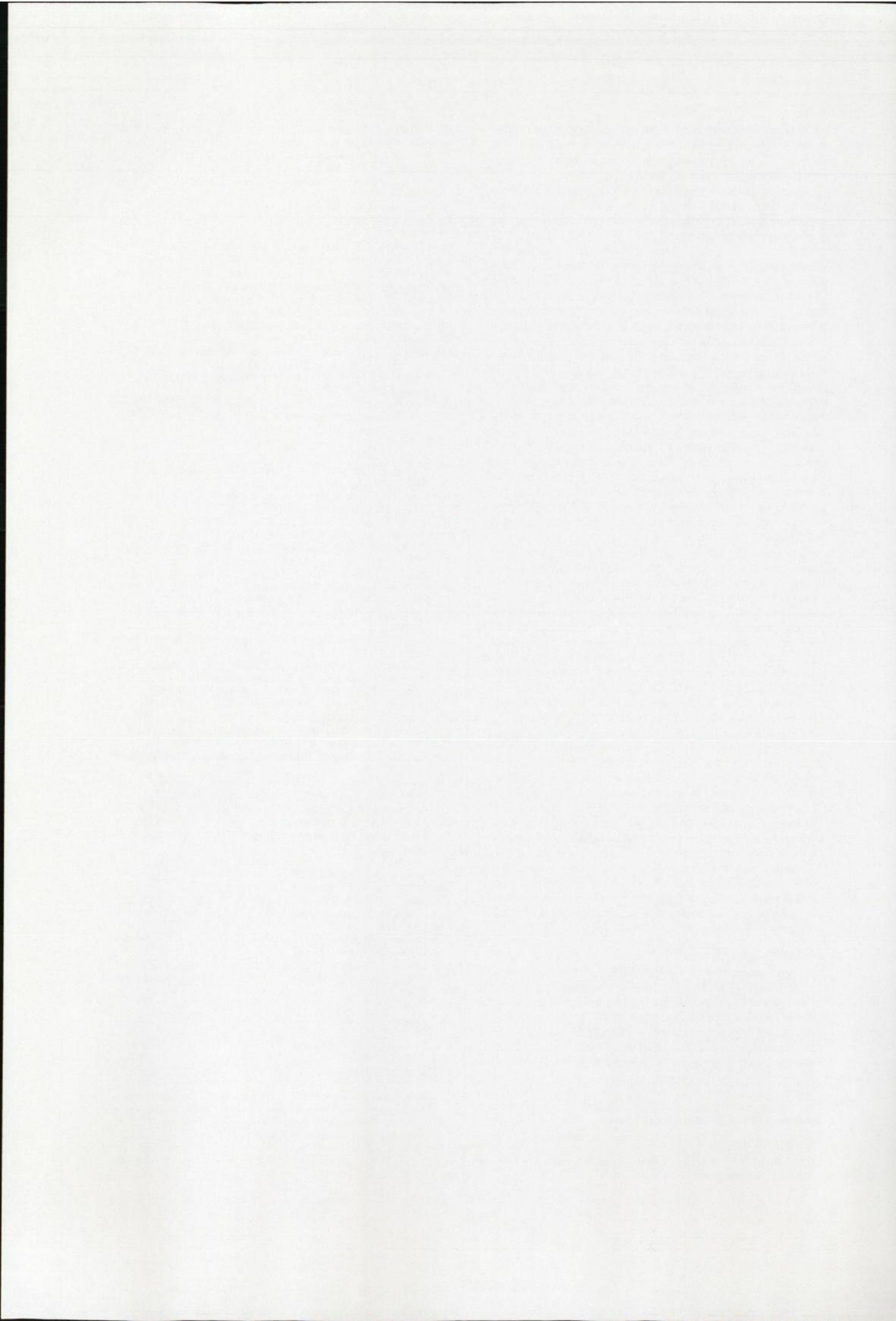

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Received for review September 30, 2003. Revised manuscript received January 30, 2004. Accepted February 6, 2004.

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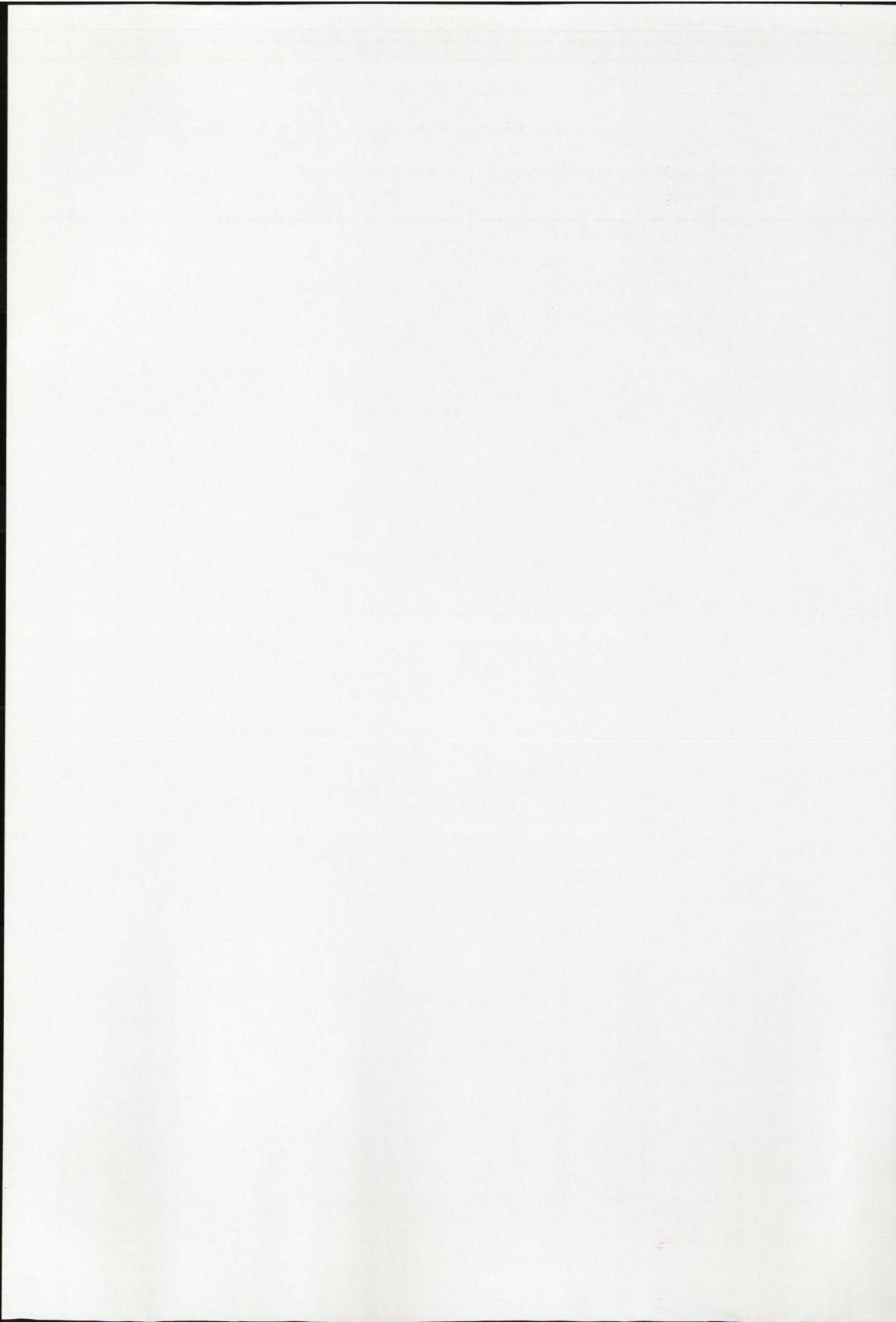

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Marine Environmental Research 58 (2004) 257–261

MARINE ENVIRONMENTAL RESEARCH

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Detecting a field gradient of PAH exposure in decapod crustacea using a novel urinary biomarker

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Abstract

Norwegian coastal waters are subject to PAH contamination from electrochemical industries such as aluminium smelters. Evidence of PAH exposure has been established in fish and bivalves. The present study tests the applicability of a novel crustacean PAH exposure biomarker to a PAH contamination gradient in the field (Karmsund Strait, SW Norway). Fluorescence analysis of urine samples collected from crabs at each site revealed 1-OH pyrene "equivalent" levels (indicative of pyrogenic PAH contamination) decreased with increasing distance from a point source of pyrogenic PAH (a large aluminium works). The assay was shown to be suitable for the detection of PAH exposure in wild crustacean populations, for discriminating between contaminated and clean sites and is also sufficiently sensitive to detect gradients of PAH contamination. The method provides a rapid, inexpensive and non-destructive measure of biologically available PAH in crustaceans.
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Keywords: Ecotoxicology; PAH; *Carcinus maenas*; Urinary exposure biomarker; Aluminium smelter; Biotransformation

Contamination of the marine environment with polycyclic aromatic hydrocarbons (PAHs), a ubiquitous group of organic pollutants, is a matter of great

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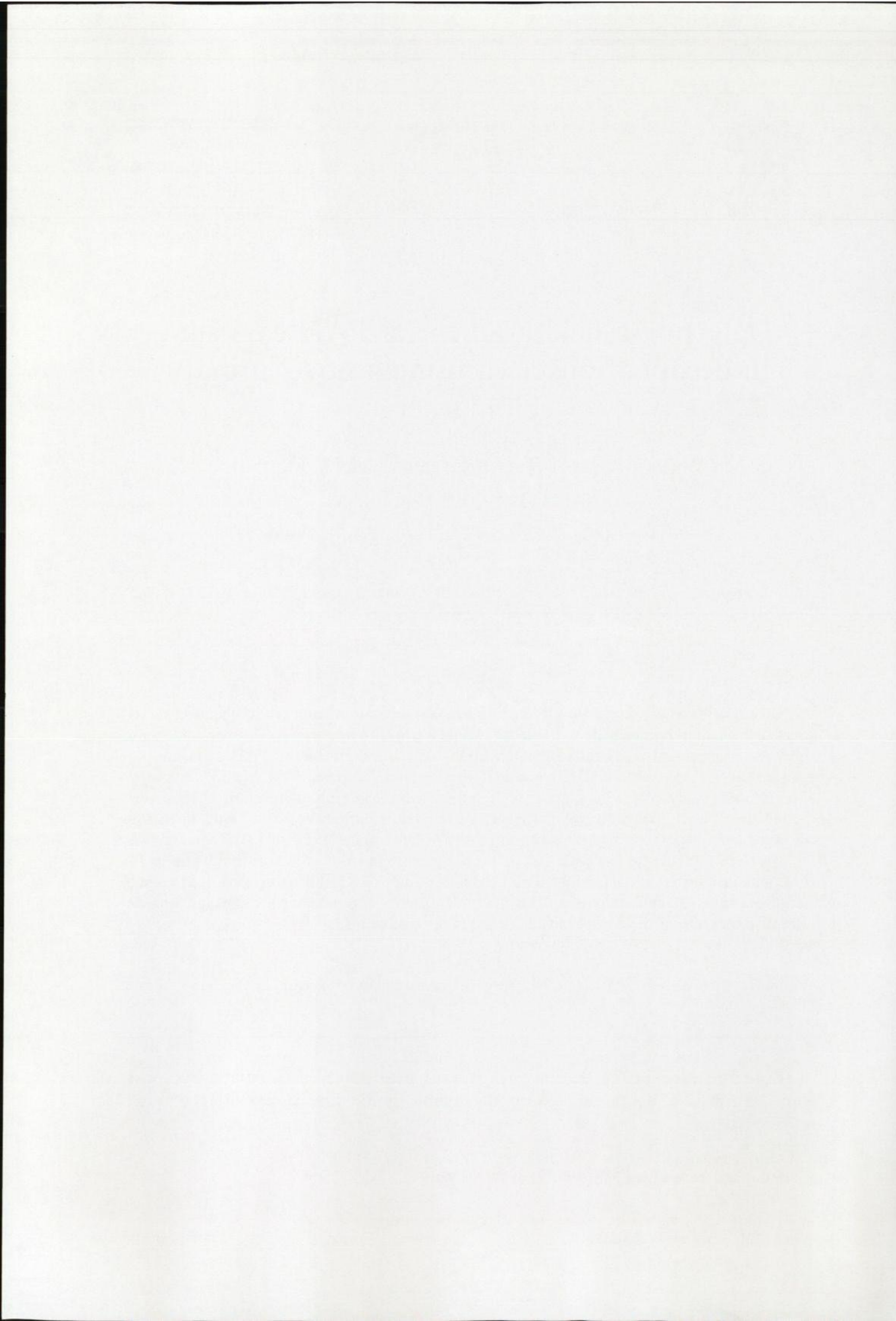
0141-1136/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marenvres.2004.03.068

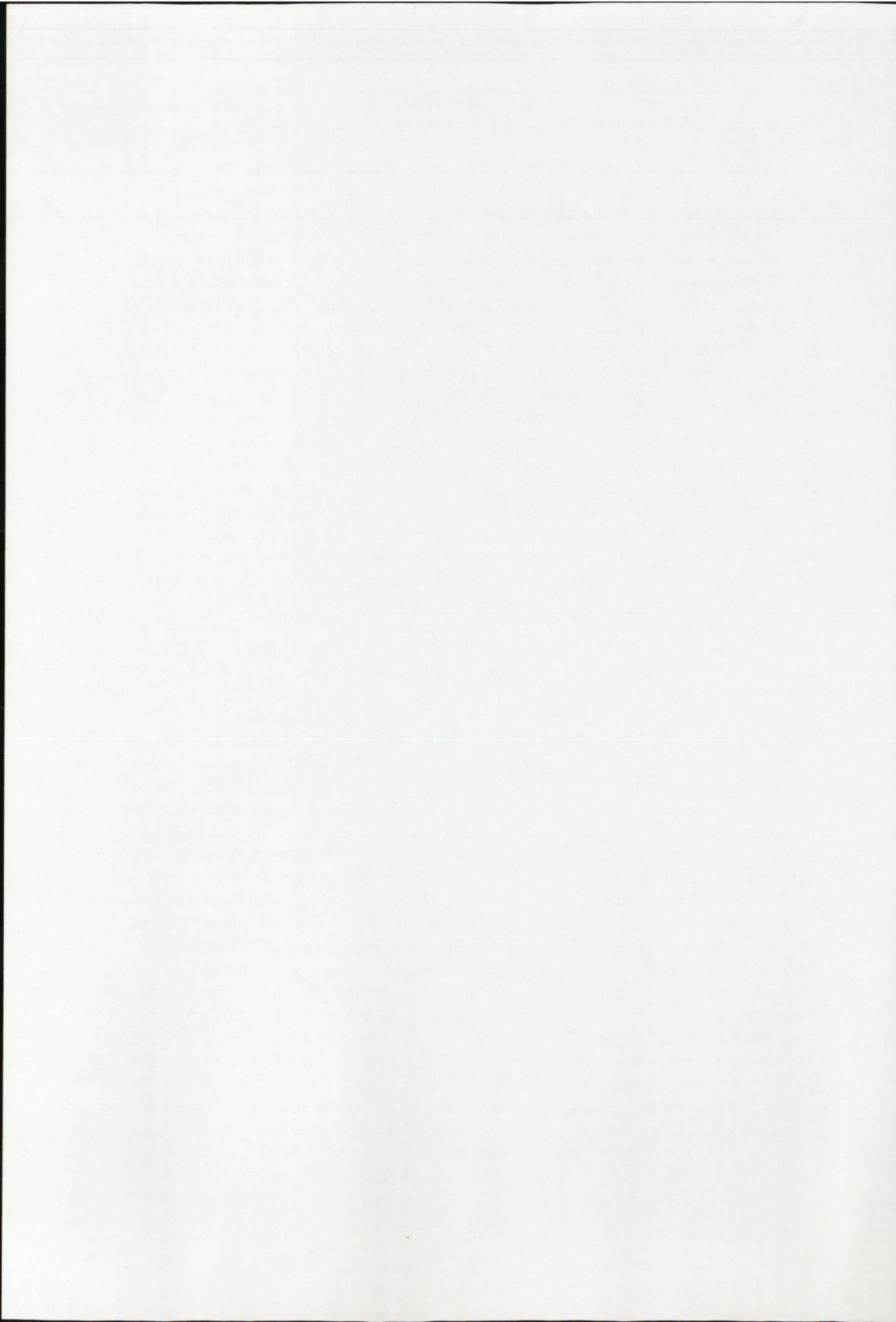


environmental concern, not least because of their often mutagenic and carcinogenic properties. Large amounts of PAH are discharged annually into Norwegian fjords and coastal areas. Electrochemical industries such as aluminium works, in particular those using "Soderberg" anode technology, are one of the most important sources of pyrogenic (combustion related) PAH pollution. The majority of such pyrogenic PAH contamination originates from the discharge of effluent from settling ponds (following "scrubbing" of gas emissions with seawater) and consumption of tar-based anodes (Aas, Beyer, Jonsson, Reichert, & Andersen, 2001). Evidence of exposure to PAH in Norwegian waters has already been established in fish and mussel studies (Aas et al., 2001; Beyer, Aas, Borgenvik, & Ravn, 1998; Naes, Knutzen, & Berglind, 1995). The present study is a preliminary field trial of a novel biomarker, developed to assess PAH exposure in a ubiquitous decapod crustacean, the shore crab *Carcinus maenas*, using urine as a sample source (Fillmann, Watson, Francioni, Readman, & Depledge, 2002; Watson, Andersen, Depledge, & Galloway, 2004). Previous studies have shown that PAHs are biotransformed in crustacean species (Fillmann et al., 2004; Sundt & Goksoyr, 1998). Urine is an important excretory route for polar metabolites of xenobiotics such as PAHs, and can be sampled non-destructively. Analysis is performed using direct fluorimetric techniques, widely employed in previous studies for detecting PAH metabolites in fish bile (Aas, Beyer, & Goksoyr, 2000).

The present study tests the ability of the assay to detect a gradient of exposure in wild crab populations with increasing distance from a point source of PAH contamination in Karmsund Strait, a shallow fjordal strait located east of Karmoy Island, SW Norway.

Green male intermoult *C. maenas* of carapace width 50–72 mm were collected by local fishermen from 5 sites within Karmsund strait (Hogevarde, Austvik, Koppervik, Krokaneset and Bukkoy) and one reference site in a nearby fjord (Salvoy) ($n = 12$ at all sites except Koppervik, $n = 8$). The selected sites within Karmsund strait increased in distance progressively from a point source of chronic and high level PAH contamination – an aluminium works (Hydro Aluminium Karmoy – HAK) at Hogevarde (Fig. 1). Evidence of a gradient of PAH contamination has been established from previous sediment analyses at sites along the strait (data not shown).

Crabs were urine sampled in situ whilst maintained in well-aerated local seawater at ambient temperature. Urine samples (70–600 μl per crab) were transferred to siliconised microcentrifuge tubes, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer. Measurements were carried out in a 3.5-ml quartz fluorescence cuvette and 50% ethanol was used as the solvent for samples, standards and blanks. All fluorescence measurements were performed with excitation and emission slit widths of 2.5 nm. A series of 1-OH pyrene standards were prepared in 50% ethanol and analysed using fixed fluorescence (FF) at an excitation wavelength of 345 nm and by synchronous fluorescence (SFS) analysis, using a $\Delta\lambda$ of 37 nm. Peak intensity was measured at E_m 387 nm for both FF and SFS. Urine samples were diluted 1:50 in 50% ethanol and analysed by FF (E_x 345 nm/E_m 382 nm) and SFS ($\Delta\lambda$ of 37 nm, E_m 382 nm) for pyrogenic PAH (predominantly pyrenes), indicative of



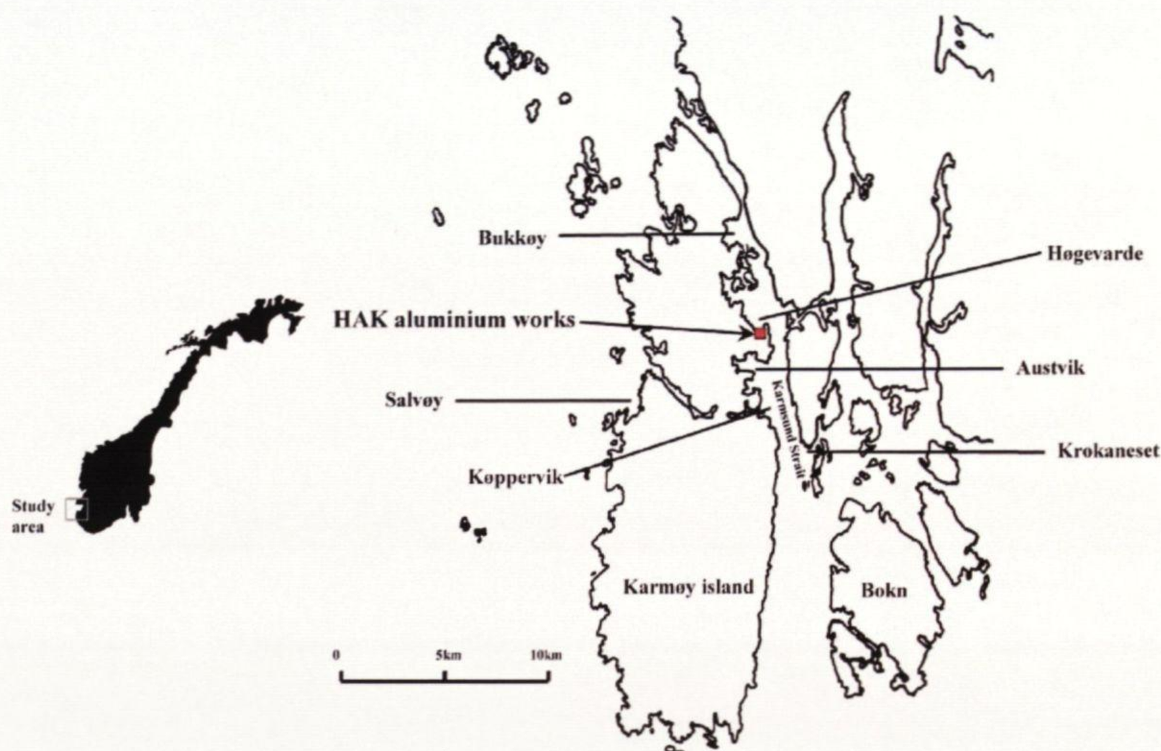
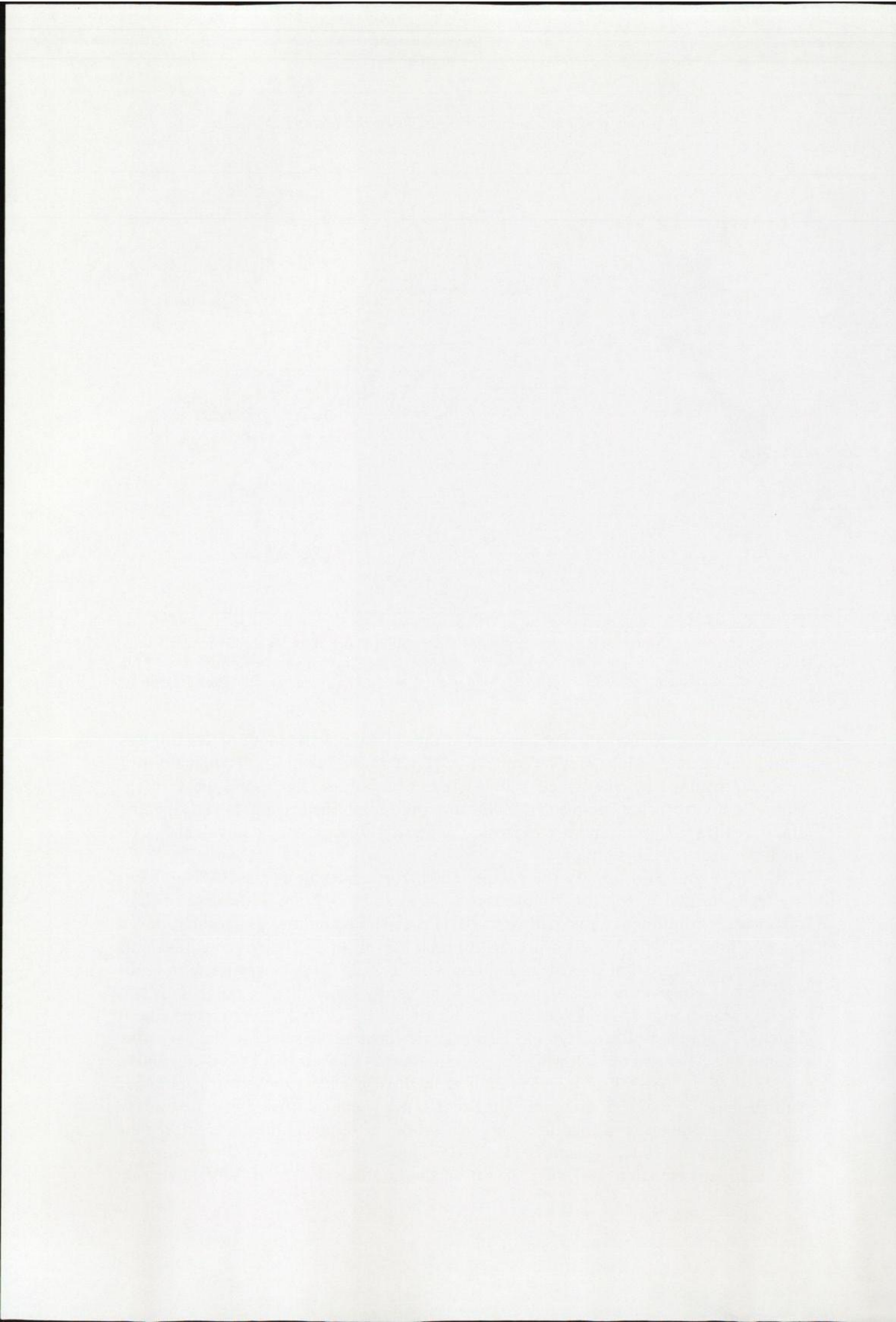


Fig. 1. Sampling sites within Karmsund Strait. The aluminium works is located between Hogevarde and Austvik. The site at Hogevarde is located next to the largest point of discharge for the aluminium works' gas scrubbing effluent. The site at Austvik is located next to a smaller discharge point. Koppervik is 2.5 km south of the aluminium works, Krokaneset is 5.5 km south whilst Bukkoy is 6 km north. The reference site is at Salvoy.

emissions from the smelter. Measurement at the emission wavelength of 382 nm was established in a previous study (Watson et al., 2004) to take into account fluorescence contributed by conjugated 1-OH pyrene metabolites (see below). Peak intensities from crab urine samples (E_m 382 nm) were quantified against 1-OH pyrene standards (E_m 387 nm) and are expressed as μg/L 1-OH pyrene "equivalents", following correction for dilution.

Both FF and SFS urinary spectra obtained from crabs along the PAH gradient are characterised by a strong fluorescence signal at E_m 382 nm, indicative of fluorescence contributions from conjugated 1-OH pyrene metabolites. The results show a marked gradient of PAH exposure, with urinary levels of 1-OH pyrene equivalents increasing with proximity to the aluminium smelter (Fig. 2). Urinary levels in crabs sampled at Hogevarde and Austvik are significantly different ($p < 0.05$, Kruskal–Wallis) from those at Krokanes (the mouth of the fjord) and the reference site at Salvoy. A gradient of exposure has also been detected at the same sites in corkwing wrasse and Atlantic cod following fluorescent analysis of their bile (Aas et al., 2001).

HPLC/F analysis of urinary metabolites from *C. maenas* exposed to pyrene has shown that the majority are conjugates of 1-OH pyrene (glucoside, sulfate and an unknown conjugate), with only negligible amounts of unchanged parent pyrene (Fillmann et al., 2004; Watson et al., 2004). This is consistent with the position of the dominant emission peak (@382 nm) on the fluorescence spectra obtained from



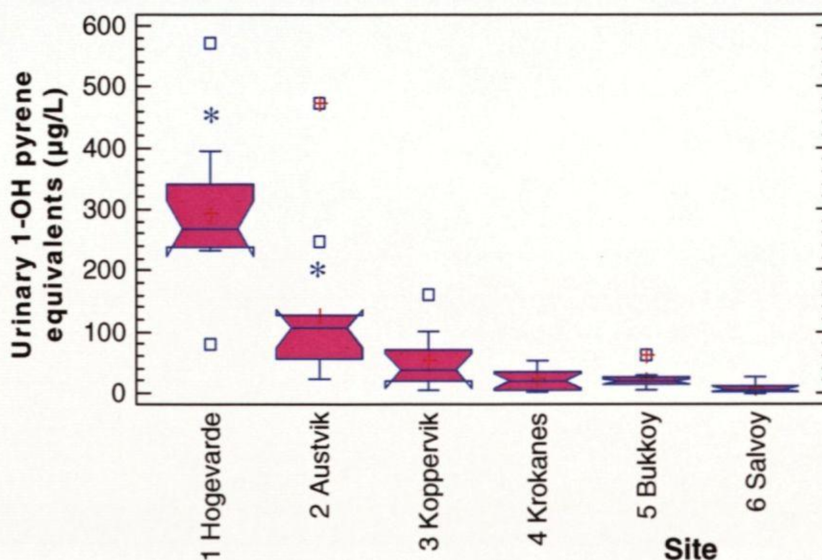


Fig. 2. Box and whisker plot of 1-OH pyrene "equivalent" levels (µg/L) in crab urine from each site (determined by SFS) - * denotes SSD ($p < 0.05$, Kruskal Wallis).

pyrene-exposed crab urine samples. Conjugation subtly changes the fluorescence characteristics of 1-OH pyrene, which in its free hydroxylated form, exhibits a fluorescence peak at 387 nm, a finding reported by Ariese et al. (1993). This fluorescence shift necessitates the expression of urinary metabolites in terms of µg/L 1-OH pyrene "equivalents". The present study confirms that *C. maenas* excretes similar conjugates following exposure to pyrogenic PAH contamination in the field, as the spectra consistently exhibit a large peak at E_m 382 nm. Without more powerful analysis (namely HPLC/F with suitable standards), the specific identity of the metabolites contributing fluorescence to this peak cannot be reported. However, it is highly likely that they are conjugates of 1-OH pyrene.

Previous studies have shown that levels of urinary pyrene conjugates determined by direct fluorescence techniques correlate closely with those determined by HPLC/F ($r^2 = 0.91$) and immunochemical techniques ($r^2 = 0.88$) (Fillmann et al., 2002, 2004).

The present study has revealed a marked gradient of pyrogenic PAH exposure in *C. maenas* along an established gradient of contamination, previously reported in fish (Aas et al., 2001). The urinary exposure biomarker is therefore suitable for the detection of PAH exposure in wild crustacean populations and is capable of discriminating between contaminated and clean sites. The method provides a rapid, inexpensive and non-destructive measure of biologically available PAH in an ecologically important crustacean.

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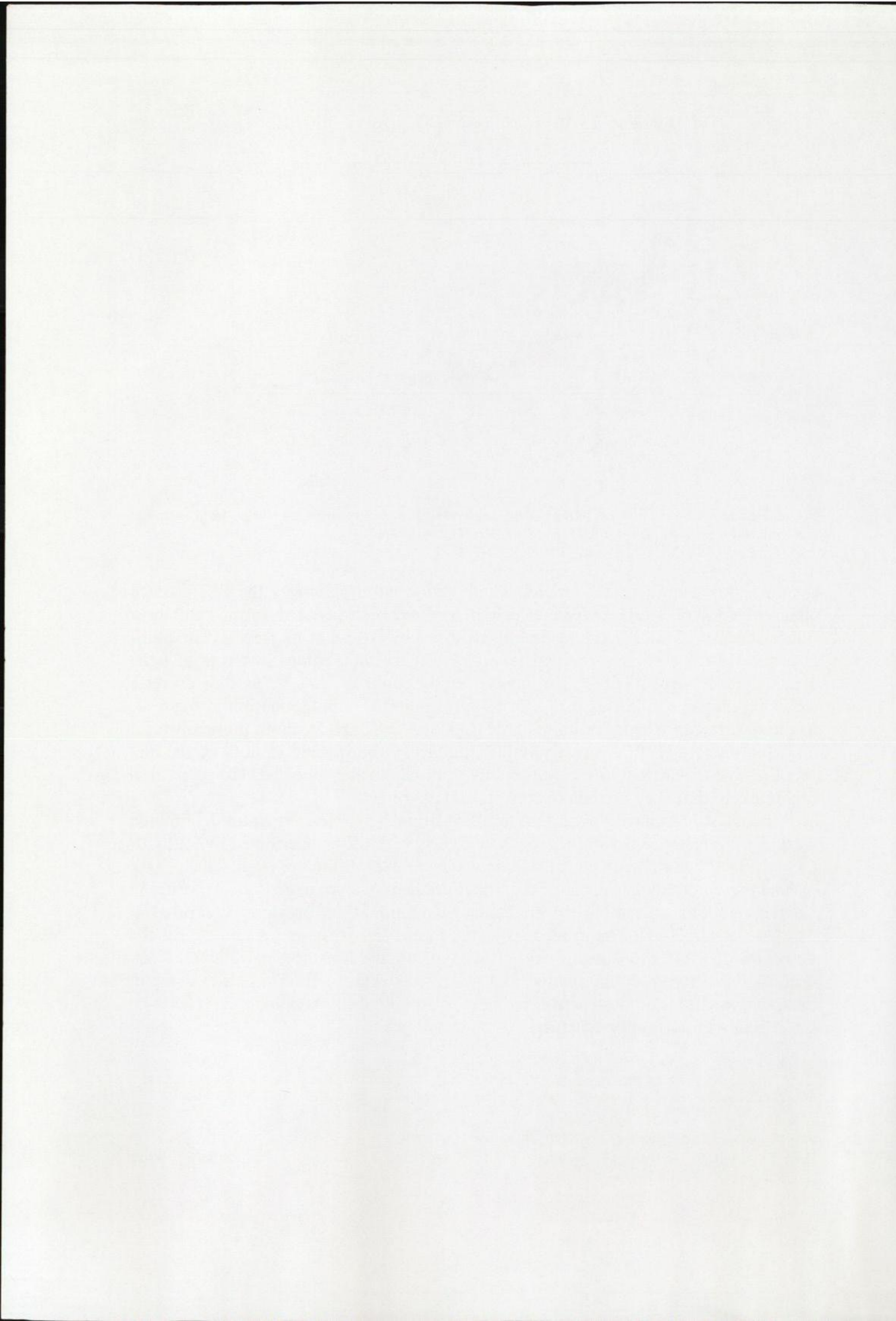

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