MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

SELECTED TECHNIQUES FOR MONITORING BIOLOGICAL EFFECTS OF POLLUTANTS IN MARINE ORGANISMS

MAP Technical Reports Series No. 71

In cooperation with

IOC

UNEP
Athens, 1993

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This volume is the seventy-first issue of the Mediterranean Action Plan Technical Reports Series.

This series contains selected reports resulting from the various activities performed within the framework of the components of the Mediterranean Action Plan: Pollution Monitoring and Research Programme (MED POL), Blue Plan, Priority Actions Programme, Specially Protected Areas and Regional Marine Pollution Emergency Response Centre for the Mediterranean.

PREFACE

The United Nations Environment Programme (UNEP) convened an Intergovernmental Meeting on the Protection of the Mediterranean (Barcelona), 28 January - 4 February 1975), which was attended by representatives of 16 States bordering on the Mediterranean Sea. The meeting discussed the various measures necessary for the prevention and control of pollution of the Mediterranean Sea, and concluded by adopting an Action Plan consisting of three substantive components:

- Integrated planning of the development and management of the resources of the Mediterranean Basin (management component);

- Co-ordinated programme for research, monitoring and exchange of information and assessment of the state of pollution and of protection measures (assessment component);

- Framework convention and related protocols with their technical annexes for the protection of the Mediterranean environment (legal component).

All components of the Action Plan are interdependent and provide a framework for comprehensive action to promote both the protection and the continued development of the Mediterranean ecoregion. No component is an end in itself. The Action Plan is intended to assist the Mediterranean Governments in formulating their national policies related to the continuous development and protection of the Mediterranean area and to improve their ability to identify various options for alternative patterns of development and to make choices and appropriate allocations of resources.

The Co-ordinated Mediterranean Research and Monitoring Programme (MED POL) was approved as the assessment (scientific/technical) component of the Action Plan.

The general objectives of its pilot phase (MED POL - Phase I), which evolved through a series of expert and intergovernmental meetings, were:

- to formulate and carry out a co-ordinated pollution monitoring and research programme taking into account the goals of the Mediterranean Action Plan and the capabilities of the Mediterranean research centres to participate in it;

- to assist national research centres in developing their capabilities to participate in the programme;

- to analyse the sources, amounts, levels, pathways, trends and effects of pollutants relevant to the Mediterranean Sea;

- to provide the scientific/technical information needed by the Governments of the Mediterranean States and the EEC for the negotiation and implementation of the Convention for the Protection of the Mediterranean Sea against Pollution and its related protocols;

- to build up consistent time-series of data on the sources, pathways, levels and effects of pollutants in the Mediterranean Sea and thus to contribute to the scientific knowledge of the Mediterranean Sea.

MED POL-Phase I initially consisted of seven pilot projects and baseline studies on the monitoring of oil, petroleum hydrocarbons and microbial pollution in sea water, heavy metals and chlorinated hydrocarbons in marine organisms as well as research on the effects of pollutants on organisms, populations, communities and ecosystems. In addition, four related projects were also included to broaden the scope of the programme or to provide ancillary support.

Based on the recommendations made at various expert and intergovernmental meetings, a draft Long-term (1981-1990) Programme for pollution monitoring and Research in the Mediterranean (MED POL-Phase II) was formulated by the Secretariat of the Barcelona Convention (UNEP), in co-operation with the United Nations Agencies which were responsible for the technical implementation of MED POL-Phase I, and it was formally approved by the Second Meeting of the Contracting Parties of the Mediterranean Sea against pollution and its related protocols and Intergovernmental Review Meeting of Mediterranean Coastal States of the Action Plan held in Cannes, 2-7 March 1981.

The general long-term objectives of MED POL-Phase II were to further the goals of the Barcelona Convention by assisting the Parties to prevent, abate and combat pollution of the Mediterranean Sea area and to protect and enhance the marine environment of the area. The specific objectives were designed to provide, on a continuous basis, the Parties to the Barcelona Convention and its related protocols with:

- information required for the implementation of the Convention and the protocols;
- indicators and evaluation of the effectiveness of the pollution prevention measures taken under the Convention and the protocols;
- scientific information which may lead to eventual revisions and amendments of the relevant provisions of the Convention and the protocols and for the formulation of additional protocols;
- information which could be used in formulating environmentally sound national, bilateral and multilateral management decisions essential for the continuous socio-economic development of the Mediterranean region on a sustainable basis;
- periodic assessment of the state of pollution of the Mediterranean Sea.

The monitoring of, and research on, pollutants affecting the Mediterranean marine environment reflects primarily the immediate and long-term requirements of the Barcelona Convention and its protocols, but also takes into account factors needed for the understanding of the relationship between the socio-economic development of the region and the pollution of the Mediterranean Sea.

Individual and collective training is provided for scientists and technicians in techniques (methods) required for their effective participation in monitoring and research envisaged in the framework of MED POL - PHASE II. This assistance is in the form of fellowships, experts, workshops, seminars,

grants for attendance to meetings, etc., and covers training in analytical and sampling techniques, data processing, interpretation of results and various research topics.

As in MED POL-Phase I, the overall co-ordination and guidance for MED POL-Phase II is provided by UNEP as the secretariat of the Mediterranean Action Plan (MAP). Co- operating specialized United Nations Agencies (FAO, UNESCO, WHO, WMO, IAEA, IOC) are responsible for the technical implementation and day-to-day co-ordination of the work of national centres participating in monitoring and research.

The seventy-first volume of the MAP Technical Reports Series contains the lecture and practical notes which have been prepared for the FAO/IOC/UNEP Training Workshop on the Techniques for Monitoring Biological Effects of Pollutants in Marine Organisms (Nice, France, 14-25 September 1992). It is divided into two parts. Part I contains the material on biochemical techniques and Part II the material on physiological techniques.

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PART I

USE OF BIOMARKERS IN THE ASSESSMENT OF CONTAMINATION IN MARINE ECOSYSTEMS. FUNDAMENTAL APPROACH AND APPLICATIONS

by

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I. Fundamental Approach

In recent decades, increased inputs of anthropogenic contaminants and other stresses such as destruction of habitats, have brought about drastic changes in aquatic ecosystems. Correspondingly, increased interest has centered on : (a) the accumulation and toxic effects of contaminants on the survival of aquatic organisms; (b) the uptake and accumulation of pathogenic organisms and contaminants in aquatic resources destined for human consumption; and (c) the release of biodegradable organic matter and nutrients, which under quiescent conditions may result in localized eutrophication, organic enrichment, and oxygen depletion (Malins and Ostrander, 1991).

In response to these and other events, research in aquatic toxicology has undergone significant changes in the past decades. The science that initially focused on body burdens of toxic environmental chemicals and acute bioassays with lethality as the principal endpoint, has expanded into studies exploring the primary steps of contaminant impact on organisms characterised by the interaction with endogenous molecules. These include studies on metabolic conversions of xenobiotics, modifications of DNA, and other biochemical processes (Malins and Collier, 1981; Moore, 1991).

1. AQUATIC ORGANISMS AND FATE OF POLLUTANTS

The relationships between pollutants and organism are summarized in Figure 1. The first step in studying aquatic toxicology is to consider the type of pollutant, its bioavailability and the pathways of its uptake by the organism.

Inputs of large amounts of toxicants into the aquatic environment occurs from a variety of anthropogenic sources and may involve literally thousands of substances, particularly in urban and industrial areas. A partial list includes most metals, polycyclic aromatic hydrocarbons (PAH), halogenated organic compounds like polychlorinated biphenyls (PCBs), as well as various nitrogen-, phophorous- and sulphur-containing organic compounds that form the large family of pesticides. The pollutant partitioning in the aquatic environment comprises (i) accumulation on the benthic substrate, (ii) distribution in the water column, and (iii) uptake by organisms (Malins and Ostrander, 1991).

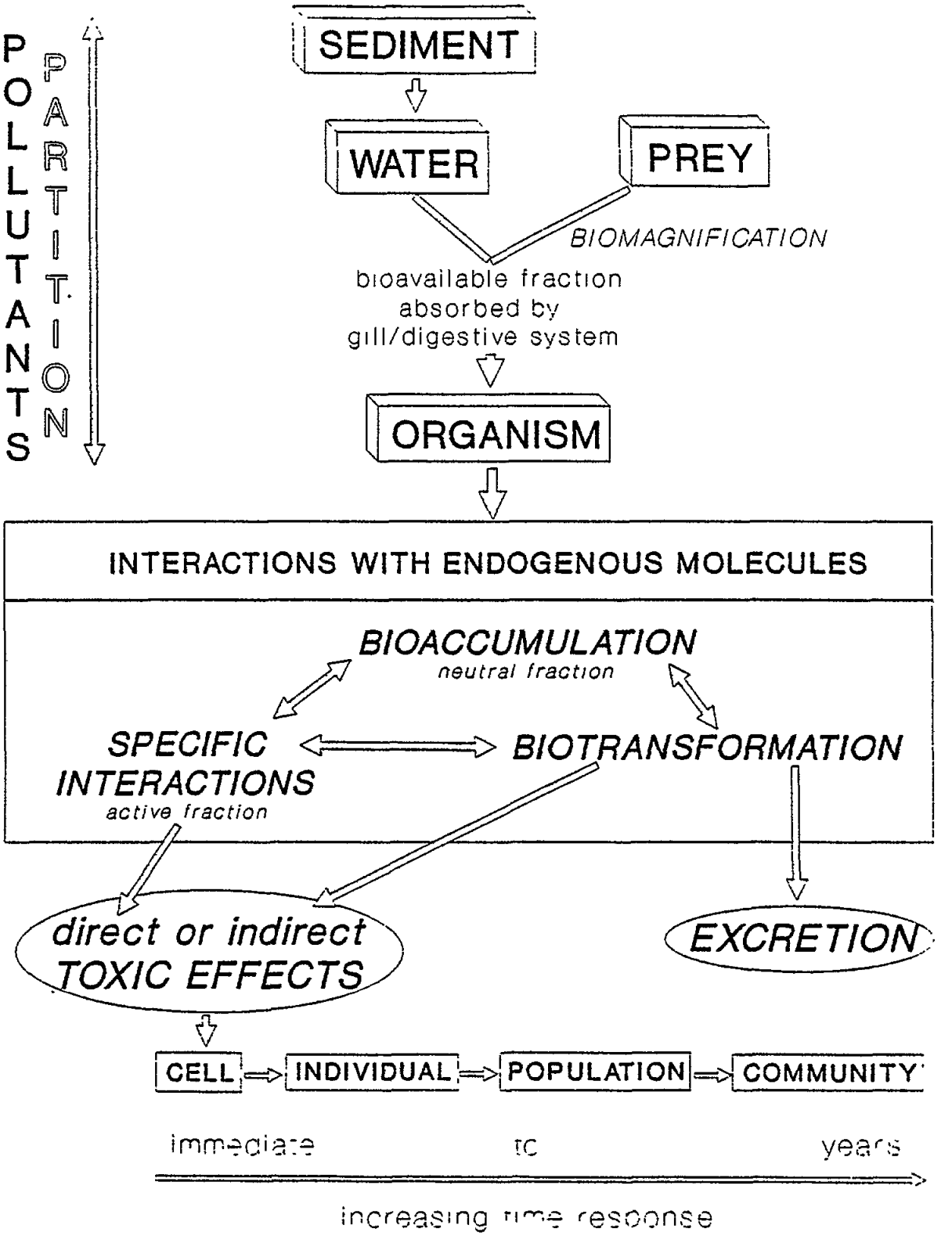

Fig. 1 Relationship between contaminants in the aquatic environment and biological effects

The fraction present in the water column (bound to colloids, particles and/or dissolved) and the food can be summarized as the pollutant fractions available for the organisms : the bioavailable fraction. Two major routes of contaminant uptake can be considered, (i) the respiratory system (gills) and (ii) the digestive system. Food as a source of uptake may be particularly important in benthic and epibenthic ecosystems where sediment-associated pollutants are a significant source of input to the aquatic ecosystem (Farrington, 1991).

The uptake of contaminants leads to their concentration in the animal tissues. The rate of bioconcentration potential of an organism is dependent on many factors such as (i) temperature, (ii) physiological status varying with sex and season, and (iii) biomagnification potential that increases within the trophic level.

The first stage of a contaminant impact towards the organism is the interaction with endogenous molecules. These interactions can be divided into three main important groups. The contaminant can be sequestrated and then neutralized like PCB or PAH in the neutral lipid fraction, and/or it can have specific interaction with endogenous molecules like the inhibition of acetylcholinesterase (AChE) by certain carbamates and organophosphates, and/or at least, it can be metabolized by the enzymes of the biotransformation systems.

All these interactions can lead (i) to a long-term storage (neutralized fraction), (ii) to direct or indirect (after biotransformation) toxic effects and/or (iii) to the excretion of the contaminants or its metabolites. Thereafter, the toxic effects can have some repercussions at the cellular, tissue, organism levels, and thus modify the population integrity, and finally perturb the whole ecosystem. The time response of impact of pollutants will vary from hours at the molecular and cellular levels, to may be years at the population and community levels.

2. MEASUREMENT OF THE BIOLOGICAL EFFECTS OF ENVIRONMENTAL POLLUTANTS: THE BIOMARKERS OF ENVIRONMENTAL CONTAMINATION

The measurement of the biological effect of an exposure to contaminants should fulfill two main criteria :

(i) The biological change must be demonstrated to be exclusively due to the contaminant(s). Thus, consideration must be given to the background variability (season, sex, temperature) in order to obtain a good signal/noise ratio.

(ii) The biological change must be related to an adverse effect on some aspect of animal physiology such as growth, reproduction or survival (Bayne, 1985). Moreover, response time should be short, of the order of hours to weeks, as the measurements are used as early-warning systems, and ideally the biological response should occur throughout the range from optimal to lethal environmental conditions.

The impact of pollutants can occur at different level of functional complexity from molecular through subcellular and from cellular to whole animal. Because each higher level of organization represents an integration of a greater number of processes at the lower level, this results in a suite

of biological effects measurements with different features of sensitivity and specificity and obvious ecological relevance (Livingstone et al., 1989a). Specificity, in terms of identifying an offending pollutant in a complex environmental situation, can be achieved only at the molecular level, whereas, for example, physiological Scope for Growth represents a non-specific response to the sum of environmental stimuli, providing a measurement of the overall impact of environmental change. The ecological relevance is obvious for Scope for Growth but requires mechanistic theory for molecular events. The consequence of the latter is that a certain amount of fundamental research is required for interpretation of a particular event. The inclusion of a particular biomarker in a programme of environmental monitoring depends on all these features of background variability, specificity etc., and also on the more practical considerations of the ease and cost of measurement.

The term of biomarker will define here all the biological parameters which can be modified in an organism exposed to environmental contaminants.

Figure 2 shows a synthetic overview of the main important biomarkers of environmental pollution.

2.1 Specific biomarkers

2.1.1 The cytochrome P-450 dependent mixed function oxidases: MFO

High levels of PAH carcinogens commonly occur in aquatic systems where neoplasms arise in fish and other animals (Carles, 1984; Couch and Harshbarger, 1985; Lafaurie et al., 1990). In mammals, enzymes that transform PAHs (MFO) can act in initiating this disease and can indicate the contamination of the animals by carcinogens and other pollutants. MFO has similar roles in activating procarcinogens in vertebrate and also in some invertebrate marine species (Anderson and Döos, 1983; Stegeman, 1985; Michel et al., a and b, in press). PAHs and many chlorinated hydrocarbons, e.g. PCBs, induce cytochrome P-450 that is the primary catalyst of PAH metabolism. The induction of MFO can accelerate the disposition of hydrocarbons, but can also enhance the formation of carcinogenic derivatives of PAHs. Invertebrates, such as mussels, have lower rates of PAH metabolism and also less inducible MFO activities than in fish (Livingstone et al., 1989b). That may limit the involvement of some procarcinogen pollutants in disease processes in invertebrates and partly explain the survival of mussels in highly polluted sites where no fishes can be found (Narbonne et al., 1991a).

The induction of MFO can indicate the exposure of fish to PAHs, PCB and other related compounds and this is not restricted to carcinogens. Environmental induction has been detected in fish and in mussel from contaminated areas by use of enzyme assays (Garrigues et al., 1990; Narbonne et al., 1991a), antibodies to fish cytochrome P-450 (Goksøyr, 1991), and cDNA probes that hybridize with P-450 messenger RNA (Stegeman and Lech, 1991). Application of these methods can provide sensitive biological monitoring tools that can detect environmental pollution of marine organisms by carcinogens and tumor promoters (Stegeman and Lech, 1991).

Moreover, some isoforms in the cytochrome P-450 family are involved in the metabolism of endogenous substrates such as steroids, prostaglandins, fatty acids and vitamin A (Coon and Koop, 1983; Gibson et al., 1990; Van den Bosshe and Willemsens, 1991). The interactions between the cytochrome P-450

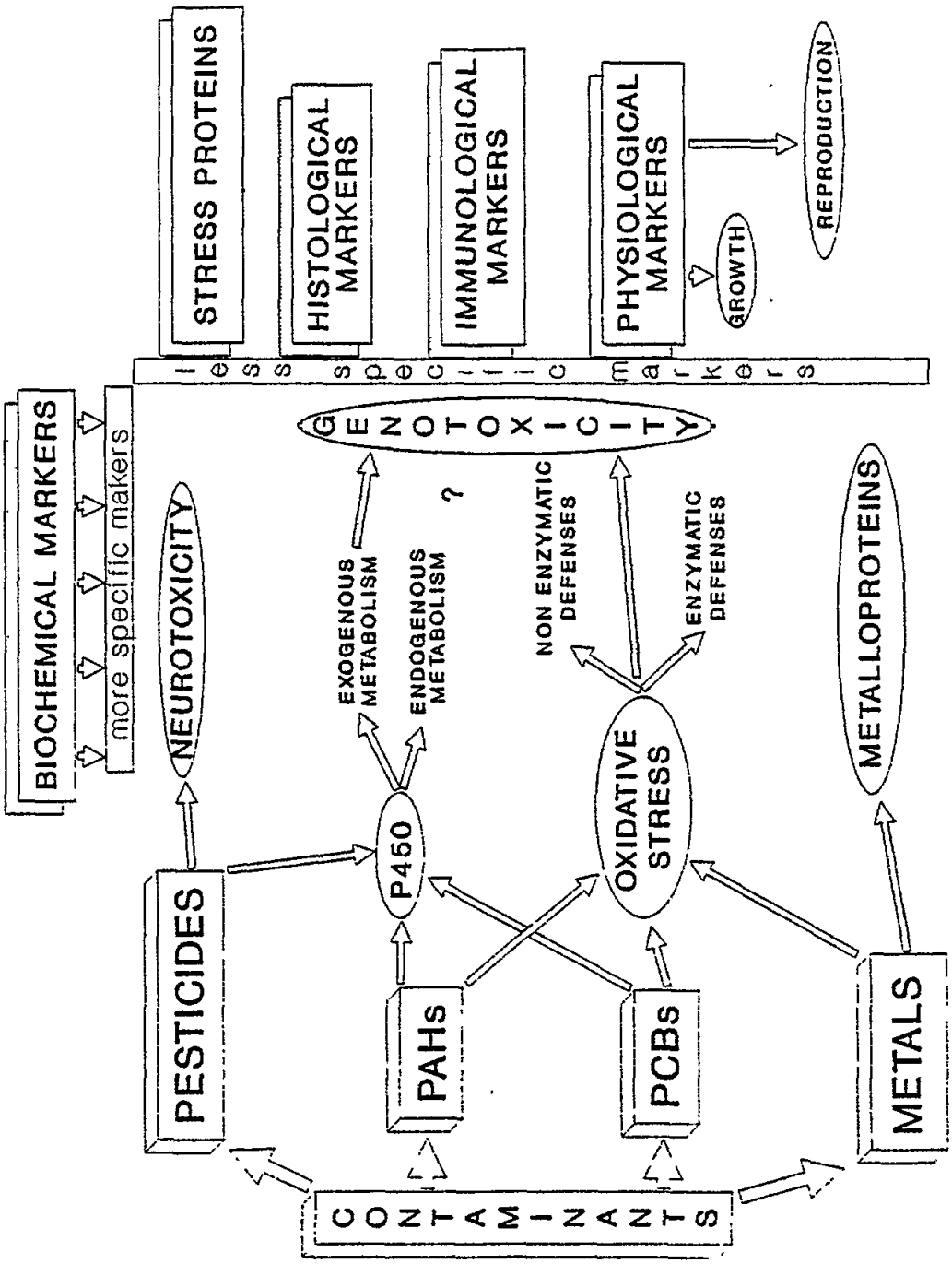

Fig. 2 Relationship between main contaminants and biological markers

exogenous and endogenous dependent metabolism have not been extensively studied in marine organisms. It is important to explore this MFO-related endogenous metabolism since many physiological processes such as reproduction may be disturbed by a change in hormonal status. This may be particularly the case for lower organisms, such as echinoderms and other marine invertebrates, in which the number of iso-enzymes is limited and the degree of substrate specificity therefore lower (Livingstone, 1990; Den Besten et al., 1991).

2.1.2 Phase II (conjugating) enzymes

A sequence of reactions is often involved in the biotransformation of foreign compounds. The first step (phase I) is frequently an oxidative process whereby a polar moiety, such as a hydroxyl group, is introduced. This reaction is usually catalyzed by the cytochrome P450 monooxygenase system. Subsequent reactions involve a second class of enzyme-catalyzed reactions known as phase II or conjugation reactions (Armstrong, 1987; James, 1987; Buhler and Williams, 1988; Foureman, 1989). Phase II enzymes serve to link metabolites to various water-soluble endogenous compounds present in the cell at high concentrations. These reactions generally result in further increases in water solubility and elimination rates, and reduced toxicity of the foreign compound (Buhler and Williams, 1988). The most widely studied and perhaps most important of the phase II enzymes are glutathione transferases, UDP-glucuronosyl-transferases, and sulfotransferases, which link metabolites to glutathione, glucuronic acid, and sulphate respectively (Armstrong, 1987; James, 1987; Buhler and Williams, 1988; Foureman, 1989). Although not always considered to be a phase II enzyme, epoxide hydrolase (EH) catalyzes the addition of a molecule of water to an epoxide formed in phase I (Armstrong, 1987; Foureman, 1989) and is included in this section.

Because some phase II enzymes appear, in some cases, to be influenced by exposure to various foreign compounds (Hammock and Ota, 1983; Andersson et al., 1985; Buhler and Williams, 1988), there has been some recent interest in their potential usefulness as indicators of biomarkers of pollution exposure and/or effects (Collier and Varanasi, 1984; Van Veld and Lee, 1988; Suteau et al., 1988; Lindström-Seppä and Oikari, 1988).

Although biomarker programmes have focused primarily on aquatic systems (Giam, 1977; McIntyre and Pierce, 1980; Bayne et al., 1988), much of our understanding of the characteristics and functions of drug metabolizing enzymes in aquatic species has evolved from the vast mammalian literature and from our knowledge of known similarities in these enzymes among aquatic species and mammals.

At the present time information on the characteristics, functions, and responses of these enzymes in natural populations of organisms may be insufficient to evaluate their full potential as biomarkers of exposure and/or effects. We know that phase II enzymes are present in most species and that assays for their activities are fast, reliable, and reproducible. Some laboratory and field studies indicate that activities of GST, EH and UDPG are influenced by exposure to various environmental contaminants. However, compared to some of the other enzymes and proteins discussed in this chapter, the phase II enzymes have a more restricted use as indicators of either pollution exposure or effects.

2.1.3 The oxidative stress

The occurrence of oxidative stress in marine organisms has been extensively reviewed by many authors (Livingstone et al., 1989c; 1990; Winston, 1991).

Oxidative stress is experienced potentially by all aerobic life when antioxidant defences are overcome by prooxidant forces. Recent evidence indicates that the health of aquatic organisms might also be linked to oxidative stress (Di Giulio et al., 1989). Of particular concern are processes by which environmental contaminants may enhance oxidative stress in aquatic organisms. The epidemiology of highly elevated rates of ideopathic lesions and neoplasia among some populations of aquatic animals inhabiting polluted environments is increasingly related to oxidative stress associated with environmental pollution (Malins et al., 1988). Reactive oxygen species (ROS) are continually produced in the biological system by a number of processes involving both endogenous and foreign compounds. ROS comprise the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$). Potential sources of pollutant-stimulated oxyradical production include redox reactions with transition metals and organic free radicals. These radicals can be formed by two principal pathways (i) redox cycling of certain compounds and (ii) production of contaminants' radical metabolite by MFO. In a redox-cycle, the xenobiotic (quinone, aromatic nitro or amine compound) is reduced by the reductase in a one-electron step to a reactive intermediate which, in turn, is able to reduce O_2 to $O_2^{\cdot-}$: a cycle is thus formed of $O_2^{\cdot-}$ generation at the expense of cellular reducing equivalents, notably NADPH. The cytochrome P-450 and MFO mediated ROS and organic free radical generation (from PAHs, PCBs) represent undesirable "side reactions" of the enzymes (Livingstone et al., 1990). The cytotoxic consequences of oxyradical production include alterations in redox balance, enzyme inactivation, lipid peroxidation, and DNA damage (Winston, 1991).

The extent to which such biological damage occurs depends upon the effectiveness of antioxidant defences which function to remove the oxyradicals. These defences include various low molecular weight free radical scavengers, such as glutathione (GSH), beta-carotene and vitamin A, E, and C, and a number of specific enzymes, viz. superoxide dismutase (SOD catalyses $O_2^{\cdot-}$ reduction), catalase (H_2O_2 reduction), glutathione peroxidase (H_2O_2 and organic peroxides reduction using 2 GSH), and DT-diaphorase which prevents redox cycling of quinones by their two-electron reduction to hydroquinones. The measurement of oxidant mediated responses including both adaptative responses (ie antioxidant inductions) and deleterious effects (such as lipid and DNA alterations) have already proved to be potential markers of oxidative stress in marine organisms exposed to certain pollutants (Wenning et al., 1988; Ribera et al., 1989; 1991; Livingstone et al., 1990; Malins et al., 1990).

2.1.4 Genotoxicity

All the processes involved in contaminant metabolism (sections 2.1.1 and 2.1.2), result in the production of highly reactive radical intermediate byproducts. Such intermediates possess an electrophilic character that confers on them an affinity for the nucleophilic sites of cellular macromolecules like DNA. The interactions between contaminant metabolites and DNA lead to the formation of DNA lesions. If these lesions are not correctly repaired by the nuclear specialized enzymes, it can result to a misexpression of the genome

(e.g. oncogene activation). This characterizes the first step in the chemical carcinogenesis processes (section 2.1.1) and thus the beginning of cell transformation to tumor cell.

Many techniques of in vivo DNA alteration measurement have been developed for mammals and then transferred to marine organism studies (Dunn et al., 1987; Shugart, 1988; Bihari et al., 1990; Herbert and Zahn, 1990). Present results are encouraging further studies, although the use of these methods might sometimes be limited by their sensitivity threshold (Kurelec et al., 1989).

2.1.5 The neurotoxicity

Heavy metals (Baatrup, 1991) and some pesticides (Galgani and Bocquené, 1990) have been reported to perturb neuro-parameters. Organophosphorus and carbamate pesticides which represent about 35% of the total pesticides used in French agriculture, are inhibitors of acetylcholinesterase (AChE), an essential enzyme in the transmission of nerve flux. Recent data reported that this activity measured in vitro in subcellular fractions of fishes were inversely correlated to a pollution gradient in the North Sea (Bocquené and Galgani, 1991). Moreover, Narbonne et al. (1991b) observed, in the Mediterranean sea, a good correlation between AChE activity inhibition in mussel and the level of pollution and particularly the level of metal contamination.

These data suggest that both pesticides (carbamates and organophosphates) and metals seem to inhibite AChE activity in the tissues of fish or mussel exposed to those contaminants. Further studies are needed and especially in the field of pesticide chemistry to perform pesticide analysis in environmental samples. Thus, it could be verified whether AChE inhibition measurement in marine organism tissues could be specifically related to pesticide contamination in the field.

2.1.6 The metalloproteins

In the last few years, emphasis has been given on studies of important biological cations such as zinc and copper, which are toxic at above normal physiological level, and of cadmium and mercury, which are among the most toxic environmental contaminants (Viarengo, 1985; 1989). Heavy metals can be stored in non-toxic forms in tissues as inorganic precipitates, in membrane limited vesicles and in lysosomes. Metals can also be trapped by cytosolic cystein rich proteins called metallothioneins, the synthesis of which increases in response to the accumulation of heavy metals in the cells.

Therefore, metal toxicity appears to be related not to its total tissue concentration, but to the amount of metal free to interact with cell structures (oxidative stress) and/or enzymes, in this way affecting metabolic pathways. Numerous authors have successfully demonstrated the induction of metallothioneins after metal(s) exposure in microcosm or mesocosm study (Viarengo et al., 1988). But this induction is less evident in field studies, and if metallothioneins are to serve as biomarkers in aquatic systems, much more direct study of specific aquatic organisms will be needed (Petering et al., 1990; Garvey, 1990).

2.2 Non specific biomarkers of environmental pollution

2.2.1 Stress proteins

Recently, it has become apparent that all cells undergo alterations in gene expression in response to environmental stressors (For rewiews see Schlesinger et al., 1982; Atkinson and Walden, 1985; Sanders, 1990). This response was initially referred to as the heat shock response because it was discovered upon exposure to elevated temperatures. It is now referred to as the stress protein response since it can be elicited by a variety of physical and chemical stressors including anoxia (Spector et al., 1986), metals (Hammond et al., 1982; Caltabiano et al., 1986), and xenobiotics (Sanders, 1990). Changes in gene expression associated with this response are extremely rapid, and result in the induced synthesis and accumulation of stress proteins.

Many of the stress proteins and the genes that code for them have been sequenced in a wide range of organisms and found to be remarkably conserved (Schlesinger et al., 1982). Although a few stress proteins are found only in cells responding to environmental stressors, most are also present at much lower concentrations under normal conditions where they play a role in normal cellular function (Sanders, 1990). Two stress proteins, hsp60 and hsp70, appear to be involved in protein homeostasis under normal conditions, taking on protective and repair roles upon exposure to adverse environmental conditions (Rothman, 1989; Welch, 1990). For example, members of the large hsp70 family are found in several subcellular compartments where they are catalysts of protein folding and are involved in intercompartmental transport under normal conditions (Chirico et al., 1988; Deshaies et al., 1988; Craig, 1989; Rothman, 1989; Beckmann et al., 1990). In contrast, members of the hsp60 family are found in the mitochondria, chloroplasts, and in procaryotes where they are involved in the folding and assembly of the numerous large enzyme-protein complexes associated with the inner membrane (Cheng et al., 1989; Ostermann et al., 1989). Under adverse environmental conditions, hsp60 and hsp70 may also perform the related functions of renaturing damaged peptides and resolubilizing protein aggregates (Rothman, 1989). It is through this renaturing capability that stress proteins facilitate the repair of proteins and protein complexes associated with critical physiological processes and protect cells from stress induced damage (Gaitanaris et al., 1990; Skowyra et al., 1990).

Stress proteins make ideal candidates as biomarkers for environmental contamination since they are (a) part of the cellular protective response; (b) induced by a wide variety of environmental stressors; and (c) highly conserved in all organisms from bacteria to man (Schlesinger et al., 1982).

Preliminary data, for example in mussels (Sanders, 1990; Sanders et al., 1991), suggest that the accumulation of stress proteins has potential in environmental monitoring and toxicological screening.

We previously mentioned the particular facilities of some aquatic species and especialy mussels to thrive in areas highly contaminated by many different pollutants. Recently Kurelec and Pivčević (1991) have observed that it exists a possible biochemical mechanism that could allow this "multi xenobiotic resistance" (MXR) in mussels. This mechanism of defence seems to be similar to the multi-drug resistance (MDR) mechanism found in tumor cells

that became refractory to treatments with a variety of chemotherapeutic agents. Futher studies are needed to investigate this very interesting mechanism of defense.

2.2.2 Histological markers

Injury to cells initiates a series of molecular/biochemical and also structural changes. Cells are able to survive many types of injury by means of adaptative physiological response. Examples of such adaptations include hypertrophy, atrophy, increased lysosomal autophagy, ageing, neoplastic transformations and accumulation of materials.

The studies on fish histogical modification after exposure to pollutants have mainly focussed on the detection of neoplastic transformation or foci development (Couch et al., 1985). These foci are the first visible modification of cell phenotypes in the process of carcinogenesis.

Moore (1991) extensively reviewed the cellular reactions to pollutants in mussel digestive gland. These reactions result mainly in modifications of the lysosomal system such as its permeability, stability, size, proliferation, and content (lipofuscin, neutral lipids, or metallothioneins). Lipofuscin are byproducts of lipid peroxidation, neutral lipid accumulation is thought to scavenge lipophilic contaminants like PAH and PCB, and metallothioneins scavenge heavy metals (Viarengo et al., 1985). These changes in functional and structural modification of intracellular membranes include also proliferation of smooth endoplasmic reticulum in induction of cytochrome P-450.

All these modifications can be characterised by morphological observations and/or are characterised and quantified using cytochemical technology (Nott and Moore, 1987; Moore, 1991).

The histopathologic alterations have immediate utility as biomarkers of effect, resulting from a variety of chemical contaminants in the environment. They are particularly appropriate since many have been validated in the laboratory and found relevant to field investigations. As such, they provide higher level response signals, since morphological alterations follow earlier biochemical and physiological alterations. The alterations present, in many instances, are an integration of physiological and chemical changes.

2.2.3 Immunological markers

The impact of pollutants on the immunological system of marine organisms has not been extensively studied. However, recent experiments correlate the decrease in immunocompetence in marine animals to the PCB content in their fats (Stone, 1992) or to the pollution gradient (Anderson et al., 1989; Secombe et al., 1991) and report that the immunological parameters of fish exposed to copper was reduced. Such effect may have important incidences on the organism physiology and its defence potential against diseases.

Measurement of the various components or integrated functioning of the immune system is a sensitive indicator that reflects exposure to low concentrations of environmental toxicants in any aquatic or terrestrial animal species. Highly reliable and reproducible tests of the immune function exist for many classes of animals and can be used as screening tools or as a mean

of investigating mechanisms of effect. As standardized reagents become more readily available, tests will become more reproducible and cost effective. Immunological biomarkers have an important role to play in monitoring the health of animals prior to the occurrence of devastating disease outbreaks and as early warning indicators of the potential harm of environmental chemicals. Since many parameters of the immune system are similar in different species, animals may serve as sentinels of potential environmental hazards for humans.

2.2.4 Growth

The rate of growth is a fundamental component of physiological fitness, and therefore represents an important index of environmental pollutant effect. Determination of the energy available for growth (Scope for Growth), based on the physiological analysis of the energy budget (Bayne, 1985; Widdows, 1985), provides an immediate assessment of the energy status of the animal as well as an insight into the individual components (respiration, filtration rate, adenylate energy charge, etc.) which affect the changes in growth rate. The concept of Scope for Growth has been widely used to assess the sublethal biological effects of pollution in marine invertebrates, particularly mussels. In bivalves, a good agreement is seen between this indirect estimate of growth and the more direct determination based on detailed population size-class analysis (Livingstone et al., 1989a).

2.2.5 Reproduction

Studying effects of contaminants' impact on reproductive success is particularly important due to the ecological consequence of such effects on population development and therefore on community. The animal is strongly vulnerable to contaminant exposure in the early stages of its development. For example, the pollution by organotins in the Arcachon bay (France), an important place of oysters farming, has perturbed during many years the settlement of oysters larvae on the substrata. Abberations in the early stages of development have been demonstrated with sea star and sea urchin embryos exposed to environmental contaminants (Pagano et al., 1985; Den Besten et al., 1989). The toxicants may disturb as yet unspecified cellular processes, resulting in embryo malformation.

II. Applications

1. CONCEPTION OF BIOMARKER DEVELOPMENT

Brouwer and coworkers (1990) (Functional Ecology, 4:275-281) proposed a scientific approach related to the successive steps in the development of a biomarker :

(a) Definition of a potential BIOMARKER based on actual data in the research field of toxicology.

(b) Definition of THE SPECIES to work with : the best suitable "sentinel" species (easy collection, representative and well-spread species) that is a function of the ecosystems considered and of the biomarker to be tested.

(c) Selection of the APPROPRIATE TECHNIQUE to measure this biological parameter.

(d) IN VITRO CHARACTERISATION of the biomarker.

(e) TISSUE DISTRIBUTION.

(f) MICROCOSM and/or MESOCOSM studies.

(g) FIELD EXPERIMENTATION.

(h) Possibly, DEVELOPMENT of the marker (automatization of the analysis).

Steps 4 and 5 are not systematicaly needed.
It is obviously a simplified view of the problem and thus a feedback phenomenon may exist at each step of the development.

2. EXAMPLE OF BIOMARKER APPLICATION : THE GICBEM EXPERIENCE

The GICBEM (Groupe Interface Chimie Biologie Ecosystèmes Marins) was formed in late 1986 to investigate the response of marine organisms to exposure to pollutants by both basic research (comparative toxicology) and applied research (establishement of indicators suitable for monitoring the quality of aquatic environments, and in particular the Mediterranean Sea). In brief, research involves identification of any correlation between the degree of contamination, evaluated by measuring a range of pollutants in water, sediment and certain organisms, and biochemical parameters which might respond to the presence of these contaminants. See the paper by Lafaurie et al. in this volume which was also presented at the FAO/UNEP/IOC Workshop on the Biological effects of pollutants on marine organisms (Malta, 10-14 September 1991).

3. BIOMARKER USE AND INTERPRETATION

The summary of information provided in Table 1 suggests that each of the systems discussed in this chapter could be employed as biomarkers. However, there are at least three important considerations which must be borne in mind regarding Table 1.

First, each of the systems in this chapter is a multicomponent system, and the attributes listed reflect the knowledge only for a limited number of these components. Cytochrome P450, for example, refers only to the cytochrome P450 1A proteins; other P450 proteins that might be used as biomarkers could have a different set of qualities. Eventually, separate tables like this should be possible for each of these systems, with entries for each component.

Second, the responses for some of the systems are based largely on knowledge obtained with traditional mammalian species, or other species not of concern in the environment. This is especially true for the heat shock proteins. These two points emphasize the need and value of continued basic research on all systems.

Table 1

Protein/Enzyme Biomarkers.

Attribute	MFO	PHASE II Enzymes	Metal Binding Protein	Heat Shock Protein	Oxidative Damage Response	Achase
Exposure marker	yes	yes	yes	yes	yes	yes
Effects marker	yes	?	?	?	yes	yes
Biological specificity	vertebrates	vertebrates	Eukaryotes	Prokaryotes Eukaryotes	broad	Eukaryotes
Chemical specificity	PAH, planar HHC	PAH, PCB	selected metals	metals organics, inorganics	oxidants, redox-active compounds	metals carbamate and O.P. pesticides
Sensitivity	high	low	high	high	high in plants untested in animals	moderate to high
Time of response	6-24h	days	hours-day	min-hours	variable	min-hours
Reliability	high	high	high	high	untested	high
General or point source	both	both	both	both	both	both
Linked to further effect	yes	yes	yes	yes	yes	yes
Field trials	positive	positive	positive	no data	positive (for those tested)	little data
Assay cost-present	moderate	moderate	expensive	moderate	low to moderate	low to moderate
-future	low	low	moderate	low		low
Ease of assay-present	sophisticated	sophisticated	sophisticated	sophisticated	variable	simple
-future	may be simplified	may be simplified	simple	may be simplified	from easy to difficult	simple
Research needed	yes	yes	yes	yes	yes	yes

Third, there are important considerations regarding the methodology for using protein systems as biomarkers, and the interpretation of changes resulting from chemical exposure. These considerations include the practical aspects involved in methods of analysis and their reliability.

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BIOCHEMICAL MARKERS IN POLLUTION ASSESSMENT: FIELD STUDIES ALONG THE NORTH COAST OF THE MEDITERRANEAN SEA

by

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A B S T R A C T

Multiple biochemical factors were measured in marine organisms representative of Mediterranean ecosystems: the mussel (Mytilus galloprovincialis), the fish (Serranus scriba and Serranus cabrilla) and the plant (Posidonia oceanica) to determine their response and sensitivity to a variety of water pollutants. Test samples were obtained at 16 sites along the north coast of the Mediterranean Sea that were considered to present varying degrees of chemical contamination. Biochemical parameters included both specific markers (induction of drug-metabolizing enzymes, inhibition of acetylcholinesterase) and indicators of cellular damage (lipid peroxidation and calcium content). Physicochemical analyses were performed on samples of sediment and sea water, and polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) residues were assayed in mussel and fish. Various statistical methods were used to compare the biochemical data with the physical/biochemical findings. Overall, contamination by PAHs was well correlated with mixed function oxidases (MFO) activities in the test organisms (mussel, fish and plant). Contamination by PCBs seems to induce the activity of detoxification enzymes and lipid peroxidation. Metal contamination increases lipid peroxidation in the mussel and the calcium concentration in fish gills. Inhibition of acetylcholinesterase activity appears correlated with the pollution gradient. Thus, it is indicated that the use of a range of biochemical markers appears to evaluate the responses of marine organisms to the complex combinations of pollutants found in actual field conditions.

1. INTRODUCTION

Utilization of biochemical factors to evaluate biological responses to pollutants has increased considerably over the past 10 years. The basic concept appeared some 20 years ago, following the discovery that marine organisms possess enzymatic systems specialized in the biotransformation of liposoluble xenobiotics (oxidation by cytochrome P-450-dependent monooxygenases and conjugation by phase II enzymes such as glutathione-transferases) (Bend and James, 1978; James et al., 1979). In addition, the activity of these enzymes can be induced in a very short period of time by exposure to the substrate or compounds with a similar conformation (Payne, 1976; Stegeman, 1981). Various studies, most concerning mammals, have shown that biotransformation enzymes include a number of isoenzymes with different catalytic activities that respond to specific inducers (Parkinson et al.,

1983). This research demonstrated the role of the molecular conformation (planar or globular) in the specificity of induction, in particular for polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). These findings prompted research for biochemical markers of the presence of different types of pollutants. For example, the induction of metallothioneins in marine animals exposed to metal contaminants has been the subject of numerous studies (Viarengo et al., 1980; Thomas et al., 1985; Overnell et al., 1987).

More recently, the severity of coastal ecosystem contamination by pesticides (Kjolholt, 1985) has led to renewed interest in acetylcholinesterase activity (AChE), which can be inhibited by organophosphorus pesticides and carbamates. AChE activity had previously been demonstrated in several marine organisms (Hilderbrand et al., 1974; Schoor and Brausch, 1980).

In addition to these specific effects, contaminants can induce alterations in cell metabolism which manifest as imbalances in oxidation-reduction reactions and oxidative stress. These cellular alterations can be assessed by evaluating lipid peroxidation and cellular calcium (Viarengo et al., 1988a). Thus identification of valid biochemical indicators requires thorough knowledge of the molecular mechanisms involved in the responses of organisms, representative of marine ecosystems, to exposure to various pollutants.

The G.I.C.B.E.M. (Groupe Interface Chimie Biologie Ecosystèmes Marins) was formed in late 1986 to investigate the response of marine organisms to exposure to pollutants by both basic research (comparative toxicology) and applied research (establishment of indicators suitable for monitoring the quality of aquatic environments, and in particular the Mediterranean Sea). In brief, research involves identification of any correlations between the degree of contamination, evaluated by measuring a range pollutants in water, sediment and certain organisms, and biochemical parameters which might respond to the presence of these contaminants.

The species studied satisfied several criteria: wide geographic distribution in the Mediterranean, sedentarity, and representativity of the coastal ecosystem. While fairly extensive information is available on biotransformation enzymes in the mussel Mytilus galloprovincialis (Suteau and Narbonne, 1988), data are much more recent for sedentary fish (Serranus scriba and S. cabrilla) found near Posidonia oceanica, a marine plant characteristic of Mediterranean coastal ecosystems (Mathieu, 1990). In addition, no study of this type had ever been performed on this marine plant. Selection of the geographic sampling sites and contaminants was based on data from the Réseau National d'Observation (1988 RNO report). The most important contaminants are PAHs, PCBs and metals. The sampling sites ranged from protected, only slightly polluted zones (natural reserves) to zones of severe pollution of various origins (river deposits, sewage, intensive port and industrial activities). Samples were collected during 5 missions on board the oceanographic research vessels "Winnaretta Singer" of the Oceanographic Museum of Monaco, the "Roselys II" (IFREMER), the "Antédon" and the "Korotneff" (CNRS). Three missions were carried out in June/July of 1987, 1988 and 1989; two autumn missions were carried out in November of 1988 and 1989. Partial results have been published previously (Ribera et al., 1989; Raoux et al., 1989; Garrigues et al., 1990; Lafaurie et al., 1990; Lemaire, 1990; Mathieu, 1990; Narbonne et al., 1991; Raoux, 1991).

2. MATERIAL AND METHODS

RNO data were used to select a total of 16 sites off the coast of Corsica and between the Gulf of Fos and the Italian Riviera (Fig. 1). Sampling stations ranged from minimally polluted sites (Corsican coast) to moderately polluted areas (continental sites near seaside resorts: Cannes, Portofino), and zones of intensive industrial and port activities (Fos, Marseilles, Toulon, Genoa).

2.1 Water and sediment samples

Sediment samples were collected by skin divers in zones around mussel beds at depths of 5 to 10 meters, in proximity to areas of Posidonia. After removal of any organic matter, the superficial layer of sediment (approximately 2 cm) was collected over an area of about 0.1 m^2. In each case, 2 samples were taken and stored at - 20°C. Sea water samples (8 to 12 litres) were taken in glass bottles opened by the divers at a depth of 5 meters, then kept refrigerated in the dark after addition of hexane.

2.2 Biological samples

Combers (Serranus scriba and S. cabrilla) were selected because of their sedentarity and their simultaneous functional hermaphrodism, which has the advantage of eliminating any sex-linked variations in biochemical factors (Mathieu, 1990). Fish were caught by fishing (10 individuals per site) and immediately sacrificed. The dissected livers were homogenized in a volume of 10 mM Tris buffer with 250 mM sucrose, pH 7.4, containing 20% glycerol and 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF). The homogenates were stored in liquid nitrogen along with the specimens of fish muscle and gills.

Mussels (Mytilus galloprovincialis) were collected by skin divers, opened and classified by sex. Dissection consisted of ablation of the foot, byssus, crystalline style, and the adductor and mantle retractor muscles. One male individual and one female individual were pooled to form a sample. Ten samples per site were conserved in liquid nitrogen. In some cases, 5 individuals were conserved for calcium assays, and the adductor muscles were stored for AChE analysis. One hundred individuals were conserved for chemical analyses.

Samples of Posidonia oceanica were collected by skin divers. On board, 25 g fractions of etiolated foliar non-chlorophyllian tissues were abundantly rinsed, washed 3 times with a buffer, and crushed into power in a mortar with liquid nitrogen. Samples were then stored in liquid nitrogen.

2.3 Physicochemical analyses

2.3.1 Treatment of sediment

After removal of organic matter by floatation, sediment samples were screened to separate the fine fractions from coarse fractions, then dried. Treatment depended on the parameter to be measured: for PAH assays, Soxhlet extraction using dichloromethane was followed by purification on a column of Florisil (Garrigues et al., 1987); for PCBs, Soxhlet extraction with hexane was followed by purification through a mixed column of Al_2O_3-SiO_2.

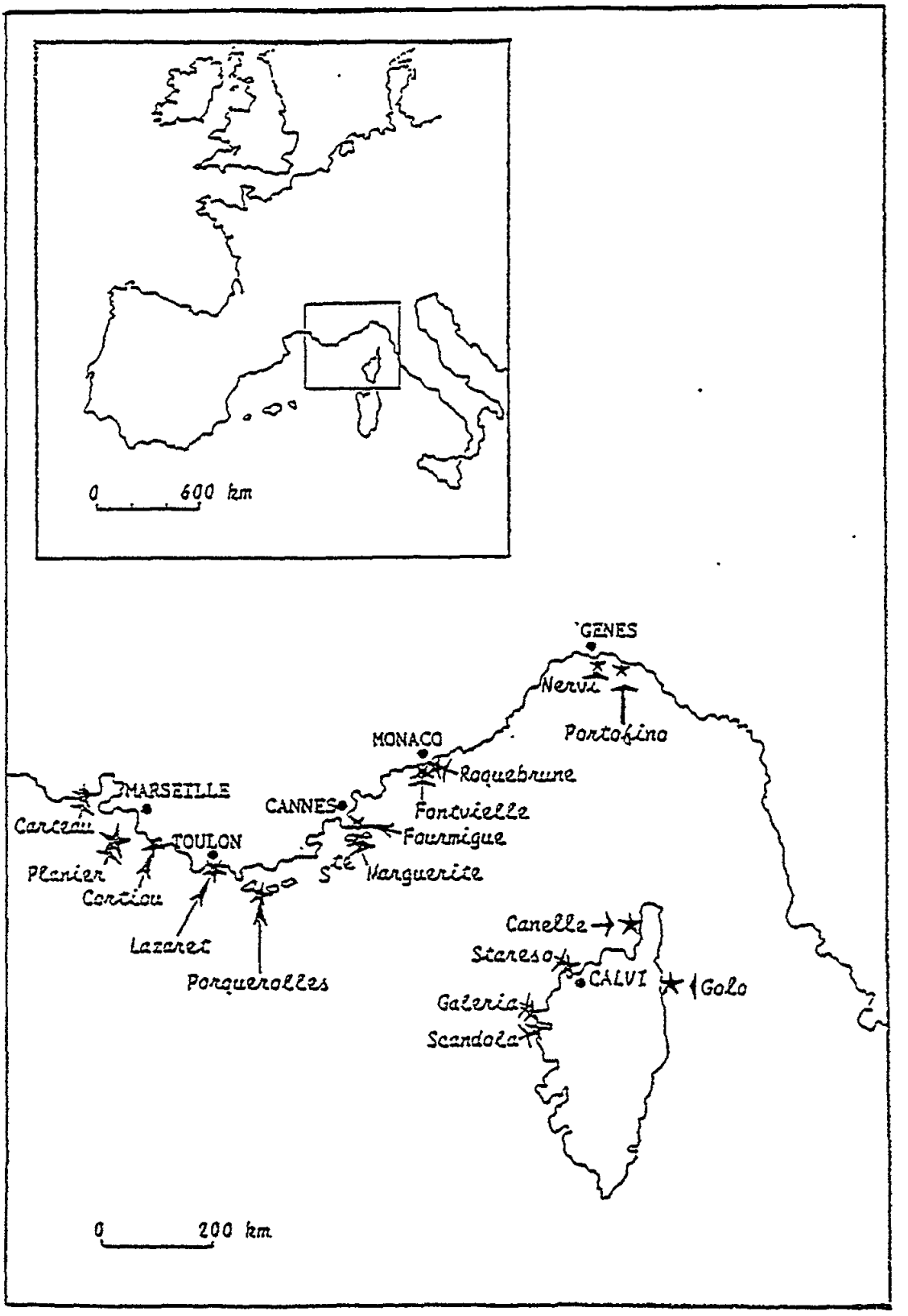

Fig. 1 Sampling sites in the north west coast of the Mediterranean sea

2.3.2 Treatment of sea water

Sea water samples (4 to 8 litres) underwent triple extraction (solvent/solvent) using pentane for PAH assays and hexane for PCB assays.

2.3.3 Treatment of biological samples

Samples of mussel and fish were freeze-dried in the laboratory. For PAH assays, Soxhlet extraction and purification were performed in a single step using pentane as the solvent; purified and deactivated Florisil was added to the sample (Michel, 1983). The extract obtained was reconcentrated dry, in a Rotavapor, under a stream of nitrogen, and recovered in acetonitrile. Deuterated benzo(a)anthracene was used as the internal standard. For PCB assays, extraction was performed as described for sediment samples.

2.3.4 Quantification of parameters

The 12 PAHs recognized as major pollutants (phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, perylene, benzo(k) fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-C,D)pyrene) were separated and assayed by high performance liquid chromatography (Vydac 201 TP 54 column, 25 cm x 4.6 mm id) using a linear gradient of solvents CH_3CN/H_2O with programed fluorimetric detection (Perkin-Elmer LS5) (Soclo et al., 1986). PCBs were analyzed by gas phase chromatography (Varian 3400, capillary column DB5 60m and DB210 30m) using a Ni 63 electron detector (Monod and Arnoux, 1979). The 7 PCB congeners used for computations (CB 28, 52, 101, 118, 153, 138 and 180, according to the classification of Ballschmitter and Zell, 1980) are the most representative of the industrial mixtures used in France (DP5 and DP6). This methodology is recommended by the EEC Community Bureau of Reference (BCR). Metals were analyzed by fluorescence X-ray spectrometry according to the method of Lapaquellerie. Particulate organic carbon in sediment was evaluated as described by Etcheber (1983).

2.4 Biochemical analyses

2.4.1 Biotransformation activities

Homogenates of thawed fish liver were centrifuged at 10,000 x g for 15 min and the supernatants collected were centrifuged at 105,000 x g for 60 min. The microsomal pellet was resuspended in a buffer of 10 mM Tris, 250 mM sucrose, pH 7.4. Ethoxyresorufin-O-deethylase (EROD) activity in this fraction was measured fluorimetrically as described by Burke and Mayer (1974); the cytochrome P-450 concentration was determined by the method of Estabrook and Werringloer (1978). Glutathione S-transferase (GST) activity in the postmicrosomal supernatant (cytosolic fraction) was assayed by the colorimetric technique of Habig et al. (1974), using 1-Chloro-2,4 Dinitro Benzene (CDNB) as the substrate. In both fractions, proteins were assayed according to Lowry et al. (1951).

Samples of mussels were homogenized in a buffer of 50 mM K_2HPO_4, pH 7.5, supplemented with 1 mM EDTA and 15% glycerol. The homogenate was centrifuged at 9,000 x g for 30 min. The supernatant was then centrifuged at 100,000 g for 60 min, and the microsomal pellet was resuspended in the buffer. Benzo(a)pyrene monooxygenase (BaPMO) activity was evaluated by the radiometric technique of Van Cantfort et al. (1977). Epoxide hydrolase (EH) activity was

measured radiometrically according to Oesch et al. (1971) using styrene oxide as the substrate. Proteins were assayed according to Lowry et al. (1951); cytochrome P-450 was assayed as described by Estabrook and Werringloer (1978).

Microsomal fractions of Posidonia were prepared using a buffer solution of 250 mM Tris, pH 8.4 containing 20% glycerol, 5 mM EDTA, 10 mM mercaptoethanol, 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF), and Polyvinyl Pyrrolidone (PVP) (0.5 g/100 ml). The microsomal pellet obtained by centrifugation at 100,000 x g was resuspended in an 0.1 M phosphate buffer, pH 7.4 containing 10% glycerol and 1.5 mM mercaptoethanol. Monooxygenase activities were measured by incubating the microsomes with NADPH and radioactive (cinnamic acid or lauric acid) or nonradioactive substrates (ethoxycoumarin). In the first case, the metabolites formed were separated from the substrates by HPLC; in the second case, the metabolite was assayed by fluorescence (Salaün et al., 1986). Cytochrome P-450 was evaluated as described by Estabrook and Werringloer (1978).

2.4.2 Lipid peroxidation activities

Samples of whole mussel were homogenized in a phosphate buffer (without EDTA) and the microsomal fraction was prepared by ultracentrifugation. The concentration of lipid peroxides in this membrane fraction (without activation) was evaluated by the reaction to thiobarbituric acid (Buege and Aust, 1978) and expressed as malonedialdehyde (MDA) equivalent (Livingstone et al., 1990).

2.4.3 Intracellular calcium

Samples of whole mussel and fish gills were thawed, freeze-dried, then mineralized by attack with nitric acid. Calcium was measured by flame spectrophotometry and the concentration was expressed in g/g dry weight.

2.4.4 Acetylcholinesterase activity (AChE)

AChE activity was measured in fractions of fish filet muscles or pools of mussel adductor muscles. Muscle samples were homogeneized in 0.1 M Tris buffer, pH 8, using an Ultraturax, and the homogenate was centrifuged at 25,000 x g. The supernatant was filtered through Millipore filters (0.45) and stored at -20°C (Galgani and Bocquené, 1990). AChE activity was measured by the Ellman method modified for the microplate technique (Galgani and Bocquené, 1991). The reaction was initiated by addition of the substrate, and enzymatic activity was measured by determining kinetics at 405 nm.

2.5 Data analysis

Biochemical factors measured at each site during each mission were expressed as the mean of all samples. Calculation of the standard deviation of the residual error (SE) for all parameters allowed evaluation of the accuracy of measurement. Means were compared by the method of the smallest significant difference (ssd) according to the formula: $ssd = SE \sqrt{(2/n.t\alpha)}$ where n is the number of repetitions and $t\alpha$ is the value of t for the threshold of significance selected (in this case 0.05) and the number of degrees of freedom corresponding to SE (Legay et al., 1966). From 1 to 5 mean values were obtained for a given site, depending on the number of missions and the number of parameters measured there.

These data allowed determination of the annual intra-site variation (% of variation between the largest and the smallest value obtained for two missions in June/July) and the seasonal intra-site variation (% of variation between the values obtained in June/July and in November of the same year). Mean annual (CAV) and seasonal variations (CSV) were calculated for all sites considered together. Inter-site variation was calculated by establishing the mean value for each parameter at each site based on data from all missions. The amplitude of response of a parameter was calculated by subtracting the lowest value thus obtained from the highest value. The inter-site discriminator factor was obtained by dividing the amplitude by the smallest significant difference (ssd).

For physicochemical measurements in water, sediment, and the various organisms, a mean value was calculated for each site using the data obtained during the different missions.

Correlations between physicochemical and biochemical parameters were calculated as indicated by Schwartz (1963). However, not all parameters were measured at all sites. Furthermore, the number of sampling sites increased between the first and the last mission, as did the number of biochemical parameters investigated. In addition, certain measurements were only performed on a preliminary basis (AChE, MFO for Posidonia), which limits the value of any conclusions. Finally, while extensive chemical data were available for sediment samples, measurements of tissue contaminant concentrations were less numerous.

3. RESULTS

3.1 Sensitivity and discriminator factor of biochemical markers (Table 1)

The values presented in Table 1 allow comparison of the sensitivities of the intra- and inter-site factors of variation for the different biochemical markers measured in the marine organisms. In the mussel, the maximum value for BaPMO activity, measured near Toulon, was 3.8 times higher than the lowest activity, measured at Scandola (Corsica). The amplitude of response was 6 times the ssd. The coefficient of intra-site variability was around 30% (approximately 2 times the ssd). For P-450, the amplitude of the inter-site response was 2.2 times the ssd; the intra-site seasonal variation was 22%. Inter-site variation in EH was low (less than 2 times the ssd) while the intra-site variability was close to 20%. For MDA, the intra-site variability was about 30%, and the inter-site amplitude was 2.4 times the ssd. Although the amplitude of response for AChE activity was low (1.7), the discriminator factor was 3.6. Intracellular calcium assays gave a high amplitude of response between the sampling sites of La Fourmigue and Stareso (6.6), with a discriminator factor of 5; intra-site variation was approximately 40%.

In fish, the amplitudes of responses for biotransformation enzymes were high (5 to 8-fold for EROD, and 5-fold for GST); the discriminator factors were around 3. For these parameters, intra-site variations ranged from 35%-50%. The inter-site amplitudes of response were relatively low for AChE and the calcium concentration in gills. Intra-site variations could not be assessed.

Table 1

Maximum and minimum site means, total ranges among site means, averages of the annual (CAV) and seasonal (CSV) site variatons, smallest significant differences (ssd) and discriminatory power for selected measures of biochemical response in some marine organisms.

	Number of measures	Maximum mean	Minimum mean	Amplitude of response	Response factor	ssd	Discriminatory power	CSV mean (%)	CAV mean (%)
Mytilus galloprovincialis									
BaPMO (pmol/mn/mg Prot)	41	57	15	42	3.8	7.0	6.0	29	35
P-450 (pmol/mg Prot)	18	115	37	78	3.1	36	2.2	22	-
EH (nmol/mn/mg Prot)	37	6.4	3.7	2.7	1.7	1.5	1.8	23	22
MDA (nmol/mg Prot)	18	3.3	1.1	2.2	3.0	0.9	2.4	32	-
AChE (U/mn/mg Prot)	7	102	58	44	1.7	12	3.6	-	-
Ca++ (µmol/g tissu)	15	185	28	157	6.6	30	5.2	-	38
Serranus scriba									
EROD (pmol/mn/mg Prot)	13	76	9	67	8.4	26	2.6	-	40
P-450 (pmol/mg Prot)	6	414	99	315	4.1	193	1.6	-	-
GST (nmol/mn/mg Prot)	21	201	35	166	5.7	56	2.9	-	51
Serranus cabrilla									
EROD (pmol/mn/mg Prot)	23	668	132	536	5.0	173	3.1	34	37
P-450 (pmol/mg Prot)	8	523	156	367	3.3	171	2.1	-	-
GST (nmol/mn/mg Prot)	23	258	49	209	5.2	70	2.9	21	35
AChE (U/mn/mg Prot)	8	12.4	6.7	5.7	1.8	2.3	2.4	-	-
Ca++ (µmol/g tissu)	9	1250	850	400	1.4	235	1.7	-	-
Posidonia oceanica									
ECOD (pmol/mn/ml)	5	7.1	2.1	5	3.4	1.7	2.9		
LAH (pmol/mn/ml)	5	19.5	5.4	14.1	3.6	4.9	2.8		
CA4H (pmol/mn/ml)	5	22.2	9.5	12.7	2.3	5.1	2.5		
P-450 (pmol/ml)	5	133	71	62	1.8	24	2.5		

MFO activities in Posidonia showed amplitudes of response of 2.3 to 3.6; the discriminator factors ranged from 2.5 to 2.9.

3.2 Correlations between biochemical markers and pollutant concentrations measured in the environment and in organisms

In order to better assess the mechanism of response of biochemical markers to various pollutants, correlations were sought with certain molecular families of PAHs and PCBs. For this purpose, we singled out heavy PAHs with a molecular weight over 228 containing more than 4 aromatic nuclei (starting with perylene) and diortho-substituted PCBs classified as globular inducers (congeners CB 52, 101, 153, 138, 180). In all cases, the correlations were calculated between the biochemical measurements and the logarithm of the pollutant concentration. Table 2 indicates the type of response expected as a function of the pollution gradient, based on laboratory studies or literature data.

3.2.1 Biochemical measurements in the mussel

As concerns BaPMO activity, a very high correlation coefficient was obtained with the total PAH content of sediment. The correlation with the PAH heavy fraction was more or less the same. A strong correlation was also observed with tissular PAH levels. By contrast, there was no relation with the PAH concentration of the water. Weaker but still significant correlations were also noted with the total PCB content, the metal concentrations, and the fine particulate content of sediment. Cytochrome P-450 and EH values were not correlated with any contaminant measurements. Lipid peroxidation activity (MDA) was significantly correlated with the PCB levels in water and sediment as well as with the presence of metals (especially lead) in sediment. AChE activity appears to decrease as a function of the gradient of sediment pollution by PAHs and especially metals. Intracellular calcium was significantly correlated with tissue PAH levels and the PCB concentration in water.

3.2.2 Biochemical measurements in fish

P-450 concentrations in both species of combers were correlated with the PAH concentration of sediment samples. However, only the EROD activity of Serranus cabrilla was significantly correlated with the PAH levels in water and sediment. In this case, a higher correlation coefficient was obtained with the PAH heavy fraction. In S. scriba, a strong correlation was observed between the PCB level in water, EROD activity, and the P-450 concentration. In S. cabrilla, P-450 values were strongly correlated with the PCB levels found in the organism. In sediment samples, the correlation coefficient was higher with the diortho-substituted congeners than with total PCB concentrations. A good correlation was also observed with metal contamination, and in particular lead and nickel. In both species of fish, GST activity was correlated with the PCB content of the sea water. In S. cabrilla, GST activity was also strongly correlated with tissue PCB levels and, to a lesser extent, with the PCB level in the sediment samples. A relation was also found between contamination by metals, and in particular lead, and the concentration of particulate organic carbon. Inhibition of AChE activity was significantly linked to the PAH content of the sea water and the metal concentration in sediment (especially nickel). The calcium concentration in fish gills was strongly correlated with sediment contamination by metals, and particularly copper and zinc.

Table 2

Rank correlations between measures of chemical parameters and concentrations of a range of contamninants in water sediments and some organisms.

		Sediments		Water	Orga-nisms	Sediments		Water	Orga-nisms	Sediments					Sediments	
	n	total PAH	heavy PAH	total PAH	heavy PAH	total PCB	diortho PCB	total PCB	total PCB	Total Metals	Copper	Nickel	Lead	Zinc	%POC	% fine
n		16	16	10	8	14	14	11	16	13	13	13	13	13	16	16
Mytilus galloprovin-cialis																
BaPMO (+)	16	.870	.876	.252	.830	.697	.677	.606	.326	.631	.528	.408	.714	.422	.547	.654
EH (+)	15	-.167	-.169	.207	.359	.386	.309	.177	-.168	-.011	-.002	-.348	-.131	0.69	.353	.179
P-450 (+)	13	-.081	-.089	.034	-.404	-.062	-.058	-.272	-.088	-.290	.161	-.270	-.351	0.22	.124	.027
MDA (+)	13	.146	.175	.276	.532	.676	.674	.627	-.234	.569	.473	-.288	.687	.524	.144	.008
AChE (-)	7	-.588	-.596	ND	ND	-.280	-.158	.307	.031	.865	-.330	-.458	-.450	-.483	-.799	-.853
Ca++ (+)	10	.444	.456	.184	.770	.255	.604	.765	.348	.092	-.176	.033	.373	-.264	.272	.299
Serranus scriba																
EROD (+)																
P-450 (+)	9	.307	.316	.259	ND	.486	.549	.866	.151	.472	.154	.626	.691	.100	.189	.176
GST (+)	6	.643	.659	-.339	ND	.417	.371	.699	-.458	-.180	-.720	-.043	-.036	-.487	.571	.791
	11	.424	.446	-.010	ND	.274	.274	.608	-.278	-.069	-.398	.177	.162	-.369	.347	.560
Serranus cabrilla																
EROD (+)	11	.647	.677	.640	ND	.413	.437	.352	.259	.500	.524	.218	.544	.415	.422	.507
P-450 (+)	6	.693	.678	.434	ND	.647	.708	.475	.936	.711	.578	.805	.771	.579	-.138	.094
GST (+)	13	.539	.539	.534	ND	.636	.623	.689	.939	.671	.588	.601	.674	.486	.692	.587
AChE (-)	8	-.367	-.333	-.666	ND	.653	.706	.006	-.164	-.614	-.034	-.971	-.358	-.090	.007	-.064
Ca++ (+)	9	-.114	-.098	.221	ND	.099	.231	.105	.244	.563	.819	.313	.522	.723	-.396	-.268
Posidonia oceanica																
ECOD (+)	5	.884	.895	-.326	ND	-.217	-.074	.551	ND	-.361	-.281	-.495	-.232	-.347	-.409	-.536
LAH (-)	5	-.416	-.491	-.343	ND	-.631	-.576	.234	ND	-.223	-.446	-.416	-.216	-.412	-.320	-.656
CA4H (+)	5	.707	.712	-.497	ND	-.268	-.243	.238	ND	-.357	-.243	-.478	-.244	-.221	-.423	-.416
P-450 (+)	5	-.152	-.212	-.728	ND	-.934	-.914	.430	ND	-.763	-.869	-.877	-.714	-.789	-.492	-.315

3.2.3 Biochemical measurements in Posidonia

In this marine plant, a strong correlation was noted between ECOD and CA4H activities and sediment pollution by PAHs, and especially by the PAH heavy fraction. The P-450 level and Laurate Hydroxylase (LAH) activity were negatively correlated with the PCB and metal concentrations in sediment.

4. DISCUSSION

4.1 Physicochemical parameters

Analysis of the physicochemical data obtained for various compartments of the marine environment calls forth several preliminary remarks. Firstly, the PAH content of sediment appears relatively independent of the percentage of fine particles smaller than 63 µ. Recent complementary research (Raoux, 1991) has demonstrated that PAH are distributed throughout all sediment fractions, but that the heaviest compounds (starting with benzo(f) fluoranthrene) are preferentially bound to the fine particles smaller than 15 µ. These fine particles have the greatest bioavailability, as reflected by the high correlation between the PAH levels in mussels and the concentrations of fine particles and particulate organic carbon in sediment. This confirms the observations of Chapman et al. (1987) on the relations between sediment toxicity, the percentage of fine particles, and the particulate organic carbon concentration. Sediment thus appears to be the major vector of contamination, as previously reported by other authors (Elder et al., 1979; Landrum, 1989).

4.2 Responses of biochemical markers

4.2.1 Cytochrome P-450 and MFO

Induction of mixed-function oxygenases (MFOs) is one of the most widely used parameters for evaluation of water pollution (Payne et al., 1987). Numerous studies have already been published on laboratory and field studies. We therefore compared our findings with recent multiparametric field studies on several test organisms, and in particular:

- Platichthys flesus and Mytilus edulis collected at different sites of Langesundfjord, presenting a gradient of contamination by PAHs and PCBs, as described at the Oslo Workshop (Bayne et al., 1988)
- Pseudopleuronectes americanus collected at various points of the Massachusetts coast (Stegeman et al., 1987)
- Platichthys stellatus collected at various points in San Francisco Bay (Long and Buchman, 1990)

We observed high amplitudes of responses for EROD activities in fish, and our values lie within the range of responses reported in the above-mentioned studies (3.05 in P. americanus, 4.6 in P. stellatus, 14 in P. flesus). A relatively limited, intrinsic variability of this parameter gives a rather high discriminator factor close to 3, which is comparable to that obtained for P. stellatus.

Seasonal variations of EROD in S. cabrilla (of the order of 35%) were higher than the ssd. Seasonal variations in MFO activity linked to changes in temperature and reproductive status have previously been demonstrated in numerous fish species in the Atlantic Ocean (Payne and Fancey, 1982), semi-tropical waters (James and Little, 1981), and the Mediterranean (Lafaurie et al., 1990; Mathieu, 1990; Mathieu et al., 1991). In the present study, assays of cytochrome P-450, a protein which catalyzes MFO activities, showed a smaller amplitude of response (from 3 to 4), which is slightly higher than the values reported by other authors (1.6 to 2.9). This gives a low discriminator factor of 2 or less, and 1 for P. stellatus. Inter-site differences in total cytochrome P-450 levels were thus of low amplitude. This is related to the existence of several isoenzymes in fish liver, including the form P-450E, which is particularly sensitive to induction by planar compounds such as PAHs, certain PCB congeners, and beta naphthoflavone (Gooch et al., 1989). This isoform accounts for less than 2% of total P-450 in P. flesus, but its amplitude of response is over 13, both in this species and in P. stellatus. This specific response is thus strongly buffered during measurement of total P-450. The results obtained at the Oslo Woskshop (1986) reveal a good correlation between MFO activities (EROD and P-450) and the gradient of contamination, in particular by high molecular weight hydrocarbons (PAH and PCB) (Addison and Edwards, 1988). These observations are confirmed by our findings, and namely data obtained for S. cabrilla.

In Mytilus galloprovincialis, BaPMO is the only cytochrome P-450-dependent enzymatic activity that appears suitable for use in field studies at this time. Our results are comparable to those reported for M. edulis at the Oslo Workshop (Suteau et al., 1988a). The lowest BaPMO value measured in Norway was 10 pmol min^{-1} mg^{-1} protein while the value at the most polluted site was 30 pmol min^{-1} mg^{-1} protein. These figures correspond to an amplitude of 3 (20 pmol) and an ssd of 16 pmol in the open environment. In our study, even the lowest BaPMO activity measured was higher (15 pmol min^{-1} mg^{-1} protein), as was the amplitude of response (3.8). The ssd, measured for individuals collected in the open environment, was 5.0 pmol owing the greater number of values utilized (41 instead of 4, and 10 repetitions versus only 4). We thus obtained a high discriminator factor (6). The intra-site variation linked to the season was around 30%; this figure is lower than the seasonal variations we measured previously in the Atlantic (approximately 50%) (Suteau et al., 1985). BaPMO activity was strongly correlated with the PAH concentration in sediment (0.870) and in tissues (0.830). Our PAH values were thus comparable to that obtained in Norway (0.876).

Recent aquarium experiments using labeled BaP (Narbonne et al., 1992) have demonstrated good pollutant transfer from water into tissues. However, when the contaminated particulate fraction was put back into suspension, accumulation from sediment pollutants became preponderant. In the field, existence of high inter-site differences in the particulate fraction contents of water explains the low correlation with PAH levels in sediment. A certain correlation exists between BaPMO activity and the PCB level in sediment (0.697), but there was no relation with tissue concentrations of PCB. This may be due to the fact that MFO activities in the mussel are not sensitive to induction by globular compounds such as phenobarbital (Livingstone et al., 1988) or diortho-substituted PCB congeners (Suteau et al., 1988b). In the mussel, P-450 gave a moderate discriminator factor, but variations were not correlated with either the pollution gradient or BaPMO activity. The absence of correlation between MFO activity and the P-450 concentration has also been

reported by other authors (Livingstone, 1988). However, available information about the isoforms of P-450 in the mussel is still insufficient to be able to specifically relate enzymatic activities and isoforms (Kirchin et al., 1987). In addition, recent laboratory studies have demonstrated that BaP metabolism in the mussel is largely dependent on oxidation pathways other than that linked to cytochrome P-450 (Michel et al., in press).

Our results reveal the existence of MFO activities in Posidonia oceanica. Russel (1971) implicated cytochrome P-450 in the conversion of trans-cinnamate into 4-hydroxycinnamate in pea seedlings. Reichhart et al. (1980) reported that this enzymatic activity is increased in higher plants by such classic inducers of P-450 as phenobarbital, ethanol, and certain herbicides. In our study, ECOD and CA4H activities were apparently induced by the presence of PAHs in sediment; they were not correlated with PCB levels. On the contrary, Laurate Hydroxylase (LAH) activity and the P-450 concentration were decreased in the presence of environmental PCB. These preliminary observations require confirmation.

In plants, ECOD and CA4H activities are generally induced by globular compounds. By contrast, in this study, they were induced by planar PAHs and not by ortho-substituted PCBs. Recent investigations by J.P. Salaün (unpublished data) on Posidonia tissue systems in the absence and in the presence of aminopyrine (a globular compound) reveal that microsomal LAH activity is reduced compared to untreated tissues. However, the interactions between planar and globular compounds at the level of P-450 isoforms in plants differ from those in animals. This is compatible with the negative relation between LAH and ortho-substituted PCBs. The good amplitude of response and discriminator factors (considerably higher than 2) have prompted us to continue investigations on these biochemical markers in this marine plant.

4.2.2 Conjugation activities

Conjugation activities with glutathione occur in numerous hepatic and extra-hepatic tissues in marine animals (James et al., 1979). In mammals, these activities can be induced by planar (PAH) or globular compounds (phenobarbital) (Clifton and Kaplowitz, 1978). In fish, induction has only been observed with planar compounds, such as 3-methyl-cholanthrene (3MC) in Pleuronectes platessa (George and Young, 1986), and BaP in P. flesus (Van Veld and Lee, 1988). However, Anderson et al. (1985) failed to observe induction of GST activity in Salmo gairdnieri 6 weeks after treatment with beta naphthoflavone (a planar compound) or Clophen A 50 (a commercial PCB mixture). Strong specific differences thus appear to exist in the GST response to inducers. The amplitude of response with S. scriba and S. cabrilla was over 5, and the discriminator factor was close to 3. When GST was induced by BaP injection i.p. in Dicentrarchus labrax (Lemaire, 1990), the amplitude of response was 2.5, with peak activity occurring within 24 hours of treatment (1-Chloro-2,4 Dinitro Benzene (CDNB) used as a substrate).

At the Oslo Workshop, Van Veld and Lee (1988) described their investigations on the response of GST activity in the intestine of P. flesus using CNDB as the substrate. The maximum amplitude of response was 2.2, with a weak correlation with the pollution gradient. A low correlation was observed in S. scriba between GST activity and the degree of environmental pollution by PAHs or PCBs. By contrast, in S. cabrilla, significant correlations were observed with the PCB levels in water, sediment, and especially tissues. Our

findings are similar to those reported by Lee (1988), who cited a coefficient of correlation of 0.795 between tissue PCB levels and GST activity in the hepatopancreas of Carcinus maenas. The response of GST to inducers thus differs as a function of the species.

In the mussel, EH activity presented an amplitude of response of 1.7 and a discriminator factor under 2. In the Norwegian mesocosm experiment, the amplitude of response was 1.9 and the discriminator factor was 1.6 (Suteau et al., 1988a). Aquarium studies on EH gave an amplitude of the order of 1.8 after exposure to planar or globular PCB congeners (Suteau et al., 1988b). In our study, the season had only a slight influence on EH activity (23%), as reported previously (Suteau et al., 1985). However, in the field, EH is not correlated with the environmental level of pollution , in agreement with the results presented at Oslo.

4.2.3 AChE activity

Measurement of AChE activity was begun in 1989 as part of a feasibility study for an environmental monitoring programme. Preliminary in vitro research (Galgani and Bocquené, 1990) had revealed slight inhibition of AChE in whole mussel exposed to carbamates and organophosphorus compounds; responses were higher with preparations of fish muscle. The results in this study concern both mussel and fish muscles. The amplitude of response was close to 1.8 in both cases, although the discriminator factor was higher in mussel than in fish. Correlations with physicochemical parameters were studied only on an experimental basis, because we did not measure the level of pesticide pollution. However, certain correlations were observed between inhibition of AChE activity and the gradient of pollution by PAHs and metals.

4.2.4 Lipid peroxidation and intracellular calcium

Several studies have demonstrated the capacity of certain marine pollutants to induce cellular oxidative stress. Wofford and Thomas (1988) reported stimulation of lipid peroxidation in extracts of Micropogonias undulatus liver exposed to cadmium and PCBs. In their comparative study of the effects of cadmium, copper and zinc on the digestive gland of the mussel, Viarengo et al. (1988b) demonstrated that only copper had a significant effect (augmentation of lipid peroxidation and reduction of glutathione). The suggested mechanisms of action implicate increased production of oxygenated radicals due to cytochrome P-450 induction by PCBs (an endogenous source of active oxygen) or to the oxidative cycle of copper (Cu++-Cu+) as well as blockage of the -SH groups of glutathione (a major radical trap), principally in the case of zinc and cadmium (Di Giulio et al., 1989).

At the Oslo Workshop, Viarengo et al. (1988a) also signalled an elevation in cellular calcium along with lipofuchsine accumulation in lysosomes in the digestive gland of mussels collected from polluted zones (Moore, 1988). Viarengo (1989) proposed the following mechanism of action : the increase in lipid peroxidation leads to an alteration in the membranes which reduces calcium exchanges, resulting in an elevation of the calcium content in cytoplasm and mitochondria.

In the mussel, a good amplitude of response was observed for MDA, and especially for calcium. The discriminator factor in this case was over 5. The results presented at Oslo by Viarengo et al. (1988a) corresponded to an

amplitude of 1.8 and a discriminator factor over 4 for calcium. In the two fish species studied, response and discriminator factors were below 2. According to the previously described mechanism of action, these parameters should have been significantly correlated with P-450 inducers (PAH and PCB) and metals such as copper and zinc. Significant correlations were in fact observed in the mussel between MDA and Ca++ and between tissue PAH and contamination of water and sediment by PCBs. In the Oslo mesocosm experiment, metals (Cu++ in particular) had a significant effect only at high doses (Viarengo et al., 1988a). By contrast, our analyses of fish gills revealed excellent correlations between cellular calcium and environmental pollution by metals (especially copper and zinc). Recent research (Gnassia-Barelli et al., 1990) has also established a relation with contamination by cadmium. However, no correlation has been found with the gradient of pollution by PAHs or PCBs. Responses thus differ not only between species but also between tissue types. For example, the gills are less sensitive to P-450 induction than the liver, but they are more exposed than the internal tissues to metal pollution because they serve as a barrier in direct contact with the outside environment.

5. CONCLUSIONS

Our study findings underscore the need to evaluate a panel of biochemical markers in several representative species in order to effectively assess the biological effects of multiagent contamination.

In the mussel Mytilus galloprovincialis, BaPMO has a good discriminator factor and is an excellent specific indicator of contamination by PAHs and planar compounds. This marker can be completed by measurement of intracellular calcium, which is sensitive to all inducers.

Serranus cabrilla appears to be the most interesting fish species for study purposes. Its EROD activity is a good indicator of the presence of planar substrates, featuring a high amplitude of response and a good discriminator factor. Measurement of GST activity allows complementary evaluation of contamination by globular compounds such as certain PCB congeners, with performances comparable to EROD assays. The cadmium content in fish gills is an indicator of contamination by certain metals (Cu, Zn, Cd), although the discriminator factor is rather low.

Preliminary data for the marine plant Posidonia oceanica reveal a good sensitivity of ECOD activity to planar compounds (induction) and sensitivity of LAH activity to globular compounds (inhibition); discriminator factors are comparable.

Similarly, preliminary investigations of AChE activity in muscle revealed the good feasibility of this parameter, which had a certain sensitivity (inhibition) to the PAH pollution gradient in sediment in both mussel and fish.

Chemical and biochemical analyses confirmed the low degree of pollution of the coast of Corsica compared to the levels along the French and Italian Riviera, as well as the high degree of pollution in other zones such as the bay of Lazaret and the cove of Carteau in the Gulf of Fos.

Comparison of these results obtained along the Mediterranean coast during three consecutive years with those obtained in Norway in 1986 reveal the reliability of biochemical markers for monitoring purposes, and underscores their suitability for use in varying environmental conditions. This constitutes a decisive step for their validation.

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BIOCHEMICAL MEASUREMENTS IN THE ASSESSMENT OF MARINE POLLUTION

by

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1. INTRODUCTION

In 1978, the International Council for the Exploration of the Sea (ICES) held a meeting in Beaufort, NC, to assess various approaches to measuring biochemical responses to marine pollution which had been developed during the 1960's and 1970's. It is interesting and instructive to review the recommendations of that meeting. It identified several biochemical measurements which seemed, at that time, to have potential as early-warning indicators of the sub-lethal impact of pollution; these were:

Measurements of blood chemistry variables in fish
Adenylate energy charge
Metallothionein induction
Mixed function oxidase (mono-oxygenase) induction
Tissue taurine-glycine ratios
Steroid metabolism disruption

Of this group, the techniques that have proved successful in the field (or which seem likely to do so) are mono-oxygenase and metallothionein induction measurements. We could add one other approach not discussed at Beaufort: measurements of acetyl-cholinesterase (AChE) inhibition, though most field experience with it comes from freshwater rather than marine studies.

Before discussing the "successful" approaches, it is worth asking why the other approaches have not been widely used. The simple (and perhaps superficial) answer is that none of these is as specific as the successful approaches, and this makes interpretation of the response difficult. For example, adenylate energy charge (AEC: the ratio of adenyne nucleotides) is a non-specific response to various environmental factors: these may include contaminants (Chevallier et al., 1989) but also natural variables such as dissolved oxygen concentrations (e.g., Reinert and Hohreiter, 1984). Similar objections apply to the use of measurements of amino acid ratios, e.g., the taurine: glycine ratio, which responds to the effect of parasitism (Soniat and Koening, 1982) and varies seasonally (Hummel et al., 1989) as well as being affected by contaminants (Scholz, 1987).

Disruptions in steroid hormone metabolism in fish appear to reflect the impact of such diverse stresses as H^+, Cd and PCBs (Freeman et al., 1984; Sangalang et al., 1990). Finally, measurement of blood chemistry variables in fish would appear, at first sight, to be an obvious technique; it is, after all, the equivalent of the clinical blood chemistry measurements used to

contribute to the assessment of "health" in humans. Its disadvantage in pollution monitoring is probably that is relatively non-specific, but also that there is not a sufficiently large data base for any of the common monitoring species that is reliable enough to show deviations or abnormalities attributable to pollution.

In contrast to these relatively non-specific measurements, the biochemical indices which have proved useful in the lab and in the field are highly specific: fish hepatic mono-oxygenase induction, for example, responds to a limited suite of organic chemicals of a fairly restricted size and shape which can interact with the Ah receptor. Metallothionein induction responds to a limited range of heavy metals (Cd, Zn and Cu). AChE is inhibited by a limited range of organophosphorus compounds which can phosphorylate a serine residue on the enzyme. One additional feature of all the "successful" biochemical indices is that their response is well understood, in chemical terms, as we shall see in the particular case of the fish hepatic mono-oxygenase system.

This presentation deals mainly with the fish hepatic mono-oxygenase system as a monitor of environmental contamination. It does not discuss AChE inhibition in any detail, since this is the subject of another paper. Metallothionein induction is dealt with only in passing as it is not the main focus of the Workshop.

2. FISH HEPATIC MONO-OXYGENASES AS INDICATORS OF ENVIRONMENTAL CONTAMINATION

Studies on this topic began in the 1970's. Previous work by mammalian toxicologists who were interested primarily in foreign compound and drug metabolism had shown that there existed in vertebrate liver an enzyme system with the following features:

- it was present in the smooth endoplasmic reticulum (SER) and could be centrifuged as microsomes at 100,000 x g;

- it required molecular oxygen;

- it involved a cytochrome "P450", so called from the absorption maximum of its CO complex;

- the system was inducible, i.e., its activity would increase if the organism were exposed to various foreign compounds, among which were various environmental contaminants.

This last point attracted the attention of environmental toxicologists, as it suggested that measurement of the enzyme activity of this system would indicate the extent to which an organism had been exposed to, or had accumulated, pollutants.

Since the mid-1970's, research on the mono-oxygenase system (as it is now called) in aquatic organisms has established the following points:

- hepatic mono-oxygenases in fish differ from those in mammals in that they are inducible mainly by organic chemicals which interact with the Ah receptor; such compounds include polynuclear aromatic

hydrocarbons (PAH), co-planar polychlorinated biphenyls (PCB), chlorinated dibenzodioxins (CDD) and chlorinated dibenzofurans (CDF) but not (e.g.) the insecticide DDT or its metabolite DDE;

- dose-response and time-course studies have shown that the induction process is a sensitive indicator of realistic environmental levels of contaminants, and that induction occurs rapidly (i.e., within hours or a few days);

- the details of the induction process have been elucidated and it is now possible to measure not only the catalytic activity of the mono-oxygenase system via marker enzymes such as ethoxyresorufin O-de-ethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) but also the concentration of the cytochrome which carries out the reaction (P4501A1) by immunochemical methods, and the mRNA which codes for P4501A1;

- finally, it appears that an inducible form of P4501A1 does not exist in marine invertebrates such as sessile molluscs.

After laboratory studies had established these points, environmental toxicologists turned their attention to field studies. A large number of field studies has now been carried out in which the fish hepatic mono-oxygenase response has been related to chemical distribution, either in the environment from which the fish were taken, or in the fish themselves. The earlier work has been summarised by Addison and Payne (1986) but here three recent studies are described --- one in Eastern Canada, and two from previous Workshops sponsored by the Intergovernmental Oceanographic Commission (IOC) and ICES.

Sydney Harbour, N.S., is heavily contaminated by PAH which have been released during the last 90 years or so from coke ovens associated with a steel mill. A PAH gradient exists in the harbour sediments, ranging from over 100 $\mu g\ g^{-1}$ (raised period) close to the plant, to less than 1 $\mu g\ g^{-1}$ at the mouth of the Harbour. We have sampled winter flounder (a local demersal flatfish, Pseudopleuronectes americanus) from Sydney Harbour and shown that its hepatic mono-oxygenase activity closely follows the PAH gradient. Furthermore, P4501A1 concentrations in liver microsomes are also well correlated with sediment PAH concentrations. (These data are summarised in Addison et al., in press).

A similar study was undertaken as part of an IOC Workshop in Langesundfjord in 1986 (Addison and Edwards, 1988; Stegeman et al., 1988). This fjord in southern Norway is contaminated by various chemicals from local industries and there exists a gradient of PAH, PCB and metals in sediments and in invertebrates. Local flounder (Platichthys flesus) were sampled from the fjord, and mono-oxygenase activity (EROD) and P4501A1 concentrations were shown to be well correlated with total PCB concentrations in the fish and with PAH in sediments or in invertebrates collected form the same sites.

In the North Sea, IOC and ICES sponsored a similar study in 1990 to assess the impact of the "plume" of the Rivers Weser and Elbe. The monitoring species used in this study was dab (Limanda limanda), and once again, mono-oxygenase activities closely followed the trend of sediment (and fish) contamination by PCBs. In this study, the mRNA which codes for P4501A1 was

also measured, and this too followed, though not as closely the PCB trend (Renton and Addison, in press).

Taken together, and with the complementary laboratory studies, these results show that measurements of mono-oxygenase activity in fish liver are well correlated with chemical contamination in the environment from which the fish were sampled.

3. METALLOTHIONEIN INDUCTION

Metallothioneins (MT) are a group of -SH rich low molecular weight proteins which bind metals such as Zn, Cu and Cd. Organisms exposed to increased concentrations of these metals have induced MT levels, and so measurement of MT concentrations provides some general information about metal exposure and accumulation. The basic biochemistry of MT structure and function is now quite well understood, and MT measurements in fish environmentally exposed to high metal concentrations are good predictors of metal exposure (e.g., Hogstrand and Haux, 1991).

4. SUMMARY

An examination of the development of biochemical measurements in response to pollution leads to some general conclusions. First, of the wide range of techniques that were identified fifteen years ago as being potential monitoring approaches, only two have proved to be routinely successful: fish hepatic mono-oxygenase induction and (to a slightly lesser extent) fish hepatic metallothionein induction. Both measurements are very specific: they respond to a very limited suite of chemicals. (In contrast, approaches which have not proved successful during the last fifteen years tend to be much less specific.) Second, the detailed biochemistry of both mono-oxygenase and metallothionein induction is well understood, and the processes relating uptake of chemicals to the induction response are well defined. Finally, both the P450 and metallothionein systems play a role in the normal biochemistry of the organism, but they also have the flexibility to respond to the presence of some exogenous compounds (in the case of the P450 system) or to unusual concentrations of metals (in the case of metallothioneins).

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MEASUREMENTS OF PROD, BROD AND CN-ECOD IN THE ASSESSMENT OF MARINE POLLUTION

by

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1. BACKGROUND AND INTRODUCTION

The previous lectures and demonstrations in this Workshop have focussed on the biochemical indicator which has been most extensively studied as an indicator of marine pollution: the hepatic mono-oxygenase (cytochrome P4501A1) system of fish. This responds to a limited range of organic contaminants which interact with the cytosolic Ah receptor, which include (usually) lipophilic compounds whose molecular structure is roughly planar with an area of about that of five fused benzene rings; such compounds include polynuclear aromatic hydrocarbons (PAH), some planar or mono-ortho-substituted chlorobiphenyls (CB) and chlorinated dibenzodioxins (CDD) and chlorinated dibenzofurans (CDF). The sequence of events in the induction process is:

(a) uptake of inducing compound
(b) interaction of inducer with Ah receptor
(c) inducer-receptor complex "switches on" CYP1A1 gene
(d) transcription of P4501A1 mRNA
(e) translation to P4501A1
(f) P4501A1 catalyses ethoxyresorufin O-de-ethylase (EROD), aryl hydrocarbon hydroxylase (AHH) etc.

(Steps underlined can be measured routinely).

2. INDUCTION OF OTHER P450s IN FISH

So far, only the P4501A1 system has been discussed. However, fish liver contains many other P450s and it is useful to consider whether any of these respond to contaminants, and whether they would be useful in pollution monitoring.

Of the many forms of P450 in fish (which have been reviewed recently by Goksøyr and Förlin, 1992) that which is of most interest (other than P4501A1) is the analogue of P4502B1. In mammals, this form is inducible by phenobarbital (PB) and related compounds including (probably) the insecticide DDT, its metabolite DDE and possibly the non-co-planar PCBs. Does an inducible P4502B1 exist in fish? The short answer is "no". By analogy with the scheme shown above, the induction process should be:

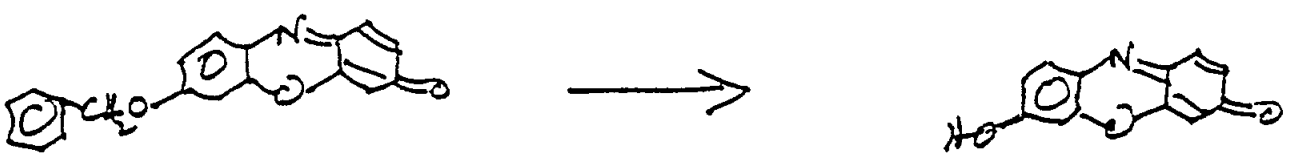
(a) uptake of inducing compound (e.g., DDT)
(b) interaction of DDT with a receptor (so far unidentified)

(c) inducer-receptor complex "switches on" CYP2B1 gene
(d) transcription of P4502B1 mRNA
(e) translation to P4502B1
(f) P4502B1 catalyses aldrin epoxidase (AE), pentoxyresorufin O-de-ethylase (PROD) or benzyloxyresorufin O-de-ethylase (BROD).

The older literature suggests that some of the marker enzymes, including AE, are indeed induced when fish are exposed to a PB-type inducer (reviewed by Kleinow et al., 1987). However, more recent studies based on more sensitive approaches to measuring PB induction via marker enzymes such as PROD have shown no evidence of induction (Addison et al., 1987; Ankley et al., 1987). However, so far there has been no direct immunochemical evidence (i.e., based on analysis of P4502B1 itself, or its mRNA) for or against an inducible P4502B1 in fish.

Since the use of marker enzymes is very simple and since it is possible that species or situations exist in which PB-type induction might be observed, the possibility of "screening" for PB-type induction might be considered. In practice, the approach is based on the use of two substrates for mono-oxygenase reactions apparently catalysed by P4502B1. These are pentoxyresorufin (PR) and benzyloxyresorufin (BR). Both undergo de-alkylation to yield resorufin; the reaction is catalysed by (apparently) P4502B1:

PR PROD (P4502B1) Resorufin



BR BROD (P4502B1) Resorufin

3. PRACTICAL MEASUREMENT OF PROD AND BROD

The approach to measuring PROD and BROD is identical to that of measuring EROD, except that the appropriate substrates are used. Pentoxyresorufin (commercially available) or benzyloxyresorufin (synthesised) are made up exactly as for ethoxyresorufin in the EROD reaction. From then on, the rates of the reactions PROD or BROD are measured similarly to that of EROD, since the product, resorufin, is the same in all cases.

In our experience, PROD or BROD activity is always very low and no field sample has shown any evidence of PROD or BROD activity (or, therefore, P4502B1 induction). However, when laboratory-maintained fish are dosed with (e.g.) ß-naphthoflavone (ß-NF) PROD or BROD may be slightly induced, usually to the same degree as EROD (e.g. Addison et al., 1991). This suggests that under these conditions, BR or PR act as poor substrates for EROD, rather than being de-alkylated by a specifically induced P4502B1.

4. MEASUREMENT OF CN-ECOD

The simplicity of the EROD assay has led to the development of another substrate which can be measured by a rapid kinetic assay: 3-cyano7-ethoxycoumarin (White, 1988). This compound is a derivative of 7-ethoxycoumarin, which was the substrate for a widely-used mono-oxygenase assay previously used to indicate P4501A1 induction, ethoxycoumarin O-de-ethylase (ECOD). ECOD itself is not measurable by kinetic fluorometric analysis because its reaction product, umbelliferone, fluoresces at pH outside the physiological range. When a 3-CN group is substituted, however, its fluorescence is detectable at physiological pH. 3-Cyano-7-ethoxycoumarin is therefore a potentially useful substrate for measuring mono-oxygenase activity:

3-cyano-7-ethoxy coumarin — CN-ECOD — 3-cyanoumbelliferone

The CN-ECOD assay is carried out exactly as is EROD, except that excitation and emission wavelengths are changed to allow the detection of 3-cyanoumbelliferone as product. The wavelengths chosen are 408 nm (excitation) and 450 nm (emission). The substrate, which is virtually insoluble in water, is prepared in dimethyl sulphoxide, and since this may inhibit the reaction, only small amounts (<10 µl) are incubated.

CN-ECOD has been introduced only recently, and it is not yet clear which P450 isozyme(s) catalyse(s) the reaction. P4501A1 certainly contributes to CN-ECOD, but other P450s may also be involved. Thus, in studies in which

EROD and CN-ECOD have been measured together (in response to ß-NF or PAH induction) each enzyme has a slightly different response to a given "dose" of inducer. Surprisingly, CN-ECOD appears to cover a wider range of responses than EROD, suggesting that it may be more sensitive to environmental induction, but perhaps less specific (e.g., Addison et al., 1991; Renton and Addison, 1992; Addison et al., in press).

5. SUMMARY

Apart from the measurement of EROD to indicate induction of P4501A1 by some organic contaminants, some other enzymatic measurements may be useful, or of scientific interest. Measurements of PROD and BROD (both closely related to that of EROD) may indicate induction of P4502B1 (if, indeed, that P450 exists and is inducible in fish); this in turn may indicate the presence of other organic contaminants. However, this approach is purely speculative, and is not supported by any field or experimental evidence. The measurement of CN-ECOD may complement that of EROD as it almost certainly is catalysed by P4501A1, but it may also indicate the presence of other P450s.

PROD, BROD and CN-ECOD therefore cannot be proposed for routine monitoring at this stage, but as their measurement involves only a small incremental effort over that required for EROD analysis, it may yield data which may eventually be valuable.

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REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME JULY 1992

Hepatic mixed function oxidase induction in fish as an environmental monitoring technique

Reference Methods For Marine Pollution Studies No. 60

Prepared in co-operation with

IOC IAEA IMO FAO

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources and has requested the development of regional action plans. The Regional Seas Programme at present includes ten regions and has over 120 coastal States participating in it (1),(2).

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment and of its resources, and of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies is being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (3). The Methods are recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared in co-operation with the relevant specialized bodies of the United Nations system as well as other organizations and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (ISO) is followed as closely as possible:

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, as analytical instrumentation and the actual need of the users. In order to facilitate these revisions the users are invited to convey their comments and suggestions to:

Marine Environmental Studies Laboratory
IAEA Marine Environment Laboratory
19, Avenue des Castellans
MC 98000 MONACO

which is responsible for the technical co-ordination of the development, testing and intercalibration of Reference Methods.

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(1) UNEP: Achievements and planned development of the UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No.1 UNEP, 1982

(2) P. HULM: A Strategy for the Seas. The Regional Seas Programme: Past and Future, UNEP 1983.

(3) UNEP/IAEA/IOC: Reference Methods and Materials: A Programme of comprehensive support for regional and global marine pollution assessments. UNEP 1990.

This Reference Method was designed to provide the user with reliable techniques for the determination of certain specific biological effects of chemical contaminants. This is the first in a series of techniques which are a product of the work of the IOC/UNEP/IMO Group of Experts on the Effects of Pollution (GEEP). GEEP has tested each of these techniques in a series of experimental workshops in temperate and tropical conditions. The techniques offer the pollution scientist a sensitive tool for examining the effect of pollutants at sub-acute levels and provide a stronger scientific basis for marine pollution assessments than "traditional" experimental studies of acute toxicity.

The present document describes the use of hepatic MFO measurements as an environmental monitoring technique. The first section gives a general description and review of hepatic MFOs in fish, and the second provides detailed procedures for measuring hepatic MFO activity using relatively simple equipment and facilities.

This first edition of the Reference Method for Marine Pollution Studies No. 60 was prepared by Dr R.F. Addison of the Department of Fisheries and Oceans, Physical and Chemical Sciences Branch, Scotia-Fundy Region, Bedford Institute of Oceanography, Dartmouth, N.S., Canada. The work was supported partly by the Panel on Energy Research and Development. The author appreciates the support of D.R. Livingstone and D.E. Willis for their comments on the manuscript. The document was edited by UNEP in co-operation with the International Atomic Energy Agency (IAEA) and the Intergovernmental Oceanographic Commission (IOC).

CONTENTS

I. THE USE OF MIXED FUNCTION OXIDASES (MONO-OXYGENASES) AS AN ENVIRONMENTAL MONITORING TECHNIQUE

1. Introduction

Our present interest in using MFO induction as an environmental monitoring technique arises from work undertaken since the early 1950s to understand detoxification processes, usually in mammals, and usually focussing on drug metabolism. By the early 1970s the following points had been established:

1. There existed in vertebrate (usually mammalian) liver a group of enzymes which catalysed the metabolism of a wide range of lipophilic substrates (both endogenous and foreign) to more polar (hence more readily excreted) products.
2. These enzymes were associated with the smooth endoplasmic reticulum and could be centrifuged as "microsomes" at 100,000 x g; they required oxygen, NADPH and involved one or more isozymes of a cytochrome P450 (so-called from the absorption maximum of its CO complex).
3. These "mixed function oxidases" were inducible: under normal conditions, their activity was relatively low, but if the organism were exposed to certain compounds, their activity would increase, apparently to accelerate the degradation and clearance of the inducing compound.

This last point attracted the interest of environmental toxicologists, because many of the more powerful inducers were compounds such as polynuclear aromatic hydrocarbons (PAH) or some polychlorinated biphenyls (PCB), which were persistent environmental contaminants. Hepatic MFO activity in organisms taken from the wild might therefore indicate exposure to ambient levels of some environmental contaminants, in other words, it may serve as a field sub-lethal bioassay.

Since the mid-1970s, research on hepatic MFOs can be considered (for the purposes of this article) to have taken two directions. Research on mammalian MFOs has focussed on the basic biochemistry of the system. This aspect of the subject has been reviewed recently (Ortiz de Montellano, 1986) and will not be discussed further here. The other objective of MFO research has been to describe in detail MFO systems in aquatic biota, and to apply measurements of MFO activity, particularly in fish, as an environmental monitoring tool.

2. Hepatic MFOs in fish

Hepatic MFO systems in fish are generally similar to those in mammals in terms of their sub-cellular localisation (centrifuged as microsomes at 100,000 x g) and co-factor requirements (O_2 and NADPH). Structurally, the fish hepatic P450s of most interest (those which correspond to the mouse P4501A1: Nebert et al., 1987) are similar in terms of amino acid sequence, structure of active site, and MW (48,000-56,000). Substrate specificity and the structure of products formed during P450-catalysed reactions are also generally similar in fish and in mammals, but may differ in detail (e.g.,

Goksøyr et al., 1986). There are differences in the rates of P450-catalysed reactions, those in fish proceeding more slowly reflecting different temperature adaptations. Since the P450s in rat and fish are structurally similar, these rate differences probably result from the organisation of the P450 + NADP-P450-reductase + phospholipid complex (Williams et al., 1983).

The main difference between fish and mammalian hepatic MFOs lies in their inducibility. In mammals, at least five representative compounds usually induce distinct P450 isozyme groups (although these P450s may catalyse the conversion of a wide range of substrates): these inducers are (i) ß naphthoflavone (BNF), (ii) phenobarbitone (PB), (iii) pregnenolone-16-α-carbonitrile (PCN), (iv) isosafrole (ISF) and (v) clofibrate. In fish, only BNF and related compounds (which include PAH, and planar chlorobiphenyls, chlorinated dibenzodioxins and dibenzofurans) have consistently induced hepatic MFOs in a wide range of species (Kleinow et al., 1987). PCN appears not to induce, or does so to only a very limited extent (Hansson et al., 1980; Vodicnik and Lech, 1983). PB and related compounds have been reported occasionally, and usually in the older literature, to induce fish hepatic MFOs, but more recent work using sensitive assays of PB induction, suggests that PB is not an inducer (Addison et al., 1987; Ankley et al., 1987). ISF has been reported (on one occasion) to induce MFO enzymatic activity in trout (Vodicnik et al., 1981). No information exists about induction in fish by clofibrate. It is worth emphasising that most experimental work on MFO induction in fish has been done with salmonids (mainly rainbow trout) and inducers other than BNF may be effective in other species. Furthermore, most of the evidence for the absence of induction by (e.g.) PC rests on measurements of enzymatic activity, rather than on immunochemical measurement of specific isozymes.

Dose-response relationships have been established between exposure to BNF inducers and MFO activity in fish. In brook trout, hepatic ethoxycoumarin O-de-ethylase (ECOD) activity was related (by a power function) to PCB residue concentrations in the fillet after experimental feeding of Aroclor 1254 (Addison et al., 1981); the form of the equation suggested that ECOD should be sensitive to PCB concentrations encountered in "naturally" contaminated environments. A similar relationship existed between ethoxyresorufin O-de-ethylase (EROD) activity or aryl hydrocarbon hydroxylase (AHH) activity and Aroclor 1242 injected intraperitoneally to rainbow trout (Elcombe and Lech, 1978). Flounder exposed to petroleum-contaminated sediment showed increases in hepatic EROD activity related to sediment hydrocarbon concentration in the range 0.3-91 ppm (Payne et al., 1988). In the tropical fish Haemulon sciurus hepatic EROD and specific P450 isozymes were related to BNF injections in the range 0-10 mg kg^{-1} body weight (Stegeman et al., 1990). Overall then, there is evidence from several species of fish exposed to various chemicals to show that MFO activity can be predictably related to petroleum or PCB exposure, though the nature of the relationship may vary from species to species.

The time-course of induction has been determined in several studies. Various species of temperate-water fish show clear induction (either enzymatic activity or P450 isozyme induction) after 3-5 days (Lidman et al., 1976; Addison et al., 1981; Fingerman et al., 1983). Changes in the mRNA which codes for P450 isozyme synthesis occur even sooner (Haasch et al., 1988). In tropical fish acclimated to higher temperatures, induction of enzymatic activity may occur within three days (Stegeman et al., 1990). Since MFO induction is related to tissue concentrations of certain chemicals (reflecting

exposure) and since these chemicals are usually cleared only fairly slowly, MFO activity usually remains elevated for some time even after a single dose (Elcombe and Lech, 1978; Addison et al., 1981; Stegeman et al., 1990).

Several natural factors influence hepatic MFO activity in fish. The most obvious of these is sex. In sexually mature fish, there are differences in MFO enzymatic activity (Stegeman and Chevion, 1980; Förlin, 1980) though these are usually not so obvious in immature fish. Not only are there sex differences in MFO activity, but also in the inducibility of MFO activity (Förlin, 1980). In addition, there were pronounced seasonal variations in MFO activity which were related to the reproductive cycle in apparently uninduced fish from a clean environment (Edwards et al., 1988). In summary, seasonal (or other) variations in the reproductive cycle appear to have a major influence on hepatic MFO activity, and should be eliminated during sampling of fish for monitoring studies. This may be achieved most easily by sampling reproductively immature specimens.

3. Selection of fish species for MFO monitoring

A fish species suitable for MFO monitoring is likely to have the following characteristics:

(i) It should be common and easily available, and its basic biology (habitat, feeding behaviour, migration and reproductive cycle) should be well defined; its migration should be short compared to the distances over which spatial comparisons are being made.
(ii) It should be robust enough to be kept in the laboratory so that its MFO system can be characterised and examined experimentally.
(iii) Reproductively immature specimens should be large enough to yield about 1 g liver for study.
(iv) It should probably (though not necessarily) be a bottom dweller, since many of the contaminants which affect MFO systems are likely to be sediment (or at least particle) bound.

In temperate marine waters, these criteria are often met by flatfish such as members of the flounder family: thus, winter flounder (Pseudopleuronectes americanus) or Pacific dabs (Citharichthys spp.) have been used successfully in North American monitoring programmes (Payne et al., 1984; Spies et al., 1980; 1982) and the European flounder (Platichthys flesus) has been used in Norwegian waters (Addison and Edwards 1988; Stegeman et al., 1988). It should be emphasised, however, that the last of the criteria listed above need not be applied rigidly: many fish which are not demersal have been used in successful monitoring programmes; these include blenny (Kurelec et al., 1977) and perch (Förlin et al., 1985).

It is worth noting that the fish hepatic MFO induction measurements which have been most useful in indicating the effects of contamination have involved comparisons of fish from suspect habitats with those from clean or reference sites. Absolute measurements of MFO induction depend to a considerable extent on technique and on factors such as the choice of buffer pH or ionic strength, enzyme: substrate ratios, etc.. As a result, comparative studies, in which samples from different sites are analysed using any standard techniques, are preferable.

4. MFOs in aquatic invertebrates

Sessile aquatic invertebrates would appear to be better candidate organisms for environmental monitoring than would fish, because of their immobility. Unfortunately, their MFO systems appear to be much less sensitive to organic pollutants than those in fish. There was some controversy about whether aquatic invertebrates in general have inducible MFO systems at all; however, the more recent literature (which is based on more sensitive and specific methods for detecting components of such systems) has shown that MFO systems are indeed present in aquatic invertebrates (Livingstone et al., 1989; James, 1989; Livingstone, 1990). It is clear, however, that invertebrate, particularly molluscan, MFO systems are in general less well characterised than are vertebrate systems, and are not yet ready to be applied as a routine environmental monitoring tool (Livingstone, 1988).

II. PRACTICAL MEASUREMENT OF MFO ACTIVITY

1. Scope and field of application

When vertebrates (and some invertebrates) are exposed to chemicals like petroleum hydrocarbons or some polychlorinated biphenyls, their hepatic microsomal mixed function oxidases (MFO, or mono-oxygenases) are induced. Increased hepatic MFO activity in wild organisms may therefore indicate contamination of their habitat; in other words, MFO induction may be a sub-lethal bioassay of environmental contamination by certain chemicals.

2. Principle

The methods summarised here are based on conventional laboratory procedures for estimating MFO activity. These have been modified for use in less well-equipped laboratories or in the field, usually by choosing robust and simple versions of more elaborate apparatus. (Obviously, if advanced instruments are available, they can be used). The basic facilities required include some bench space and a source of electrical power (in North America, 110V 60 Hz single phase, maximum 10A). The methods and equipment described have been used in such diverse environments as aboard ship (CSS Hudson Arctic cruises), in a tent at Resolute Bay, (North West Territories, Canada), using power supplied from a gasoline generator and on Sable I., Nova Scotia.

It is convenient, particularly in field work, to prepare in advance as many reagents and solutions as possible. Most, like those required for protein determinations, are stable and will withstand freezing and thawing if kept in plastic bottles. It is usually not possible to prepare nucleotide co-enzyme solutions in advance, however, and since (usually) small amounts of these are needed and as they are relatively expensive, it is desirable to pre-weight appropriate amounts of these, and keep them (cooled and desiccated) in small vials. The instruments described are simple and robust examples of their type and which have simple maintenance or support requirements, but which are adequately sensitive or precise.

Weighing presents special problems, especially aboard ship unless a weighing table on gimbals is available. A partial solution to the problem is

to use an "averaging" balance of the sort that integrates over a period of several seconds; even this, however, is not satisfactory for weights below 1g. (If liver size must be estimated, its volume can usually be measured accurately by displacement in a small measuring cylinder). Fortunately, most variables of interest in MFO assays can be expressed in terms of protein contents, which in turn can be measured aboard ship using only volumetric manipulations.

The following pages describe methods for preparing MFO suspensions, for two MFO assays (ethoxyresorufin O-de-ethylase: EROD and benzo(a)pyrene hydroxylase, also known as aryl hydrocarbon hydroxylase: AHH), and for protein estimation. These procedures have been chosen since they have been well tested both in the field and the laboratory as monitors of contamination, mainly by PAH, PCB and chlorodibenzodioxins. The general procedures for both MFO assays can be applied to different substrates. The procedure for EROD, for example, can be applied to pentoxy- or benzyloxy-resorufin O-de-alkylase (PROD and BROD) to indicate induction of other P450 isozymes (Burke and Mayer 1983; Lubet et al., 1985). Similarly, the EROD assay can be modified by changing only the fluorimeter excitation and emission wavelengths to detect 3-cyano-7-ethoxycoumarin O-de-ethylation (CN-ECOD), which may also indicate induction of other P450 isozymes (White, 1988). The excitation and emission wavelengths used for benzo(a)pyrene hydroxylation can be modified to allow the use of diphenyloxazole as a safer substrate. It must be emphasised, however, that CN-ECOD, PROD and BROD have not been "field tested" as indicators of contaminant-induced stress.

Although the assays described here are "classical" indicators of MFO induction, other indicators are being developed continuously. Protein determinations can be carried out by various methods: the bicinchoninic acid (BCA) reagent has been introduced recently, for example, and promises to be a convenient one-step process (Anon., 1989). However, as it has not yet been evaluated extensively, the more conventional Lowry method is described here. Immunochemical probes for P450 isozymes are now available (e.g., Stegeman et al., 1988) and the mRNAs which code for some of the P450s can now be detected (e.g., Haasch et al., 1988). However, such approaches are still at the research stage and as they require the use of reagents not available commercially, they are not described in detail.

Finally, it should be emphasised that the methods described here only illustrate the procedures for measuring MFO induction. They should not be regarded as immutable; indeed, it is desirable that investigators should modify the methods for enzymatic activity measurements, e.g., by varying pH, substrate concentration etc., to optimise incubation conditions for their sample types.

3. Important note on safety

Safety deserves special attention, especially if these techniques are to be used in the field where medical help is not available. Most of the chemicals and equipment recommended below are relatively harmless, provided they are not abused. It is simply good laboratory practice to treat any chemical or equipment item with respect, even if it is known to be harmless; furthermore, the toxicology of some chemicals (such as ethoxyresorufin) has not been investigated. Disposable items should be used wherever possible, as

should safety items such as "propipettes", gloves, bench-top soakers etc. The benzo(a)pyrene hydroxylase assay presents special problems, since the substrate is carcinogenic: the assay is best carried out in an area dedicated to the work (which is desirable in any case, as the assay should be done under safe lights since the products are photo-sensitive); protective clothing **MUST** *be worn, pipetting by mouth* **MUST** *be avoided, and the working area* **MUST** *be protected with soakers which* **MUST** *be combined with carcinogenic waste for special disposal.*

4. Preparation of samples for analysis

The procedures for isolating MFOs are shown schematically in Fig. 1. This section describes the steps for preparing a sample prior to the measurement of MFOs.

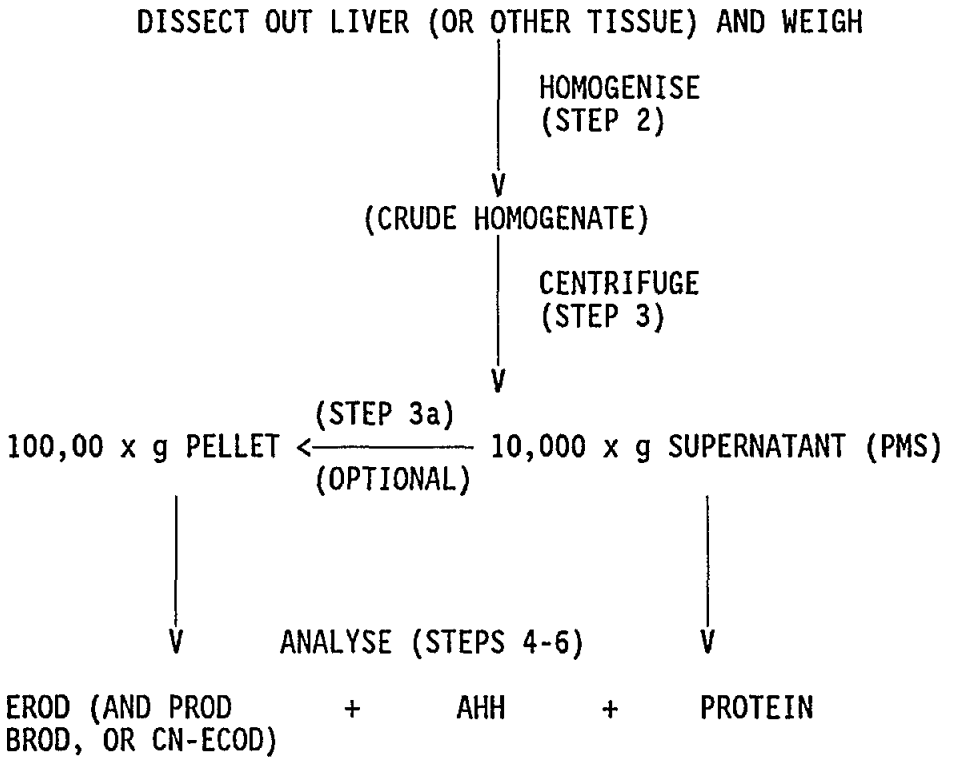

Figure 1 Scheme for preparation of mixed function oxidases from fish tissue. Numbers identify steps described in the text

4.1 Reagents

4.1.1 Homogenisation solution. 1.15% KCl prepared in advance and stored frozen or cooled in plastic bottles

4.1.2 Ice (for cooling tissues)

4.2 Equipment

4.2.1 Top-pan balance weighing to 0.1g
4.2.2 Conventional dissection instruments
4.2.3 Bench space with adequate lighting
4.2.4 Ice bucket
4.2.5 Range of small beakers
4.2.6 Electric drill capable of 1750 rpm
4.2.7 Potter-Elvehjem teflon-glass homogeniser (5 or 15 ml)
4.2.8 Measuring cylinder (10 or 25 ml capacity)
4.2.9 Scissors
4.2.10 Beckman "Microfuge" or equivalent (capable of achieving at least 10,000 x g) and tubes. This instrument is not self-cooled, but it may be run in a domestic freezer. (If a conventional refrigerated centrifuge is available it should be used: sample volumes should be modified as required)
4.2.11 Refrigerated ultracentrifuge, 100,000 x g, if available
4.2.12 Graduated tubes, 5-15 ml

4.3 Procedure

4.3.1 Dissection and isolation of tissue

Kill fish, usually by a blow to the head and/or severing spinal cord; weigh fish with appropriate accuracy (± 1%). Dissect out tissue (usually liver) avoiding rupturing the gall bladder, since bile may contain MFO inhibitors. Weight the liver (± 1%) and place in a beaker on ice, pending homogenisation.

4.3.2 Homogenisation of tissue (Step 2, Fig. 1)

Mince weighed tissue (ideally 1g minimum, weighed to ± 0.1g) with scissors and place in homogeniser tube on ice; add KCl solution in ratio 4:1, v:w. Homogenise with 6 vertical strokes at 1750 rpm, keeping tube cooled in ice. This yields the "crude homogenate".

4.3.3 Preparation of 10,000 x g homogenate (Step 3, Fig. 1)

Place 1.5 ml aliquots of crude homogenate in "Microfuge" tubes and spin for 15 min. The supernatant is nominally 10,000 x g supernatant ("post-mitochondrial supernatant", PMS) or 12,500 x g supernatant, depending on instrument. Retain the supernatant for analysis, or process further as described in paragraph 4.3.4 (Note: protein concentrations in supernatants prepared in this way do not differ significantly from those prepared by conventional centrifuging (5 min at 500 x g followed by 15 min at 10,000 x g) in refrigerated centrifuges).

A single "Microfuge" tube usually provides sufficient supernatant (>1 ml) for benzo(a)pyrene hydroxylase, ethoxyresorufin O-de-ethylase and protein determinations. If activity or protein concentrations are low, or if subsequent samples are required for, say, electrophoretic studies of P450 isozymes, further aliquots of the crude homogenates can be centrifuged.

4.3.4 *[Optional]* Preparation of 100,000 x g pellet (microsomes) (Step 3a, Fig. 1)

This is optional, if 5-10 ml 10,000 x g supernatant have been prepared, and ultracentrifuge is available. Transfer the 10,000 x g supernatant (or an aliquot of it) to an ultracentrifuge tube. Spin at 100,000 x g for 40 min. at 4°C. Discard the supernatant ("cytosol"). Loosen the microsomal pellet with a glass rod, and transfer it quantitatively to a tissue homogeniser. Resuspend the pellet in 3-5 ml 1.15% KCl solution, keeping the homogeniser cooled in ice. Rinse the homogenate into a graduated tube and record its volume. Hold on ice.

5. Protein determination (Lowry et al., 1951) (Step 4, Fig. 1)

The reader is referred to the bibliography for full details of this widely-employed method which will only be described briefly here.

5.1 Reagents

5.1.1 Distilled water
5.1.2 0.5% aq. copper sulphate ($CuSO_4.5H_2O$) in 1% aq. sodium or potassium tartrate solution
5.1.3 2% aq. sodium carbonate in 0.1 N sodium hydroxide
5.1.4 Mixed reagent "A", prepared not more than one day in advance, as described by Lowry et al. (1951). Add 1 ml reagent 5.1.2 to 50 ml reagent 5.1.3
5.1.5 Folin-Ciocalteu phenol reagent, diluted to 1 N. (The reagent available commercially from (e.g.) Sigma is 2 N and must be diluted 1:1 v/v with distilled water
5.1.6 Protein standard, e.g., bovine serum albumin, diluted (accurately) to approx. 100-200 $\mu g\ ml^{-1}$ (so that appropriate volumes can be pipetted conveniently to prepare standard curves).

5.2 Equipment

5.2.1 15 ml test-tubes and rack
5.2.2 Micropipettes with disposable tips to deliver 10, 25 or 500 µl
5.2.3 Graduated glass pipettes to deliver 2 ml and 10 ml
5.2.4 250 ml glass beakers
5.2.5 Vortex mixer
5.2.6 Spectronic 20 or similar simple robust single beam spectrophotometer with wavelength range to 660 nm and cuvettes.

5.3 Procedure

Prepare a standard curve for each set of determinations. Use four points (plus a blank) in the range 20-200 µg protein. Make up to final volume 1 ml with distilled water. To duplicate 10 µl (PMS) or 25 µl (microsomal) samples in test tubes add 1 ml distilled water. Prepare reagent "A" as above and add 5 ml reagent "A" to each standard and sample tube. Mix on vortex mixer and allow to stand about 20 mins. Then, while each tube is being mixed on the vortex mixer, add 0.5 ml diluted Folin-Ciocalteu phenol reagent and allow to stand a further 20 min. Read at 660 nm v. distilled water.

6. Ethoxyresorufin O-de-ethylase (EROD) determination (Step 5, Fig. 1)

This is based on the method of Burke and Mayer (1974) in which the substrate (ethoxyresorufin) is incubated in a fluorimeter cuvette with enzyme preparation and cofactor (NADPH) in appropriate buffer, and the fluorescence increase due to resorufin production is recorded.

6.1 Reagents

6.1.1 0.1 M phosphate buffer in range 7.5-8.5
6.1.2 Ethoxyresorufin (substrate) 100 µM in 2.5% aqueous Tween 80
6.1.3 Resorufin (product) in range 10 µM in ethanol (both substrate and product should be protected from light)
6.1.4 NADPH, 5 mg in pre-weighed vial

6.2 Equipment

6.2.1 Micropipettes with disposable tips to deliver 10, 25, 50, 100, 200 µl
6.2.2 Glass pipette to deliver 2 ml
6.2.3 Fluorimeter functioning in range excitation 510 nm and emission 585 nm with 15 nm bandwidth in each beam (Turner 430 or similar is suitable), and connected to strip chart recorder or other recording device (temperature control of the cuvette is optional)

6.3 Procedure

6.3.1 Ethoxyresorufin O-de-ethylase (EROD)

Place 2 ml buffer in fluorimeter cuvette (maintained at an appropriate temperature if possible) and add NADPH to a final concentration of 200 µM; add appropriate amount of enzyme (10,000 x g supernatant, or microsomal preparation) and mix. Record fluorescence with excitation at 510 nm and emission at 585 nm. (There should be no increase over time). After 1 min add ethoxyresorufin to a final concentration of 1.25 mM and record increase in fluorescence for 2 min. Finally add a known concentration of resorufin and note increase in fluorescence; express gradient of enzymatic fluorescence increase in terms of product concentration. A typical run is shown in Fig. 2.

6.3.2 Pentoxy- and benzyloxy-resorufin O-de-ethylase (PROD and BROD)

PROD and BROD are sensitive indicators of the P450 isozymes which are induced by PB in some species (Burke and Mayer, 1983; Lubet et al., 1985). Although there is some doubt as to whether fish hepatic MFOs are inducible by PB (see Part I) the only species in which these enzyme activities have been measured are trout (Addison et al., 1987; Ankley et al., 1987) and flounder (Addison et al., 1991). The assays are described in the event that they may be useful in other species.

These assays are carried out exactly as described for EROD (since the same product, resorufin, is measured) except that pentoxyresorufin or benzyloxyresorufin is substituted for ethoxyresorufin.

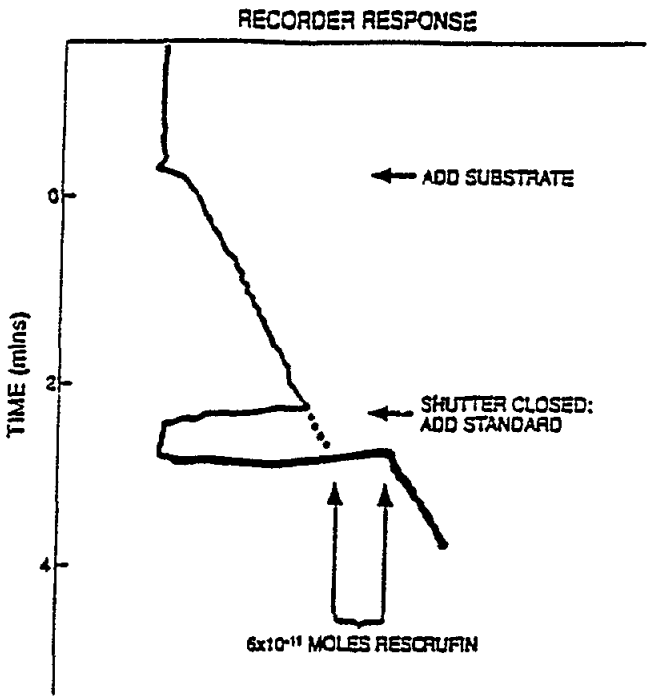

Figure 2 Strip chart record of ethoxyresorufin O-de-ethylase (EROD) activity in a flounder (Pseudopleuronectes americanus) liver preparation, illustrating (a) constant fluorescence due to buffer, enzyme preparation and co-factor; (b) increase in fluorescence due to resorufin production after addition of substrate, and (c) increase in fluorescence after addition of a known amount of product for calibration

6.3.3 3-Cyano-7-ethoxycoumarin O-de-ethylase (CN-ECOD) determination

7-ethoxycoumarin O-de-ethylase (ECOD) is a popular and sensitive indicator of induction of (probably) a fairly wide range of P450 isozymes. Since the product of the reaction, umbelliferone, does not fluoresce at physiological pH it is difficult to determine by "kinetic" methods. White (1988) has described the preparation of the 3-cyano derivative which is easily detectable in the pH range 6-9, and has shown that CN-ECOD, like ECOD, is a sensitive indicator of MFO induction though probably less specific than the resorufin derivatives.

The assay is carried out exactly as described for EROD except that the wavelengths are 400 nm (excitation) and 455 nm (emission).

7. Benzo(a)pyrene hydroxylase determination (Step 6, Fig. 1)

This is based on the procedure of Nebert and Gelboin (1968) in which the substrate (benzo(a)pyrene) is incubated with an enzyme preparation and base-extractable phenolic products are isolated and determined fluorimetrically.

READ THE PARAGRAPH ON SAFETY. BENZO(A)PYRENE IS CARCINOGENIC. IF SUITABLE FACILITIES FOR HANDLING CARCINOGENS ARE NOT AVAILABLE, USE AN ALTERNATIVE SUBSTRATE SUCH AS DIPHENYLOXAZOLE (PRO) TO ASSESS AROMATIC HYDROCARBON HYDROXYLASE (AHH) ACTIVITY.

7.1 Reagents

7.1.1 Buffer solution 0.1M phosphate pH 7.5
7.1.2 1.15% KCl solution
7.1.3 Acetone
7.1.4 Hexane, spectroscopic grade (redistilled from $KMnO_4$ if necessary)
7.1.5 Benzo(a)pyrene, 19mM in acetone
7.1.6 NADPH, 10 mg ml^{-1} in phosphate buffer pH 7.5
7.1.7 Quinine sulphate (fluorescence standard) in range 0-100 µg ml^{-1}

7.2 Equipment

7.2.1 Disposable polypropylene test-tubes (16 ml) (Falcon #2006 is suitable)
7.2.2 Micropipettes with disposable tips, 10 µl to 100 µl
7.2.3 Waste pail dedicated to carcinogenic waste and lined with plastic bag
7.2.4 Clinical centrifuge, preferably with disposable liners in buckets
7.2.5 Vortex mixer
7.2.6 Fluorimeter capable of functioning at 395 nm (excitation) and 520 nm (emission) (corresponding wavelengths for assay based on PPO are 345 and 520 nm).
7.2.7 Water bath set at 27°C
7.2.8 Safe light (25W red lamp)
7.2.9 Test-tube racks
7.2.10 Pasteur pipettes
7.2.11 Disposable tissue, gloves and bench-top soaker
7.2.12 Timer
7.2.13 Long wave UV lamp (for monitoring spills of benzo(a)pyrene)

7.3 Procedure

To a 16 ml polypropylene tube add 500 µl phosphate buffer, 100 µl NADPH solution, 10 µl 19 mM benzo(a)pyrene, and bring to 27°C. Add 10-100 µl 10,000 x g homogenate or microsomal preparation. Prepare a "zero-time" blank with pooled enzyme preparations and stop the reaction immediately with 0.5 ml acetone.

Carry out the following steps under safelights: incubate all samples and blank with gently shaking for 15 min. Stop reaction by addition of 0.5 ml acetone and add 2 ml hexane to each tube. Mix 30 sec. on Vortex mixer and centrifuge 3 min. (Wrap the tubes and use liners to prevent leakage at these steps). Transfer hexane phase (Pasteur pipette) as completely as possible to second tube and back extract with 2 ml 1N NaOH. Mix 30 sec. and centrifuge 3 min. Remove hexane layer to incubation tube to minimise waste. Measure fluorescence of the NaOH phase against a curve prepared with quinine sulphate in the range 100 µg ml^{-1}. This secondary standard is standardised against 3-hydroxygenzo(a)pyrene (stock 100 µM in acetone, diluted to 10-200 nM in NaOH).

Seal all disposable tubes and add to waste. Monitor area with long wave UV lamp and clean with solvent and/or NaOH-soaked tissues. Incinerate all waste.

8. Calculations

It is conventional to calculate the following:

(i) Liver % body weight: $\frac{\text{Liver wt (g) x 100}}{\text{Fish wt. (g)}}$

(ii) PMS or microsomal protein content: mg protein g liver^{-1}

(iii) Enzymatic activities as:

(a) moles product formed min^{-1} (mg protein)$^{-1}$

(b) moles product formed min^{-1} (g fresh tissue)$^{-1}$

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IMMUNOASSAYS FOR CYP 1A1 INDUCTION IN FISH: NEW ANSWERS WITH WESTERN BLOTTING, ELISA AND IMMUNOHISTOCHEMISTRY

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1. POLLUTION, BIOTRANSFORMATION AND AQUATIC TOXICOLOGY

As a result of a great variety of human activities, the aquatic environment is becoming increasingly threatened by an alarming number of foreign chemicals or xenobiotics. This pollution is a threat to the health of organisms inhabiting the seas (Murchelano, 1990), as well as to human consumers of such organisms (Dawe, 1990). Fish populations living in highly polluted areas often have high incidences of gross pathological lesions and neoplasms (Malins et al., 1988), associated with elevated levels of toxic contaminants in the sediments.

Biotransformation or metabolism of lipophilic chemicals to more water soluble compounds is a requisite for detoxification and excretion. In addition, certain steps in the biotransformation pathway are responsible for the activation of foreign chemicals to the reactive intermediates that ultimately result in toxicity, carcinogenicity and other adverse effects (see Nebert and Gonzalez, 1987). Many of the enzyme systems involved in biotransformation are also engaged in critical physiological functions such as steroid hormone biosynthesis and inactivation (ibid.), fatty acid metabolism etc., making interactions between foreign chemicals and physiological processes possible (Nebert, 1991).

The first step in the biotransformation process is usually an oxidative step, catalyzed by the cytochrome P-450 (*CYP*) monooxygenase system, called "*phase I*" *metabolism*. *In* "*phase II*" *larger endogenous groups* are conjugated to the oxygenated xenobiotic with the aid of different families of transferase enzymes, thereby transforming a lipophilic xenobiotic into a polar and water-soluble end-product which can be excreted from the organism through bile or urine or over the gill.

Several of the components in biotransformation enzymes in fish have been the subject of reviews over the past years (Stegeman and Kloepper-Sams, 1987; Buhler and Williams, 1988; Buhler and Williams, 1989; Foureman, 1989; Stegeman, 1989; Stegeman et al., 1990), while Goksøyr and Förlin (1992) provide a more extensive discussion of some of the topics treated below.

2. CHARACTERIZATION OF BIOTRANSFORMATION ENZYMES IN FISH: THE CYP1A SUBFAMILY

2.1 Molecular properties of the cytochrome P-450 system

The cytochrome P-450s belong to a superfamily of structurally and functionally related hemoproteins. The cytochrome P-450 isoenzymes all consist of a single polypeptide chain with iron-protoporphyrin IX loosely bound by hydrophobic forces, electrostatic and covalent bonds. The molecular weight of the isoenzymes are in the range of 45,000-60,000 daltons.

Even though the basal features of the monooxygenase reaction are the same in all P-450-mediated transformations, there are often large differences in the chemistry of both substrate and product, from large and bulky molecules to small and planar ones, from complex steroids and polyunsaturated fatty acids to simple benzenes. The reactions catalyzed with xenobiotic substrates have still been grouped into a limited number of categories, displayed in Table 1. A focal point for these studies has been the development of prototype substrates for the measurement of specific monooxygenase activities in samples from various tissues and species (cf. Addison, this report). Ideally, such measurements should reflect the activities of individual P-450 isoenzymes, since several forms may be present in tissues and these have a certain degree of substrate specificity due to differences in the topography of the active site. Although this does not seem to hold true for more than a few of the several isoenzymes isolated so far, certain reactions (e.g. benzo(a)pyrene hydroxylation, AHH; ethoxyresorufin O-deethylation, EROD) seem specific for P-450s in the polyaromatic hydrocarbon (PAH)-inducible subfamily (*CYP*1A), and other (e.g. ethylmorphine N-demethylation, EMND, pentoxyresorufin O-dealkylation, PROD) seem specific for the phenobarbital (PB) inducible forms (see below and Table 2). Such catalytic probes have been important tools in the studies of environmental effects of pollutants, as will be discussed later. It is however important to recognize that these catalytic activities *in vitro* do not necessarily reflect the functional role of the P-450 form *in vivo*.

Table 1

Cytochrome P-450 monooxygenase reactions catalyzed with xenobiotic substrates.

Aromatic epoxidation and hydroxylation (e.g. benzo(a)pyrene and other PAHs)
Aliphatic hydroxylation (e.g. n-propylbenzene)
N-dealkylation (e.g. aminopyrin, ethylmorphine)
O-dealkylation (e.g. 7-ethoxyresorufin, 7-ethoxycoumarin)
S-dealkylation (e.g. some thioethers like methylmercaptan)
N-oxidation (e.g. aniline, amphetamine)
S-oxidation (e.g. thioethers in general, insecticides)
P-oxidation (e.g. trisubstituted phosphines)
Desulphuration and breaking of ester bonds (e.g parathion, insecticides)
Oxidative deamination
Oxidative dehalogenation
Reductive dehalogenation

2.2 P-450 genes and nomenclature

In a recent update, 27 P-450 gene families containing a total of 154 P-450 genes were described, all belonging to the P-450 superfamily (Nebert et al., 1991). According to the recommended nomenclature for naming a P-450 gene, the italicized root symbol *CYP*, denoting cytochrome P-450, followed by an Arabic number designating the P-450 family, a letter indicating the subfamily, and an Arabic numeral representing the individual gene (e.g. *CYP*1A1) should be used (ibid.). At the mRNA and protein level, nonitalicized letters are recommended (i.e. CYP1A1), but P-450 1A1 may also be allowed for the protein. This standardization of nomenclature will be of tremendous help in a field that has been confused by the use of non-standardized naming of individual P-450 forms in different laboratories. The P-450 families mostly encountered in the literature are presented in Table 2, together with prominent inducers and model catalytic activities used to study these enzymes.

Table 2

Nomenclature of selected cytochrome P-450 gene subfamilies, prominent inducers, and prototype monooxygenase activities used to assess their activity.

P-450 (*CYP*) subfamilly	Prominent inducers	Prototype activities
1A	Aromatic hydrocarbons, ß-naphthoflavone, chlorinated dibenzo-p-dioxins and dibenzofurans, certain (planar) PCBs	EROD, AHH, ECOD
2B	phenobarbital certain (non-planar) PCBs	PROD, EMND, APND, AE
3A	pregnenolone-16a-carbonitrile glucocorticoids	EMND, T6H
4A	clofibrate, phthalate esters 2,4,5-T	FAH

Abbreviations: EROD, 7-ethoxyresorufin O-deethylase; AHH, aryl hydrocarbon hydroxylase; ECOD, 7-ethoxycoumarin O-deethylase; PROD, pentoxyresorufin O-dealkylase; EMND, ethylmorphine N-demethylase; APND, aminopyrine N-demethylase; AE, aldrin epoxidase; T6H, testosterone 6ß-hydroxylase; FAH, fatty acid ω-hydroxylase
(Note: these activities are not necessarily catalyzed solely by the indicated P-450 subfamily).

Of the 154 P-450 genes that have been reported sequenced, mammalian genes predominate, and only a single fish *CYP* gene has been described; the *CYP*1A1 from rainbow trout, Oncorhynchus mykiss (Heilmann et al., 1988). This

gene was reported to be the only member of the *CYP*1A subfamily in this species. Its sequence displayed a remarkably large number of conserved regions compared with mammalian *CYP*1A forms, and specifically showed greatest similarity with the *CYP*1A1 forms in mammals (57-59% amino acid similarity compared to 51-53% for *CYP*1A2, ibid.). The strong conservation of these sequences over more than 350 million years, i.e. since the divergence of fish from the vertebrate line leading to mammals, implicates important roles in catalytic function and/or structure. Also, the presence of a single gene in rainbow trout related to both members of the *CYP*1A subfamily in mammals, is in accordance with the opinion that the duplication of this subfamily took place after the divergence of fish and mammals (Nebert and Gonzalez, 1987).

2.3 Characterization of P-450s in fish

Purification and characterization of P-450 proteins in fish have progressed somewhat faster than the study of teleost *CYP* genes. Still only a limited set of freshwater and marine fishes have been studied. Multiple P-450 forms have been purified from the freshwater species rainbow trout and perch (Perca fluviatilis), and from the marine species scup (Stenotomus chrysops), cod (Gadus morhua), and plaice (Pleuronectes platessa). Many of these forms have been discussed in recent reviews (see Goksøyr and Förlin, 1992) and are summarized in Table 3. In some cases, the isozymes were purified from fish treated with PAH-type inducers like ß-naphthoflavone (BNF) or 3-methylcholanthrene (3-Mc), in others the source was untreated fish taken directly from the environment.

Several of the forms purified from rainbow trout and scup have been reported to possess steroid monooxygenation capacity (Table 3), indicating important physiological roles for these enzymes. Such roles and regulation mechanisms are discussed in more detail by Andersson and Förlin (1992).

2.4 Characterization of *CYP*1A1 in fish

In mammals, the members of the *CYP*1A subfamily and their response to exogenous inducers such as BNF, PAHs or 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) are among the best characterized and well studied parts of the whole P-450 superfamily (Nebert et al., 1989), because of their role in metabolism and activation of aromatic hydrocarbon carcinogens and other toxic chemicals. Two genes, *CYP*1A1 and 1A2, resulting in two distinct protein products, are known to exist in all mammals studied to date.

In all fish species studied so far, only a single gene (rainbow trout) or purified protein with properties related to the *CYP*1A subfamily in mammals have been found. Using the criteria of inducibility with BNF or PAH compounds, reconstitution of AHH or EROD activity, and immunochemical relatedness to *CYP*1A forms in mammals, a *CYP*1A1 (or P-450 1A1) form has been described in rainbow trout (P-450LM4b, assuming that P-450LM4a is identical), in scup (P-450E), in cod (P-450c), and in perch (P-450 V), as shown in Table 3. Antibodies against the former three of these have been used in a number of studies to investigate the presence of immunochemically related forms in other fish species as well as in mammals (Varanasi et al., 1986; Park et al., 1986; Van Veld et al., 1990; Collier et al. manuscript in prep.). In these studies, and in a comprehensive reciprocal analysis with all these antibodies comprising several fish species spanning the phylogenetic tree of teleosts and rat (Goksøyr et al., 1991a), evidence for but a single *CYP*1A form in fish has

been found. Furthermore, these antibodies usually recognize two distinct proteins in mammalian species, consistent with the view that the mammalian *CYP*1A gene has undergone a gene duplication event after the radiation of fishes from the vertebrate line of evolution.

Table 3

Microsomal P-450 forms purified from teleost tissues (see Goksøyr and Förlin, 1992).

Species	P-450 designation	M_r	Family/subfamily (known/putative*)	Prominent activity
Rainbow trout (Oncorhynchus mykiss)	P-450LM1	50,000	-	-
	P-450LM2	54,000	CYP2B	AFB1, LA
	P-450LM3	56,500	-	-
	P-450LM4a	58,000	CYP1A	EROD, AHH
	P-450LM4b	58,000	CYP1A	EROD, AHH
	P-450LMC1	50,000	CYP2B	
	P-450LMC2	54,000	CYP2B	AFB1, TH
	P-450LMC3	56,000	-	-
	P-450LMC4	58,000	-	-
	P-450LMC5	59,000	CYP3A	-
	P-450con	54,000	CYP2*	-
	P-450KM1	54,000	-	-
	P-450KM2	52,000	-	-
Scup (Stenotomus chrysops)	P-450A	52,700	-	-
	P-450B	45,900	CYP2B	-
	P-450C	49-51,000	-	-
	P-450D	50,000	-	-
	P-450E	54,300	CYP1A	EROD, AHH
Cod (Gadus morhua)	P-450a	55,000	-	-
	P-450b	54,000	CYP2*	-
	P-450c	58,000	CYP1A	EROD, AH
	P-450d	56,000	-	-
Perch (Perca fluviatilis)	P-450 V	58,000	CYP1A	

In Table 4, we have summarized much of the available data on *CYP*1A1 cross-reacting forms in different fish species. It is apparent that most species contain a P-450 1A1 form in the 54,000-59,000 Da range, and the molecular mass of this form does not seem to have changed a lot during teleost evolution. The strong interspecies cross-reactions seen with fish P-450 1A1 antibodies, even across to mammals, suggest a strong conservation of molecular structure during the 350 million years since fish diverged from mammals (Goksøyr et al., 1991a). Furthermore, an amino acid sequence comparison of the deduced N-terminus of the rainbow trout *CYP*1A1 gene (Heilmann et al., 1988), and the sequence reported for scup P-450E (Klotz et al., 1983), demonstrates a strong conservation of this portion, with only a single amino acid differing in 10 residues (see Stegeman, 1989). Such a remarkable conservation over 70 million years of evolution, i.e. since Salmoniformes was established as a separate order in the geologic record, suggest a retained signal sequence of importance in the insertion of the protein into the endoplasmic reticulum membrane, as discussed by Stegeman (1989). More full

sequences of *CYP*1A1 genes or proteins from teleosts are needed to evaluate the structure and function relationships of these proteins in fish.

Table 4

P-450 1A1 cross-reacting proteins in different teleost species, relative molecular mass and inhibitory effect of fish P-450 1A1 antibodies.

Superorder/ order/species	M_r[a]	EROD INHIBITION[b]			Ref.[c]
		A	B	C	
Clupeomorpha					
Clupeiformes					
Herring (Clupea harengus)	55,000	++	+	+++	1
Protacanthopterygii					
Salmoniformes					
Rainbow trout	59,000	++	+	+++	1
Atlantic salmon (Salmo salar)	58,000	-	+++	-	2
Brook trout (Salvelinus fontinalis)	57,000	-	-	++++	3
Northern pike (Esox lucius)	58,000	+++	-	-	4
Paracanthopterygii					
Gadiformes					
Cod	58,000	+++	++++	++	1
Batrachoidiformes					
Toadfish (Opsanis tau)	56,000	-	-	-	5
Acanthopterygii					
Cyprinodontiformes					
Killifish (Fundulus heteroclitus)	54,000	-	-	-	3
Perciformes					
Perch	58,000	+	++++	-	1
Scup	54,000	++	++	++++	1
Pleuronectiformes					
Plaice (Pleuronectes platessa)	58,000	+++	++++	++++	1
Winter flounder (Pseudopleuronectes americanus)	55,000	-	-	-	3
English sole (Parophrys vetulus)	55,000	++++)*	++	-	5
Rock sole (Lepidopsetta bilineata)	56,000	-	++	-	5
Starry flounder (Platichthys stellatus)	55,000	-	--	-	5
Flounder (Platichthys flesus)	58,000	-	-	-	6
Dab (Limanda limanda)	58,000	-	-	-	6

a) Relative molecular mass of cross-reacting protein band in Western blots of fish liver microsomes probed with an anti-fish P-450 1A1 antibody.

b) Inhibitory effect of anti-fish P-450 1A1 antibody on liver microsomal 7-ethoxyresorufin O-deethylase (EROD) activity: + 0-25% inhibition, ++ 25-50%, etc. (-, no data); (A) polyclonal anti-rainbow trout P-450LM4b IgG (or equivalent, see ref. 4); (B) polyclonal anti-cod P-450c IgG; (C) monoclonal anti-scup P-450E MAB 1-12-3.

c) Data adapted from (1) Goksøyr et al. (1991a); (2) Goksøyr and Larsen (1991); (3) Klaepper-Sams et al. (1987); (4) Förlin et al. (1992); (5) Collier et al., manuscript in prep.; (6) Goksøyr et al. (1991d).

*) Inhibition of AHH activity (Varanasi et al., 1986)

Observations that typical *CYP*1A activities such as AHH, EROD, and ECOD (7-ethoxycoumarin O-deethylase) may be differentially induced by different inducers like diesel oils, isosafrol (Leaver et al., 1988), and piperonylbutoxide (Erickson et al., 1988), compared with 3-MC and BNF, have lead to suggestions that there may be a second inducible *CYP*1A gene present in fish, as discussed in Buhler and Williams, 1989; Stegeman, 1989. An apparent support for such postulations is the differential inhibitory effect of antibodies seen on these same activities (e.g. Stegeman et al. 1985; Kloepper-Sams et al., 1987). A similar difference in the induction response of Atlantic salmon (Salmo salar) EROD and ECOD activities to BNF was seen between immature and mature fish (Goksøyr and Larsen, 1991). In this case, immunodetectable P-450 1A1 was better correlated with ECOD than with EROD in the sexually mature fish, and separate experiments demonstrated that estradiol may directly inhibit EROD activity at physiological concentrations in vitro. (Goksøyr, unpublished results). The cloning of chimaeric mammalian P-450s in the *CYP*1A subfamily has revealed that different catalytic activities, e.g. EROD and AHH, are differently located on the P-450 protein's secondary structure (Pompon and Cullin, 1989). These findings together suggest a different explanation to the observations of differential induction and inhibition, i.e. that they rather represent different susceptibilities of a single P-450 1A1 protein's catalytic activities towards inhibitory effects of endogenous or exogenous agents, including effects of the inducing agent itself.

3. FACTORS INFLUENCING THE CYTOCHROME P-450 SYSTEM

3.1 Induction

Many chemically different compounds induce de novo synthesis of cytochrome P-450. The inductive response is generally a process by which a chemical stimulates the rate of gene transcription, resulting in increased levels of messenger RNA, new synthesis of cytochrome P-450 protein, and subsequently processing and folding to yield the catalytically active enzyme. Each of these steps i.e. mRNA, protein, and catalytic activity, can be analyzed with a suitable probe to detect induction (Fig. 1).

Classically, inducers of the cytochrome P-450 system were divided into PAH-type inducers and PB-type inducers. However, it soon became evident that many compounds induced specific patterns of cytochrome P-450 isoenzymes. Today inducers are classified according to the family or subfamily of cytochrome P-450 genes that they activate, as described in Table 2.

The induction response is not fully characterized in all *CYP* families, the best studied being the *CYP*1A subfamily. The induction response in this subfamily is known to occur via the high affinity binding of aromatic hydrocarbons to an intracellular receptor (the Ah receptor), translocation of the inducer-receptor complex to the nucleus, and transcriptional activation of the genes in the Ah battery (i.e. the two mammalian *CYP*1A forms *CYP*1A1 and 1A2, among several others).

The fact that many of the inducers of fish P-450 activities are known aquatic pollutants has greatly stimulated research in the P-450 system or fish. Pollutants of major concern in the aquatic environment are polyaromatic

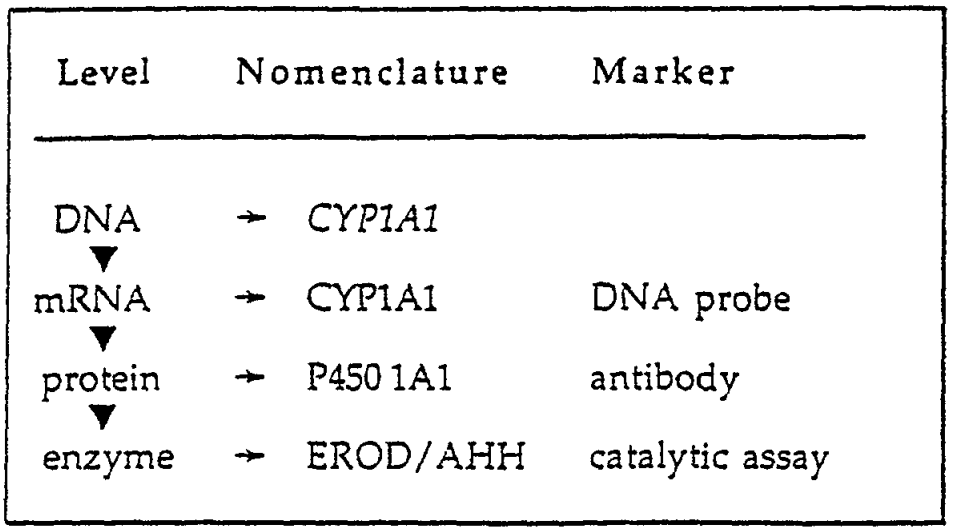

Fig. 1 Induction of P-450 1A1 involves several steps, from the activation and transcription of the *CYP*1A1 gene, translation of *CYP*1A1 mRNA to P-450 1A1 protein, and posttranslational modification (including heme insertion) to give the catalytically active enzyme. Each of the steps in the process can be studied by using the appropriate molecular probes (cDNA or antibodies) or catalytic measurements (e.g. aryl hydrocarbon hydroxylase, AHH; 7-ethoxyresorufin O-deethylase, EROD)

Table 5

Examples of xenobiotics with inducing properties on the cytochrome P-450 system of fish.

Benzo(a)pyrene
3-methylcholanthrene
ß-naphthoflavone
Pyrene
Chrysene
7,12-dimethylbenz(a)anthracene
2,3,7,8-tetrachlorodibenzo-p-dioxin
Polychlorinated biphenyls (PCBs)
Butylated monochlorobiphenyl ethers
Hexabromobenzene
Butylated hydroxyanisole
Butylated hydroxytoluene
tert-Butylhydroxyquinone
Ethoxyquin
Cyclopropenoid fatty acids
Isosafrole
Endosulfan

hydrocarbons (PAH), polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/PCDF), polyhalogenated biphenyls (PCB/PBB) and other halogenated organic compounds such as pesticides and herbicides. Examples of compounds that have demonstrated induction effects in fish are shown in Table 5.

As mentioned above (Fig. 1), the inductive response is not only detected by catalytic activity, but also with antibody probes of protein levels and with DNA probes of mRNA levels. These studies not only confirm, but also complement the results of earlier catalytic studies on inducibility by PAH type inducers in fish. Some inducers (especially some organochlorines) can for example inhibit the catalytic activity of induced P-450 (Gooch et al., 1989; Miranda et al., 1990; Boon et al., 1992). In such cases analysis of catalytic activity alone might show no response, but strong induction can still be seen by immunochemical analysis of the P-450 1A1 protein or hybridization studies with *CYP*1A1 mRNA. Apparently different types of inducers can also modulate the catalytic activity, as can endogenous compounds (discussed above). In other cases the catalytic activity may be lost due to bad storage conditions, e.g. in field sampling situations, or the sample or tissue may be too small to give measurable catalytic activity, as with fish eggs and larvae (Goksøyr et al., 1991b). In all of these cases, immunodetection of P-450 1A1 protein has been able to detect inductive responses that would not have been possible with catalytic measurements alone (Goksøyr et al., 1991b).

3.2 Other factors

Other chemicals may also affect the biotransformation enzymes, either as inhibitors or modulators of enzyme induction or catalytic activity, giving antagonistic effects. Heavy metals such as Cd has been shown to lower P-450 activities (Förlin et al., 1986), as well as immunodetectable P-450 1A1 protein (George, 1989). The mechanism of this effect is not known, and should be studied to give a basis for evaluating the response of biotransformation enzymes in mixed contamination situations, i.e. with organic chemicals and heavy metals present in the environment at the same time.

In addition to such effects of exogenous agents, endogenous physiological factors and processes, as well as changes in the physical environment, may strongly influence the P-450 system and give large natural variations in catalytic activities. Among the most important of these are the differences between the sexes of maturing fish, probably regulated by steroids, and the effects of temperature in seasonal variations of activities. These factors are discussed elsewhere (Andersson and Förlin, 1992).

There is a range of other environmental as well as physiological factors that may influence the activities of the P-450 system, such as light (photoperiod), pressure, salinity, and other stress factors like handling, noise, ionizing radiation, etc. Studies on the effects of these factors in fish are almost completely absent from the literature, while there is a great need of information on such effects to delineate background variabilities in the enzyme system.

4. BIOTRANSFORMATION AND ENVIRONMENTAL MONITORING: THE CYP1A1 CASE

4.1 *CYP*1A1 induction and environmental pollution

The induction response of some biotransformation enzymes in fish to certain classes of organic contaminants was the basis for early proposals that these biochemical responses may be used as biomarkers in monitoring environmental pollution (Payne, 1976). The marine environment today is loaded with maybe 60,000 different chemicals, for which we have very limited analytical capabilities. The link between contaminant levels and biological effects is therefore known in only a minority of these cases. Studies of molecular effects such as the induction of biotransformation enzymes by certain pollutants may give us an integrated signal of contaminant levels, their bioavailability, co-acting effects, and defence responses of the organism. Such investigations will be of tremendous help in delineating the environmental quality of an area (Stebbing, 1989).

From a number of studies carried out during the last 15 years it has become clear that P-450 1A1 mediated monooxygenase activities (EROD and AHH) are often elevated in fish from polluted waters (for reviews, see Payne, 1984; Lindström-Seppä et al., 1985; Payne et al., 1987; Haux and Förlin, 1988; Vindimian and Garric, 1989). In the work of Payne and Penrose (1975), increased hepatic AHH activity was observed for the first time in brown trout taken from a small urban lake in Newfoundland with a history of hydrocarbon contamination. Subsequent studies have shown that fish caught in waters contaminated with oil hydrocarbons, industrial or municipal wastes exhibit increased P-450 monooxygenase activity. The interpretation of most of these field studies require a reference station located in an unpolluted area with fish of similar age and sex. In addition, the interpretation in most cases relies upon the fact that the response is relatively specific with respect to the inducing chemical. However, the *CYP*1A1 induction response can provide very important information even when the inducing chemical is not identified. This may require suitable sampling strategies.

The *CYP*1A1 response, measured at the catalytic level as EROD or AHH monooxygenase activities, has already been incorporated into some major monitoring programmes, as for example the National Status and Trends Program in USA, and the North Sea Task Force Monitoring Master Plan of the North Sea nations.

4.2 Immunodetection of *CYP*1A1

4.2.1 Western blotting

Since the success in purifying *CYP*1A1 forms from fish sources (see section 2.3), antibodies towards these forms have been used to detect the induction response in several field and laboratory studies. Using the Western blotting technique, such antibodies have been used to demonstrate correlations between *CYP*1A1 levels and contaminant burden in deep-sea fish from the Northern Atlantic (Stegeman et al., 1986), in English sole (Parophrys vetulus) from Puget Sound, WA, USA (Varanasi et al., 1986), in winter flounder (Pseudopleuronectes americanus) from Northeastern US areas (Stegeman et al., 1987; Elskus et al., 1989), and in flounder from Frierfjorden in Norway (Stegeman et al., 1988). This technique implies running SDS-PAGE with subsequent transfer to a membrane where cross-reacting protein bands are

detected. The intensity of the bands are determined densitometrically, and sometimes compared to a series of standards with known amounts of purified P-450 1A1. However, only in the cases where the standard protein is from the same species as the sample, can the determination be quantitative. Usually, antibodies and standards are used across species borders (in so-called "heterologous assays"), and the results are semi-quantitative. In many cases, such results are presented as "P-450 1A1 equivalents".

4.2.2 ELISA

Recently, simpler techniques for immunodetection of P-450 1A1 levels in fish using indirect ELISA with anti-fish P-450 1A1 have been developed (Celander and Förlin, 1991; Goksøyr, 1991). The ELISA technique is better suited for handling large series of samples, and is much less time- consuming compared to Western blotting and catalytic assays. In the technique of Goksøyr (1991), microtiter plates are coated with a dilute solution of sample (10 $\mu g\ ml^{-1}$), and the intensity of cross-reaction is analyzed by reading the absorbance of the solution in each well, after proper incubation with antibodies and colour reagents. Since each well is saturated with protein, the absorbance will be related to the same amount of protein in each well. Results are usually presented as absorbance, as standard proteins, which are purified and in a different microenvironment than the microsomal P-450 1A1 in the sample, seem to behave differently than sample proteins in this assay. At this stage, the technique is therefore of limited value in quantifying P-450 levels, but it still shows a good reflection of relative levels in individual fish samples compared to each other (Goksøyr et al., 1991c).

This technique has been used to assess environmental induction in flounder, plaice and dab (Limanda limanda) from the Hvaler Archipelago in Norway (Goksøyr et al., 1991d), in rock sole (Lepidopsetta bilineata), English sole and starry flounder (Platichthys stellaius) from Puget Sound (Collier et al., manuscript in prep.), in pike (Esox lucius) from Lake Vänern, Sweden (Förlin et al., 1992), and, in the framework of the ICES/IOC Workshop on Biological Effects Techniques in Bremerhaven, March 1990, in dab from the German Bight (Goksøyr et al., in press).

Under controlled laboratory exposure, the anti-cod P-450 1A1 antibody has also been used to demonstrate the ability of low levels of water-soluble compounds from crude oil to induce P-450 1A1 in cod larvae and juveniles (Goksøyr and Solberg, 1987; Goksøyr et al., 1991b), and to demonstrate effects of pesticides, PCB congeners and mixtures, and 2,3,7,8-TCDD in different fish species (Gram Jensen et al., 1991; Skåre et al., 1991; Van der Weiden et al., 1992; Boon et al., 1992). In some of these cases, catalytic assays showed no induction response due to small sample sizes (Goksøyr et al., 1991b) or inhibitory effects of contaminants (Boon et al., 1992).

Another critical feature of the fish P-450 system that may affect catalytic activity measurements, is its susceptibility to protein denaturation under field sampling or suboptimal storage conditions (Förlin and Andersson, 1985). Although such samples are useless for catalytic measurements, the denaturation and proteolytic breakdown of the P-450 1A1 does not destroy the antigenic epitopes recognized by the antibody (Goksøyr, 1991). Accordingly, the P-450 1A1-ELISA has demonstrated induction effects in trout (Salmo trutta) living in a lake nearby and aluminium plant and a village, where the inducing agents are unidentified. In this case, the liver samples were taken from fish

that had been stored for several months at -20°C, i.e. with zero EROD activity. Still, the immunoquantitation using ELISA revealed a strong and significant induction effect compared to fish from nearby reference lakes (Skaala et al., unpublished results).

4.2.3 Immunohistochemistry

In immunohistochemistry, tissues are fixed immediately at sampling using proper fixation solutions, embedded in paraffin or plastic, and sliced into sections where the cellular integrity of the sample has been conserved. Now, antibodies can be added to study the presence and localization of specific proteins at the cellular or subcellular level using the appropriate microscope. Cellular localization of P-450 1A1 using specific antibodies has been evaluated in liver and extrahepatic organs of both mammals (Dees et al., 1982; Anderson et al., 1987) and fish (Goksøyr et al., 1987; Lorenzana et al., 1988; Miller et al., 1988; Stegeman et al., 1989; Miller et al., 1989; Smolowitz et al., 1991, Stegeman et al., 1991). Endothelium has been identified as a site of induction in several organs of these vertebrate groups (references as above).

In our laboratory, P-450 1A1 induction was evaluated by immunohistochemistry in fixed tissue samples from liver and several extrahepatic organs of Atlantic cod (Husøy et al., in press). The results are summarized below.

Liver. P-450 1A1 induction was evident in hepatocytes and endothelial cells of hepatic arterioles and portal veins. There was also mild staining in biliary epithelial cells. In some control fish, (moderate to) weak staining was evident in hepatocytes, although there was no staining of the vascular endothelium.

Heart. P-450 1A1 staining was intense in the endothelial cells of endocardium in both ventricle and atrium. The staining in endocardium of the atrium was more intense than that of the ventricle. The endothelial cells in the atrium were also weakly stained in some control cod, but no staining was evident in the ventricle.

Head kidney. All BNF-induced fish demonstrated strong staining with anti-cod P-450 1A1 in the tubular epithelial cells of the proximal segment. The endothelium of glomeruli and endothelium of vessels stained moderately. There was no staining in collecting ducts, opisthonephric ducts and hematopoietic tissue.

Spleen. Positive staining was seen in endothelial cells of larger blood vessels, as well as the endothelial cells lining the ellipsoids in BNF-treated fish.

Pyloric ceacae and intestine. Induced cod showed positive staining in the vascular endothelium in both pyloric caecae and gut.

Gill. Strong positive staining of the pillar (endothelial) cells was present in gills from the BNF-treated group. Further, endothelial cells of the branchial arteries and veins showed significant staining. No staining was evident in control fish.

Using immunohistochemistry (and also the ELISA technique) this study verified P-450 1A1 induction in multiple organs of Atlantic cod. The strong staining in endothelial cells of the vasculature adds to recent recognition that endothelium may be a common site of P-450 1A1 induction in vertebrates from fish to mammals (Dees et al., 1982; Anderson et al., 1987; Stegeman et al., 1989; Miller et al., 1989, Smolowitz et al., 1991; Stegeman et al., 1991). Induction of P-450 1A1 detected in S9-fractions or microsomes of many organs by ELISA may possibly reflect the P-450 1A1 induced in the endothelial cells only of these organs, and may therefore give a "diluted" result compared to immunohistochemistry.

Nebert (1991) propose a new understanding of drug-metabolizing enzymes: That the steady state level of many important growth effector ligands might be regulated by P-450 and related enzymes. Induction of P-450 1A1 in endothelial cells may indicate that these cells have important regulatory functions in these processes.

The observations presented by Husøy et al. (in press) and by Smolowitz et al. (1991) demonstrate that immunohistochemistry can be a useful indicator of P-450 1A1 induction in extrahepatic tissues (kidney, gill, spleen and heart) where EROD activity is low.

5. CONCLUSIONS

Summarizing the studies with *CYP*1A1 antibodies, they have demonstrated the usefulness of immunoquantifying the P-450 1A1 protein level as an important complement to catalytic studies in environmental monitoring. Such information is in fact of crucial importance in cases were catalytic activity (a) is inhibited by compounds (exogenous or endogenous) present in the sample, (b) is lost due to denaturation in improper sampling or storage conditions, or (c) is not measurable due to small sample sizes, as in fish eggs and larvae. ELISA seem to be a useful technique when large numbers of samples are to be analyzed, as in monitoring programmes. However, the results obtained by immunohistochemistry indicate that only specific cell groups (endothelium) are able to be induced in the different organs, thereby resulting in a dilution effect when using techniques where the tissue is homogenized, as in catalytic measurements, Western blotting, and ELISA. This also points to a role of immunohistochemical studies in monitoring programmes.

In natural fish populations the combined influences of biotic (e.g. species, sex and age) and abiotic (e.g. temperature and season) factors are known to cause background variations in P-450 levels and activities (Table 6). In biochemical monitoring of environmental contaminants, the effects of such biotic and abiotic factors have to be taken into account. This can be done by achieving an understanding of the mechanism by which these factors affect the P-450 system and by characterizing the magnitude and timing of the changes. Finally, it should be noticed that the susceptibility of the P-450 system to degradation e.g. during sample handling and storage, imply the use of well characterized and well functioning techniques in studies of biotransformation and effects of foreign compounds in fish.

Table 6

Factors affecting the cytochrome P-450 system in fish.

Species
Strain
Sex
Reproductive stage
Temperature
Age
Dietary factors
Inducing agents
Antagonistic agents

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PRACTICAL NOTES ON IMMUNODETECTION OF CYTOCHROME P-450 1A1 INDUCTION IN FISH LIVER AFTER ß-NAPHTHOFLAVONE TREATMENT

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1. INTRODUCTION

1.1 General

Antibodies (immunoglobulins) are proteins produced by the organism's immune system as a response to exposure to a foreign compound or structure. The antibodies have a specific ability to recognize and bind with high affinity to the molecule (antigen/immunogen) that produced the response in the organism (e.g. by immunization). Information on how antibodies are produced, how they look and how they work can be found in any textbook in biochemistry or immunology.

Because of their ability to bind strongly and specifically to particular molecules, antibodies have become an increasingly important tool in modern experimental biochemistry, anatomy, physiology, microbiology, toxicology as well as in medical and industrial applications. Antibodies can be generated against specific proteins, DNA, carbohydrates or other cellular structures, and against small molecules or pieces of larger structures, if they are conjugated to a carrier protein (hapten-carrier complex).

Antibodies can be used to study the presence of specific proteins in certain clinical conditions, to localize a protein in histochemical studies, to quantify an antigen, determine structural similarities, study protein synthesis etc. The affinity between antigen and antibody can also be utilized in immunoaffinity purification of proteins. Applications of antibodies cover a large area.

The key words are high sensitivity and high specificity, but it all depends on the quality of the antibodies we are working with. The principles of detection is normally to use a secondary antibody conjugated with an enzyme, radioactivity or a fluorescent group as a "piggy-back" on the primary antibody. These conjugates can subsequently be detected using the appropriate system.

1.2 Monoclonal and polyclonal antibodies

Normally we immunize a rabbit (or a larger animal such as a goat, sheep or horse), and let the rabbit produce the antibodies. In this case, we get polyclonal antibodies in the rabbit serum. If we immunize a mouse and remove the spleen cells from this animal, each spleen cell will produce a different antibody. After fusion of the spleen cells and immortal myeloma cells, we can produce cell clones that can be cultured and harvested, and that

will produce antibodies of one specific type. These are called monoclonal antibodies.

2. PROCEDURE

Note: Keep liver samples on ice until processing!
See Annex for information on buffers.

2.1 SDS-polyacrylamide gel electrophoresis

Samples are to be separated in a 4-20% precast polyacrylamide gel (non-gradient gels of 7-10% can also be used). The liver samples must be treated with SDS and 2-mercaptoethanol in order to solubilize protein aggregates and disulfide bonds, and to give the proteins a similar charge/surface ratio.

I. From each sample, a volume corresponding to 100 µg of protein is mixed with 50 µl of "Boiling mixture" (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.05% bromophenol blue), 3 µl 2-mercaptoethanol (wear gloves, work in the hood!) and dist. water to a final volume of 100 µl in eppendorf tubes. After mixing, pinch a small hole in the lid and heat the tubes in water bath at 95°C for 3-4 minutes (in the hood!). Pre-stained molecular weight standards (Bio-Rad) are prepared by mixing 20 µl with 20 µl "Boiling mixture" and heating the same way as the samples. After boiling, electrophoresis can start.

II. 10 µl from each sample is carefully applied in the well (avoid overfilling), and the buffer chambers are filled with "Reservoir buffer" (6g Tris, 1g SDS, 28.8 g glycin, pH 8.3, per litre dist. water). Check for leakage, adjust voltage on the power supply to 200 V, and start electrophoresis (make sure the poles are correctly connected).

III. Let electrophoresis proceed until the blue front is near the bottom of the gel (normally 40-45 min.). When finished, turn off the power supply, take out the gels from the system (carefully, wear gloves), and put them in a small tray containing "Blotting-buffer" (3.03 g Tris, 14.4 g glycin, pH 8.3, 200 ml methanol + 800 ml dist. water) for equilibration for 10 min.

2.2 Western blotting

After the equilibration we are ready to start the blotting procedure. If we leave the gels too long, proteins will diffuse into the gel, and the blotting will result in diffuse bands. The gel is now packed into a sandwich system with close contact with the nitrocellulose membrane for electrophoretic transfer of proteins in the sample. Wear gloves all the time, both for your own and the result's sake!

IV. Open the cassette and put the dark part down into a tray with "blotting buffer". Put one of the sponges down, and place a wet sheet of filter paper (e.g. Whatman 3MM) on top of this. The gel is placed mirror-side up on top of the sheet, and a piece of nitrocellulose membrane, cut to cover the gel and carefully dipped into buffer first (hold only in corners, and avoid trapping air inside the membrane), is placed on top over the gel. Use a clean glass tube as a roller to carefully squeeze out any air bubbles trapped between the gel and the membrane and to get good contact between the

two. Place a new sheet of wet filter paper on top of the membrane, and the other sponge as the final part of the sandwich, before the cassette is closed (keep in buffer).

V. The cassettes are placed into the blotting cell with the dark side facing the dark side of the container. Put the frozen cooler in place, and fill up the "Blotting buffer". Put on the lid (check poles!), adjust voltage to 100 V, and start blotting.

VI. After 1.5 h transfer, blotting is terminated by turning off the power supply, cassettes are opened (wearing gloves) and membranes taken out and placed in TTBS-buffer (TBS=Tris-buffered saline: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl + 0.05% Tween-20 = TTBS) (PBS=phosphate-buffered saline may also be used for this purpose) for 5 min. equilibration. Change to "Blocking buffer" (TTBS + 3% gelatin or 5% low fat dry milk) and leave for 30-45 min. to block sites on the membrane not occupied by sample protein.

VII. Wash the membrane 2 x 5 min. in TTBS (or TPBS).

VIII. Add 1° Antibody solution (rabbit anti-cod P-450 1A1 IgG 1:300 in TTBS w/1% gelatin or 5% low fat dry milk) to the tray containing the membrane. More membranes can occupy the same tray to save antibody solution. Incubate 2 h at room temperature, or leave overnight at 4°C.

IX. Wash the membrane 2 x 5 min. in TTBS.

X. Add 2° Antibody solution (Goat anti-rabbit) IgG conjugated with horseradish peroxidase, GAR-HRP, 1:3000 in TTBS w/1% gelatin or 5% dry milk). Incubate 1 h at room temperature.

XI. Wash the membrane 2 x 5 min. in TTBS, and once with dist. water.

XII. Prepare HRP Colour Reagent solution just prior to use: (A) 30 mg HRP Color Development Reagent (1 tablet) is dissolved in 10 ml methanol. Protect against light. (B) Add 30 µl cold 30% H_2O_2 (hydrogen peroxide) to 50 ml TBS (do not use PBS or TTBS!). Mix (A) and (B) and pour immediately over the membrane.

XIII. After 5-30 min. cross-reacting bands should appear. If weak staining, shift to dist. water and leave in the tray protected from light. If strong staining, wash twice with dist. water and leave in the tray protected from light. Photographs or densitometric analyses are made when staining is most intense.

XIV. Plot the mw of the pre-stained standards vs. migration on a semilogarithmic paper. Place the main cross-reacting P-450 1A band on the standard curve according to migration and calculate mw of the band.

3. ELISA

I. For ELISA, samples are diluted with "Carbonate buffer" (50 mM Na-bicarbonate, pH 9.5) to a concentration of approx. 10 µg ml^{-1}. Using micropipettes take out a volume corresponding to 100 µg of microsomal protein and dilute in 10 ml "Carbonate buffer". Mix well. Repeat for each sample. Keep on ice.

II. Make a diagram of a 96-well microplate, avoid the wells along the edges, and mark 3 wells for each sample (triplicates). Put 100 µl in each well according to the diagram. Put "Carbonate buffer" in all other wells not containing sample. The plates are wrapped in aluminium foil and placed at 4°C overnight. This will allow the proteins in the sample to adsorb to the well surface ("coating").

III. After coating, wash the wells 3 times in TTBS (or TPBS), leaving the last washing solution in the wells for 3-5 min. as a soaking step.

IV. Block wells with 200 µl TBS w/5% dry milk for 45 min. at room temperature.

V. After blocking, wash the wells again 3 times in TTBS, leaving the last washing solution in the wells for 3-5 min. as a soaking step.

VI. To all wells used in step IV, add 100 µl 1° antibody solution (rabbit anti-cod P-450 1A1 IgG 1:300 in TBS w/5% dry milk). Wrap in aluminium foil and place in incubator at 37°C for 2 h (or overnight at 4°C).

VII. After incubation, wash the wells 3 times in TTBS as above.

VIII. Add 100 µl 2° antibody solution (GAR-HRP 1:3000 in TBS w/5% dry milk). Wrap in aluminium foil and place in incubator at 37°C for 1 h.

IX. Wash the wells 5 times in TTBS as above.

X. Prepare developing solution just prior to use by dissolving 1 tablet (15 mg) o-phenylenediamine (OPD, carcinogen, wear gloves) to 40 ml "OPD buffer" (150 mM Na-phosphate, 50 mM Na-citrate pH 5.7) and adding 15 µl 30% H_2O_2 (hydrogen peroxide). Add 100 µl to each well.

XI. Let the colour reaction develop for 5-15 min. depending on the intensity of the reaction. Stop the reaction by adding 50 µl 4N H_2SO_4.

XII. Read absorbance in the plates on a microplate reader at 492 nm. Subtract blanks (take the mean of the blank row).

XIII. Make histograms of ELISA absorbance in each group (mean ± SD), i.e. controls and ßNF-treated.

ANNEX TO PRACTICAL NOTES

BUFFERS FOR ELECTROPHORESIS, WESTERN BLOTTING AND ELISA

30% Acrylamide solution

4.10 M Acrylamide	29.2 g
0.05 M Bis-acrylamide	0.8 g
Total volume (dist. water)	100 ml

Note: Add distilled water carefully and wait until all the acrylamide is dissolved before you fill up to the final volume. Filter the solution and store at 4°C.

Gel buffer, pH 8.8

1.5 M Trizma base	18.17 g
0.4 % SDS	0.40 g
Total volume (dist. water)	100 ml

Mix and adjust to pH 8.8 with HCl.

Stocking buffer, pH 6.8

0.5 M Trizma base	3.02 g
0.4 % SDS	0.20 g
Total volume (dist. water	50 ml

Mix and adjust to pH 6.8 with HCl.

Boiling mixture

0.25	M Trizma base	3.03 g
20	% Glycerol	20 ml
4	% SDS	4.0 g
0.05	% Bromphenol blue	0.05 g
Total volume (dist. water)		100 ml

Mix and adjust to pH 6.8 with HCl.

Polyacrylamide gels:

	7.5%	9%	15%	Stacking
30% Acrylamide	2.5 ml	3.0 ml	5.0 ml	1.25 ml
Dist. water	5.0 ml	4.5 ml	2.5 ml	3.25 ml
Gel buffer (*stock b)	2.5 ml	2.5 ml	2.5 ml	0.50 ml*
10% Amm. persulphate	33.3 µl	33 µl	33 µl	15.0 µl
TEMED	6.6 µl	10 µl	10 µl	25.0 µl
Total volume	10 ml	10 ml	10 ml	5 ml

Acrylamide, water and buffer is mixed and degassed before freshly made Ammonium persulphate (10%) and TEMED is added. Gently fill up between

glassplates and let polymerize for 20 min. (with 2-3 drops of butanol on top). Then make the stacking gel, and place appropriate combs to mark the samples well.

Reservoir buffer

0.02 M Trizma base	6.0 g
0.2 M Glycine	28.8 g
1 % SDS	2.0 g
Total volume (dist. water)	2 litre

Stir for 10 min. Check that pH=8.3.

Blotting buffer

0.025 M Trizma base	3.03 g
0.2 M Glycine	14.4 g
20 % Methanol	200 ml
Total volume (dist. water)	1 litre

Stir and check pH=8.3

Tris-buffered saline, TBS

20 mM Trizma base	4.84 g
500 mM NaCl	58.48 g
Total volume (dist. water)	2 litre

Stir and adjust to pH=7.5 with HCl.

TTBS

TBS-buffer + 0.05 % Tween-20	1 ml/2 litre

Phosphate buffered saline, PBS

5 mM di-Sodium hydrogen phosphate Dihydrate (Na_2HPO_4 x 2 H_2O)	1.7 g
2 mM Potassium dihydrogen phosphate	0.5 g
150 mM Sodium chloride (NaCl)	17.0 g
Total volume (dist. water)	2 litre

Stir and adjust to pH 7.2 with NaOH.

Or:
10 tablets per litre of dist. water (TPBS: Add 0.05% Tween-20 to the PBS buffer)

HRP Colour Reagent Solution

(A)
30 mg HRP Colour Development Reagent (or 1 tablet), 4-chloronaphthol
10 ml Methanol

(B)
30 µl cold 30% H_2O_2 (hydrogen peroxide)
50 ml TBS

Protect solutions against light. Mix (A) and (B) and pour immediately over the membrane.

Sodium citrate / Phosphate buffer (diluant for OPD)

150 mM Sodium dihydrogen phosphate	10.35 g
50 mM Sodium citrate	7.40 g
Total volume (dist. water)	500 ml

Mix and check pH=5.7.

N.B. If available use 1 capsule of "Phosphate-citrate buffer with sodium perborate capsules" (SIGMA) per 100 ml dist. water.

Coating buffer

50 mM Sodium hydrogen carbonate	9.5 g
Total volume (dist. water)	1 litre

Stir and adjust to pH 9.5 with NaOH.

N.B. If available use 5 capsules of "Carbonate-bicarbonate buffer capsules" (SIGMA) per 500 ml dist. water.

Blocking solution and Antibody-diluant (ELISA)

5 % non-fat dry milk	5 g
TBS-buffer	100 ml

Stopper

2 M Sulphuric acid (H_2SO_4, 95-97 %)	53 ml
Dist. water	bring to 500 ml

ASSESSMENT OF THE BIOLOGICAL WATER QUALITY USING ACETYLCHOLINESTERASE (AChE) INHIBITION MEASUREMENT

by

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1. INTRODUCTION

Inhibition of acetylcholinesterase (AChE) activity has been described as evidence of organophosphate and carbamate chemical poisoning (Williams and Sova, 1966; Holland et al., 1967; Coppage and Matthews, 1974; Lee and Serat, 1977; Zinckl et al., 1987). The determination of fish acetylcholinesterase as a monitoring tool for marine pollution by organophosphate and carbamate pesticides has been proposed as a biochemical indicator of pollution by these chemicals (Weiss and Gakstatter, 1964; Weiss, 1965; Holland et al., 1967; Coppage and Braidech, 1976).

The characterization of AChE from different marine species, has been studied by many workers (Coppage, 1971; Bocquené et al., 1990). Recently the International Workshop on Biological Effects (Bremerhaven, March 1990) has shown the feasibility of using such a method in field monitoring.

2. AChE INHIBITION MEASUREMENTS

2.1 Species selection

Requirements for a species to be used as biological material in a monitoring programme are as follows:

- the species must be widely distributed and very common;
- its biology should be well described;
- it must show a strong AChE activity;
- it should be as sedentary as possible (pelagic fishes for instance should be discarded).

Following these requirements, flat fishes, crustaceans and some bivalves seem to offer potential for monitoring of AChE activity.

Most of the enzyme characterization has been done on the plaice (Pleuronectes platessa), the dab (Limanda) and the common prawn (Palaemon serratus) that revealed sensitive species. The highest activity was found in the muscle of these species. In spite of the fact that molluscs had very low activity, the adductor muscle of the mussel has been successfully used.

2.2 Enzyme preparation and storage of samples

Extraction is performed on fresh muscle (1 g of tissue is enough) using TRIS 0.1 M pH 8 buffer. The tissues are homogenized (1/4 w/v) and then

centrifuged at 15000 g for 30 mn. Crude supernatants are used as enzyme source but samples can be stored either at -85°C without significant activity loss.

2.3 Protein determination

Bradford's method (1976) can be used for quantitative determination using Bovine Serum Albumin (BSA) as standard. This method is based on the differential colour change of a dye in response to various concentrations of proteins. This method has been adapted to be used with a microplate reader (Galgani and Bocquené, 1988). For each microplate well, 280 µl of Bradford's reagent (Coomassie brilliant Blue G-250, phosphoric acid and methanol) are added to 100 µl of appropriate dilution of sample. Absorbance is read at 595 nm and compared to a standard curve. Results are given as mg ml^{-1} or µg $µl^{-1}$ of crude extract.

2.4 Enzyme assay method

The method used to measure acetylcholinesterase activity has been described by Ellman et al. (1961) and adapted to microplate reading. Using acetylthiocholine (ACTC) as specific substrate, Ellman's method is based on the increase of yellow colour produced from thiocholine when it reacts with dithiobisnitrobenzoate (DTNB) ion.

For each well of the microplate, 340 µl of TRIS buffer (0.1 M, pH 8), 20 µl of DTNB (0.01 M) and 10 µl of crude enzyme solution are successively added. Acetylthiocholine (substrate) is added before enzymatic reaction is started. The enzyme kinetic is monitored on a microplate reader at 405 nm, for 4 minutes. One unit (U) of AChE activity corresponds to 0.001 variation of the optical density (O.D.). Specific activity can be expressed as Units/minute/mg of protein. Assays can be easily multiplied.

2.4.1 Enzyme assay instrument

A microplate reader is employed using a 96 wells plate. This instrument is attached to a spectrophotometer offering the possibility of reading eight different optical densities at the same time so the reading of the whole plate takes only 6 seconds. The system gives results that are accurate and reproducible. The correlation obtained by the conventional method using a classical photometer, with those obtained by a microplate reader is 0.98 for acetylcholinesterase assay (Galgani and Bocquené, 1989). In each well of the microplate, a total volume of 380 µl is needed to respect a 1 cm optical path. Using an automatic multichannel pipette the distribution of reagents takes only a few minutes.

3. ROUTINE DETERMINATION

The most important advantage in using the AChE activity for routine work is the use of the microplate reader. The perfection of this equipment enables simultaneous assays of a large number of colorimetric and enzymatic reactions.

The strategy for field work is to prepare in advance as many reagents as possible. Except for the acetylthiocholine that must be pre-weighed and

kept desiccated (because of a possible spontaneous hydrolysis), all the chemicals needed for the AChE assay can be prepared in small vials. For the protein determination, only the standard BSA must be desiccated and resuspended in buffer just before the assay.

The reduced volume (380 µl) of each well of the microplate allows the use of very small quantities of these reagents.

From the time that one gets the biological material to that of obtaining the results, the different steps for the measurement of AChE activity would only take about 30 or 40 minutes. Finally, the whole equipment can be easily shipped on board during a scientific cruise.

4. SOURCES OF ERROR

4.1 Sampling conditions

Some variations of AChE activity may occur under natural conditions but compared with many other enzymatic activities, the AChE activity is rather stable. Therefore, test measurements should be performed on population with calibrated age, sex and maturation state and all the samples should be analysed at the same time.

4.2 Analytical procedures

The main sources of variation during the analytical procedure may be due to:

- a storage temperature of samples higher than 25°C;
- a change in pH buffer;
- a too low temperature of measurement.

5. INTERPRETATION OF RESULTS

Pesticides from organophosphorus and carbamate compounds are known to be strong inhibitors of acetylcholinesterase activity (Holland et al., 1967; Coppage and Braidech, 1976; Lee and Serat, 1977; Williams et al., 1966; Zinckl et al., 1987).

Among the inorganic chemicals, arsenite and arsenate ions, metal cations in general are also inhibiting this enzyme. In the same way cholinergic acting neurochemical agents (atropine - nicotine), D.D.T. and lindane (organochlorine pesticides) have a moderate inhibitory effect (Olson and Christensen, 1980). But, if some of these compounds are not important pollutants (atropine), the concentration required to cause inhibition of AChE from those that are known to be highly toxic (DDT, Lindane, arsenic) is so large that it is unlikely to be found in the environment. Indeed, concentrations of organophosphorus or carbamate compounds required to cause 50% reduction of in vivo enzyme activity (I_{50}) are in the range of 10^{-5} to 10^{-7} M (Malaoxon, Carbaryl, Neostigmine, Eserine). Previous work (Galgani and Bocquené, 1989) has demonstrated that a detection level of 10^{-10} M was obtained for Paraoxon, using bovine erythrocyte acetylcholinesterase (Fig. 1). Recent work (Bocquené, unpublished data) has showed that the sublethal

concentration of 10^{-9} M Carbaryl resulted in 40% inhibition of AChE from common prawn, (Palaemon serratus) after 29 days of continuous exposure.

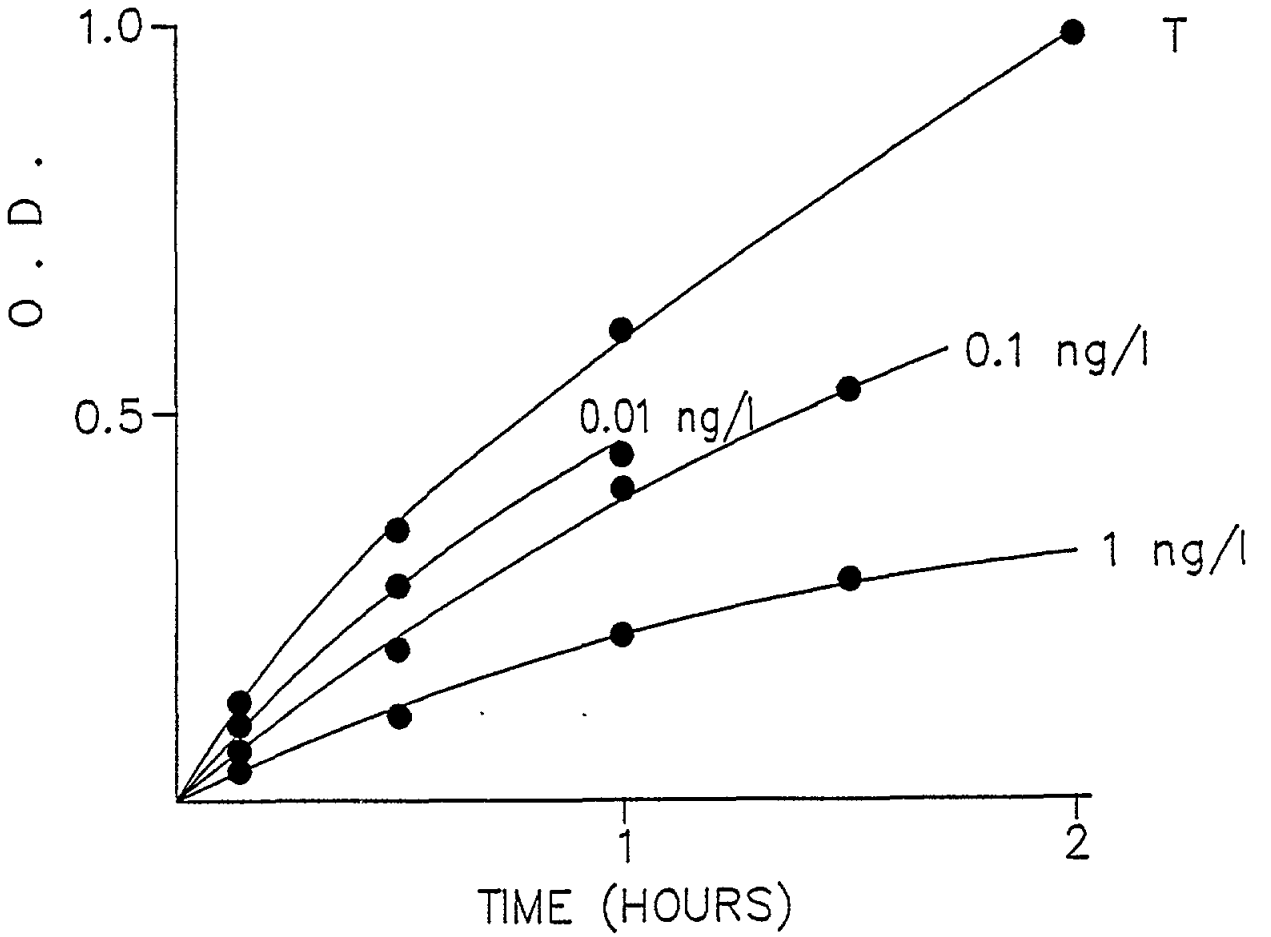

Fig. 1 Effects of DNP (Paraoxon) in sea water on the activity of bovine erythrocyte AChE, in vitro

On the other hand, inorganic chemicals such as arsenic and copper that show an important inhibitory effect (I_{50} is in the range of 10^{-3} to 10^{-5} M) cannot be found at realistic environmental levels.

Unfortunately, only a few data on the contamination level by those pesticides are available as the measurement of several thousands of different pesticide formulations (and their metabolites) at ng or pg level is a considerable problem.

FIELD EXPERIMENT

The I.C.E.S. - I.O.C. International Workshop on Biological Effects of contaminants that took place in Bremerhaven (Germany) during March 1990 gave us the opportunity to prove that AChE inhibition could be applicable as an index of sea water contamination.

One of the two areas of study was the German Bight Transect that consisted of seven points from station 1, located close to the Elbe and the Wesel estuaries, to station 9 that is situated in the Dogger Bank area. The distance of this gradient is 200 nautical miles (370 km) from station 3 to station 9 (Fig. 2). The amount of AChE activity has been determined using ten fishes (L. limanda) from each station. Results are given in Fig. 3. Consistent levels of AChE inhibition occurred in fishes from site 1, 3 and 5 while the enzymatic activity increases from station 3 to station 8 where the highest activity is found. The AChE activity from dabs from station 9 is quite interesting as it decreases significantly. This inhibition is probably in relation with the fact that the Dogger Bank area is known for its high fish disease rates and contamination.

As shown in Fig. 3 there is an evidence of a decreasing inhibition with increasing distance from the coast.

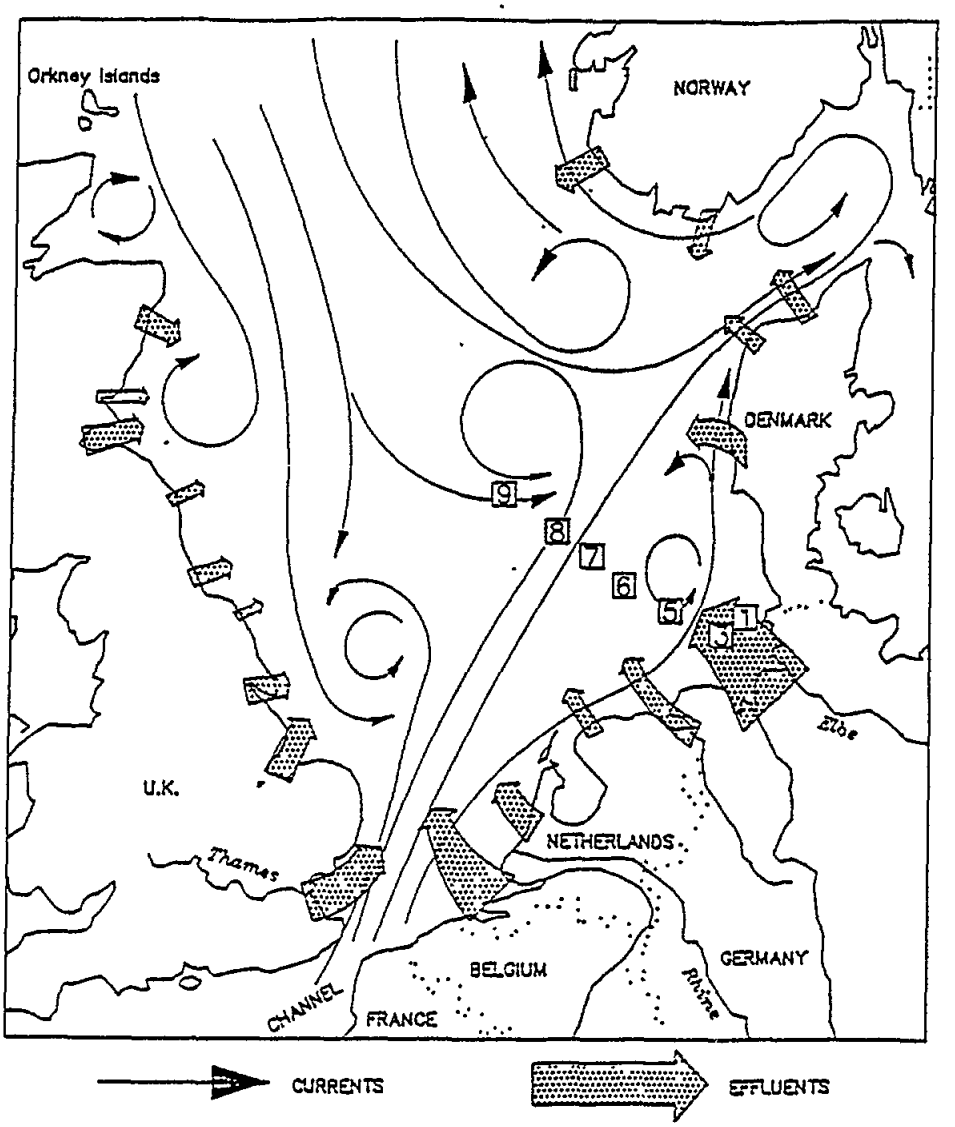

Fig. 2 Location of the different fishing stations of the German Bight Transect during the International Workshop of Bremerhaven (March 90)

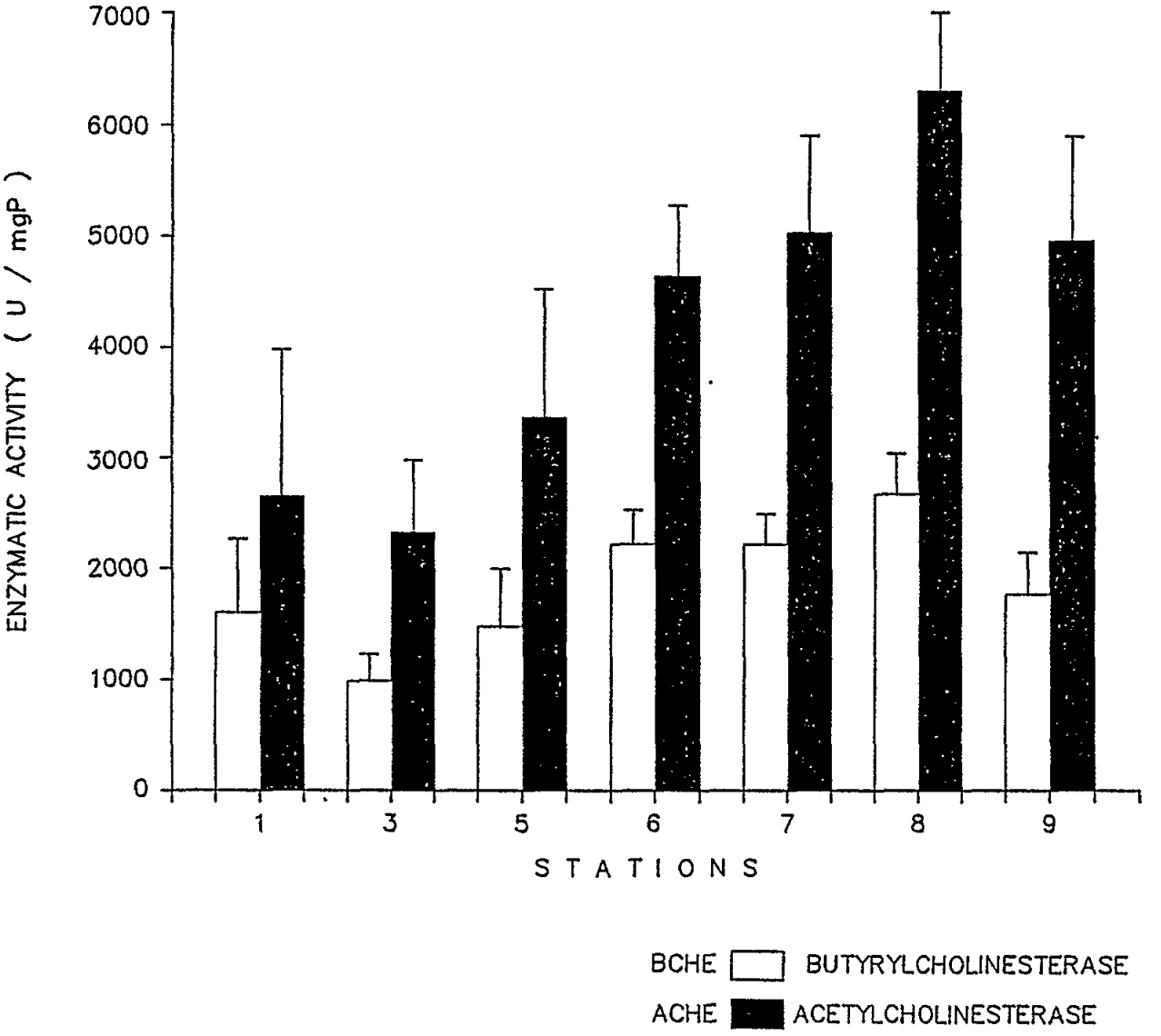

Fig. 3 AChE activity from dabs (Limanda limanda) from the seven stations of the German Bight Transect - March 90

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PART II

ROLE OF SCOPE FOR GROWTH IN ENVIRONMENTAL TOXICOLOGY AND POLLUTION MONITORING

by

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Contents

Lecture 1:
Role of Physiological Energetics in Environmental Toxicology

1. INTRODUCTION

1.1 Primary objectives of Agencies concerned with aquatic environmental protection and pollution control are:-

- To ensure increasing quality of controlled water (rivers, lakes, coastal waters and ground waters);
- To establish a full picture of any spatial and temporal changes in quality and the causes of such changes.

(Stated objectives of the newly established U.K. National Rivers Authority [NRA], 1990)

1.2 How do we achieve these objectives?

- Due to environmental complexity there is no single / simple method;
- Must ultimately be a combination of physical-chemical (cause) and biological (effect) measurements.

1.3 Current emphasis primarily on :-

- Monitoring of selected chemical contaminants (< 1% of potential contaminants)
- Aspects of Benthic Community Structure

While these are important aspects of pollution assessment, they do have significant limitations (see below) and need to be complemented by more sensitive biological effects measurements which can be used to establish cause - effect (*concentration - response*) *relationships*.

1.4 'Biological Effects Measurements' for assessing and monitoring environmental pollution (and also for laboratory toxicity testing) should ideally fulfill the following criteria:-

- They should be sensitive to a wide range of chemical contaminants at environmentally realistic concentrations;
- They should be responsive and have a large scope for response throughout the range from optimal to lethal conditions;
- They should reflect a quantitative and predictable relationship with toxic contaminants (i.e. pollutants);
- They should have a relatively short response time, in the order of hours to weeks, so that pollution impact can be detected in its incipient stages;
- They should provide either an integrated response to the 'total pollutant load', as a measure of overall impact, and / or a

contaminant - specific response reflecting the underlying cause and mechanism of toxicity;
- The technique should be applicable to both laboratory and the field studies in order to relate laboratory based concentration - response relationships to field measurements of environmental quality;
- The biological response should have ecological relevance and be shown to have deleterious effects on growth, reproduction or survival of the individual, population and ultimately the community;
- They should be easily and cost-effectively measured by trained personnel.

1.5 Brief review of the methods available for assessing marine pollution, highlighting the main examples and their advantages and disadvantages

Cause(s)

Chemical Contaminants -

Examples: Metals, Organometals, Hydrocarbons, Polychlorinated biphenyls (PCBs), Phenols, Organophosphates, Carbamates, Organochlorines etc.

Attributes: Chemical contaminants have to be analysed because they are the primary cause of deleterious effects. Selected contaminants are analysed by many laboratories world-wide.

Limitations: Due to high chemical diversity (i.e. >20000 potentially toxic contaminants and their degradation products), the time-consuming, costly (equipment and staff) and complex methodology, only a few 'selected' comtaminants are analysed and quantified (i.e. <1%).

Biological Effects

Community Structure -

Examples: Diversity indices, Indicator species abundance, 'ABC' curves, Hierarchical classification, Multidimensional Scaling etc.

Attributes: High ecological relevance because it is the community which one is trying to protect. Widespread application to 'fine sediment' communities.

Limitations: Low sensitivity (dependent on lethal effects and loss of individuals / species). Descriptive and retrospective (i.e. used for damage assessment after major pollution incidents) but is not predictive and anticipatory. Slow response and recovery to environmental change (based on mortality and recruitment). Labour intensive and time-consuming.

Lethal Bioassays -

Examples: Many species (Microtox, Copepods, Bivalves embryos / larvae are commonly used).

Attributes: Low costs, short exposures (<24 h) and relatively simple procedures.

Limitations: Low sensitivity and therefore limited to in vitro toxicity testing of chemicals and complex effluents.

Sublethal Responses -

Examples: Bivalve energetics (Scope for Growth).

Attributes: Sensitive, quantitative and integrated stress response to environmental levels of pollutants, ranging from optimal to lethal conditions. Rapid measurement (24 h) and applicable to laboratory and field studies. Growth is readily interpretable and ecologically relevant.

Limitations: Initial cost of specialised equipment and relatively complex methodology.

Biomarkers - (Responses to specific classes of contaminants)

Examples: Mixed function oxygenases (MFO), Metallothioneins, Sister Chromatid Exchange (SCE).

Attributes: Sensitive, quantitative and a degree of contaminant specificity.

Limitations: Inducibility of biomarkers limited to certain taxa (often higher invertebrates and vertebrates). Interpretacion and ecological significance is uncertain. Initial cost of equipment and relatively complex methodology.

Additional comment: It is important that EPAs do not select biological effects methods primarily on the basis of simplicity and low cost, thus sacrificing sensitivity. For example, the use of insensitive lethal bioassays in environmental monitoring will produce 'false negative' results, and significant deleterious effects will not be detected until serious damage occurs at the community level.

2. PHYSIOLOGICAL ENERGETICS OF BIVALVES

2.1 Concept of Scope for Growth

Growth provides one of the most sensitive measures of stress in an organism, since growth represents an integration of major physiological responses and specifically the balance between processes of energy acquisition (feeding and digestion) and energy expenditure (metabolism and excretion). Each of these physiological responses can be converted into energy equivalents (J h-1) and alterations in the energy available for growth and reproduction (termed scope for growth) can be quantified by means of the balanced energy equation (Fig. 1):-

$$C - F = A = R + E + P$$

or

$$P = A - (R + E)$$

where C = total consumption of food energy;
F = faecal energy loss;
A = absorbed food energy;
R = respiratory energy expenditure;

E = energy lost is excreta;
P = energy available for growth and reproduction.

Therefore scope for growth (SFG) provides an instantaneous measure of the energy status of the animal (mussel), which can range from maximum positive values under optimal conditions, declining to negative values when the animal is severely stressed and utilizing body reserves (Fig. 2). While this energetics approach can (in theory) be applied to any animal, suspension feeding bivalves and particularly mussels, represent good experimental animals because the major components of the energy budget are readily quantified.

Determination of SFG based on the energy budget, rather than direct measurement of growth itself, has proved to be particularly useful in assessing the biological effects of pollution. Direct measurement of bivalve growth is usually difficult to quantify and interpret, especially in relation to environmental pollution, due to the lack of coupling between shell and other growth components (i.e. somatic and gonadal), and the difficulty in separating 'nutrient effects' from 'toxicant effects' in the field. However, there is good agreement between indirect estimates of growth (e.g. SFG) and direct measurement of tissue and shell growth and also determination of production based on detailed population size-class analysis.

2.2 Why use mussels as indicators of pollution?

Mussels are ideal 'sentinel' organisms for monitoring chemical contamination and pollution stress, because they are :-

- sedentary and therefore cannot escape from pollution (although they can temporarily isolate themselves from high contaminant exposures by valve closure);
- abundant and widely distributed for mapping regional and global pollution;
- commercially important shellfish;
- suspension - feeders that pump and 'sample ' large volumes of water (>4 L h-1);
- bioconcentrators of chemical contaminants by factors of 10 to 100000 and so facilitate analytical detection of contamination;
- responsive to sublethal levels of pollutants, yet generally tolerant of a wide range of conditions, thus enabling stress effects to be detected before mortalities occur;
- unaffected by handling stress, so can be transplanted to sites without indigenous mussel populations;
- amenable to laboratory and field study, thus results from laboratory experiments can be used to interpret data from the field.

Although most of the research has been carried out using the common mussel (Mytilus edulis) the approach described here applies equally well to other bivalves.

A number of the attributes of mussels in relation to environmental toxicology will be highlighted below.

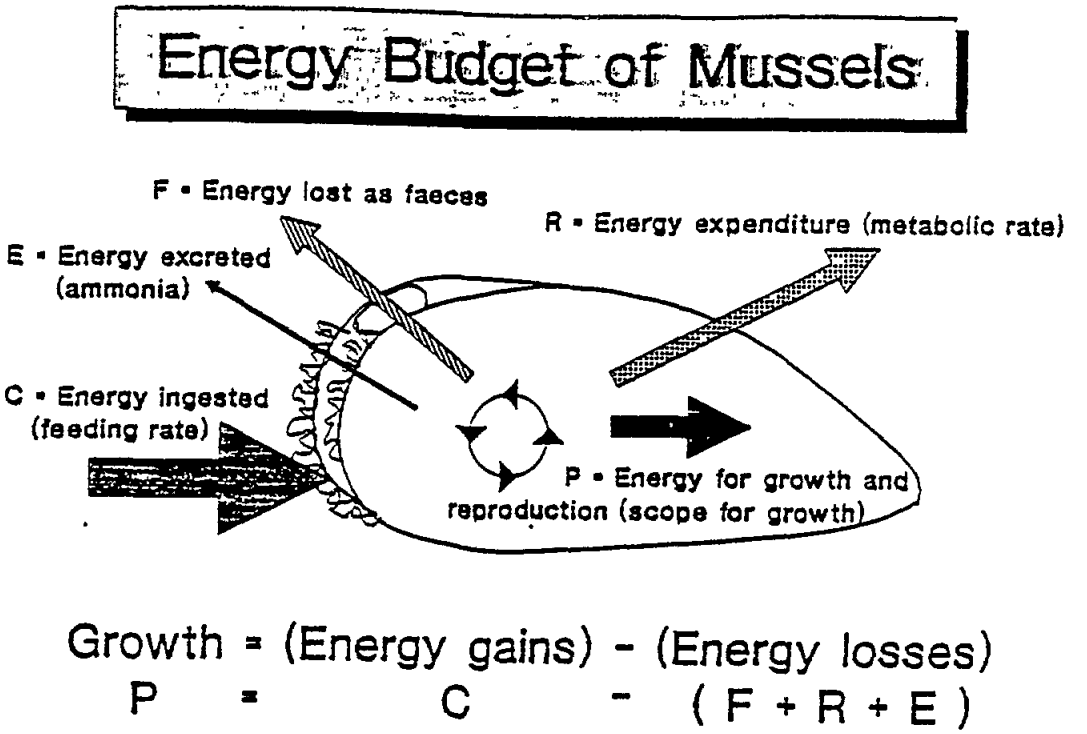

Fig. 1 Diagrammatic representation of the energy budget of a mussel

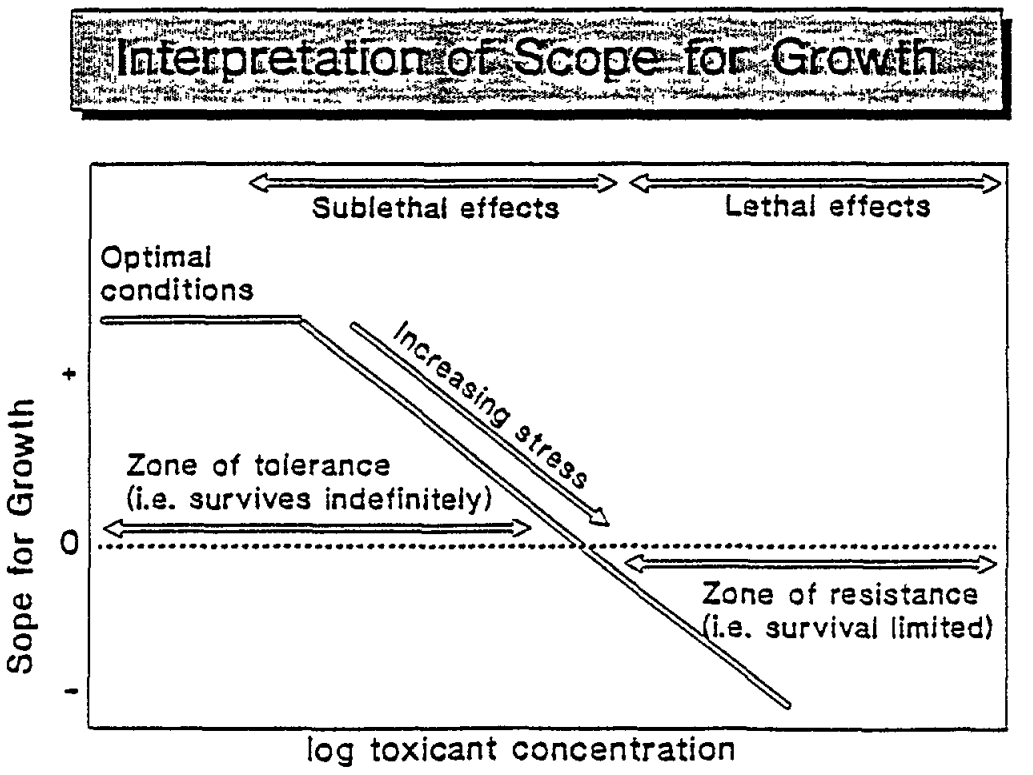

Fig. 2 Interpretation of physiological energetics (scope for growth) in response to a wide range of conditions

2.3 Bioaccumulation

Mussels readily bioaccumulate organic contaminants with minimal metabolic transformation and excretion. This is clearly demonstrated when comparing mussels and crabs collected from the same site (Fig. 3 - Langesundfjord). Mussels show markedly higher polyaromatic hydrocarbons (PAH) levels (note different scales). Therefore it is easier to monitor contaminants in the tissues of mussels and relate biological effects to body burdens. In addition, it probably explains the greater sensitivity of mussels to organic contaminants such as PAHs.

2.4 Sensitivity

In comparison with other species, mussels and other bivalves have proved to be particularly sensitive to environmental levels of pollutants. This is probably due to their ability to accumulate contaminants in their tissues to high concentrations.

In addition, physiological energetic responses are consistently more sensitive than lethal responses of bivalve larvae and adults and other sublethal responses, such as shell growth and valve gape (Fig. 4).

For example in the case of toxicants representing metals, organometals and organics - Copper (Cu) :- EC50 values (i.e. exposure concentration inducing a 50% reduction) are similar for all sublethal responses, but are considerably (>10x) more sensitive than the lethal responses (LC50) of larvae and adults.

Tributyltin (TBT a biocide used in anti-fouling paints) :- SFG is slightly more sensitive than the 15d LC50 values for larvae and 100x more sensitive than short-term response of shell growth and valve movement.

Petroleum Hydrocarbons :- EC50 values for feeding rate and SFG show that they are 10-100x more sensitive than other sublethal and lethal responses.

In field monitoring and pollution assessment sensitivity is an important feature of the biological response, to avoid recording false negatives when there may be a significant underlying effect. It is critical to establish not only the degree of growth inhibition but also how close the prevailing conditions are to the lethal limit.

2.5 Mechanistic interpretation

Physiological energetics and the disturbance of the mussel's energy balance provide insight into, and integration of, some of the primary mechanisms of toxicity, that are both biologically and environmentally important (Fig. 5). Major mechanisms of toxicity reflected in physiological energetics include:

- Non-specific narcosis inhibiting ciliary feeding of bivalves (e.g. hydrocarbons);
- Neurotoxic effects on the neural control of the gill cilia (e.g. TBT, dinoflagellate toxins);
- Uncoupling of oxidative phosphorylation resulting in an increase in the metabolic rate to maintain adenosine triphosphate (ATP) production (e.g. TBT, phenols);

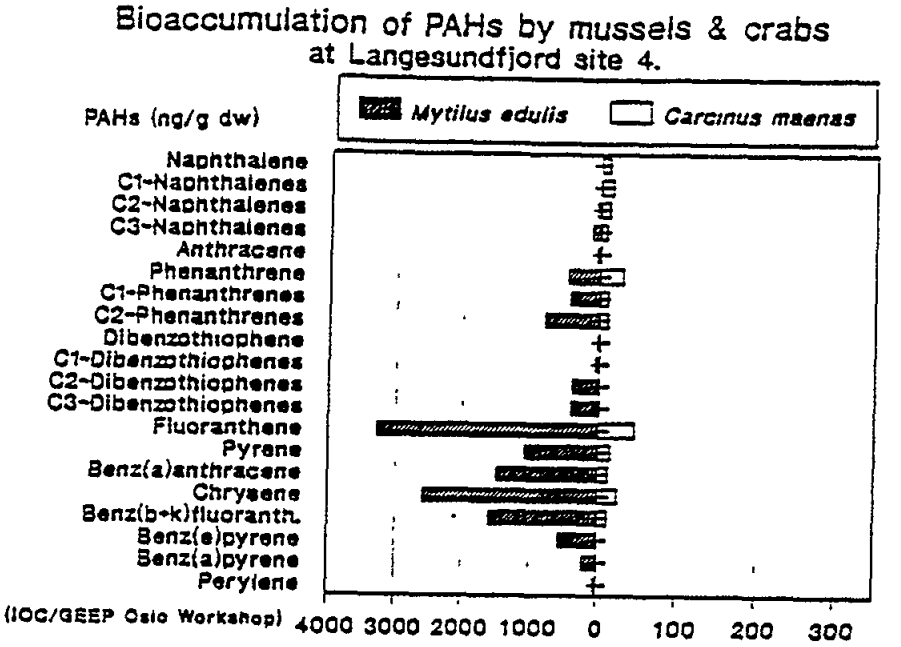

Fig. 3 Bioaccumulation of PAHs by mussels and crabs living in the same environment

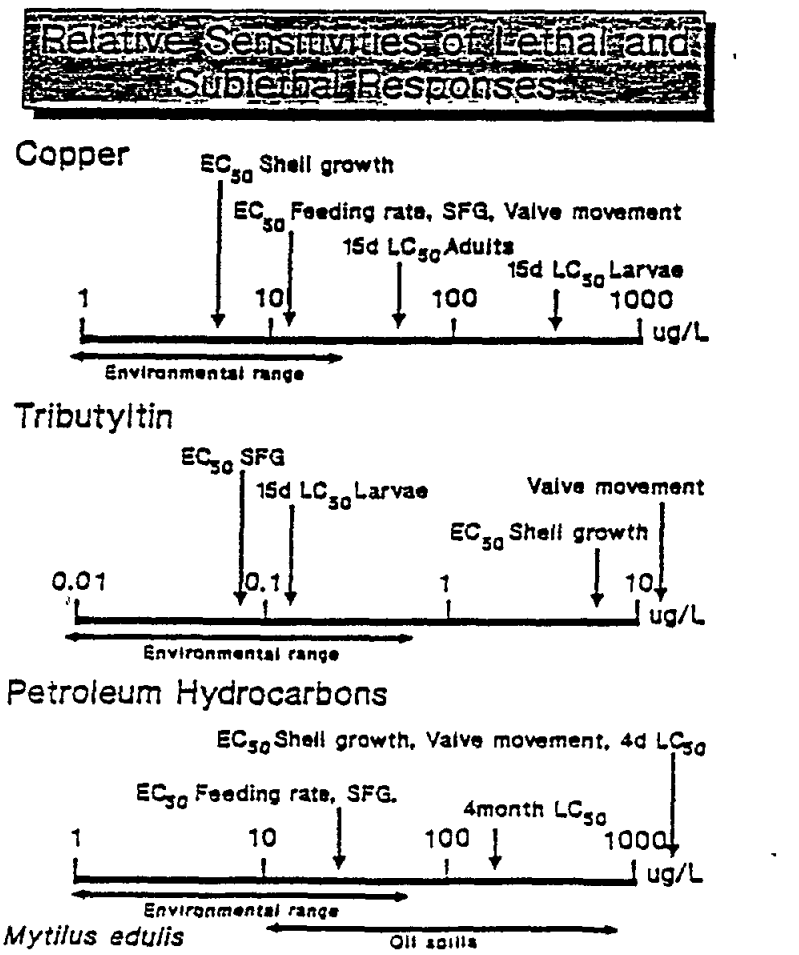

Fig. 4 Relative sensitivities of lethal and sublethal responses of mussels to copper, tributyltin and petroleum hydrocarbons

- Inhibition of metabolism thus reducing respiration rate (e.g. DBT-dibutyltin, hypoxia);

Physiological energetic responses are sensitive to important classes of contaminants because they reflect these primary mechanisms of toxicity.

2.6 Integration of multiple mechanisms of toxicity

Another important feature of the energy budget approach is that it can provide a means of integrating different mechanisms of toxicity induced by a mixture of contaminants, or in the case of TBT a single toxicant. At low concentrations, TBT induces uncoupling of oxidative phosphorylation which causes an increase in respiration rate (i.e. energy expenditure increases to maintain ATP production); while at higher concentrations TBT induces a neurotoxic effect on feeding. The resultant integrated effect is shown as a decline in growth with a pronounced inflexion at tissue concentrations of c. 2-3 µg TBT g^{-1} d.w. (Fig. 6).

2.7 Additivity

In the majority of cases the toxic effects of mixtures of structurally related and unrelated compounds are simply additive (on a proportional basis i.e. 50%+50%=25% of control). An example of the effects of Cu and oil on the feeding rate and growth of mussels is illustrated in Fig. 7.

2.8 Ecological relevance

Energetics offers a common currency [energy] enabling the consequences of primary toxic mechanisms at the cellular level to be translated into effects on growth, reproduction and survival at the individual and population levels. A decline in growth, or SFG, is therefore readily interpretable and understood as representing a deleterious effect. Field and mesocosm studies have provided confirmation that the long-term consequences to growth and survival of individuals and the population can be predicted from measured effects on energy balance at the individual level. For example, in a long-term (18 month) mesocosm experiment a littoral community was exposed to two oil concentrations. Mussels at the lower concentration (28 µg L^{-1}) had a slightly negative SFG (-0.35 J g^{-1} h^{-1}) which resulted in a gradual population decline after 12 months, whereas mussels at the higher concentration (125 µg L^{-1}) had a severely negative SFG which caused a rapid population decline over 6 months (Fig. 8).

3. ECOTOXICOLOGICAL FRAMEWORK

3.1 Detecting effects and identifying causes

The ultimate objective of ecotoxicological studies is both to predict and diagnose the causes of biological / ecological effects resulting from exposure to chemicals and other stressors in the environment. To meet this objective it is necessary to establish:-

(i) relationships between the concentration of chemical contaminants in the environment and in the tissues of biota (e.g. mussels);

Energetics reflects major mechanisms of toxicity		
Rates of:-	*Mechanism*	*Toxicants*
Feeding (↓)	Narcosis	Hydrocarbons Phenols
Feeding (↓)	Neurotoxic	TBT Toxic algae
Metabolism(↑)	Uncoupling ATP	Phenols TBT
Metabolism(↓)	Inhibition	Hypoxia DBT
Digestion (↓)	Narcosis	Hydrocarbons

Fig. 5 Some of the major mechanisms of toxicity reflected in physiological energetics

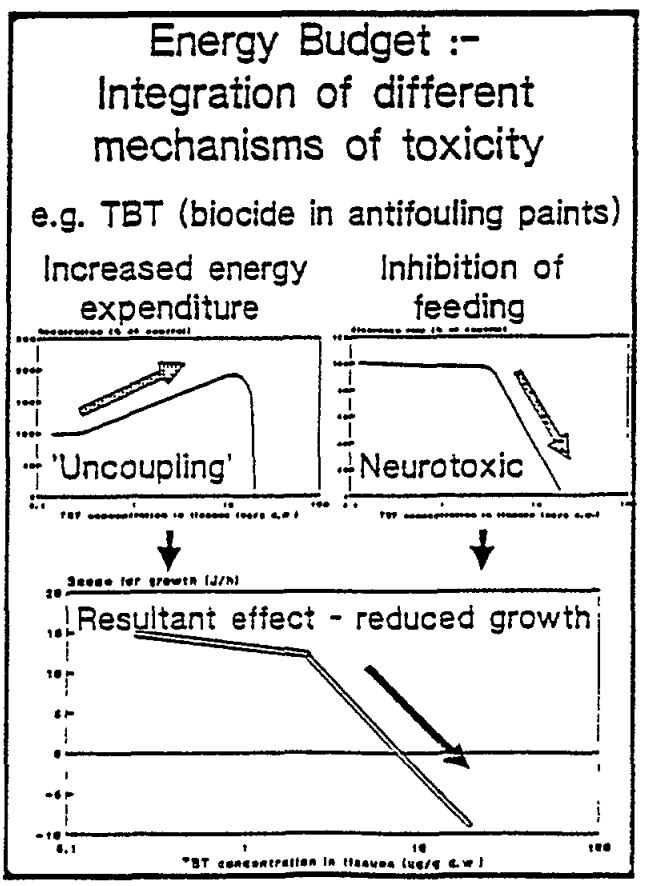

Fig. 6 Integration of different mechanisms of toxicity by the energy budget

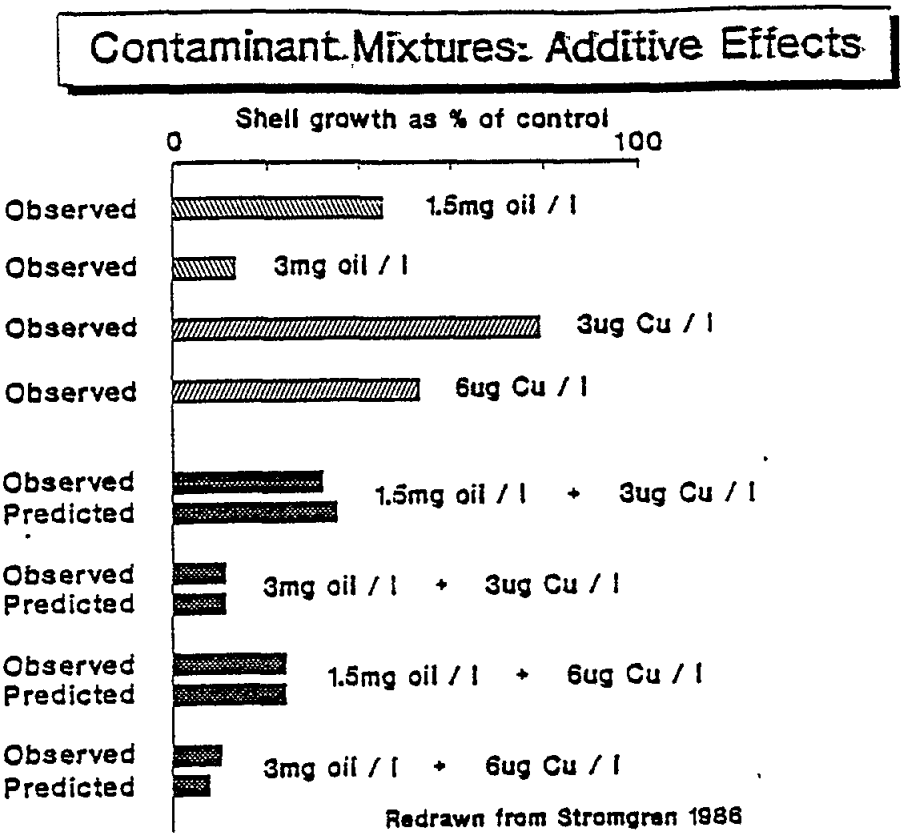

Fig. 7 Example of the additive effects of structurally unrelated toxicants, copper and hydrocarbons, on the growth of mussels (after Strømgren, 1986)

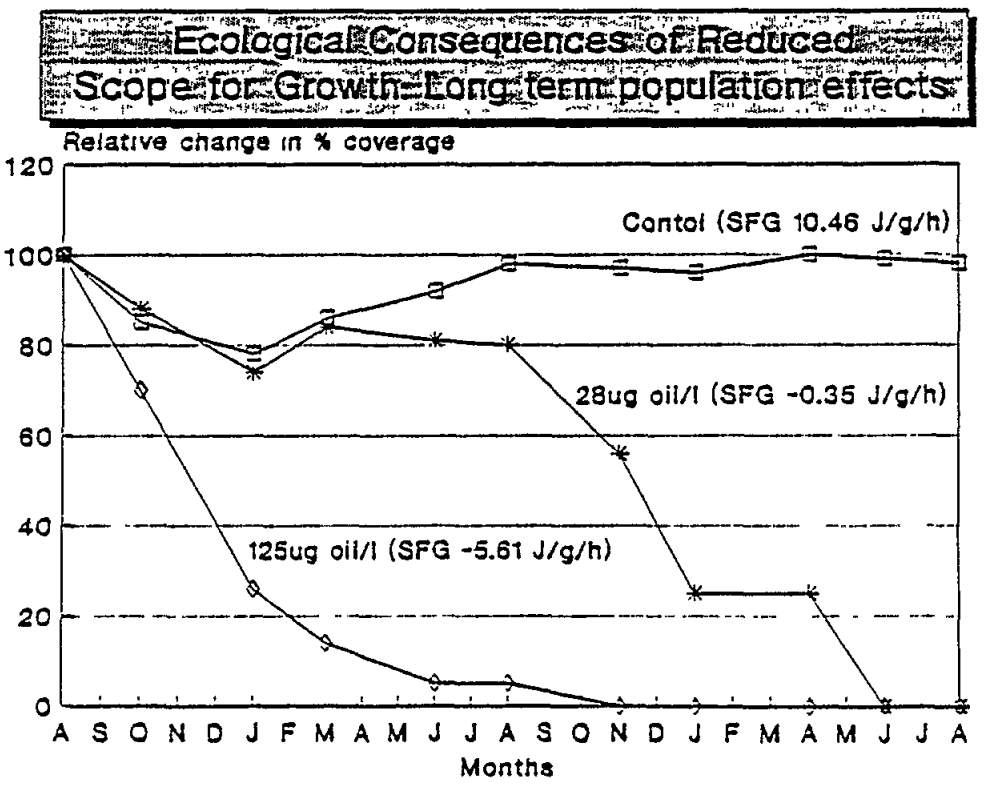

Fig. 8 Prediction of long-term population effects from Scope for Growth measurements (from Solbergstand mesocosm experiments)

(ii) cause - effect relationships between the [tissue] contaminant concentration and the resultant biological effects.

Interpretation of such relationships requires knowledge of the bioconcentration factors for chemicals and some understanding of their mode of toxic action.

The combination of chemical analysis of contaminant levels in body tissues of mussels and the measurement of physiological energetics is ideally suited to such a 'cause - effect' framework (Fig. 9). Once established, these relationships allow the prediction of effects from contaminant levels and a diagnosis of the cause from combined measurement of chemical and biological effects in the field.

3.2 Quantitative tissue concentration - Response relationships

These relationships, derived from controlled laboratory and mesocosm studies, provide an appropriate toxicological database that can be used in the prediction of effects from tissue residue chemistry data and in the identification of chemical contaminants causing effects recorded in the field.

For individual contaminants with specific mechanisms of toxicity, such as metals and organometals, it is feasible to determine tissue concentration - response relationships. Typically toxicants show a linear decline in SFG with a log increase in toxicant concentration in the body tissues. There is a zone of tolerance where effects are measurable but sublethal, but when SFG declines below zero the animal can only survive by utilizing body reserves.

For example, Cu and TBT show a decline in SFG over an environmentally realistic range of concentrations (Fig. 10). In contrast, Cd has no effect on SFG over a wide range of concentrations (>100 µg Cd L^{-1} that are above those found in contaminated environments. Consequently, Cd in seawater is not considered toxic to bivalves.

3.3 Diversity and complexity of mixtures of organic contaminants

While it may be feasible to establish tissue concentration - response relationships for individual contaminants, it is clearly unrealistic to examine the sublethal effects of more than 20,000 individual organic contaminants which are released into the environment.

For example, within oil there are many thousands of individual compounds and mussels accumulate complex mixtures (Fig. 11). How do we assess the 'total toxic load' in the animal, and which chemicals are toxic and causing the adverse effects?

3.4 Quantitative structure - Activity relationships (QSARs)

One way of overcoming this problem is to study compounds which represent particular chemical groups, and to establish which molecular characteristics give rise to the biological response (Widdows and Donkin, 1991; 1992). The toxicity of untested chemicals can then be predicted form a knowledge of their structure or related physico-chemical properties (e.g. solubility, hydrophobicity). Such a QSAR approach has been successfully used in drug and agrochemical development for many years.

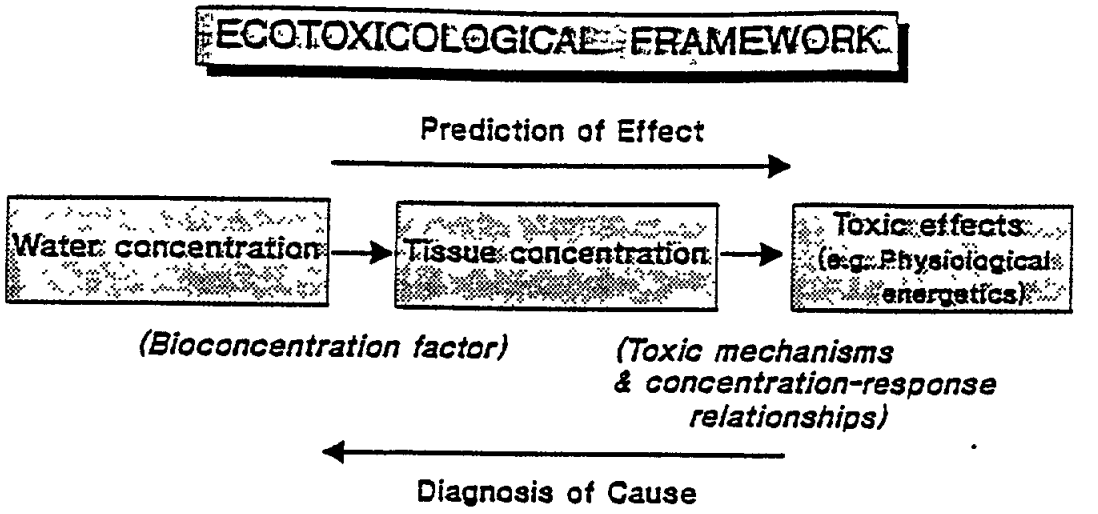

Fig. 9 Ecotoxicological framework for bioaccumulation and sublethal effects studies

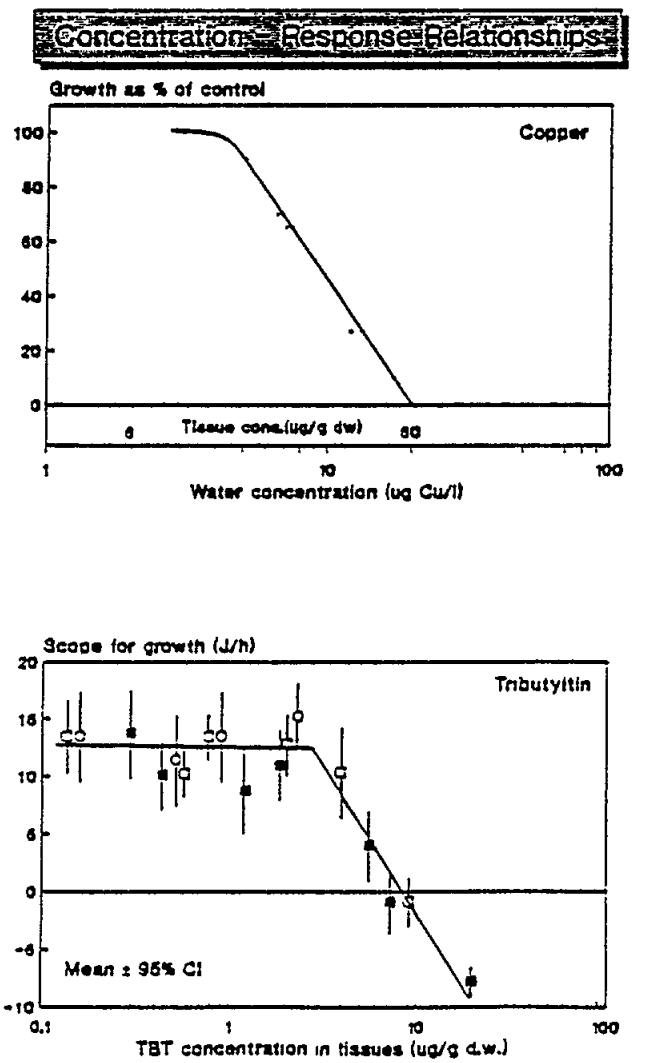

Fig. 10 Concentration-response relationships for individual toxicants such as copper and tributyltin (from Redpath, 1985; Widdows and Page, in press)

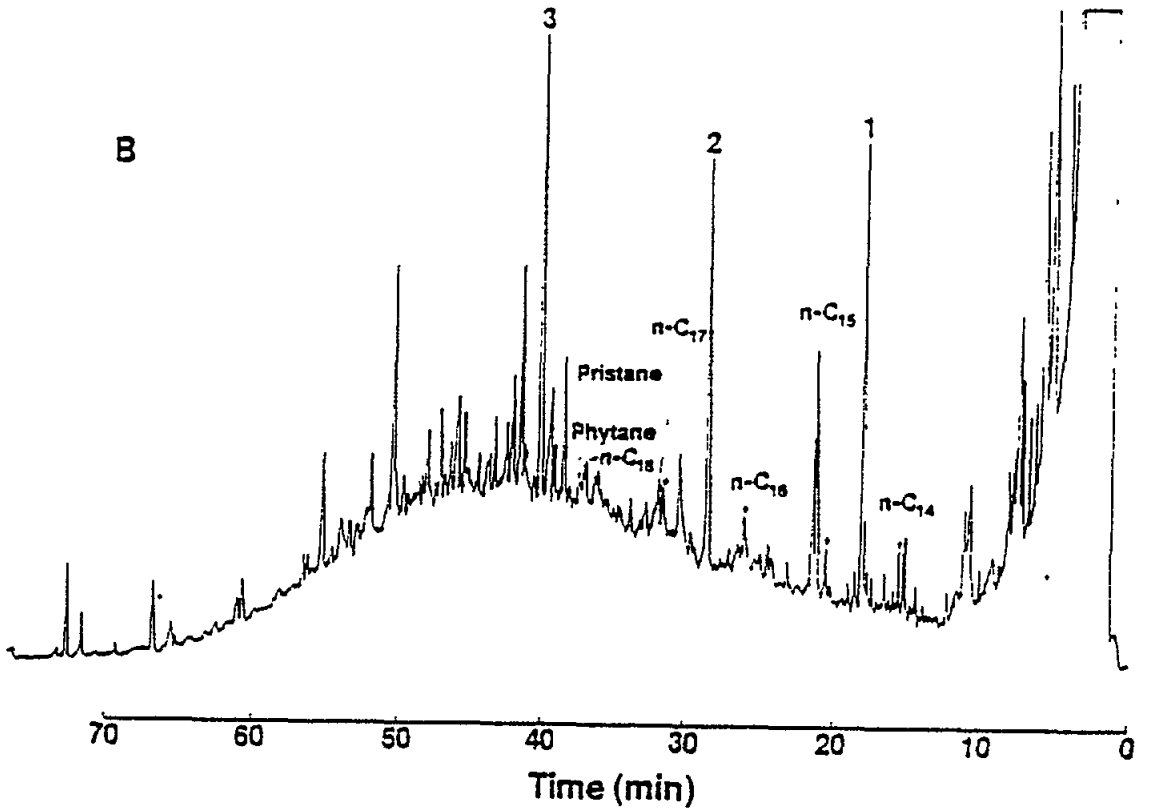

Fig. 11 Example of gas chromatogram of aliphatic hydrocarbons accumulated by mussels

A QSAR for the effect on mussel feeding activity of a range of hydrophobic organic chemicals commonly found in industrial/urban environments is represented by Fig. 12. Such QSARs show that:-

(i) As hydrophobicity (measured in terms of the octanol-water partition coefficient: log K_{ow}) increases (or water solubility declines) there is an apparent increase in the toxicity of these chemicals, when expressed in terms of the water concentration inducing a 50% reduction in feeding rate;

(ii) As hydrophobicity increases the bioconcentration factor (BCF - contaminant concentration in the organism relative to the concentration in the water) increases; and

(iii) As a consequence of these two relationships, when toxicity is expressed in terms of toxicant concentration accumulated in the tissues, most compounds are found to have equal toxicity;

(iv) However, this relationship ceases to apply for extremely hydrophobic compounds and above a critical point (termed the molecular weight cut-off) compounds are readily accumulated, but they do not exert toxic effects.

Such QSARs are of considerable practical importance, for example in interpreting the toxicity of oil, and identifying which compounds are toxic and need to be included in chemical monitoring programmes.

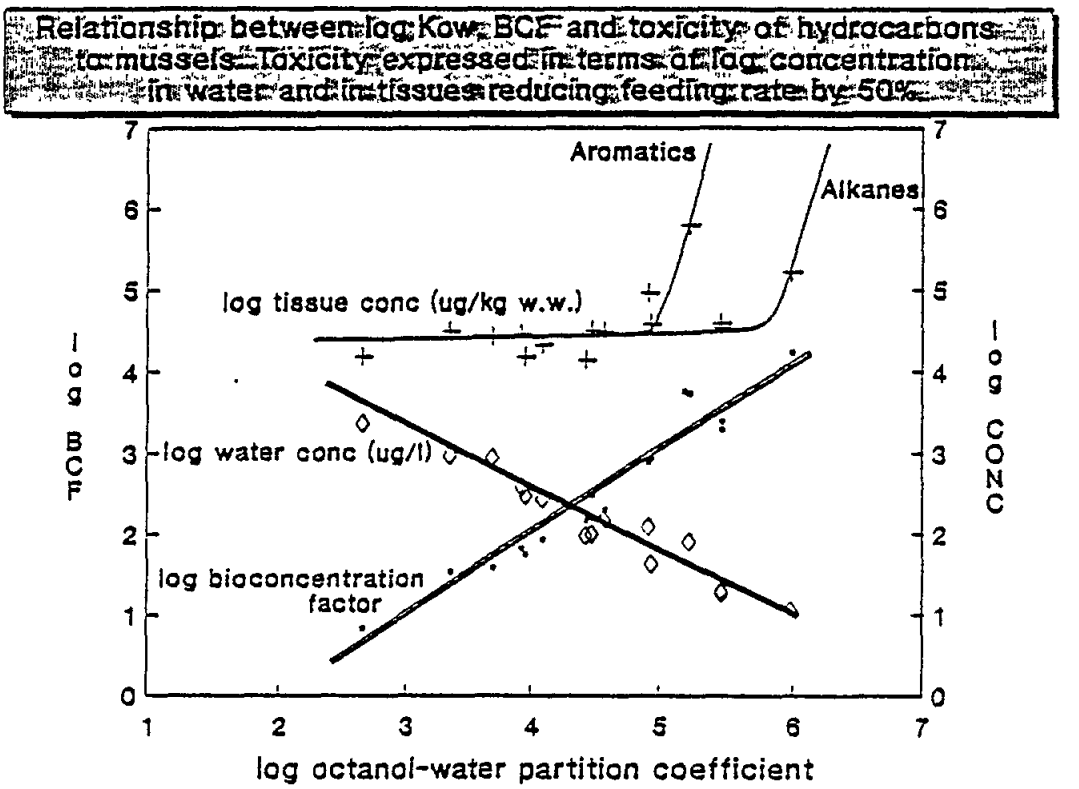

Fig. 12 QSARs showing relationships between log K_{ow}, bioconcentration factors and toxicity of hydrocarbons to mussels. Toxicity is expressed in terms of log concentration in water and in tissues reducing feeding rate by 50%

3.5 Summary of main features of QSARs

- Groups organic compounds with similar structures and mechanisms of toxicity;
- Identifies outliers with different mechanisms of toxicity;
- Predicts BCF, toxicity and additivity;
- Identifies potentially toxic compounds for chemical monitoring;
- Identifies molecular weight cut-off separating toxic from non-toxic compounds;
- Method of comparing sensitivities of different species to toxicants;
- Basis for modelling fate and effects of contaminants.

3.6 Toxicological database

Ultimately it is possible to develop a toxicological database (Fig. 13) relating growth effects to tissue concentration of toxicants. Such a database can then be used to interpret field derived body burden and effects measurements, and identify those contaminants which are sufficiently elevated to cause deleterious effects.

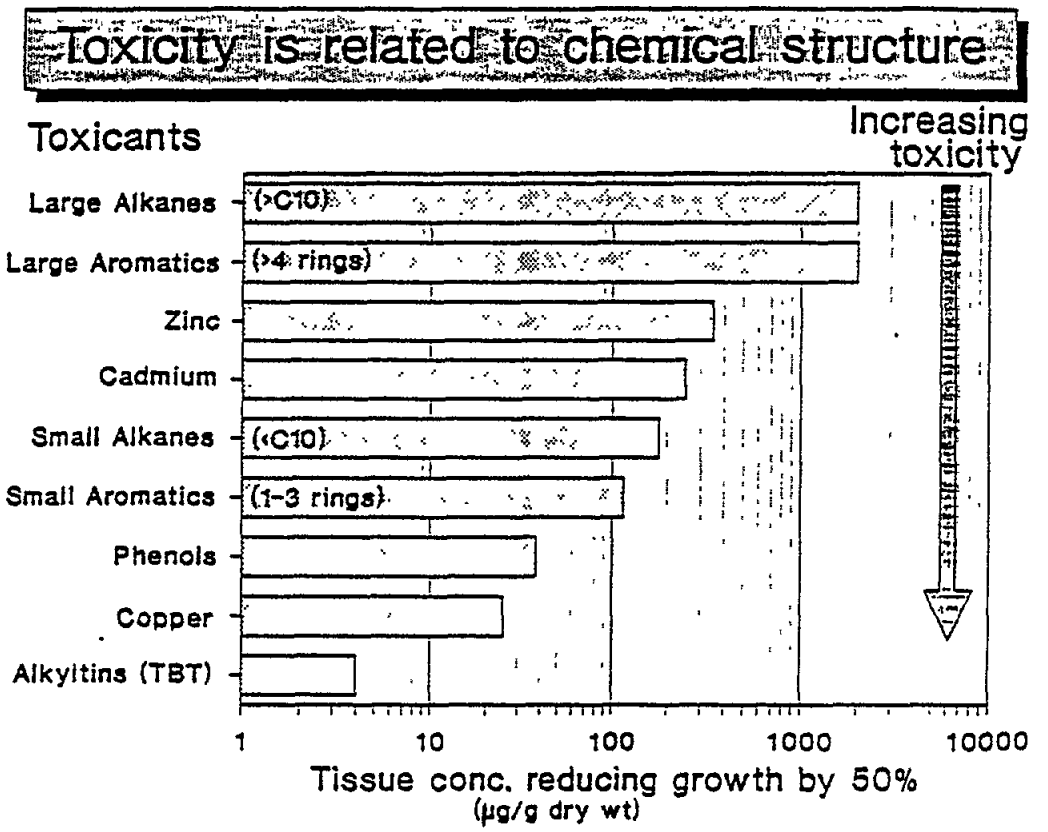

Fig. 13 Graphical representation of a toxicological database

Lecture 2:
Pollution Assessment: Field Application of Scope for Growth

1. INTRODUCTION

The assessment of environmental pollution using physiological energetic measurements of mussels, in conjunction with chemical analysis of contaminants in their tissues, has been successfully carried out in many field situations, detecting and quantifying pollution effects in both temperate and tropical environments.

1.1 Studies using combined chemical and Scope for Growth measurements of mussels over contaminant gradients of c. 10 km:-

- Narragansett Bay, USA (Widdows et al., 1981)
- San Francisco Bay, USA (Martin, 1985)
- Plymouth sewage dump site, UK (Lack and Johnson, 1985)
- Shetland oil terminal, UK (Widdows et al., 1987)
- Langesundfjord, Norway (Widdows and Johnson, 1988)
- Hamilton Harbour, Bermuda (Widdows et al., 1990)
- Greenwich sewage treatment plant, USA (Nelson, 1990)
- Cochin Harbour, India (Widdows, unpublished)
- Western Scheldt, Netherlands (Smaal et al., unpublished)

Application of this approach and the environmental questions addressed are illustrated with reference to a field monitoring programme in the vicinity of the North Sea terminal at Sullom Voe in the Shetlands, and two IOC/GEEP Biological Effects Workshops which have investigated pollution gradients in sub-tropical and temperate environments. Finally, a recent study in the North Sea will be used to demonstrate how the approach has been extended form 10 km pollution gradients in estuaries to a larger spatial scale of >1000 km of coastline.

2. NORTH SEA OIL TERMINAL IN THE SHETLAND ISLANDS

A major industrial development in a previously very clean environment was completed and opened in 1980. The objectives of the mussel monitoring programme were to assess the spatial and temporal impact of oil pollution in the vicinity of the Sullom Voe oil terminal. Combined measurements of SFG and selected polyaromatic hydrocarbons (PAH) in mussels were carried out annually during the period 1982-1989. In this study a mobile laboratory was used for the physiological measurements.

(Views of mobile laboratory, power generator, apparatus inside laboratory).

2.1 Spatial and temporal changes in environmental quality were assessed by sampling mussels from established populations around Sullom Voe in July each year (i.e. during the summer period of growth after spawning). Site 1 (Fig. 14) was the 'clean' reference site outside of Sullom Voe. Site 5 represents the oil loading jetties (i.e. the source of the oil). This was consistently contaminated by PAHs,

typically an order of magnitude higher than the reference site, and consequently mussels had a reduced SFG. Site 4 was c. 2 km south of the oil jetties and mussels were moderately contaminated and affected. Site 3 was towards the head of the Voe and relatively clean. Site 2 at the southern end of the Voe showed significant PAH contamination and the mussels had reduced SFG. However, this was not the result of oil spills from the terminal but was due to a localised and gradual seepage of hydrocarbons following a land spillage of diesel oil at a nearby quarry before 1980. Therefore the results demonstrate the absence of a simple pollution gradient along Sullom Voe and highlight the ability to detect additional pollution events (e.g. site 2).

2.2 A synthesis and interpretation of Sullom Voe results

It is necessary to place the Sullom Voe results into a broader geographical and ecological context, in order to address the questions posed by environmental managers, such as:- 'How bad or good are the conditions in Sullom Voe?' and 'At what level of impact should managers start to be concerned and take remedial action?'

A synthesis of data derived from Sullom Voe and Solbergstrand mesocosm oil studies provides a semi-log relationship between SFG and log (2+3 ring PAHs) in the tissues (Fig. 15) and illustrates a number of points:-

(i) There is an inverse relationship between SFG and log PAH over 3 orders of magnitude without an apparent threshold effect - suggesting that toxic effects are simply dependent on PAH loading of the tissues with no evidence of physiological compensation.

(ii) Mussels from the reference site near Sullom Voe are very clean (subsequently established to be the cleanest site studied).

(iii) Mussels at the tanker loading jetties, nearest the source of pollution are only moderately contaminated (c. 10-fold higher than reference site) and no greater than other areas near urban development (e.g. Tamar estuary, Plymouth; Solbergstrand, Oslofjord).

(iv) In comparison with other sites, mussels in Sullom Voe experience only moderate levels of pollution and appear to have sufficient capacity to grow, reproduce and maintain a viable population.

It provides a unique study in which there has been quantification of contaminant inputs (oil spills), subsequent bioaccumulation in mussels and resultant effects with high correlations of >0.85.

3. BERMUDA BIOLOGICAL EFFECTS WORKSHOP

The approach has also been applied in a more tropical, non-industrial region with low levels of contaminants and using a native mussel. Arca zebra were transplanted in cages to two areas of Bermuda (Fig. 16):-

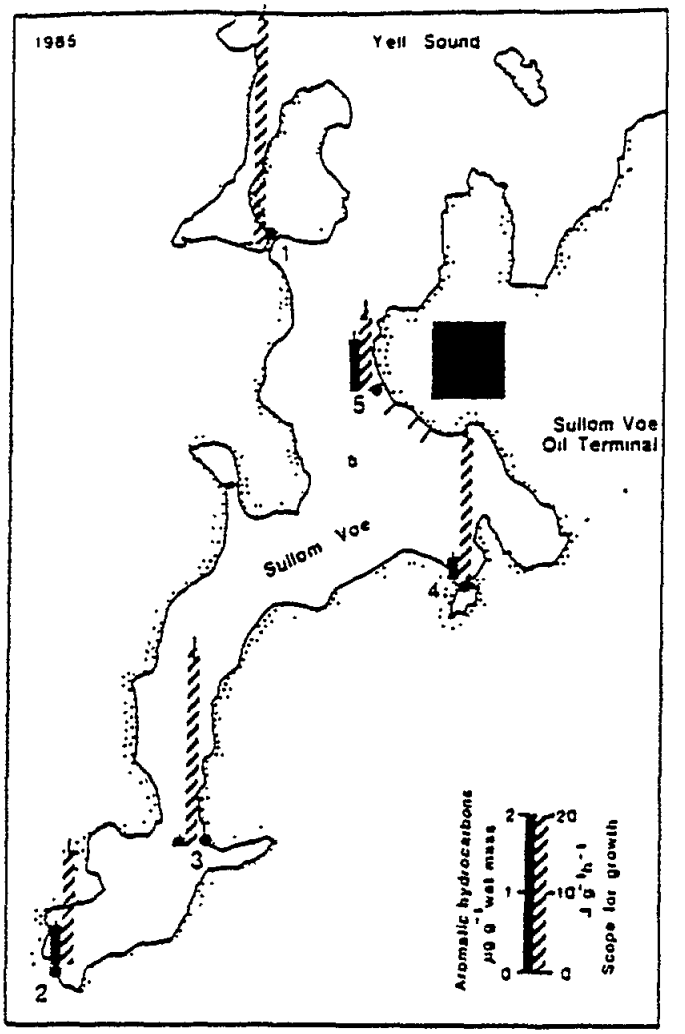

Fig. 14 Location of mussel populations/sampling sites in the vicinity of the North Sea oil terminal in the Shetlands

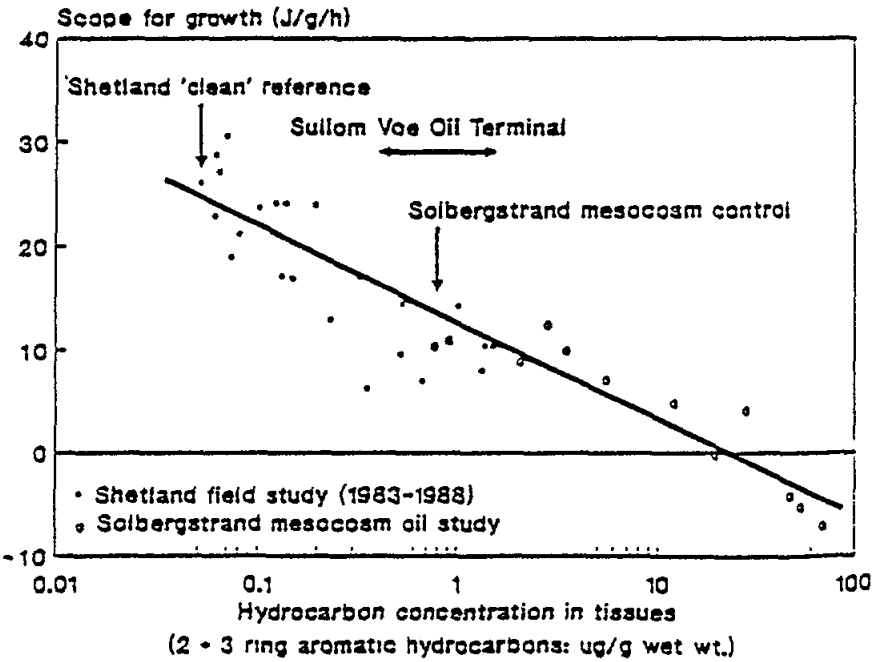

Fig. 15 Relationship between Scope for Growth and the log concentration of 2+3 ring PAHs in the mussels. Synthesis of results from Shetland study and Solbergstrand mesocosm experiments

(i) Two sites in Castle Harbour with a shore based 'dump site'; and
(ii) Five sites along a contamination gradient in Hamilton Harbour which is influenced by the small town of Hamilton, yachts and cruise liners.

3.1 Castle Harbour

Despite the visible 'dump site', there was no significant decline in SFG or accumulation of contaminants in the Arca transplanted to the Castle Harbour 'dump' site.

3.2 Hamilton Harbour

Mussels transplanted to Hamilton Harbour showed a significant decline in SFG towards the head of Hamilton Harbour, primarily as a result of an enhanced metabolic rate and a reduced clearance (=feeding) rate (Fig. 17).

Also note that any reduction in growth potential, caused by relatively low levels of pollution, will have a greater impact on animals living in an oligotrophic sub-tropical environment compared to a more eutrophic environment. The low SFG shifts the animal closer towards the limit of growth and reproduction; consequently the effects of pollution are likely to be more marked in a food-limited oligotrophic environment.

3.3 Toxicological interpretation beyond statistical correlations

The results show significant negative correlations between SFG and the log concentration of the major classes of contaminants, including PCBs ($r=-0.95$), hydrocarbons ($r=-0.92$), their polar oxygenated derivatives ($r=-0.89$), TBT ($r=-0.91$) and Pb ($r=-0.76$). There was no significant accumulation of other metals (Cd, Cu, Zn). However, toxicological interpretation of tissue residue data extends the analysis beyond statistical correlations and indicates that hydrocarbons and their polar oxygenated derivatives, and TBT can explain the observe decline in SFG.

3.4 Identification of the cause(s) and partitioning of effects

The overall reduction in SFG at the two inner sites of Hamilton Harbour can be partitioned (Fig. 18) and fully explained in terms of the inhibition of clearance rate by hydrocarbons (e.g. 65% of the reduction in SFG at H2) and the enhancement of respiration by TBT (e.g. 35% of the reduction in SFG at H2).

4. OSLO BIOLOGICAL EFFECTS WORKSHOP

In contrast to Bermuda, the Workshop in Oslo examined a contaminant gradient associated with an industrialised area in temperate waters.

The Langesundfjord receives contaminants from the industrial area of Porsgrunn and these remain in the freshwater surface layer in Frierfjord and mix with the seawater in Langesundfjord at the sill of the fjord near site D (Fig. 19). Mussels as sites C and D had the lowest SFG and were severely stressed. Those at the reference site A at the mouth of the fjord had the highest SFG, and mussels at site B were intermediate.

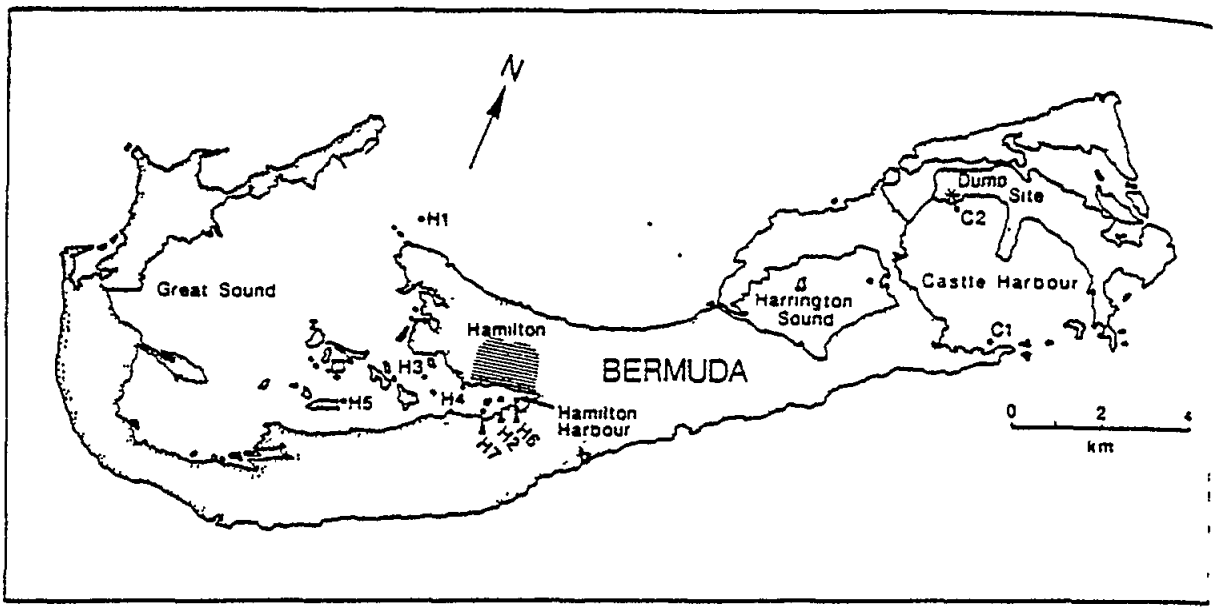

Fig. 16 Location of field sampling sites in Bermuda

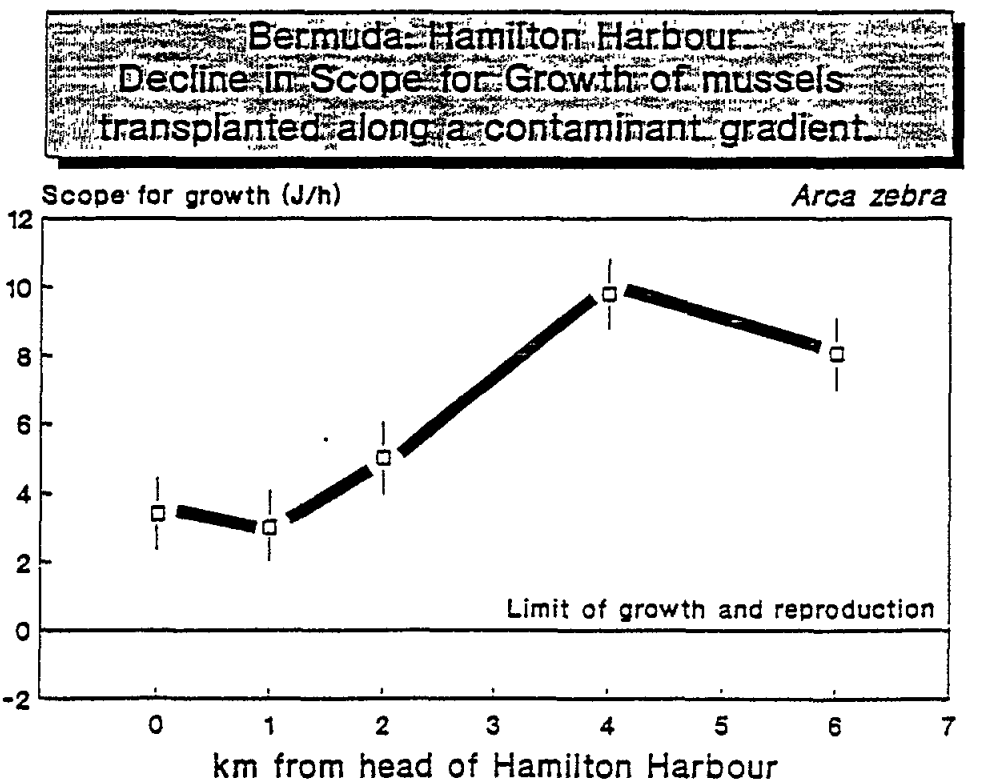

Fig. 17 Scope for growth of Arca zebra at sites along contaminant gradient in Hamilton Harbour, Bermuda (after transplantation and 12 d exposure).

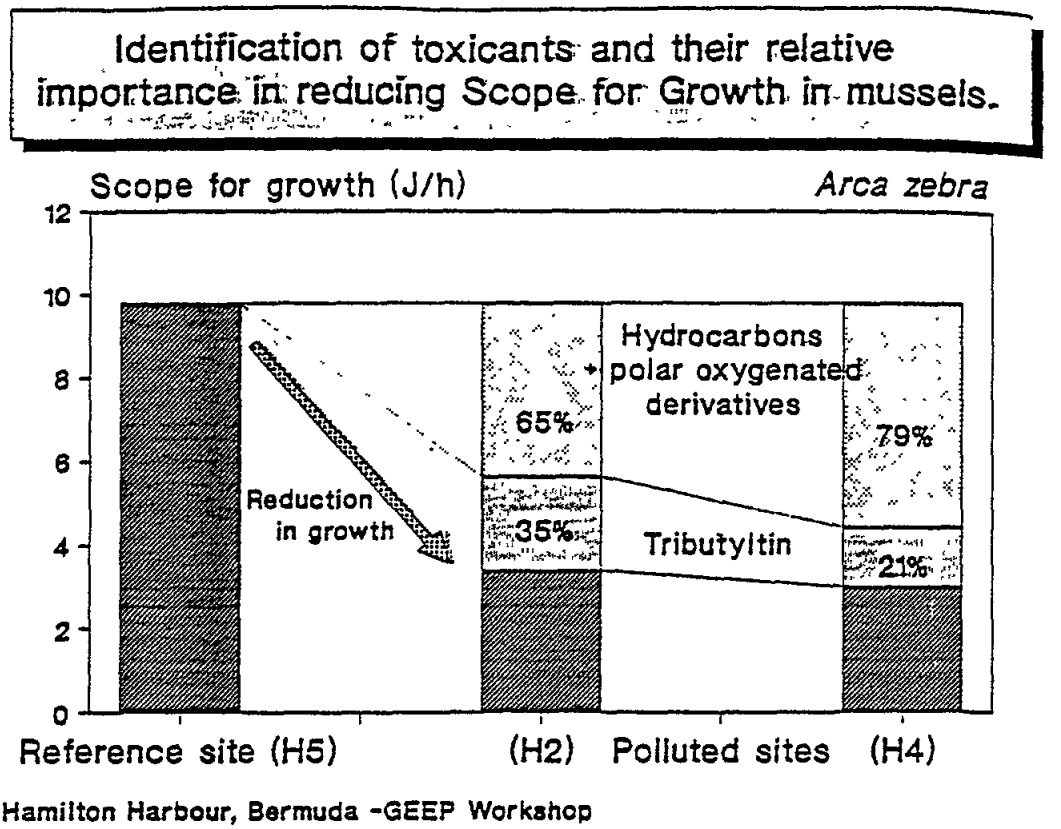

Fig. 18 Toxicological interpretation of scope for growth and contaminant concentrations in Arca zebra

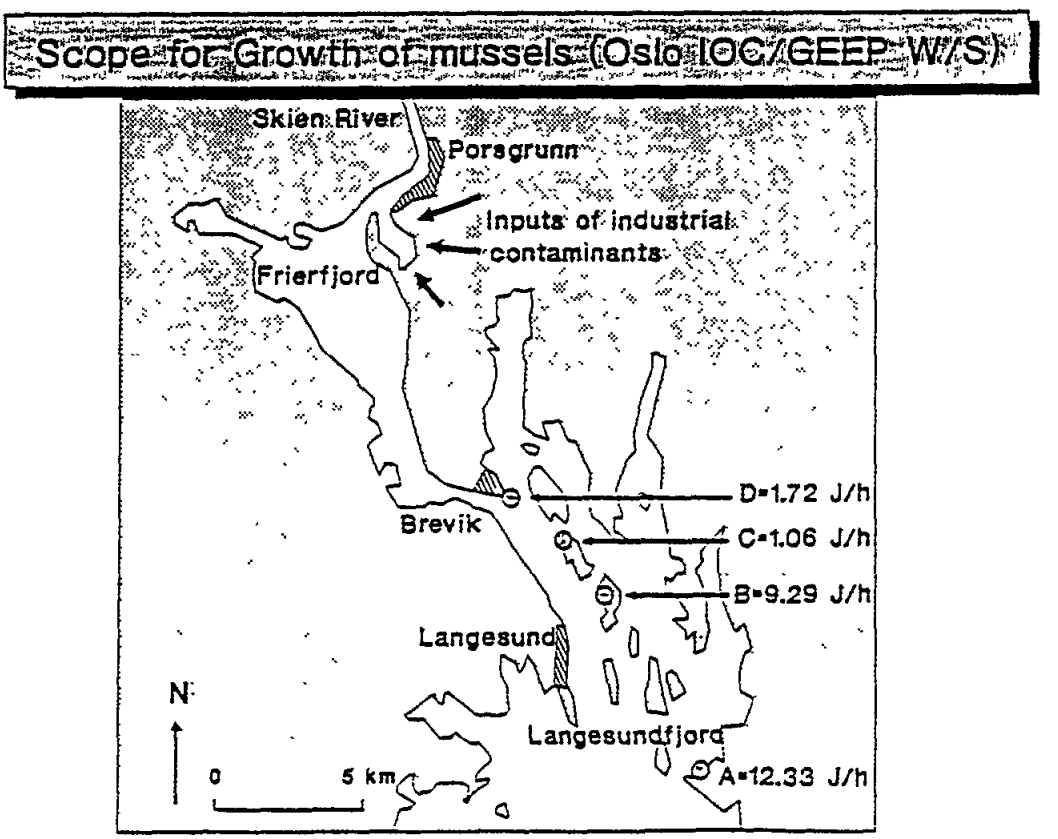

Fig. 19 Location of sampling sites in Langesundfjord, Norway

4.1 Toxicological interpretation of energetic responses

Toxicological interpretation of the contaminant levels in mussel tissues and SFG demonstrate a number of important points:-

(i) The reference site was significantly contaminated by PAHs and TBT and was not a sufficient distance from the source of pollution. When placed in a broader context and compared with the clean reference site in the Shetlands, the lowered SFG at site A can be explained in terms of the PAH and TBT levels in the mussels (Fig. 20). This highlights the need to select appropriate 'clean' reference sites in any field programme, otherwise the true pollution impact will be under-estimated.

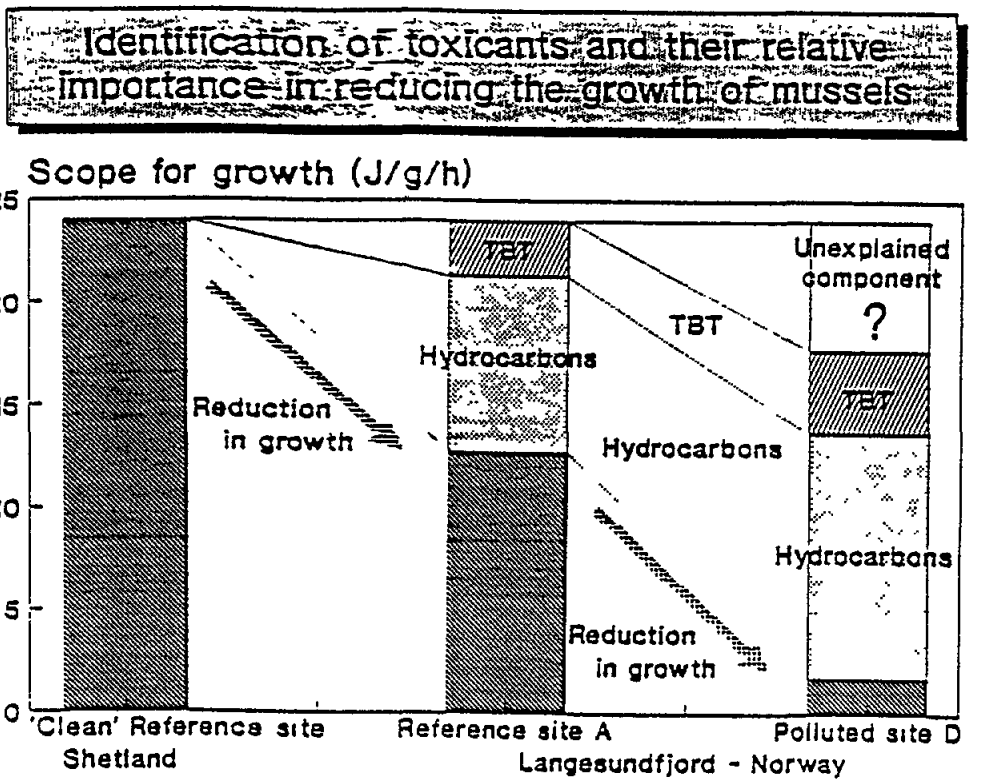

Fig. 20 Effect of pollution on scope for growth of mussels in Langesundfjord in comparison to a 'clean reference site' in the Shetlands

(ii) The effects on SFG at the inner sites (C & D) were far greater than could be explained in terms of measured contaminants. Partitioning of effects showed that metals were below the threshold of effect, PAHs explained 55%, TBT explained 15% and 30% of the reduction in SFG was left unexplained. This unexplained component is due to both identified contaminants for which we have no toxicological data and unidentified contaminants (only a few classes of contaminants were measured). This investigative approach allows you to then:

- Analyse stored tissue samples for other contaminants, and
- Conduct further toxicological studies to derive concentration - response relationships to help explain any observed discrepancies.

(Following this Workshop it has been established that this fjord has high levels of chlorinated hydrocarbons, dioxins and Hg).

5. NORTH SEA MUSSELS MONITORING PROGRAMME

Recent studies have extended the approach from relatively small scale gradients (i.e. c. 10 km) in estuaries to larger spatial scales (i.e. >1000 km) along the North Sea coastline.

The primary aim of the North Sea study was to use mussels to assess:
- The extent to which the North Sea is contaminated (e.g. Mussel Watch); and
- The degree of impact or stress experienced (measured in terms of SFG).

However, before such a programme could be undertaken it was necessary to establish appropriate procedures for a large scale study.

5.1 Factors to consider when comparing responses over a large spatial scale (c.1000 km)

(i) Genetic differences - Transplantation between the N and S showed differences between mussel populations reflected differences in environmental quality rather than genetic differences (i.e. no significant genetic differences).

(ii) Environmental Factors - Mussel performance is independent of:-
Temperature (constant and fluctuating <6-20°C; Widdows, 1973; 1976)
Salinity (constant and fluctuating 19-33ppt; Widdows, 1985)
Food / Particulates (<1 to 20 mg seston L^{-1}; Widdows *et al.*, 1979; Kiørboe *et al.*, 1980).

(iii) Procedural Effects - No significant effects on performance after:-
Transportation / 24 h air exposure + 24 h recovery
Comparison between field and laboratory measurements.

5.2 Method of transportation

Before the study was undertaken it was important to establish appropriate procedures for collection, transportation and measurement. Mussels can be collected from any site in the UK and transported to Plymouth within 24 h, via a national carrier. We therefore tested the effect of 24 h air exposure on mussels (cooled to 7-8°C by means of freezer packs in an insulated box and using disposable nappies as an absorbent buffer between the freezer blocks and the mussels).

5.3 Time-course of recovery from 24 h air exposure

Clearance rates of 16 mussels collected from four sites were measured over 48 h of recovery (Fig. 21). Mean clearance rates show that mussels recover from 24 h of air exposure within 12 h and certainly by 24 h.

5.4 Pollution induced effects retained

Comparison between mussel populations confirmed that after 24 h of recovery they still retained the pollution induced stress effects. The Exmouth and Tamar populations showed a marked decline in SFG with increasing PAH concentration (Fig. 22).

Furthermore there was good agreement between Gluss Voe mussels measured in the field in the Shetlands and Gluss Voe mussels transported to Plymouth, recovered and measured in clean offshore sea water.

5.5 Summary of sampling procedures

The sampling procedures for chemical analysis and physiological measurements are summarised in Fig. 23.

5.6 Photographs of mussel labelling, clearance rate apparatus, particle counting, respirometry, filtration apparatus of food and faeces

5.7 North sea sampling sites

Mussels were collected from 28 sites along the North Sea coastline of Scotland and England in July 1990 (Fig. 24). Shetland sites, previously established as 'clean and uncontaminated', formed appropriate reference sites at the northern entrance where the Atlantic water enters the North Sea. All sites were selected to reflect the coastal environment rather than estuarine hot-spots. Many of the sites had previously been sampled as part of the MAFF 'Mussel Watch' chemical monitoring programme.

Biological effects were measured in terms of SFG (by PML) and tissues were stored and later analysed for chemical contaminants (by MAFF, Burnham and PML). Chemistry data is still not available, therefore a toxicological interpretation of the results is not yet possible.

Phase II, carried out in 1991, measured the SFG and chemical contaminants in mussels transplanted offshore to lightvessels anchored in the southern part of the North Sea.

5.8 Preliminary results (Phase I - Coastal Sites)

Highlight of main trends (Fig. 25):-

(i) High SFG in north (i.e. low stress) with significantly reduced SFG in the south. This would appear to reflect increasing contamination further south with increasing population density and industrialisation. Clean North Atlantic water moves from north to south along the coast.

(ii) Marked decline in SFG in major estuaries (Tees, Humber / Wash, Thames)

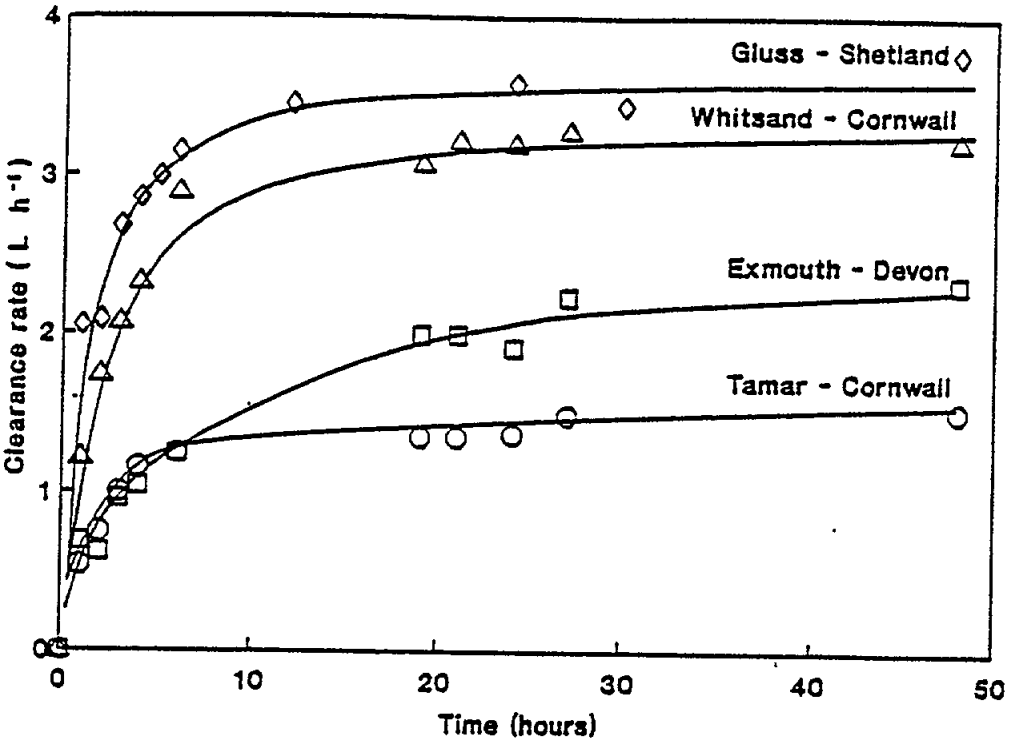

Fig. 21 Time course of recovery of clearance rate after 24 h air exposure

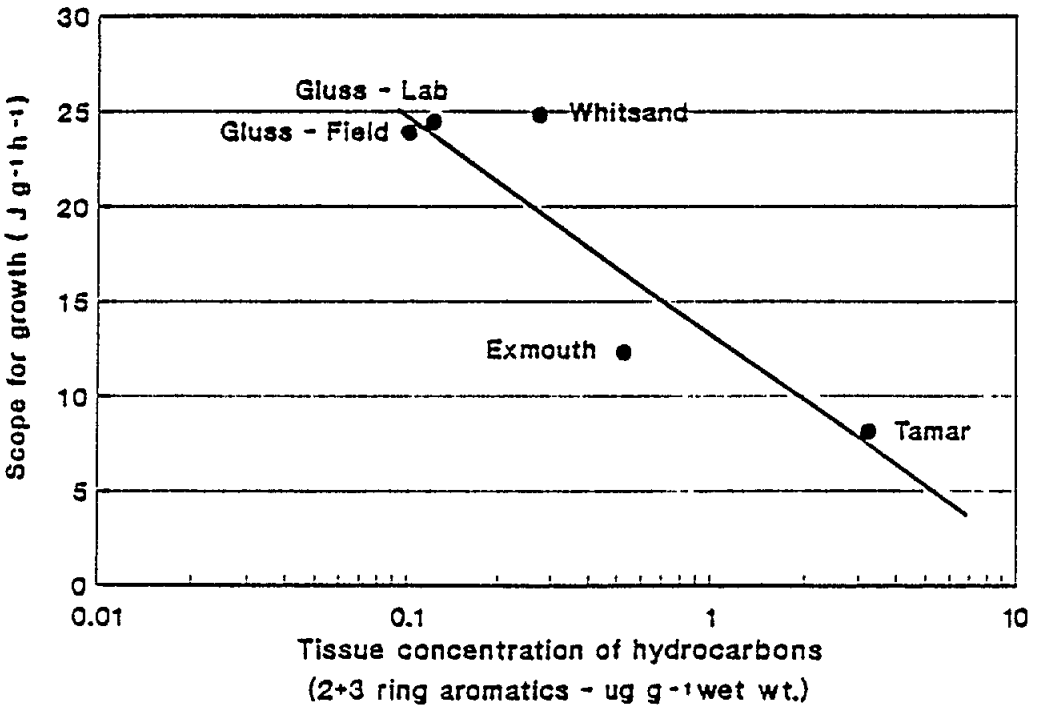

Fig. 22 Pollution induced effects on scope for growth are retained after 24 h air exposure and 24 h recovery period

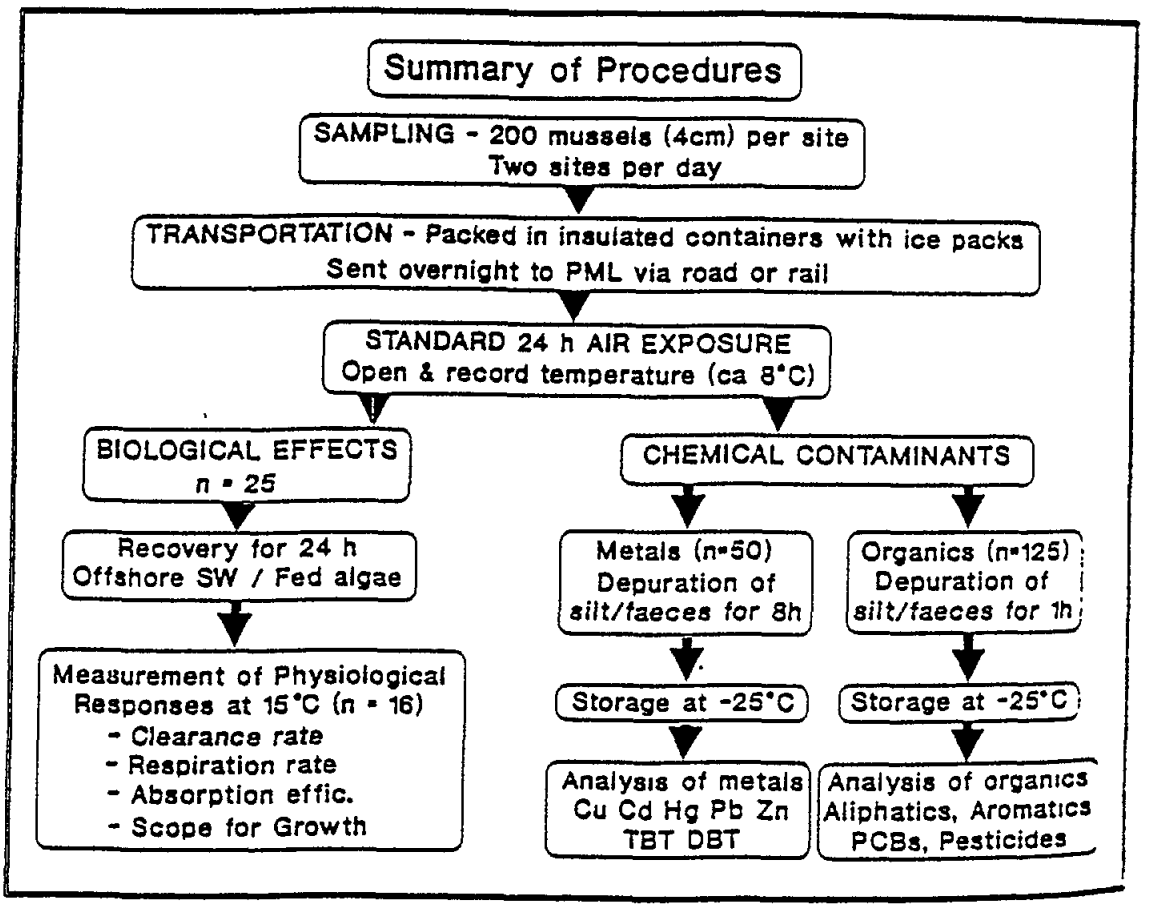

Fig. 23 Summary of sampling procedures for North Sea study

(iii) Identified unexpected low SFG in Ythan, where mussels gave a response specific to algal toxins. It has since been confirmed (MAFF) that the area north of Montrose was affected by PSP at the time of our sampling.

5.9 PAH in mussels

Preliminary analysis of 2+3 ring PAHs confirms the general trend in increasing PAH contamination from north to south (Fig. 26). However, this chemical component only explains 25% of the total variance in the SFG. This is considerably less than in previous studies, involving small scale pollution gradients in estuaries / bays, where contaminants are generally diluted, dispersed and degraded along the axis length. Over a larger spatial scale the contaminant mixture is more complex, with a wider and changing composition. Consequently, contaminants will not necessarily co-vary or correlate with a single 'marker' of overall contamination.

In the final analysis we will attempt to provide a toxicological interpretation of the results and examine how much of the reduction in SFG we can explain in terms of the chemical contaminants analysed.

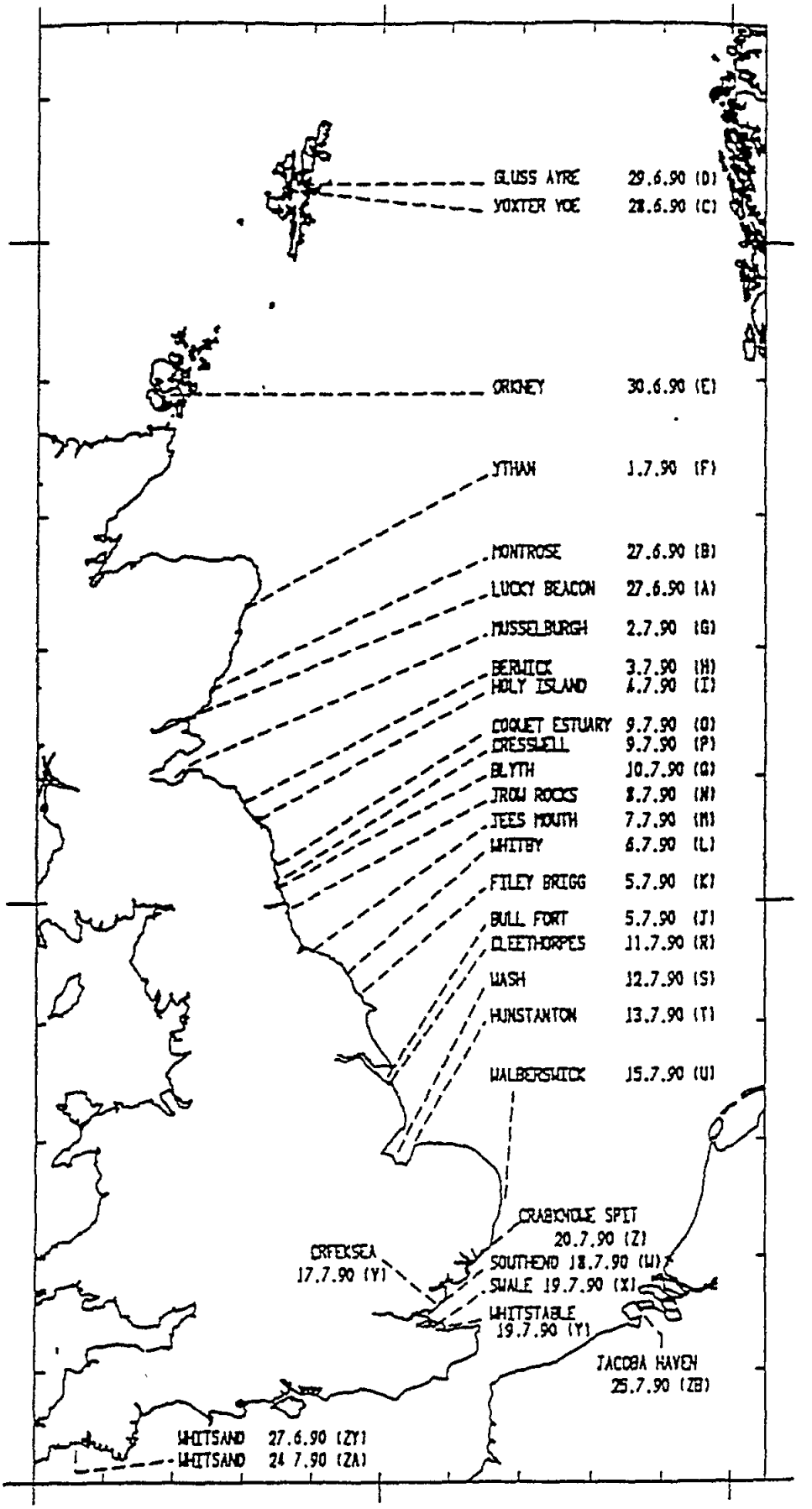

Fig. 24 Location of mussel sampling sites along North Sea coast

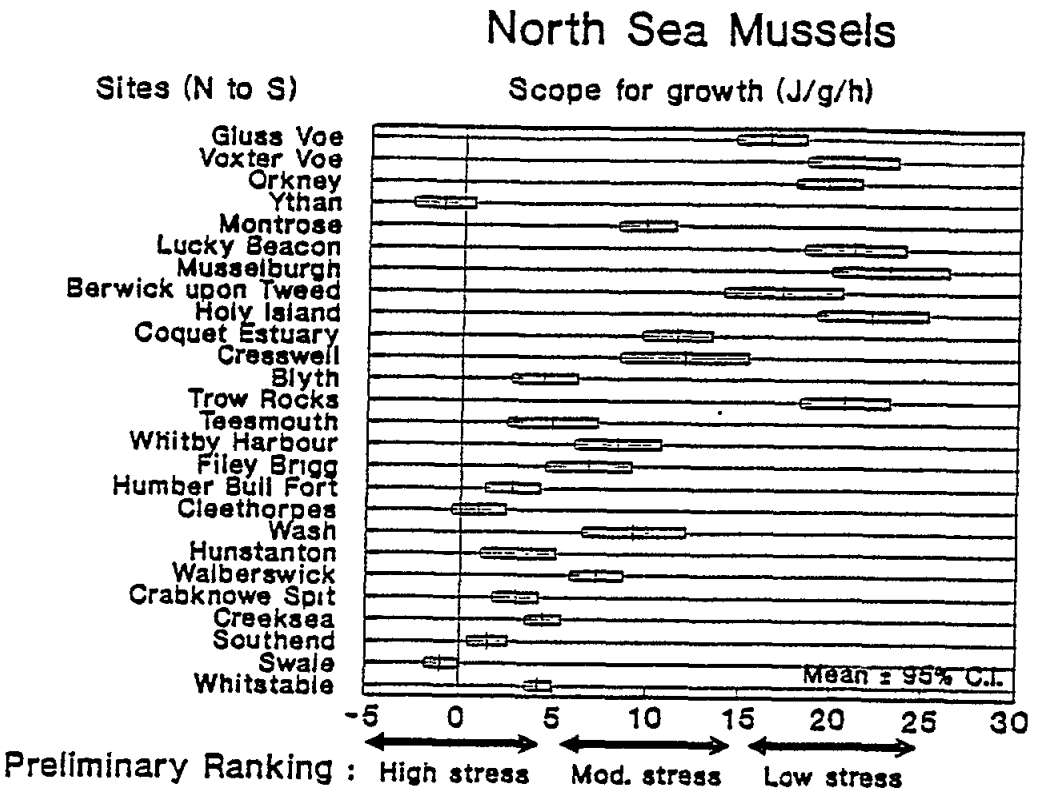

Fig. 25 Scope for growth of mussels sampled from North Sea coastal sites

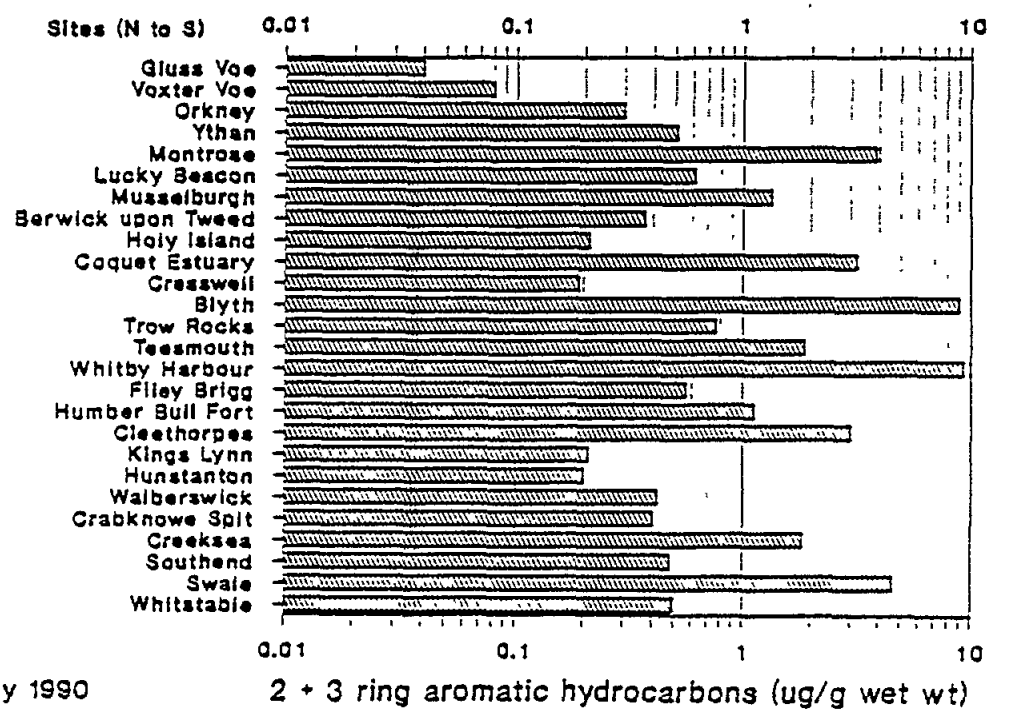

Fig. 26 Bioaccumulation of 2 + 3 ring PAHs in the mussels sampled from North Sea coastal sites

6. SUMMARY

Measurement of SFG combined with analysis of chemical contaminants in the tissues of mussels (bivalves) provides a means of:-

i) Detecting and quantifying sublethal pollution effects along small scale contaminant gradients as well as over larger spatial scales (SFG offers a rapid and low cost screening method compared to chemical analyses and therefore supports the case for measuring biological stress responses first, to identify the degree of impact and the hot spots, before investing in considerable time and money in chemical analyses).

ii) Interpreting tissue residue chemistry data in terms of toxicological significance.

iii) Identifying the cause(s) of stress effects and their relative contribution to the overall reduction in growth.

PRACTICAL PROCEDURES FOR THE MEASUREMENT OF SCOPE FOR GROWTH

by

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Contents

1. INTRODUCTION

1.1 Test Organisms: Bivalves / Mussels (Mytilus edulis)

Mussels, like most bivalves, are suspension feeders that are capable of pumping considerable volumes of water through their large gills and filter out small particulate food items from the water column. Mussels are particularly efficient filter feeders, removing particles from 2 µm to c. 50 µm diameter with 100% efficiency. The total amount of particulate matter present in suspension (= seston) contains several food types which are potentially utilizable by mussels. The major utilizable component is phytoplankton, but bacteria and fine organic detritus are also important in supplementing the algal diet. Mussels undergo a seasonal cycle of somatic and shell growth, gametogenesis and spawning of gametes. The eggs are fertilized externally in the water, where they develop into a free-swimming pelagic larval stage which feeds and grows in the water column for 2-3 weeks prior to settlement and then metamorphosis, which marks the start of a sessile mode of life.

2. GENERAL TEST PROCEDURES

2.1 Source of animals

Mussels to be used in laboratory toxicity testing, and mussels chosen to represent 'clean reference' sites in field studies, should be collected from a location that is known to be free from significant chemical contamination (i.e. removed from urban development and industry). It is advisable to analyse body tissues for contaminants, particularly organics such as hydrocarbons, to confirm that the site is not significantly contaminated (visual assessment of the site is not sufficient).

2.2 Native or Transplanted Animals

Mussels can be collected from:-
Native populations, or
Transplanted from 'clean' reference sites and caged at particular sites of interest.

2.3 Laboratory or Field Measurement

Physiological energetic responses of mussels can either be measured in the field using a mobile laboratory (e.g. Widdows et al., 1987) or in the laboratory under 'standardised' conditions (e.g. Widdows and Johnson, 1988). Comparative studies (Widdows, 1983; Salkeld & Widdows - unpublished data) have found no significant difference between laboratory and field measurements of SFG if the former are made in high quality seawater and within 24 h of collection from the field (i.e. before they recover from pollution induced stress). When the objective is primarily to quantify the decline in growth potential or the degree of stress induced by environmental pollution, then measurement under 'standardised' laboratory conditions is recommended on the basis of convenience, cost and efficiency. Under controlled laboratory conditions, natural environmental variables (such as food availability, temperature, salinity and dissolved oxygen) are held constant, so that the physiological responses reflect the underlying effects of toxic contaminants accumulated in the tissues. The basic physiological responses of mussels (such

as feeding and respiration rate) are maintained relatively independent of short-term changes in natural environmental variables over a wide range of conditions; for example food / seston concentration (0.1-10 mg seston L^{-1}; Widdows et al., 1979; Kiørboe et al., 1980), temperature (6-20°C; Widdows, 1976) and salinity (20-33 ppt; Widdows, 1985). In addition, transplantation experiments over >1000 km have shown that any measurable differences in physiological responses and growth rates of different populations reflect environmental factors rather than genetic differences (Kautsky et al., 1990; Widdows and Salkeld, unpublished data), thus enabling the direct comparison of mussels over a wide geographical area.

In field studies where mussels are collected from various sites to assess pollution effects, all measurements should be made at a 'standard' temperature (within 2°C of the mean ambient seawater temperature), in air-saturated, high quality sea water at full salinity, and at a constant algal food concentration representative of field conditions (e.g. 0.4 mg particulate organic matter [POM] L^{-1} for northern temperate coastal waters; Widdows et al., 1987).

2.4 Collection of animals

Mussels of a standard body size (e.g. 4 cm shell length) should be collected from field sites, taking care to cut byssus threads, packed in insulated containers and then transported in air under moist / cool conditions. SFG measurements should ideally be made during the summer period of active growth after the spawning season.

Twenty mussels from each site are cleaned of detritus and fouling organisms, numbered (e.g. white permanent marker pen) and placed in flowing sea water for a period of recovery and depuration of silt / faeces prior to physiological measurement. The period of recovery necessary prior to physiological measurement is dependent upon the duration of air exposure and should be evaluated for the particular species. In the case of Mytilus edulis, recovery is completed within 2 h of reimmersion after 5 h air exposure (Widdows and Shick, 1985), and within 12 h after 24 h air exposure at 7°C (Widdows and Salkeld, unpublished data). It is important that the mussels are measured after recovery from any transportation and handling stress, but before they begin to depurate significant quantities of contaminants and recover from any pollution induced stress.

Before applying physiological measurements to a new (bivalve) species, it is important to carry out some preliminary studies to establish the particular species requirements and the appropriate protocols. For example, a species may be sensitive to light or exhibit a diurnal cycle of activity (e.g. Arca zebra; Widdows et al., 1990). However, in the case of Mytilus edulis there is no evidence of a diurnal cycle or sensitivity to light.

2.5 Physiological Measurements: Static vs. Flow-through

Physiological measurements can be performed in either static or flow-through systems. There are advantages and disadvantages associated with each approach:-

Static

The main advantages of using a static (closed) system are:- (1) It generally requires less equipment; (2) Large changes in food or O_2 are recorded; and (3) Smaller quantities of seawater and food are utilised (also less toxicant is required in toxicological studies). The disadvantages are:- (1) Experimental conditions (e.g. food, oxygen, toxicant concentration) are not held constant, but decline with time and may therefore affect the physiological rate if they fall below a specific concentration, and (2) Faeces are generally not produced in sufficient quantities and are not the result of a steady-state food concentration.

Flow-through

The main advantages of using an open flow-through system are:- (a) Experimental conditions are held constant; (b) Continuous and simultaneous monitoring of physiological responses is possible; and (c) Less physical disturbance of animals and more natural conditions. The disadvantages are:- (a) More complex experimental systems requiring pumps and tubing / plumbing; (b) Precise control of flow rates and inflow concentrations (of food, O_2, and toxicant) are required; (c) Large quantities of seawater are required (recirculated or to waste); and (d) Accuracy of measurement and the detection limit is dependent on the flow rate because there is an inverse relationship between the flow rate and the difference between inflow and outflow concentration.

In the procedures outlined below both static and flow-through techniques will be described where appropriate.

3. MEASUREMENT OF FEEDING RATE (CLEARANCE RATE)

Clearance rate, which is defined as the volume of water cleared of suspended particles (i.e particles > 3 μm equivalent spherical diameter) per hour, can either be determined in a flow-through system or a static system by measuring the removal of suspended algal cells (e.g. Isochrysis galbana, Phaeodactylum tricornutum, Tetraselmis suiecica), added to filtered seawater (FSW down to 1 μm). A flow-through system is generally preferred in field monitoring programmes as mussels can be held under conditions of constant algal concentration, thus enabling continuous monitoring of clearance rate and the collection of faeces. However, in most toxicological studies a static system is used, to avoid consuming and disposing of large quantities of toxic chemicals.

3.1 Flow-through approach

The clearance rate measuring system (Fig. 1A) consists of a small centrifugal pump (or sufficient pressure from a reservoir) discharging FSW into a mixing chamber (1.5 L volume) with a magnetic stirrer and thence via narrow (i.e. 2.5 mm) bore tubing through 18 identical chambers in parallel (16 experimental chambers with individual mussels and 2 control chambers without mussels).

Flow rates through each chamber are maintained constant at c. 180 mL min^{-1}. The inflow into each chamber is at the bottom, adjacent to the mussel's inhalent mantle edge, and the outflow is via an overflow tube at the top of the chamber (volume c. 500 mL). A variable speed peristaltic pump introduces algal cells into the mixing chamber to achieve the required cell concentration (c. 7000 cells mL^{-1}). Accurate estimates of clearance rate are only achieved by using appropriate flow rates; low enough to record a significant difference between the inflow and outflow cell concentration, yet sufficient to prevent any significant recirculation of water by the mussel in a small chamber. As a general guide, flow rates through each chamber should be approximately 2 to 3 times the clearance rate of the mussels (i.e. the cell concentration in the outflow should be >50 and <80 % of the inflow concentration). After placing mussels in the experimental chambers they are left undisturbed for at least 60 min (length of recovery period is dependent upon period of transportation / air exposure) to allow their valves to open and feeding to be resumed.

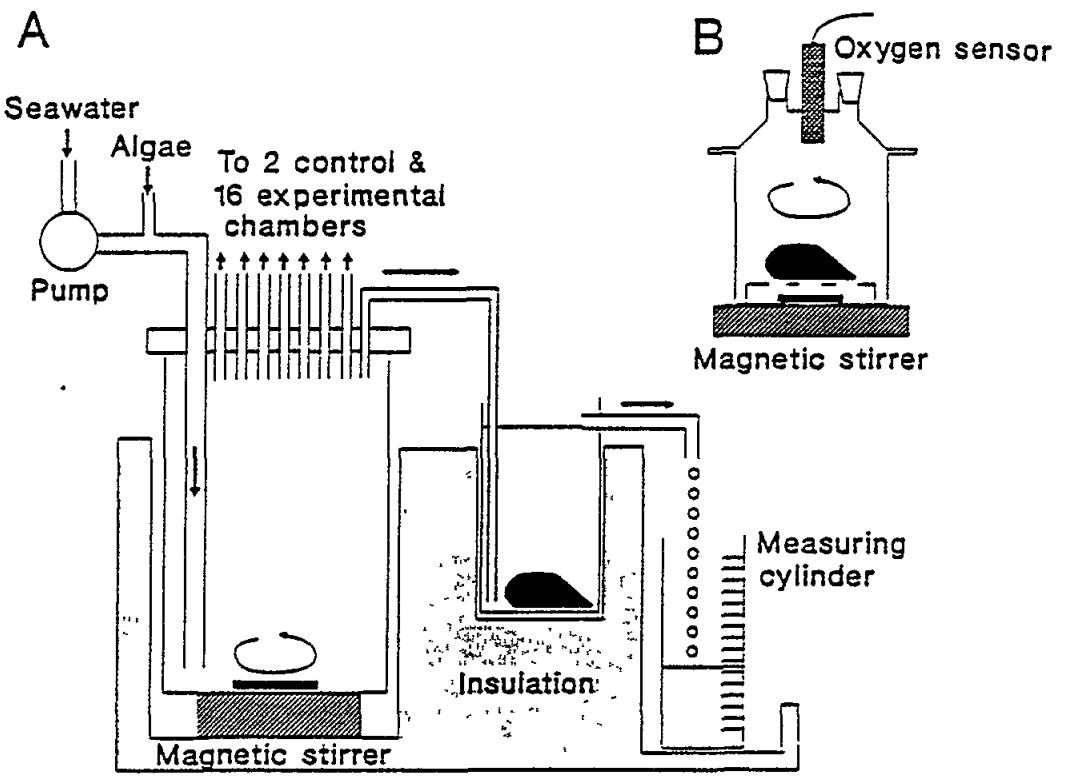

Fig. 1A Flow-through apparatus for measurement of clearance rate by bivalves
Fig. 1B Glass respirometer for measurement of oxygen consumption

Water samples from all chambers are collected simultaneously by moving a rack of measuring cylinders (> 200 mL volume) directly under the outflows and removing them after a period of 60 seconds. It is important to avoid disturbing the animals by shading or vibration in the vicinity of the apparatus. The flow rate through each chamber is recorded. The concentration of algal cells in each water sample is then measured using an electronic

particle counter (e.g. Coulter Counter Model D; for details see Appendix IX) with a 100 or 140 µm orifice tube and set to count all particles > 3 µm diameter (spherical equivalent) in a 0.5 mL sub-sample. Four replicate counts are made on each sample and the mean calculated. Clearance rate is then calculated as follows (see Appendix I):-

$$\text{Clearance rate (L h}^{-1}\text{)} = \text{Flow rate (in L h}^{-1}\text{)} \times (CI - CO) / CI$$

where CI is the inflow concentration represented by the cell concentration in the control, and CO is the outflow concentration from each experimental chamber. The clearance rate of each mussel is determined on four occasions at 45-60 min intervals. An important feature of mussels is their ability to open rapidly and maintain a relatively constant pumping / feeding rate; therefore any individual that fails to open and produce faeces should be replaced.

In circumstances where measurement of clearance rate in a flow-through system is not possible (e.g. limited supply of seawater and algal food, or toxicological studies) then clearance rate can be determined in a static system by measuring the exponential decline in cell concentration over a period of time (see below).

3.2 Static approach

Sixteen mussels are placed in separate beakers each containing 2L of FSW and a magnetic stirrer bar. An additional beaker without a mussel acts as a control. The water is mixed by means of a magnetic stirrer base plate. To avoid any physical disturbance, position each mussel to one side of the beaker away from the stirrer bar. An alternative and less costly method of mixing is to 'gently' aerate each beaker. However, aeration is not recommended for toxicological exposure studies due to the loss of more volatile toxicants from sea water.

After a period of 15 min, to allow for the mussels to open their shell valves and to resume pumping, algal culture (e.g. Isochrysis galbana or Phaeodactylum tricornutum) is added to each beaker to give an initial concentration of 25000 cells mL^{-1}. It is important not to exceed this maximum concentration in order to avoid pseudofaeces production and the inhibition of clearance rate. Allow 5 min for the algal cells to be thoroughly mixed in the 2 L and then sample a 20 mL aliquot from each beaker (using a large syringe). Place this T0 sample in a numbered vial and count the cell concentration (mean of 4 counts) using an electronic particle counter (e.g. Coulter Counter Model D). An alternative and less precise method is to measure the algal concentration by means of fluorescence spectrophotometry. Take four subsequent 20 mL samples at 30 min intervals over period of 2 h. The clearance rate (CR, volume of water cleared of particles per hour) by individual mussels is then calculated using the following equation (see Appendix II; Coughlan, 1969):-

$$CR\ (L\ h^{-1}) = (\text{Volume of water e.g. 2L}) \times (\log_e C_1 - \log_e C_2) / \text{time interval in h}$$

where C_1 and C_2 are the cell concentrations at the beginning and end of each time increment (i.e. 0.5 h). Generally the control beaker does not show a significant change in cell concentration. However, if there is a change in the control (due to either cell division or settlement) then this needs to be calculated as a rate using the above equation and subtracted from the experimental rates.

The maximum clearance rate of each mussel is then calculated based on a 1 h period (i.e. two consecutive time increments) during which the decline in cell concentration was greatest. This avoids the inclusion of periods when individuals may be totally or partially closed.

Note, that large mussels (i.e. >4-5cm) with higher clearance rates will reduce the algal cell concentration in 2L of sea water down to <1000 cells mL^{-1} in less than 90-120 min. Such low concentrations may inhibit clearance rates or be difficult to quantify accurately. Consequently, when measuring the clearance rates of larger individuals it will be necessary to use a larger volume of water (e.g. 5L).

4. MEASUREMENT OF FOOD ABSORPTION EFFICIENCY

Absorption efficiency is measured by the ratio method of Conover (1966); it represents the efficiency with which organic material is absorbed from the ingested food material.

$$\text{Absorption Efficiency} = (F - E) / [(1 - E)F]$$

where F=ash-free dry weight:dry weight ratio of food, and
E=ash-free dry weight:dry weight ratio of the faeces.

Faeces are collected after mussels have been held for 24 h in the laboratory at a constant algal cell concentration; thus allowing gut contents reflecting their previous diet to be evacuated and discarded.

Algal culture of known cell concentration is filtered through washed, ashed and pre-weighed 4.5 cm glass fibre (Whatman GFC) filters (a minimum of five 'food' samples should be collected). The filters should not be 'over-loaded' so that salts can be readily washed out with 0.5M ammonium formate (3x10 mL). Care should also be taken to wash salts out of the edges of the filters. Ammonium formate at 0.5M is approximately iso-osmotic with sea water and provides a means of washing algal cells and faeces without inducing osmotic stress. It has a low melting point and can be sublimated at 110°C.

Faeces accumulated in the flow-through clearance rate chambers (or in the static exposure tanks) are sampled by pipetting onto washed, ashed and pre-weighed GFC filters. The salts are then washed out of the filters with 0.5M ammonium formate (3x10 mL). If faecal production is low then faeces from two or three mussels may have to be pooled.

The filters are oven dried at 110°C for >24 h and weighed. They are then ashed in a furnace at 450°C for 6 h and weighed again in order to calculate the weight of organic material combusted. In spite of careful handling of filters with forceps and storing in desiccators, GFC filters can show significant changes in weight, presumably due to daily changes in humidity. Therefore, blank GFC filters are weighed at each stage for each batch of filters in order to correct for any weight change. The calculation of absorption efficiency is shown in Appendix III.

5. MEASUREMENT OF RESPIRATION RATE

5.1 Static approach

Rates of oxygen consumption by individual mussels are measured in 'closed' glass respirometers (e.g. 500 mL Quickfit flasks) held in a temperature controlled water bath mounted on a multi-point magnetic stirrer. Air-saturated seawater is added to each respirometer and stirred by means of a magnetic stirrer bar beneath a perforated glass plate supporting a mussel (Fig. 1B). The rate of decline in oxygen partial pressure (Po_2) in each chamber is measured by a calibrated oxygen electrode (e.g. Radiometer E5046 or Strathkelvin 1302; for details see Appendix X) connected to an oxygen meter (e.g. Strathkelvin Model 781b). Eight respirometers are usually run simultaneously and each oxygen meter is coupled to a multichannel chart recorder. Twenty minutes are allowed for the mussels to open and to resume pumping, then oxygen uptake is measured over the next hour. The rate of oxygen consumption should not be measured below a partial pressure of c. 100 mm Hg (13 kPa) because the rate then becomes dependent on the external Po_2.

The preferred method is a continuous monitoring of oxygen uptake by means of an oxygen sensor in each respirometer (Fig. 1B). However, a less convenient but less costly alternative utilizes a single oxygen meter and sensor mounted in a thermostated cell (e.g. Strathkelvin 1302 electrode and MC 100 microcell). At c.40 min intervals, small volumes (c. 1 mL) are sampled from each respirometer by means of a glass (i.e. gas impermeable) syringe coupled to a stainless steel needle passing through a silicone stopper in the top of each respirometer. Each sample is then slowly injected into the thermostated microcell and a steady Po_2 reading is obtained after c. 4 min. The decline in Po_2 is measured by sampling from each respirometer on at least two, ideally three occasions. Ensure that the animals are not disturbed when taking the samples.

The rate of oxygen consumption is calculated as follows (see Appendix IV):

$$\text{Rate of } O_2 \text{ uptake } (\mu\text{moles } O_2\ h^{-1}) = [C(t_0) - (C(t_1)] \times (V_r) \times 60 / (t_0 - t_1)$$

where t_0, t_1 = start and finish times (min) of the measurement period;
$C(t)$ = concentration of oxygen in the water (μmoles $O_2\ L^{-1}$) at time t;
V_r = volume of respirometer minus the animal.

Oxygen solubility values (see Appendix V) are used to convert Po_2 (mm Hg) values to oxygen concentration in μmoles $O_2\ L^{-1}$ as follows:

$C(t)$ = [(Exptl. Po_2 in mm Hg) / (Po_2 at air saturation)] x 259.6 μmoles $O_2\ L^{-1}$
(e.g. 259.6 μmoles $O_2\ L^{-1}$ is concentration and 156.6 mmHg is Po_2 at air saturation; when 15°C, 32ppt & 760mm Hg or 101.325 kPa).

The partial pressure of oxygen representing air saturation varies as a function of temperature and atmospheric pressure according to the following equation:-

Po_2 (mm Hg) = [Barometric press. - (5.7 + 0.03 x (Temp °C)2)] x 0.20946
e.g. 160 mmHg = (776 mmHg - (5.7 + 0.03x152)) x 0.20946

[Conversion factors for pressure: 1 atm = 101.325 kPa; 1 mm Hg = 0.133322 kPa]

5.2 Flow-through approach

Rates of oxygen uptake can also be determined using a flow-through approach. This consists of individual mussels held in small volume (i.e. <100 mL) glass respirometers with an inflow from a reservoir of aerated seawater and an outflow to a peristaltic pump via a thermostated cell containing an oxygen sensor (e.g. Strathkelvin MC 100 microcell and 1302 electrode). The volume of the respirometer chamber and the diameter / length of the tubing to the oxygen sensor / pump should be minimised in order to reduce residence time and thus increase the equilibrium / response time of the system. For example, the tubing should be non-permeable (butyl rubber) with an internal diameter of c. 2mm. The flow rate required will vary depending on animal size and its rate of oxygen uptake. It should be sufficient to provide a 15 - 20% difference between the inflow and outflow oxygen concentrations (e.g. c. 360 ml h^{-1} for a 1 g animal). Eight respirometers can be run simultaneously and measured by a single thermostated oxygen sensor, switching the outflow from each chamber via the sensor at intervals and allowing c. 5-10 min for the oxygen meter to achieve a new steady state. The inflow oxygen concentration is obtained from the outflow of a control chamber without an animal. A temperature controlled water bath is used to maintain the respirometer chambers, reservoir and oxygen sensor at the required experimental temperature. Constant flow rates should be maintained by a multichannel peristaltic pump. The rate of oxygen consumption is calculated as follows:

Rate of O_2 uptake (μmoles of O_2 h^{-1}) = [C(t)(in) - C(t)(out)] x flow rate (L h^{-1}).

5.3 Use of oxygen sensors

The oxygen sensors are calibrated in solutions of known oxygen tension. After renewing the membrane on an oxygen sensor, it should be placed in Po_2 zero solution and the meter adjusted to zero. Before using the probe it should be left to stabilize for >12 h with the polarising current on. Each day the probe should be calibrated in air-saturated (i.e. aerated) seawater at the required experimental temperature. The oxygen meter is then set at the appropriate Po_2 for air saturation (see above) when they have stabilised (i.e. become temperature and oxygen equilibrated).

A silicone tubing sleeve (or several layers of parafilm wrapped around the sensor) provides a seal between the sensor and the orifice in the respirometer chamber. However, this should be kept to a minimum to avoid diffusion of oxygen into the respirometer.

6. AMMONIA EXCRETION

The rate of ammonia excretion is usually closely coupled to the respiration rate and forms a relatively small proportion (<5%) of the metabolic energy expenditure. Therefore it can generally be omitted from physiological energetic measurements and the calculation of scope for growth (see Appendix VII for details of the method of ammonia analysis).

7. CALCULATION OF SCOPE FOR GROWTH

After all physiological measurements have been completed the shell length and dry tissue weight of each mussel are recorded. Body tissues are dissected from the shell and dried to constant weight at 90°C. Physiological rates are corrected to a 'standard body size' (e.g. 1 g dry weight) using appropriate weight exponents (e.g. b=0.67; see Appendix VIII). The measured physiological responses are then converted into energy equivalents (J h^{-1}) and used in the balanced energy equation to calculate the energy available for growth and reproduction (SFG):

a) Energy consumed or ingested (C)
C = clearance rate (L g^{-1} h^{-1}) x (mg POM L^{-1}) x (23 J mg^{-1} POM) where the energy content of POM or algal food is c. 23 J mg^{-1} ash-free dry weight (Slobodkin & Richman, 1961; Widdows et al., 1979).

b) Energy absorbed (A)
A = (C) x absorption efficiency

c) Energy respired (R)
R = (µmoles O_2 g^{-1} h^{-1}) x 0.456 where the heat equivalent of oxygen uptake is 0.456 J µmole^{-1} O_2 (Gnaiger, 1983)

d) Energy excreted (U)
U = (µmoles NH_4 -N h^{-1}) x 0.349 where the excretion of 1 µmole NH_4-N h^{-1} is equivalent to an energy loss of 0.349 J h^{-1}.

e) Scope for Growth (P)
P = A - (R + U)

Examples of the spreadsheets used for calculating components of the energy budget and scope for growth are presented in Appendices I to VI.

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Appendix I
Example of spreadsheet for calculating clearance rate in a flow-through system

CALCULATION OF CLEARANCE RATE
EXPERIMENT :- N Sea Mussels Site E Orkney
DATE :-Jul 2 1990

	1st C.R.	Time:-	15.00	2nd C.R.	Time:-	16.15	3rd C.R	Time:-	17.30
ANIMAL	FLOW RATE (l/min)	CELL CONC.	C.R. (l/h)	FLOW RATE (l/min)	CELL CONC.	C.R. (l/h)	FLOW RATE (l/min)	CELL CONC.	C.R. (l/h)
Control	-	3197	-	-	3201	-	-	3204	-
1	0.180	2147	3.550	0.179	2156	3.510	0.179	2132	3.590
2	0.183	2362	2.870	0.181	2097	3.750	0.182	2183	3.470
3	0.170	1902	4.130	0.169	1983	3.860	0.169	1871	4.220
4	0.181	2278	3.120	0.180	2156	3.530	0.179	2195	3.380
5	0.175	1954	4.080	0.174	1909	4.210	0.174	1786	4.620
6	0.181	2164	3.510	0.180	2116	3.660	0.180	1931	4.280
7	0.171	2125	3.440	0.171	2078	3.680	0.171	2337	2.770
8	0.172	2377	2.650	0.172	2329	2.810	0.172	2209	3.200
Control	-	3197	-	-	3202	-	-	3199	-
9	0.176	2165	3.410	0.175	2101	3.610	0.178	2153	3.500
10	0.166	3156	0.130	0.164	2237	2.960	0.167	2233	3.030
11	0.162	2318	2.670	0.160	2206	2.990	0.163	2131	3.270
12	0.165	2331	2.680	0.163	2279	2.820	0.166	2334	2.700
13	0.178	2608	1.970	0.176	2401	2.640	0.179	2404	2.660
14	0.170	2268	2.960	0.169	2100	3.490	0.172	1865	4.310
15	0.177	2365	2.760	0.175	2224	3.210	0.178	2113	3.630
16	0.175	2355	2.770	0.172	2199	3.230	0.175	2237	3.160

	4th C.R.	Time:-	18.45	5th C.R.	Time:-	20.00	
ANIMAL	FLOW RATE (l/min)	CELL CONC.	C.R. (l/h)	FLOW RATE (l/min)	CELL CONC.	C.R. (l/h)	Maximum C.R. (l/h)
Control -	-	3176	-	-	3213	-	
1	0.180	2082	3.690	0.181	1791	4.800	4.800
2	0.183	1942	4.230	0.180	1974	4.160	4.230
3	0.172	2090	3.500	0.173	2059	3.720	4.220
4	0.182	2009	3.980	0.181	2164	3.540	3.980
5	0.176	1909	4.180	0.178	1962	4.150	4.620
6	0.183	2029	3.930	0.183	1915	4.430	4.430
7	0.172	2128	3.370	0.173	2059	3.720	3.720
8	0.174	2219	3.110	0.176	2149	3.490	3.490
Control -	-	3144	-	-	3207	-	
9	0.177	2311	2.850	0.179	1969	4.150	4.150
10	0.168	2185	3.110	0.170	1869	4.260	4.260
11	0.164	2036	3.500	0.165	2148	3.270	3.500
12	0.167	2232	2.940	0.167	1996	3.790	3.790
13	0.180	2364	2.720	0.178	2131	3.590	3.590
14	0.172	2123	3.390	0.173	2047	3.760	4.310
15	0.176	2159	3.380	0.176	2118	3.590	3.630
16	0.177	2148	3.400	0.178	2119	3.630	3.630

Appendix II
Example of spreadsheet for calculating clearance rate in a static system

CALCULATION OF CLEARANCE RATE (STATIC SYSTEM)
EXPERIMENT: TC2 Control
DATE:May 18 1989 TIME INTERVAL(MIN): 20 VOLUME (L): 2

TIME 0	COUNTS			MEAN	TIME 1	COUNTS			MEAN	TIME 2	COUNTS			MEAN
ANIM	1	2	3	4	ANIM	1	2	3	4	ANIM	1	2	3	4
1	11832	11531	11597	11653	1	7410	7208	7240	7286	1	4417	4422	4604	4481
2	12166	12239	12239	12215	2	6360	6394	6343	6365.7	2	3323	3324	3399	3348.7
3	11104	11348	11156	11203	3	6337	6516	6433	6428.7	3	4005	4090	4119	4071.3
4	10245	10502	10617	10455	4	5655	5497	5604	5585.3	4	3671	3842	3840	3784.3
5	11169	11175	11078	11141	5	6514	6392	6507	6471	5	3103	3021	3038	3054
6	12923	12991	12655	12856	6	8509	8398	8510	8472.3	6	4835	4707	4973	4838.3
7	12488	12554	12452	12498	7	6414	6118	6282	6271.3	7	2709	2616	2919	2748
8	10772	10657	10752	10727	8	6842	6674	6540	6685.3	8	4008	3968	3954	3976.7
9	11791	11761	11913	11822	9	6482	6198	6426	6368.7	9	2899	2817	2830	2848.7
10	12069	12015	12053	12046	10	7469	7469	7465	7467.7	10	3842	3904	3887	3877.7
11	11988	11912	11986	11962	11	6875	6807	6670	6784	11	3817	3802	3674	3764.3
12	11656	11733	11739	11709	12	6463	6431	6571	6488.3	12	3321	3091	3014	3142
13	11511	11382	11606	11500	13	6357	6555	6354	6422	13	2944	3046	2931	2973.7
14	12428	12582	12512	12507	14	7721	7606	7501	7609.3	14	5091	5030	5039	5053.3
15	11341	11456	11265	11354	15	5758	5655	5597	5670	15	2577	2500	2575	2550.7
16	12950	12905	12919	12925	16	7437	7363	7366	7388.7	16	4569	4662	4648	4626.3
CONTR	12725	12729	12736	12730	CONTR	12760	12933	12688	12794	CONTR	12726	12971	12758	12818

TIME 3	COUNTS			MEAN	TIME 4	COUNTS			MEAN
ANIM	1	2	3	4	ANIM	1	2	3	4
1	2753	2688	2749	2730	1	1483	1439	1366	1429.3
2	1906	1940	1933	1926.3	2	965	907	865	912.33
3	2160	2153	2151	2154.7	3	1124	1057	1101	1094
4	1314	1183	1227	1241.3	4	552	537	538	542.33
5	1620	1481	1473	1524.7	5	788	792	739	773
6	2623	2490	2511	2541.3	6	1497	1380	1473	1450
7	1158	1160	1114	1144	7	469	487	510	488.67
8	2566	2515	2417	2499.3	8	2058	1984	1968	2003.3
9	1460	1407	1399	1422	9	796	763	780	779.67
10	1991	1984	1981	1985.3	10	1048	1046	1043	1045.7
11	1913	1914	1876	1901	11	1031	1050	1013	1031.3
12	1541	1481	1515	1512.3	12	768	773	748	763
13	1460	1553	1489	1500.7	13	731	737	676	714.67
14	2802	2757	2684	2747.7	14	1672	1518	1492	1560.7
15	1180	1153	1110	1141	15	623	617	589	609.67
16	2501	2480	2452	2477.7	16	1234	1299	1221	1251.3
CONTR	12581	12568	12569	12569	CONTR	12767	12456	12525	12583

CONTINUED BELOW

CLEARANCE RATE (l/h)					CR (l/h) OVER 2 INTERVALS			MAX CR (l/h) OVER 2 INTERVALS	
ANIM	T0-T1	T1-T2	T2-T3	T3-T4	T0-T2	T1-T3	T2-T4	ANIM	
1	2.818	2.917	2.973	3.883	2.867	2.945	3.428	1	3.428
2	3.91	3.854	3.318	4.484	3.882	3.586	3.901	2	3.901
3	3.332	2.741	3.818	4.067	3.037	3.279	3.942	3	3.942
4	3.761	2.336	6.688	4.968	3.049	4.512	5.828	4	5.828
5	3.26	4.505	4.168	4.076	3.882	4.337	4.122	5	4.337
6	2.502	3.361	3.863	3.367	2.932	3.612	3.615	6	3.615
7	4.137	4.951	5.258	5.104	4.544	5.104	5.181	7	5.181
8	2.837	3.117	2.787	1.327	2.977	2.952	2.057	8	2.977
9	3.711	4.827	4.169	3.606	4.269	4.498	3.887	9	4.498
10	2.869	3.932	4.017	3.847	3.4	3.974	3.932	10	3.974
11	3.403	3.534	4.099	3.669	3.468	3.817	3.884	11	3.884
12	3.542	4.351	4.387	4.105	3.947	4.369	4.246	12	4.369
13	3.496	4.62	4.103	4.451	4.058	4.361	4.277	13	4.361
14	2.982	2.456	3.656	3.394	2.719	3.056	3.525	14	3.525
15	4.166	4.793	4.827	3.76	4.48	4.81	4.294	15	4.81
16	3.355	2.809	3.747	4.099	3.082	3.278	3.923	16	3.923
CONTR	-0.03	-0.01	0.118	0.03	-0.02	0.053	0.074		

MEAN	4.16	N=	16
S.E.	0.17		
2 S.E.	0.34		
LOWER 95% C.I.	3.82		
UPPER 95% C.I.	4.499		

Appendix III
Example of spreadsheet for calculating absorption efficiency

CALCULATION OF FOOD ABSORPTION EFFICIENCIES

Experiment:- North Sea Study Pop I

Date :- Jul 3 1990

BLANK FILTERS

Filter No.	(A) Initial Wt Filter (mg)	(B) Dry Wt + Filter (mg)	(B-A) Dry Wt of Sample (mg)	(D) Ash Wt + Filter (mg)	(D-A) Ash Wt of sample (mg)
1	5.013	5.013	0	4.961	-0.052
2	4.567	4.574	0.007	4.523	-0.044
3	4.873	4.911	0.038	4.84	-0.033
4	5.447	5.489	0.042	5.413	-0.034
5	4.915	4.909	-0.006	4.885	-0.03
Mean			0.0162		-0.0386

FOOD

Filter No.	(A) Initial Wt Filter (mg)	(B) Dry Wt + Filter (mg)	(B-A) Dry Wt of Sample (mg)	(C) Dry Wt after blk corr(mg)	(D) Ash Wt + Filter (mg)	(D-A) Ash Wt of sample (mg)	(E) Ash Wt after blk corr(mg)	FOOD [(C-E)/C] Ash-free dry wt to Dry wt ratio
1	5.819	6.902	1.083	1.0668	5.856	0.037	0.0755	0.9291
2	5.082	6.137	1.055	1.0388	5.109	0.027	0.0656	0.9369
3	4.966	5.945	0.979	0.9628	4.991	0.025	0.0636	0.9339
4	5.393	6.389	0.996	0.9798	5.452	0.059	0.0976	0.9004
5	5.767	6.624	0.857	0.8408	5.802	0.035	0.0736	0.9125
Mean			0.994	0.9778		0.0366	0.0752	0.9226

FAECES

Filter No.	(A) Initial Wt Filter (mg)	(B) Dry Wt + Filter (mg)	(B-A) Dry Wt of Sample (mg)	(C) Dry Wt after blk corr(mg)	(D) Ash Wt + Filter (mg)	(D-A) Ash Wt of sample (mg)	(E) Ash Wt after blk corr(mg)	FAECES [(C-E)/C] Ash-free dry wt to Dry wt ratio	ABSORPTION EFFICIENCY
1	5.1	7.216	2.116	2.0998	5.322	0.222	0.2606	0.8759	0.4073
2	4.366	6.761	1.395	1.3788	5.083	0.217	0.2556	0.8640	0.4669
3	4.357	7.545	2.388	2.3718	5.133	0.276	0.3146	0.8823	0.3719
4	4.34	6.773	1.933	1.9163	5.057	0.217	0.2556	0.8667	0.4544
5	4.993	7.142	2.149	2.1328	5.221	0.228	0.2666	0.8750	0.4124
6	5.294	7.511	2.217	2.2008	5.548	0.254	0.2926	0.8670	0.4525
7	4.713	6.865	2.152	2.1358	4.951	0.238	0.2786	0.8705	0.4358
8	5.104	6.903	1.799	1.7828	5.298	0.194	0.2326	0.8695	0.4405
9	5.093	7.223	2.14	2.1238	5.327	0.234	0.2726	0.8716	0.4299
10	5.3	7.244	1.944	1.9278	5.473	0.173	0.2116	0.8700	0.4382
11	5.361	7.482	2.121	2.1048	5.586	0.225	0.2636	0.8748	0.4137
12	5.513	7.633	2.12	2.1038	5.724	0.211	0.2496	0.8814	0.3794
13	5.294	6.708	1.414	1.3978	5.429	0.135	0.1736	0.8758	0.4092
14	5.415	6.346	0.931	0.9148	5.492	0.077	0.1156	0.8736	0.4198
15	5.291	7.211	1.93	1.9138	5.503	0.222	0.2606	0.8633	0.4673
16	5.308	7.091	2.083	2.0668	5.217	0.209	0.2476	0.8802	0.3832

MEAN = 0.4223
N = 15.0000
S.D. = 0.0677

Appendix IV
Example of spreadsheet for calculating respiration rate

CALCULATION OF OXYGEN CONSUMPTION
EXPERIMENT :- M Sea Mussels Site 8 Orkney
DATE :- Jul 2 1990

O2 SAT. umol O2/l= 259 STAND MASS= 0.500 g WT EXP = 0.670

ANIMAL	DRY MASS (g)	RESP VOL (litres)	START (mm Hg)	END (mm Hg)	TIME INTERVAL (min)	O2 UPTAKE umol/h	O2 uptake umol/h/g
1	0.277	0.485	151.3	144.0	40	8.646	12.843
2	0.391	0.500	151.5	144.3	40	8.913	10.510
3	0.392	0.500	151.5	148.5	20	7.377	8.683
4	0.358	0.510	150.0	143.0	50	7.023	8.784
5	0.513	0.510	152.3	141.0	50	11.336	11.143
6	0.372	0.490	148.5	139.0	50	9.157	11.163
7	0.354	0.480	149.0	143.0	50	5.665	7.140
8	0.407	0.460	153.5	144.8	50	7.918	9.088
9	0.474	0.485	151.5	141.5	60	7.950	8.240
17	0.499	0.500	150.5	139.0	60	9.426	9.438
11	0.388	0.500	157.0	145.5	60	9.426	11.171
12	0.363	0.510	151.5	142.0	60	7.942	9.843
13	0.369	0.510	156.7	147.5	60	7.725	9.469
14	0.371	0.490	151.0	141.0	60	8.032	9.810
15	0.315	0.480	152.0	143.0	50	8.498	11.581
16	0.401	0.460	154.5	144.0	50	9.501	11.015
				MEAN=		8.408	9.995
				S.E.=		0.309	0.353
				N =		16.000	16.000
				L95%CI=		7.790	9.289
				U95%CI=		9.027	10.701

Appendix V

Table of oxygen solubilities (μmoles O_2 L^{-1}) with temperature and salinity (from Gnaiger and Forstner, 1983)

Values calculated for the standard pressure of 101.325 kPa

The values presented in the table below are derived from Gnaiger (1983) having the units umoles oxygen/litre

	Salinity											
Temp °C	0	10	15	25	26	27	28	29	30	31	32	33
0	457.08	425.85	411.00	382.84	380.13	377.44	374.77	372.12	369.49	366.88	364.28	361.71
1	444.32	414.33	400.07	372.99	370.38	367.80	365.23	362.68	360.14	357.63	355.13	352.65
2	432.18	403.36	389.64	363.58	361.07	358.58	356.11	353.65	351.21	348.79	346.38	343.99
3	420.61	392.89	379.68	354.58	352.16	349.76	347.38	345.01	342.66	340.32	338.00	335.70
4	409.58	382.89	370.16	345.97	343.64	341.32	339.02	336.74	334.47	332.22	329.98	327.76
5	399.04	373.32	361.06	337.72	335.47	333.23	331.01	328.81	326.62	324.44	322.28	320.14
6	388.98	364.17	352.34	329.80	327.63	325.47	323.33	321.20	319.08	316.98	314.89	312.82
7	379.35	355.41	343.98	322.21	320.11	318.02	315.95	313.89	311.85	309.81	307.80	305.79
8	370.13	347.01	335.96	314.91	312.88	310.86	308.86	306.87	304.89	302.92	300.97	299.03
9	361.30	338.95	328.27	307.90	305.93	303.98	302.04	300.11	298.19	296.29	294.40	292.52
10	352.84	331.21	320.87	301.15	299.25	297.35	295.47	293.61	291.75	289.90	288.07	286.25
11	344.72	323.78	313.76	294.65	292.81	290.97	289.15	287.34	285.54	283.75	281.97	280.20
12	336.92	316.63	306.92	288.39	286.60	284.82	283.05	281.29	279.55	277.81	276.09	274.37
13	329.43	309.76	300.34	282.35	280.61	278.88	277.17	275.46	273.77	272.08	270.40	268.74
14	322.22	303.13	293.99	276.52	274.83	273.16	271.49	269.83	268.18	266.55	264.92	263.30
15	315.29	296.75	287.87	270.90	269.25	267.62	266.00	264.39	262.79	261.20	259.61	258.04
16	308.62	290.60	281.97	265.46	263.86	262.28	260.70	259.13	257.57	256.02	254.48	252.95
17	302.19	284.67	276.27	260.20	258.65	257.10	255.57	254.04	252.52	251.02	249.52	248.03
18	295.99	278.94	270.76	255.12	253.60	252.10	250.60	249.12	247.64	246.17	244.71	243.26
19	290.02	273.41	265.44	250.20	248.72	247.25	245.80	244.35	242.91	241.47	240.05	238.63
20	284.25	268.06	260.30	245.43	243.99	242.56	241.14	239.72	238.32	236.92	235.53	234.15
21	278.68	262.90	255.32	240.81	239.41	238.01	236.62	235.24	233.87	232.51	231.15	229.80
22	273.31	257.90	250.50	236.33	234.96	233.60	232.24	230.89	229.55	228.22	226.90	225.58
23	268.11	253.06	245.83	231.99	230.65	229.32	227.99	226.67	225.36	224.06	222.77	221.48
24	263.08	248.37	241.31	227.77	226.46	225.16	223.86	222.58	221.29	220.02	218.75	217.50
25	258.22	243.83	236.92	223.68	222.40	221.12	219.85	218.59	217.34	216.09	214.86	213.62
26	253.51	239.43	232.67	219.70	218.45	217.20	215.96	214.72	213.50	212.28	211.06	209.86
27	248.95	235.16	228.54	215.84	214.61	213.39	212.17	210.96	209.76	208.56	207.38	206.19
28	244.54	231.02	224.53	212.08	210.88	209.68	208.49	207.30	206.12	204.95	203.79	202.63
29	240.26	227.01	220.64	208.43	207.25	206.07	204.90	203.74	202.58	201.43	200.29	199.15
30	236.11	223.10	216.86	204.88	203.71	202.56	201.41	200.27	199.14	198.01	196.89	195.77
31	232.09	219.32	213.18	201.42	200.28	199.14	198.01	196.89	195.78	194.67	193.57	192.47
32	228.18	215.63	209.60	198.05	196.93	195.81	194.70	193.60	192.51	191.42	190.34	189.26
33	224.39	212.05	206.13	194.78	193.66	192.57	191.48	190.39	189.32	188.25	187.18	186.12
34	220.71	208.57	202.74	191.58	190.48	189.40	188.33	187.27	186.21	185.15	184.11	183.07
35	217.13	205.19	199.45	188.44	187.38	186.32	185.26	184.22	183.17	182.14	181.11	180.08
36	213.65	201.89	196.24	185.40	184.35	183.31	182.27	181.24	180.21	179.19	178.18	177.17
37	210.28	198.68	193.11	182.44	181.40	180.37	179.35	178.33	177.32	176.32	175.32	174.32
38	206.99	195.56	190.07	179.54	178.52	177.51	176.50	175.50	174.50	173.51	172.52	171.54
39	203.79	192.52	187.10	176.72	175.71	174.71	173.71	172.73	171.74	170.76	169.79	168.83
40	200.68	189.55	184.20	173.96	172.96	171.98	171.00	170.02	169.05	168.08	167.12	166.17

Salinity							
34	35	36	37	38	39	40	Temp °C
359.15	356.61	354.08	351.58	349.09	346.62	344.17	0
350.19	347.74	345.31	342.90	340.51	338.13	335.77	1
341.62	339.26	336.92	334.60	332.29	330.00	327.72	2
333.41	331.14	328.88	326.64	324.41	322.20	320.01	3
325.55	323.36	321.18	319.01	316.86	314.73	312.61	4
318.00	315.89	313.78	311.69	309.62	307.55	305.51	5
310.76	308.71	306.68	304.66	302.65	300.66	298.68	6
303.80	301.82	299.85	297.90	295.96	294.03	292.11	7
297.10	295.18	293.28	291.39	289.51	287.64	285.79	8
290.65	288.80	286.95	285.12	283.30	281.49	279.69	9
284.44	282.64	280.85	279.08	277.31	275.56	273.82	10
278.45	276.70	274.97	273.25	271.54	269.83	268.14	11
272.67	270.97	269.29	267.62	265.96	264.31	262.67	12
267.09	265.44	263.81	262.18	260.57	258.96	257.37	13
261.69	260.09	258.51	256.93	255.36	253.80	252.25	14
256.48	254.92	253.38	251.84	250.32	248.80	247.29	15
251.43	249.92	248.41	246.92	245.44	243.96	242.49	16
246.54	245.07	243.61	242.15	240.71	239.27	237.84	17
241.81	240.38	238.95	237.53	236.12	234.72	233.33	18
237.23	235.83	234.44	233.05	231.68	230.31	228.95	19
232.78	231.41	230.06	228.71	227.36	226.03	224.71	20
228.46	227.13	225.80	224.49	223.18	221.87	220.58	21
224.27	222.97	221.67	220.39	219.11	217.84	216.57	22
220.20	218.93	217.66	216.40	215.15	213.91	212.67	23
216.24	215.00	213.76	212.53	211.31	210.09	208.88	24
212.40	211.18	209.97	208.77	207.57	206.38	205.20	25
208.66	207.47	206.28	205.10	203.93	202.76	201.60	26
205.02	203.85	202.69	201.53	200.38	199.24	198.11	27
201.47	200.33	199.19	198.06	196.93	195.81	194.70	28
198.02	196.90	195.78	194.67	193.57	192.47	191.38	29
194.66	193.56	192.46	191.37	190.29	189.21	188.14	30
191.38	190.30	189.22	188.15	187.09	186.03	184.98	31
188.19	187.12	186.07	185.01	183.97	182.93	181.89	32
185.07	184.03	182.98	181.95	180.92	179.90	178.88	33
182.03	181.00	179.98	178.96	177.95	176.94	175.94	34
179.06	178.05	177.04	176.04	175.04	174.05	173.07	35
176.16	175.17	174.17	173.19	172.21	171.23	170.26	36
173.33	172.35	171.37	170.40	169.43	168.47	167.52	37
170.57	169.60	168.64	167.68	166.72	165.78	164.84	38
167.86	166.91	165.96	165.01	164.07	163.14	162.21	39
165.22	164.28	163.34	162.41	161.48	160.56	159.64	40

Appendix VI
Example of spreadsheet for calculating Scope for Growth

CALCULATION OF SCOPE FOR GROWTH
EXPERIMENT :- North Sea Mussels Site 3 Orkney
DATE:- Jul 2 1990

Weight exponent = 0.670 Food energy value= 9.200 Joules/L
Standard weight = 1.000 Absorption effic = 0.420

ANIMAL	DRY MASS g	LENGTH mm	C.R. l/h	C.R. l/g/h	C J/g/h	A J/g/h	Resp umol/h	Resp umol/g/h	R J/g/h	S.F.G. J/g/h	S.F.G. J/h/indiv
1	0.277	41.280	4.800	11.344	104.368	43.835	8.646	20.434	9.318	34.517	14.605
2	0.391	42.920	4.230	7.936	73.008	30.663	8.913	16.722	7.625	23.038	12.280
3	0.392	42.170	4.220	7.903	72.711	30.539	7.377	13.815	6.300	24.239	12.942
4	0.358	40.820	3.980	7.921	72.873	30.607	7.023	13.976	6.373	24.234	12.176
5	0.513	41.640	4.620	7.225	66.474	27.919	11.336	17.729	8.085	19.834	12.682
6	0.372	41.660	4.430	8.593	79.055	33.203	9.157	17.762	8.099	25.104	12.942
7	0.354	40.290	3.720	7.460	68.628	28.824	5.665	11.360	5.180	23.643	11.791
8	0.407	40.400	3.490	6.374	58.639	24.628	7.918	14.460	6.594	18.035	9.875
9	0.474	41.500	4.150	6.843	62.960	26.443	7.950	13.110	5.978	20.465	12.410
17	0.499	40.710	4.260	6.787	62.441	26.225	9.426	15.017	6.848	19.377	12.163
11	0.388	42.510	3.500	6.600	60.721	25.503	9.426	17.774	8.105	17.398	9.226
12	0.363	40.110	3.790	7.473	68.753	28.876	7.942	15.660	7.141	21.735	11.023
13	0.369	41.330	3.590	7.001	64.413	27.054	7.725	15.065	6.870	20.184	10.349
14	0.371	41.770	4.310	8.375	77.052	32.362	8.032	15.608	7.117	25.244	12.991
15	0.315	41.070	3.630	7.871	72.415	30.414	8.498	18.426	8.402	22.012	10.151
16	0.401	41.960	3.630	6.696	61.601	25.872	9.501	17.525	7.992	17.881	9.694
Mean =	0.390	41.284	4.022	7.650	70.382	29.560	8.408	15.903	7.252	22.309	11.706
S.E. =	0.015	0.193	0.100	0.287	2.637	1.108	0.309	0.562	0.256	1.008	0.362
N =	16.000	16.000	16.000	16.000	16.000	16.000	16.000	16.000	16.000	16.000	16.000
U 95%CI =	0.420	41.770	4.222	8.224	75.656	31.776	9.027	17.026	7.764	24.325	12.431
L 95%CI =	0.360	40.797	3.822	7.077	65.107	27.345	7.790	14.780	6.740	20.292	10.982

Appendix VII
Procedures for measuring ammonia excretion into seawater

Five litres of sea water are filtered through a membrane filter (0.45 µm). The batch of filtered sea water (FSW) is then returned to full air saturation by aeration at the experimental temperature.

Animals are placed in individual beakers containing 200 mL of FSW (larger volumes may be necessary for larger animals). An additional beaker containing 200 mL of FSW, but without an animal, acts as a control. Following a 2 h incubation period in a water bath held at the experimental temperature, samples are taken from each beaker and analysed for ammonia using the phenol-hypochlorite method of Solorzano (1969).

Elevated ammonia concentrations (above c. 100 µM NH_4 - N L^{-1}) can inhibit the rate of NH_4 excretion. The incubation time and volume of water should therefore be adjusted when necessary.

Ammonia Test

Into each tube place:
5mL sea water sample
0.2mL phenol solution
Mix well and add 0.2mL nitroprusside solution
Mix and add 0.5mL oxidising solution (made fresh)
Mix well; cover tubes and place in dark.

Read on spectrophotometer at 640nm after 2-24 h. Carry out analysis in duplicate with clean test tubes (acid-wash tubes or heat tubes at 450°C in muffle furnace).

For standards, use distilled water as blank, and make up:
1 µM (5 µL of stock in 5 mL DW)
5 µM (25 µL of stock in 5 mL DW)
10 µM (50 µL of stock in 5 mL DW)
20 µM (100 µL of stock in 5 mL DW)
40 µM (200 µL of stock in 5 mL DW)

Use 5 mL of standards and treat same as samples.

Reagents

1. Phenol solution:

Dissolve 10 g of phenol in 100 mL of 95% v/v ethyl alcohol. Store at 5°C.

2. Sodium nitroprusside:

Dissolve 1 g of sodium nitroprusside in 200 mL DW. Store in amber bottle at 5°C for not more than 1 month.

3. Oxidizing solution:

Mix 25 mL of fresh domestic bleach (commercial hypochlorite) with 100

mL alkaline solution. This solution is only stable for c. 12 h and has to be made up fresh each day.

4. Alkaline solution:

   Dissolve 100 g trisodium citrate and 5 g NaOH in 500 mL DW. Store at 5°C.

5. Standard stock solution:
   0.05349 g NH_4Cl per litre = 1 mM NH_4-N. Add a few drops of chloroform to preserve.

Calculation of Ammonia Excretion

Construct a standard curve and convert optical density (O.D.) readings for samples and control to µM NH_4-N. Subtract controls from the samples and express rate of excretion as µmoles NH_4-N h^{-1}.

$$\mu M\ NH_4\text{-}N \text{ excreted } h^{-1} = (\text{Test } \mu M - \text{Control } \mu M) \times (V / 1000) \times 1/t$$

where V = volume of seawater in which animal is incubated (e.g. 200 mL) and t = incubation time (e.g. 2 h).

Appendix VIII
Correction for Body Size

Body size is an important variable affecting most physiological responses, but one that can be largely eliminated by selecting and transplanting animals of similar body size. It is inevitable, however, that there will be slight differences in the dry body mass and this effect can be removed by correcting rates of feeding, respiration, excretion and growth to a 'standard body size' by means of the allometric equation:

$$Y = \alpha X^b \quad (1)$$

or

$$\log Y = \log \alpha + b \log X \quad (2)$$

where Y = physiological rate, X = dry body mass (g), and α and b are the intercept and slope, respectively. Physiological rates are converted to an appropriate weight-specific rate using the exponent b.

The equations describing the relationships between each physiological rate and dry body mass are first established for a base-line reference population. Approximately 30 individuals covering a wide size range are measured and the data are then analysed by linear regression of log transformed data (X, Y). The weight exponent or slope of the regression for each physiological response is then used to correct for differences in dry body mass found within any sample. If animals of approximately 1g dry mass are selected and measured, then rates can be corrected to a 'standard 1 g animal'.

For example:-

The slope (b = 0.65) describing the relationship between oxygen uptake and dry body mass is substituted in equation 2. Therefore, if an individual has an oxygen uptake of 12.54 µmoles O_2 h^{-1} and a dry mass of 0.83 g then:

$$\log \alpha = \log Y - b \log X$$
$$\log \alpha = \log 12.54 - 0.65 (\log 0.83)$$
$$\alpha = 14.16 \text{ µmoles } O_2 \text{ } g^{-1} \text{ } h^{-1}$$

If the average body mass of the animals is markedly different from 1 g dry mass, then a standard body size equivalent to the mean body mass is chosen and the corrections for any weight differences are made in a similar manner but using the following equation:

$$\log Y_c = \log Y_o - (b \log X_o - b \log X_c)$$

where Y_c is the corrected value for a standard body mass (X_c) and Y_o and X_o are the individual's measured rate and body mass, respectively.

Appendix IX
Principle of Particle analysis using a Coulter Counter

Particles suspended in an electrolyte solution (e.g. sea water) are made to flow through a small orifice (or aperture) in the wall of a glass tube (acting as an electrical insulator). The orifice creates the sensing zone. A current path is established between two immersed electrodes, across this orifice, setting a certain base impedence to the electrical detection circuitry. As each particle enters the orifice, it has effectively displaced a volume of electrolyte solution equal to its own immersed volume, and the base impedence is therefore modulated by an amount proportional to the volume of the particle. This results in an electrical pulse of short duration being created by each particle; the height of the pulse being proportional to the volume of the particle. It is conventional to report the equivalent spherical diameter from the volume. The pulse may be measured as the change in the resistance, current or voltage across the electrodes.

The passage of a number of particles produces a train of pulses which can be observed on an oscilloscope and analysed by counter and pulse height circuits to produce a number against particle volume, or equivalent spherical diameter, distribution. A volume or mass against size distribution can also be computed.

Simple models (e.g. Model D) have only one counter and size level circuit; ore complex models can obtain number an/or mass distributions automatically in up to 256 size channels in seconds. Counting and sizing rates up to some 10000 particles per second are possible with each pulse height being measured within one or two percent.

Coulter Counters are widely used in the medical (blood cell analysis) and industrial fields, but have been used increasingly in marine research over the past 20 years.

Particle Size Range is a function of the Aperture Tube (i.e. 2-60% of the orifice size).

Appendix X
Principles of measuring oxygen using a polarographic oxygen sensor

A typical 'Clark-type' oxygen electrode consists of a membrane permeable to oxygen (e.g. polypropylene) spread over a flat or convex electrode, usually clamped in place with an O-ring. Between the membrane and the surface of the electrode there is a layer of electrolyte which provides a high conductance path between the silver anode and the platinum cathode, but the anode need not directly face the membrane. The cathodic reaction is summarized by the following equation:

$$O_2 + H_2O + 4e^- = 4OH^-$$

An electrical current is generated and this is proportional to the oxygen reaching the electrode. The surface area of the cathode needs to be small to avoid a significant oxygen consumption by the probe itself. In order to obtain a steady reading most oxygen sensors require stirring.

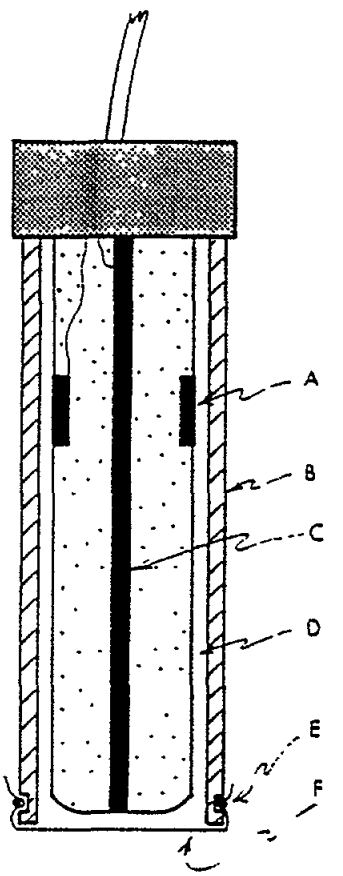
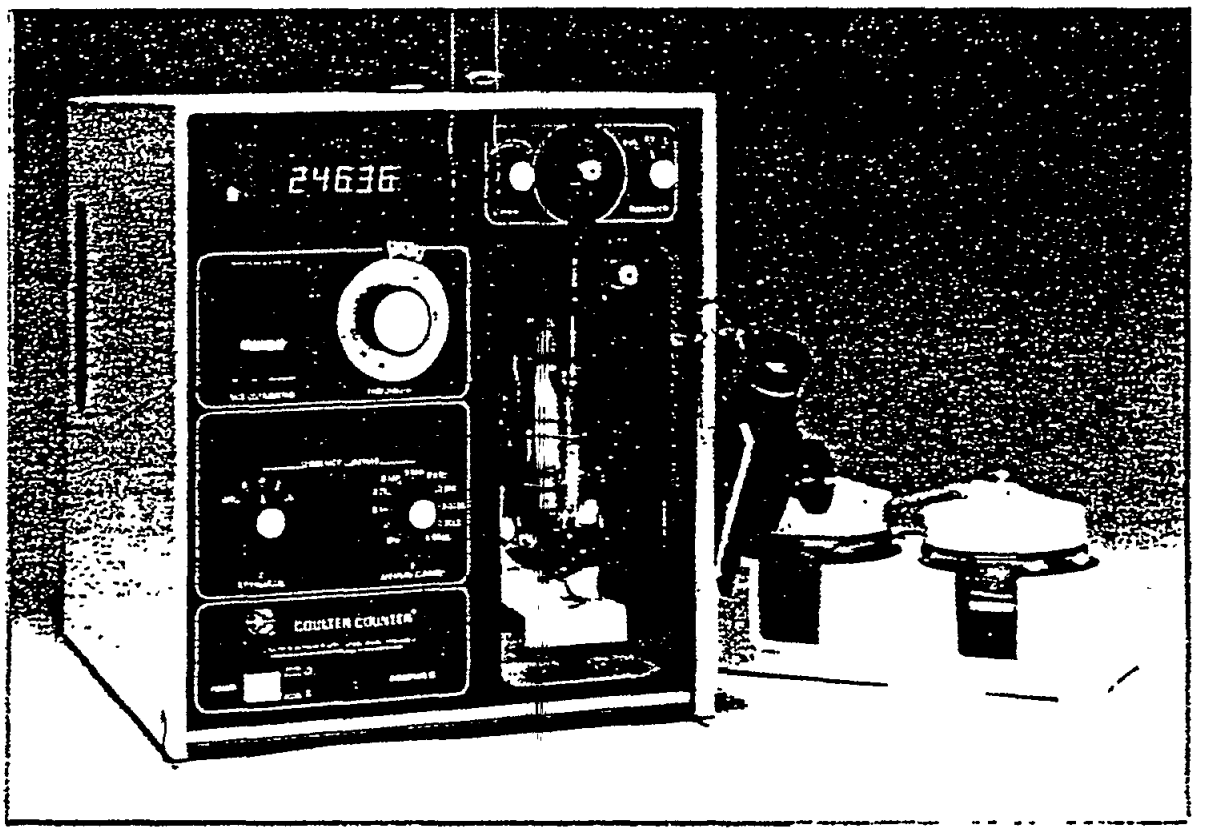


Diagram of cross-section of a typical oxygen electrode showing the silver anode (A), electrode housing (B), platinum cathode (C), electrolyte space (D), and the O-ring (E) holding the membrane (F) to the end of the housing.

COULTER COUNTER

Economical Analyser for the control of simple Industrial/Biological Processes



The Coulter Counter® Model D Industrial is a low-cost, general purpose particle counter designed to count and size particles within the range of 1μm – 112μm. Materials to be analysed should be confined to those that may be dispersed and suspended in an aqueous-based electrolyte. The Model D Industrial is ideally suited to liquid contamination monitoring such as the analysis of intravenous fluids and seawater, as well as for water treatment and filtration studies. It is widely accepted and used in hospitals and pharmaceutical companies for basic biological cell counting, such as yeasts, spermatozoa, tissue culture cells and milk cells. Other applications include the analysis of powdered materials such as ceramics, toners and plastics. The instrument is self-contained, robust and ideally suited to the laboratory requiring a low-cost, simple-to-use method for particle counting and sizing that is reliable, accurate and reproducible. It is the ideal teaching instrument for technical colleges and universities.

Technical Specifications

Measurement Range
1 – 112μm

Aperture Tube Range
30 – 280μm

Reproducibility
± 1 wt.% (1.s.d.) at any point on the size distribution

Operating Temp. Range
10°C – 30°C

Sample Volumes
1ml, 500μl & 50μl

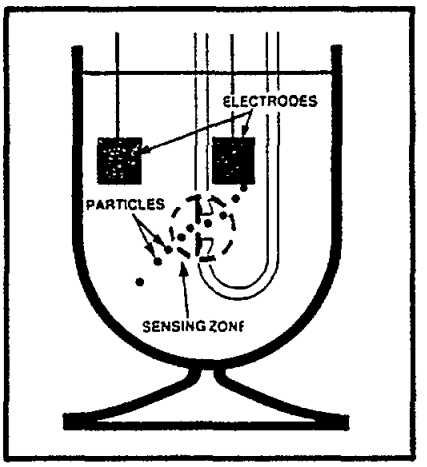
Counting Time
Typically 15 seconds for 100μm aperture

Dimensions
39cm High × 33cm Wide × 39cm Deep
Weight 15kg

Power Requirement
220 V a.c. ± 10% 50 or 60Hz
110 V a.c. ± 10% 50 or 60Hz
Power consumption 110 Watts.

The Coulter Principle

(W.H. Coulter, 1949)



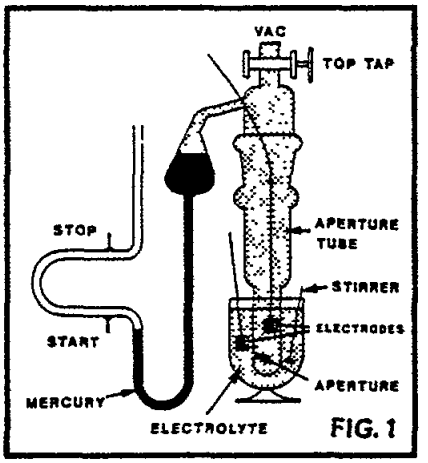


FIG. 1

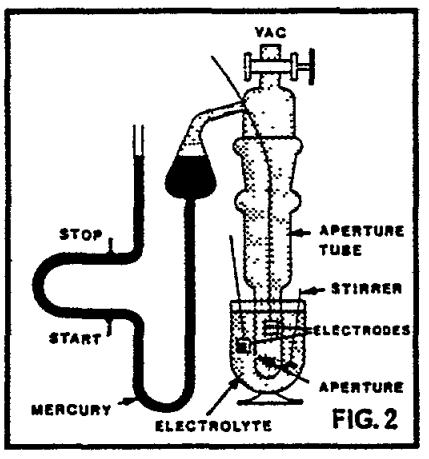
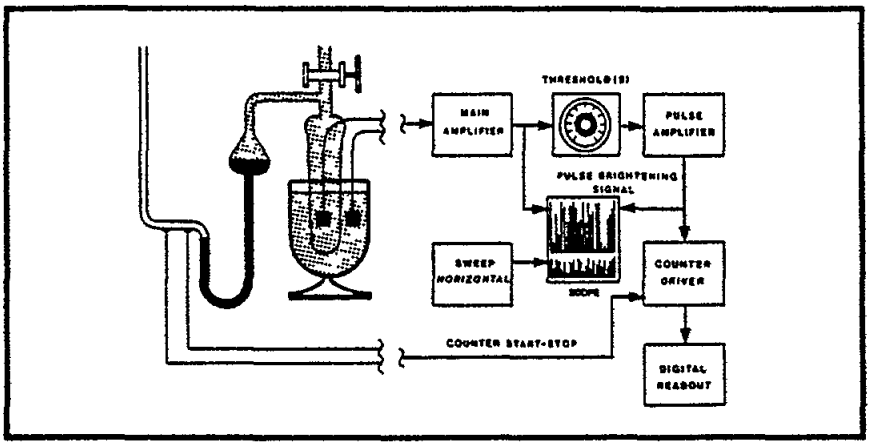


FIG. 2

A dilute suspension of particles to be analysed is made in an electrolyte solution. This suspension is then stirred and drawn through a small aperture by means of a tap being opened to a vacuum source (Fig. 1). A current, passing through the aperture between two electrodes, enables the particles to be sensed by the momentary changes in the electrical impedance as they pass through the aperture, as each particle displaces its own volume of electrolyte within the aperture itself. These changes in impedance may be detected and presented as a series of voltage pulses, the height of each pulse is essentially proportional to the volume of the particle that produced it

At the same time, a mercury siphon becomes unbalanced by the applied vacuum. Closing the tap isolates the vacuum source and allows the sample suspension to continue to be drawn through the aperture as the mercury returns to equilibrium (Fig. 2) Electrical contacts in the mercury column allow the apparatus to count the particles in a precise, reproducible, volume of suspension passing through the aperture These particle-generated pulses are amplified and measured. By this technique, several thousand particles per second may be individually counted and sized, to great accuracy. Almost any type of particle can be analysed and choice of electrolyte will depend upon the particle composition.

Particle size analysis may be performed in the overall size range of 0.4-800μm, the upper limit being dependent on particle density. To achieve this range, a number of different sensors (aperture tubes) are required. Table 1 gives details of the operating range of each aperture by instrument model.



The patented "constant current" feature of the Coulter Counter®, as used on most models, ensures maximum accuracy by eliminating calibration drift due to changes in electrolyte conductivity and temperature. The mercury siphon provides the most reproducible and accurate method of sampling from a suspension with an accuracy of better than ±0.5% on the 0.5ml volume position.

The Coulter Principle is used as the reference method for several National Standards in particle size analysis throughout the world. It is the subject of British Standard 3406: Part 5: 1983

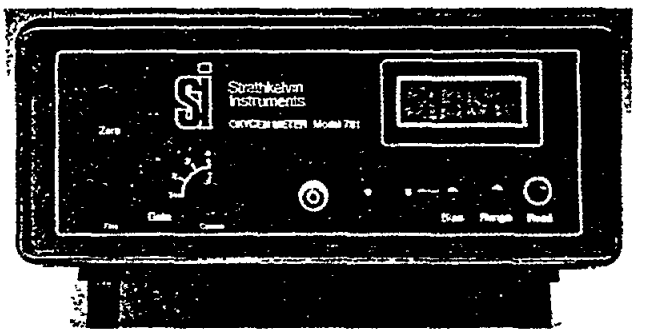
PRECISION DISSOLVED OXYGEN MEASUREMENTS WITH THE STRATHKELVIN SYSTEM

The Strathkelvin Instruments dissolved oxygen measurement systems take advantage of the characteristics of microcathode oxygen electrodes, which operate in unstirred media. This is of particular value where the sample size is very small, as in blood oxygen measurements, in microflowcell operations or in monitoring oxygen change in respirometers containing delicate organisms which would be disturbed by vigorous stirring of the water surrounding them.

Model 781 Oxygen Meters

These are precision meters incorporating high impedance, high gain amplifiers to measure the very low currents generated by microcathode electrodes, and offer very high stability. The digital display can be set to read in units of ppm (mg/l), ml/l, % saturation or mm Hg. A range switch allows oxygen concentration to be displayed in hyperoxic conditions up to 1999 mm Hg.

Model 781b operates on internal rechargeable batteries. The fully charged batteries give approximately 100 hours of readings. When used in the laboratory, the meter still operates from the batteries which are kept continuously charged, and this model is particularly advantageous where the mains supply is unreliable or badly contaminated with voltage transients. Model 781 operates on mains power supplies.

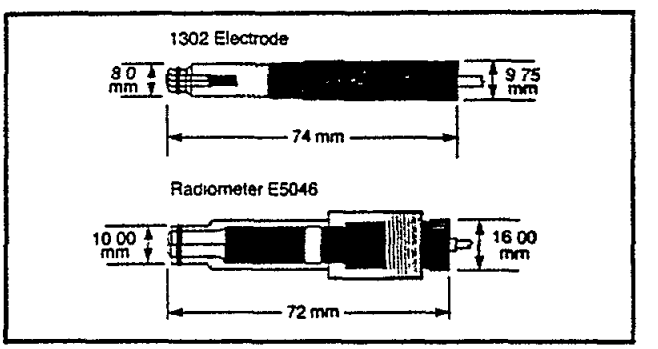


Features

- **Digital display — resolution to 0.1%.**
- **Selection of display units.**
- **Range extension facility.**
- **Recorder output.**
- **Range of gains to allow use of other electrodes.**

Microcathode Oxygen Electrodes

These are Clark type polarographic electrodes and were originally designed to meet the exacting requirements for oxygen determinations of blood in clinical laboratories. Because of the small surface area of the cathode, the rate of oxygen consumption by the electrode is extremely low. This in turn means that oxygen replacement can take place by diffusion processes, so that unlike the situation with conventional oxygen electrodes, it is not necessary to stir the sample liquid adjacent to the electrode tip.



Specifications

	mm Hg or % saturation	ppm or ml/l
Digital display normal	0-199 9	0-19 99
extended	0-1999	0-199.9
Recorder output:	1V for 199 9 disp	1V for 19 99 disp.
Readability:	0.1	0.01
Repeatability:	0 2	0.02
Temperature coefficient:	c 2% per °C	

Response time (at 37°C)· Polypropylene membranes	c 18 sec for 90% change
Teflon membranes	c 6 sec for 90% change
Oxygen consumption·	c 1-5 x 10^{-11} mg O_2/sec
Polarising voltage:	Adjustable 500-900 mv referred to ground
Power requirements	120 or 240v AC, 40-60 Hz
Dimensions·	275 x 258 x 117mm
Weight·	2.2Kg (Model 781) 3.2Kg (Model 781b)

A complete oxygen measurement system requires an oxygen meter, a microcathode oxygen electrode, and a service kit containing spare membranes, O rings and electrolyte. As it is necessary to maintain the electrode and sample at constant temperature, it may be desirable to order an oxygen measuring cell or chamber.

OXYGEN MEASUREMENT CELLS

In order to prevent gaseous exchange between the sample and atmospheric oxygen, it is necessary to enclose the sample in a gas-tight chamber. Strathkelvin Instruments have developed a number of chambers and cells for use with the 1302 electrode in specific applications. In each case it is necessary, in order to exploit the high precision of the electrode and meter, to maintain the sample chamber at a set temperature, using water from a constant temperature bath.

MC 100 MICROCELL

For micro O_2 determination or microflowcell use

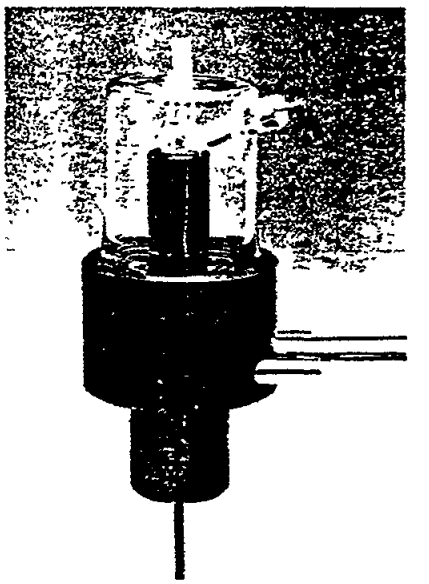
The all-glass sample chamber has a capacity of approximately 70µl Samples are injected into the chamber via a 1mm stainless steel tube fitted with a fine p.v c. needle-locating collar. The sample leaves the chamber through a glass Luer fitting to which a small bore flexible drainage tube can be connected.

The sample chamber is completely surrounded by water circulated through the glass water jacket from a constant temperature bath. This provides good temperature regulation whilst providing excellent visibility of the contents of the chamber. The water jacket screws into an acetal base section and can be removed easily for cleaning the inside surfaces.

The microcathode electrode fits into the central electrode holder and the membrane at its tip forms the floor of the sample chamber.

Specifications

Sample chamber	·70µl
Diameter (base)	:50mm
Height	:106mm
Jacket and cell	·glass
Base section	:black acetal
Mounting rod	·10mm dia stainless steel



Features

- Small sample volume.
- Flow through or spot measurement.
- Exceptional visibility.
- Easy access for cleaning.

RC 200 MICRORESPIRATION CELL

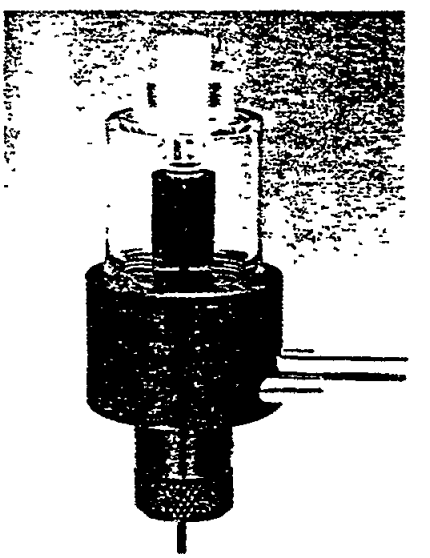
For respiration rate measurements of free swimming plankton and microorganisms

The 6mm diameter glass microrespiration cell is an integral part of the water jacket. The volume of the cell is set by a polycarbonate plunger and is adjustable between c 0.05 and 0.18 ml. The water jacket screws into an acetal base section and is connected to a constant temperature water bath. The microcathode electrode fits into the central electrode holder, and its tip forms the floor of the microrespiration cell.

In use, the organisms are introduced into the precalibrated volume of water in the cell and the plunger is inserted, displacing air upwards through the fine bore in its centre. The swimming movements of the organisms mix the water and provide a homogeneous distribution of oxygen If required, experimental solutions can be injected into the chamber through the bore in the plunger.

Specifications

Cell diameter	:6mm
Cell volume	0.05-0.18ml
Overall height	.125mm
Jacket and cell	:glass
Plunger	:clear acrylic
Base section	·black acetal



Features

- Respiration measurements under natural conditions; no need to shake or stir the organisms.
- Small sample size.
- Exceptional visibility of the organisms in the cell.

RC 301 RESPIRATION CELL

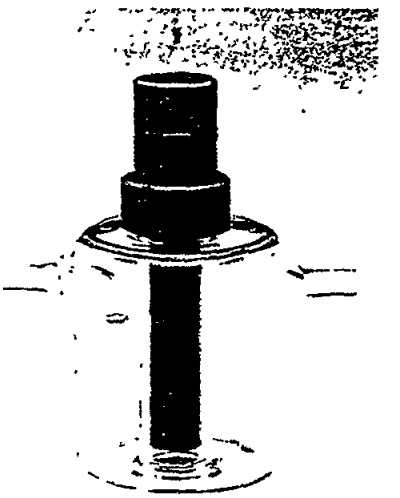
For respiration rate measurements of small free moving aquatic animals; or for oxygen consumption of microbial suspensions, mitochondria or enzyme preparations.

The microcathode electrode is located within a holder which slides into a 13mm precision bore glass tube fused into the glass water jacket. The volume of the cell can be varied between 0.3 and 1.0ml by rotating the electrode holder through the threaded collar which sits on the top surface of the water jacket. A fine slot in the side of the holder allows air to escape when the holder is inserted into the cell, and may also be used to introduce other solutions during the course of a respiration run.

When used to measure whole animal respiration rate, it is important that the animals are free swimming, in order to give a homogenous distribution of oxygen. For determination of oxygen depletion by preparations of mitochondria, microorganisms etc., it will be necessary to use a magnetic spinbar.

Specifications

Cell diameter	: 13mm
Cell volume	: 0.3-1 0ml
Cell	. glass
Electrode holder	· black acetal



Features

- Small sample volume.
- Good visibility of cell contents.
- May be used stirred or unstirred.

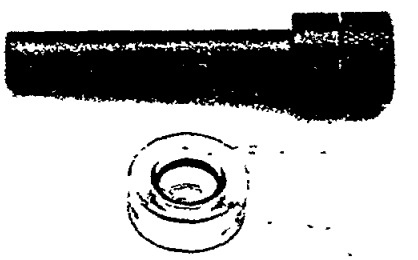
EH 100 ELECTRODE HOLDER

To enclose and protect the 1302 electrode.

The electrode holder is a black acetal tube in which the electrode is located so that its tip projects from the end. The holder gives protection to the electrode in many applications and also facilitates the insertion of the electrode into custom-built respirometer chambers. A perspex collar containing an O ring on its inside surface is also provided. This is cemented in place over a 13mm hole in the wall of the respiration chamber. The electrode holder can then be inserted through the collar, so that the O ring makes a watertight seal.

Specifications

Electrode holder:		Perspex collar:	
Length	85mm	Length	: 10mm
Diameter	. 13mm	Diameter	. 26mm
Material	: black acetal		



Features

- Allows easy insertion of electrode into custom-built respirometer chambers.
- Protects electrodes when in use.

RC 400 RESPIRATION CHAMBER

For respiration rate measurements of larger animals.

Originally developed to meet the requirements of a research project on the respiration rate of mussels when exposed to pollutants, the chamber has a screwed lid which seals against an O ring. The lid has a central hole for the electrode holder provided and two tapered holes for the insertion of sealing bungs. There is a perforated false floor to separate the animal from the magnetic follower which is slowly rotated to mix the oxygen in the water. In use, the chamber would be placed in a constant temperature water bath, with a magnetic stirrer beneath it.

Chambers of differing size can be produced to special order.

Specifications

Chamber diameter	: 102mm
Chamber volume	: c 730ml
Chamber	· clear acrylic
Electrode holder	: black acetal

Features

- Respiration rate measurements without requirement for vigorous stirring of the water.
- Minimal disturbance to animals during measurement.
- Custom-built chambers to order.

PUBLICATIONS OF THE MAP TECHNICAL REPORTS SERIES

1. UNEP/IOC/WMO: Baseline studies and monitoring of oil and petroleum hydrocarbons in marine waters (MED POL I). MAP Technical Reports Series No. 1. UNEP, Athens, 1986 (96 pages) (parts in English, French or Spanish only).

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