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Molecular, Cellular and Physiological Effects of Oil-Derived Hydrocarbons on Molluscs and Their Use in Impact Assessment [and Discussion]

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Molecular, cellular and physiological effects of oil-derived hydrocarbons on molluscs and their use in impact assessment

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The impact of pollutants on an organism is realized as perturbations at different levels of functional complexity. This presentation considers responses to oil-derived hydrocarbons at the molecular, subcellular, cellular and whole animal levels of organization, with particular emphasis on the use of marine molluscs as sentinel organisms for assessing pollutant effects.

A number of biological effects measurements are described which have been used in the development of early-warning systems based upon reactions to hydrocarbon-induced damage. These include those of the microsomal cytochrome P-450 dependent monooxygenase system involved in metabolism of organic xenobiotics, functional and structural responses of lysosomes to hydrocarbons, quantitative structural alterations in the cells of the digestive and reproductive systems and effects on physiological scope for growth and parallel correlations of this latter parameter with ecological parameters such as species diversity. Aspects of recovery processes are also considered.

Examples of both laboratory and field studies are cited to illustrate both the application of these approaches and the functional integration of the responses at the various levels of biological organization. This ability to link the various parameters in a functional manner is believed to strengthen the rationale for their use in impact assessment.

Introduction

The measurement of the impact of pollutants on marine organisms can be considered at different levels of functional complexity, namely, from the molecular level to the levels of the individual or population (Bayne *et al.* 1985). This presentation is concerned with the determination of the biological effects of petroleum-derived hydrocarbons in marine molluscs at the molecular, sub-cellular, cellular, tissue and individual physiological response levels, and their use in impact assessment.

The biological and toxicological bases of such responses have been described in considerable detail in several recent reviews and will not be reiterated in this presentation (Livingstone 1985; Moore 1985; Widdows 1985; Moore *et al.* 1987). Rather, we intend to focus on the use of such responses as indices of effect in relation to the environmental impact of petroleum hydrocarbons by using examples related to the North Sea where possible. The measurements of alterations in functional responses of organisms should form an important component of any environmental assessment programme; these range from relatively specific responses at the molecular and cellular levels, to more general (non-specific) responses to the sum of the environmental stimuli at the level of whole-animal physiological status (Livingstone 1985; Moore 1985; Widdows 1985). In this respect, measurements at the various levels of organization

are considered to be complementary, and where possible they should have ecological significance, by implication, in terms of an adverse effect on growth, reproduction or survival of the individual and the population.

The detailed rationale for this approach has been outlined elsewhere on numerous occasions and does not require repetition (Bayne *et al.* 1979, 1982, 1985; Livingstone 1985; Moore 1985; Widdows 1985; Moore *et al.* 1987). Instead, we will outline a number of examples taken from laboratory experiments, experimental mesocosms and field situations, which will serve to exemplify the use of certain marine molluscs as 'indicator species' or 'sentinel organisms' in environmental monitoring. The fundamental concepts involved and many of the practical procedures are probably applicable to a wide range of species.

Molecular responses of the microsomal cytochrome P-450 monooxygenase system

Many contaminant organic compounds including petroleum hydrocarbons enter the marine environment and are dispersed by a variety of processes including uptake into the tissues of organisms (see review by Stegeman (1981)). Such compounds are often lipid soluble and toxic, and consequently mechanisms have evolved for their detoxication or elimination or both from the animal. This is facilitated by a number of apparently universally distributed enzyme systems that function to convert non-polar (lipid-soluble) compounds to more water-soluble and, hence, readily excretable metabolites. The metabolism of compounds such as polycyclic aromatic hydrocarbons, for example, has been classified into biotransformation (phase I) and conjugation (phase II) reactions. Phase I involves oxidation by various monooxygenase reactions including epoxidation, hydroxylation and dealkylation and is catalysed by the cytochrome P-450 monooxygenase or mixed function oxidase (MFO) system; the resulting products may then be converted to dihydrodiols or conjugated with glutathione and certain other small molecules or both (Stegeman 1981).

Paradoxically, during the course of these metabolic transformations, reactive electrophilic intermediates may be formed which are more toxic, mutagenic or carcinogenic than the parent compounds and the phase I and phase II systems must be viewed as part of a detoxication–toxication system, the protective value of which rests on the balance of the enzymes present and the reactive chemistry of the metabolites produced (Stegeman 1981; Bresnick 1982). An important feature of the system is that the activities of the enzymes and the concentration of cytochrome P-450, for example, may be increased by exposure of the animal to certain organic chemicals. This phenomenon is termed induction and it has been proposed to use this stimulation of components of the detoxication–toxication system as a possible method of monitoring the biological impact of organic chemical contaminants, particularly polycyclic aromatic hydrocarbons, in marine organisms (Payne 1977; Moore 1979; Stegeman 1981; Livingstone 1985).

There have been relatively few studies of the responses of the detoxication–toxication system of molluscs to organic chemical pollution but these encompass several species of mollusc and a number of contaminants, some of them oil-derived (see recent reviews by Livingstone (1985); Moore *et al.* (1987)). Increases in the content of cytochromes P-450 and b_5 in digestive gland microsomes (fragments of endoplasmic reticulum) of *Mytilus galloprovincialis* have been indicated in response to laboratory exposure to paraffins, anthracene, perylene and 3-methyl-

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cholanthrene, and in field exposures to hydrocarbons (Gilewicz *et al.* 1984). Enhanced aryl hydrocarbon hydroxylase activity has been reported in the digestive gland of oysters (*Crassostrea virginica*) in response to benzo(*a*)pyrene, 3-methylcholanthrene and PCBs (Anderson 1978), and in the digestive gland of mussels and other bivalves exposed to PCBs, polybrominated biphenyls and petroleum hydrocarbons (Payne *et al.* 1983).

The only investigation in which the responses both of the components (cytochrome P-450 and b_5 and cytochrome P-450 reductase) of the mixed function oxygenase (MFO) system and of the MFO activity (benzo(*a*)pyrene hydroxylase (BPH)) have been examined involved the exposure of mussels for four months to 30 p.p.b.† by volume diesel oil under conditions closely resembling the field situation (outdoor flow-through tanks, natural sea water and food, wave and tidal simulation) (Livingstone *et al.* 1985). The results are summarized in table 1 and show a doubling in the concentrations of cytochromes P-450 and b_5 and increased activities of NADPH cytochrome *c* reductase (NADPH-CYTCRED) and NADPH-neotetrazolium reductase (NTR). Cytochemically measured NTR activity and biochemically measured NADPH-CYTCRED activity are generally thought to measure some aspect of the *in situ* catalytic activity of NADPH-cytochrome P-450 reductase (Masters & Okita 1982). Although these findings are encouraging in the search for relatively specific effects indices, considerable work is still required before a generally acceptable diagnostic test for the effects of petroleum-derived chemical inducers is obtained from the responses of the molluscan detoxication–toxication system, as well as an understanding of the biological consequences of induction (Livingstone 1985).

The activity of NTR has been used extensively as a cytochemical indicator of induction by toxic organic chemicals in mammals (Smith & Wills 1981). Cytochemically determined activity of this enzyme in molluscan blood cells and digestive cells has been used as a relatively specific index of response to certain organic chemical contaminants, and can be measured in sections of supercooled tissues by microdensitometry (Chayen 1978; Moore 1979). It has been shown to be stimulated experimentally by a number of polycyclic aromatic hydrocarbons (PAHs), and by contamination in the immediate vicinity of a major oil terminal at Sullom Voe (Shetland Islands, U.K.) (Moore 1979; Moore *et al.* 1982, 1984, 1986; Widdows *et al.* 1984). These latter findings are illustrated in table 2.

A recent field investigation involving both biochemical and cytochemical approaches was done after the *Sivand* oil spill in the Humber estuary, U.K., in 1983. Cockles (*Cerastoderma edule*) were used in this study and the sample sites are illustrated in figure 1 and the methodology is described in Appendix 1.

The biochemical results are presented in table 3. Biochemical similarities were obvious between Horseshoe Point and Humberstone Fitties (the two sites on the southern coast farthest from the oil spill) and between Cleethorpes and Grimsby (the two sites nearest the oil spill and also located in industrialized areas). For statistical analysis the data were, therefore, pooled for each pair of sites and considered to be representative of a clean site (Horseshoe Point–Fitties) and a contaminated site (Cleethorpes–Grimsby). The data for Cleethorpes–Grimsby and for Spurn Bight (the previously pristine but oil-impacted site as a consequence of the spill) were compared with those of Horsehoe Point–Fitties by one-way analysis of variance. Microsomal protein yield was reduced at the contaminated sites ($p < 0.05$) and possibly also at Spurn Bight. Cytochrome P-450 was detected only at the contaminated sites but the '416' peak of

† In this paper one billion is used to represent 10^9.

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TABLE 1. RESPONSES OF THE DIGESTIVE GLAND MICROSOMAL MFO SYSTEM OF (*MYTILUS EDULIS*) EXPOSED TO *CA.* 30 μg l^{-1} DIESEL-OIL HYDROCARBONS FOR FOUR MONTHS (MEAN ± STANDARD ERROR OF MEANS WITH NUMBER OF SAMPLES IN BRACKETS: EACH SAMPLE IS THE POOLED TISSUE OF EIGHT MUSSELS)

microsomal parameter	control	exposed
total protein†	5.21 ± 0.60 (6)	5.21 ± 0.29 (7)
NADH-FERRIRED‡	742 ± 33 (6)	772 ± 18 (7)
NADH-CYTCRED‡	95.4 ± 8.1 (4)	116.4 ± 4.3** (7)
NADPH-CYTCRED‡	10.3 ± 1.2 (4)	13.5 ± 0.9** (5)
P-450§	46.6 ± 14.3 (5)	92.0 ± 10.9** (7)
b_5§	25.8 ± 2.3 (5)	40.2 ± 5.8* (5)
BPH¶	54.1 ± 21.7 (6)	56.4 ± 3.8 (5)
NADPH-NTR‖	11.1 ± 1.0 (5)	15.3 ± 0.6** (5)

NADH-FERRIRED, NADH-ferricyanide reductase activity; NADPH-CYTCRED, NADPH-cytochrome *c* reductase activity; BPH, benzo(*a*)pyrene hydroxylase activity; NADPH-NTR-neotetrazolium reductase.

* $p \leqslant 0.1$.

** $p \leqslant 0.05$ (one-way analysis of variance).

† Milligrams per gram wet mass.

‡ Nanomoles per minute per milligram protein.

§ Picomoles per gram protein.

¶ Arbitrary fluorescence units per unit time.

‖ Relative units of absorbance.

Data from Livingstone *et al.* (1985). Mussels (4–5 cm length) were exposed in outdoor flow-through tanks at Solbergstrand Experimental Station, Norway.

the cytochrome P-450 spectra (there is evidence to suggest this is cytochrome P-420, the denatured form of P-450 (Livingstone *et al.* 1985)) was greatly elevated at Spurn Bight, i.e. sevenfold and fourfold greater than at Horseshoe Point–Fitties and Grimsby–Cleethorpes respectively. Cytochrome b_5 content and NADH-FERRIRED ($p < 0.1$) and NADH-CYTCRED ($p \leqslant 0.05$) activities were higher at Spurn Bight than at the two pooled groups, whereas NADPH-CYTCRED activity was elevated both at the contaminated sites ($p \leqslant 0.05$) and at Spurn Bight ($p \leqslant 0.1$). No differences were seen in BPH activities although only one measurement was available for Spurn Bight. The whole tissue PAH concentrations were markedly higher at both the contaminated sites and at Spurn Bight than those at Horseshoe Point (table 4, Oct. 1983). Elevated polycyclic aromatic hydrocarbon (PAH) concentrations, however, were also present at Humberside Fitties (table 4). The biochemical differences observed between the sites are consistent with results obtained so far for other gastropod and bivalve molluscs exposed to hydrocarbons, i.e. digestive gland microsomal cytochrome P-450 and b_5 content and NADPH-cytochrome *c* reductase activity increased in *Mytilus edulis* and *Littorina littorea* exposed to diesel-oil (Livingstone & Farrar 1985; Livingstone *et al.* 1985) and NADPH-cytochrome *c* reductase activity increased in the dog-whelk *Thais haemastoma* exposed to a water-soluble fraction of Louisiana crude-oil (Livingstone *et al.* 1986). The increased levels

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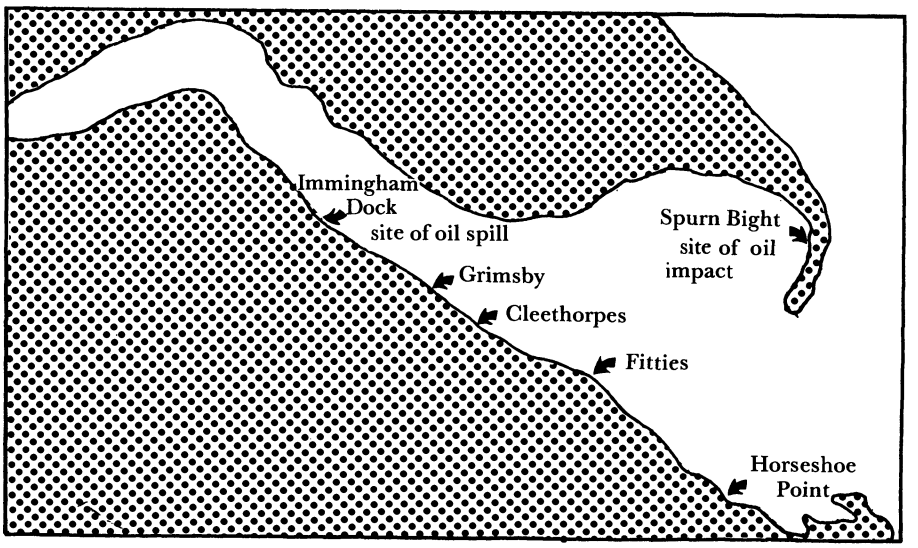

FIGURE 1. Humberside region showing location of populations of *Cerastoderma edule* at Grimsby, Cleethorpes, Humberstone Fitties, Spurn Bight (all sites of oil contamination), Horseshoe Point (uncontaminated reference site), and the site of the *Sivand* oil spill at Immingham Dock (October 1983).

TABLE 2. RESPONSES OF NADPH-NEOTETRAZOLIUM REDUCTASE (NTR) IN THE DIGESTIVE CELLS OF THE PERIWINKLE *LITTORINA LITTOREA* FROM THE VICINITY OF THE SULLOM VOE OIL TERMINAL

(Adapted from Widdows *et al.* 1984.)

sample site (July 1984)	NTR activity†
Gluss Voe (clean reference)	9.1 ± 5.9
Mavis Grind (contaminated reference)	18.3 ± 6.8†
Scatsta Voe	14.2 ± 3.6
Tanker Jetty 4 (contaminated reference)	30.7 ± 5.5‡
Ronas Voe	17.3 ± 7.5

† Relative absorbance ± standard deviation.
‡ $p \leqslant 0.05$, (Mann–Whitney *U*-test, comparing with Gluss Voe reference site) ($n = 5$).
§ $p \leqslant 0.01$.

of the '416' peak in Spurn Bight cockles are interesting and may be due to elevated concentrations of cytochrome P-450 that has subsequently been denatured to cytochrome P-420, either *in vivo* or *in vitro* during homogenization, etc., possibly as a result of the action of hydrolases released from lysosomes destabilized by hydrocarbons. The correlation of elevated P-450 and P-450 reductase with tissue PAH body-burden was only partly due to the high PAH concentrations at Fitties. However, this site was nearer to the oil spillage than Horseshoe Point (figure 1) and it may be that some impact was made at the site but it was insufficient, or too early, to have affected the MFO system.

The activity of NTR was determined cytochemically in the digestive cells of cockle digestive glands. Sites were not pooled like the biochemical data. The results are presented in table 5. There were significant increases in activity at the Cleethorpes and Grimsby sites compared with

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TABLE 3. DIGESTIVE GLAND MICROSOMAL MFO COMPONENTS AND ENZYME ACTIVITIES IN POPULATIONS OF THE COMMON COCKLE, *CERASTODERMA EDULE* L., FROM THE HUMBER ESTUARY, SAMPLED WITHIN 8 DAYS OF THE *SIVAND* OIL SPILL

microsomal parameter	Horseshoe Point	Humberstone Fitties	Cleethorpes	Grimsby	Spurn Bight
microsomal protein†	2.00±0.48 (2)	3.37±0.54 (3)	1.47±0.13 (3)	1.80±0.14 (3)	1.87±0.26 (3)
P-450‡	n.d. (1)	n.d. (2)	82.4±2.4 (2)	39.0±18.7 (2)	n.d. (2)
'416' peak§	25 (1)	24±5 (2)	61±12 (2)	36±3 (2)	178±76 (2)
b_5‡	79.7 (1)	92.8±17.1 (2)	92.5 (1)	93.7±2.1 (2)	147±19 (2)
NADH-FERRIRED¶	712±131 (2)	746±36 (3)	518±20 (3)	669±103 (3)	949±86 (3)
NADH-CYTCRED¶	109±8 (2)	98.3±9.9 (3)	90.2±7.0 (3)	110±5.6 (3)	142±16.3 (3)
NADPH-CYTCRED¶	6.74±2.16 (2)	5.26±1.29 (3)	10.54±1.19 (3)	9.13±1.66 (3)	10.56±2.14 (3)
BPH‖	9.1±6.9 (2)	5.8 (1)	8.2±2.3 (2)	10.2±0.3 (2)	6.1 (1)

Values are means±standard error of means or ±range (numbers of samples in parentheses).
† Milligrams per gram wet mass.
‡ Picomoles per milligram protein.
§ Arbitrary units mg^{-1} protein.
¶ Nanomol per minute per milligram protein.
‖ Picomoles of total metabolites per minute per milligram protein.
n.d. Not detectable.

TABLE 4. CONCENTRATION OF TWO- AND THREE- RINGED POLYCYCLIC AROMATIC HYDROCARBONS IN WHOLE TISSUES OF *CERASTODERMA EDULE* FROM THE HUMBER

site	date	concentration of two- and three-ringed aromatic hydrocarbons†
Horseshoe Point	Oct. 83	2.35±0.01
	May 84	0.48±0.03
Humberstone Fitties	Oct. 83	20.1±3.0
Cleethorpes	Oct. 83	27.7±2.0
	May 84	2.36±0.11
Grimsby	Oct. 83	35.5±1.4
Spurn Bight	Oct. 83	14.5±1.2
	May 84	0.76

† Micrograms per gram wet mass (measured in terms of 2,3-dimethylnaphthalene and 1-methylphenanthrene).

the clean reference site at Horseshoe Point. Both of these sites had highly elevated levels of two- and three-ring polycyclic aromatic hydrocarbons (table 4). These findings for NTR are broadly similar to the effects reported for NADPH-cytochrome *c* (P-450) reductase (table 3).

Samples collected seven months after the *Sivand* oil spill showed that there was still significant elevation of NTR activity at Cleethorpes. However, the data should be interpreted with caution as there is apparently a seasonal change in activity based upon the results for the clean reference site at Horseshoe Point (table 5). Both two- and three-ring PAH values were still elevated at Cleethorpes compared with the clean reference site (table 4).

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TABLE 5. NADPH-NEOTETRAZOLIUM REDUCTASE (NTR) IN HEPATOPANCREATIC DIGESTIVE CELLS IN *CERASTODERMA* FROM THE HUMBER

site	NTR activity† Oct. 1983	May 1984
Horseshoe Point	12.2±4.5	0±0
Humberstone Fitties	10.2±4.5	
Cleethorpes	26.6±5.4§	4.8±3.4‡
Grimsby	30.8±16.1‡	
Spurn Bight	20.5±14.3	16.5±15.6 ($n = 3$)

† Relative absorbance ± standard deviation.
‡ $p \leqslant 0.05$, Mann–Whitney U-test, comparing with Horseshoe Point reference ($n = 5$).
§ $p \leqslant 0.01$.

These findings are comparable with data from laboratory experiments, mesocosm studies (Solbergstrand) and Sullom Voe investigations on both mussels (*M. edulis*) and periwinkles (*L. littorea*) and are indicative of a definite effect of the oil spill on the cockles (Livingstone *et al.* 1985). Owing to the severe limitations in the data available, however, it would be imprudent to attempt to glean too much concerning the longer-term effects of the oil spill on cockle NTR activity.

FUNCTIONAL DISTURBANCES OF SUBCELLULAR ORGANIZATION: RESPONSES OF THE LYSOSOMAL SYSTEM

Subcellular and cellular pathology reflect perturbations of structure and function at the molecular level, hence, responses of lysosomes represent a logical hierarchical progression in terms of biological organization from the previous section. In many instances, the earliest detectable changes or 'primary events' are associated with a particular class of subcellular organelle (Slater 1978) such as the lysosome or endoplasmic reticulum (the microsomes of the preceding section). Investigations in mammals have revealed that much of the damaging action of environmental contaminant chemicals (xenobiotics) is produced by highly reactive metabolic derivatives. It is these activated chemical forms, generally produced by the cytochrome P-450 system, that are responsible for the initiation of what may be termed the primary intracellular disturbances. These may spread rapidly into a complex network of associated secondary and higher order disturbances which become progressively more difficult for the cell to reverse or modify. The degree to which metabolic derivatives of PAHs are responsible for toxicity, as opposed to the parent compounds, is difficult to ascertain given the current limitations of understanding of the rates and patterns of metabolic production in molluscs (see review by Moore *et al.* 1987). The numerous ways in which the structure and function of organelles and cells can be perturbed by contaminants such as PAHs have been reviewed in detail by Moore (1985) and Moore *et al.* (1987). In this presentation we are mainly concerned with injury induced by PAHs to the lysosomal system where membrane damage results in major changes in the structure and function of the lysosomal compartment (Moore 1985).

Mammalian lysosomes are noted for their responsiveness to many types of cell injury and molluscan lysosomes have also been shown to compartmentalize and accumulate a wide range of injurious agents including petroleum-derived PAH (Allison 1969; Baccino 1978; Hawkins 1980; Ericsson & Brunk 1975; Moore 1985; Moore *et al.* 1987). The cells of many molluscan

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tissues are especially rich in lysosomes (Sumner 1969; Owen 1972; Moore *et al.* 1987), particularly the digestive cells of the digestive gland.

There are a number of ways by which lysosomes can react to cellular injury; basically, these can be divided into three categories of decrease or increase in:

(1) lysosomal contents such as hydrolytic degradative enzymes and lipofuscin pigment;

(2) rate of membrane fusion events with either the cell membrane or other components of the vacuolar system, and

(3) lysosomal membrane permeability (Hawkins 1980).

For a number of reasons, however, lysosomal reactions to cell injury are not well understood owing to the many forms of cell injury and the wide variety of organisms studied. Moreover, one type of lysosomal alteration may assume the appearance of another type (Hawkins 1980; Ericsson & Brunk 1975). These three categories of lysosomal response have been discussed in detail by Moore *et al.* (1987) and consequently will only be summarized here.

Exposure of *L. littorea* to PAH has been demonstrated to result in increases in the activities of certain lysosomal enzymes, notably β-glucuronidase and acid phosphatase (Moore *et al.* 1982, 1986). A consequence of this pattern would be to prepare the cell for the degradation of particular macromolecules, hence making the products available for maintenance of the cell (Hawkins 1980; Moore *et al.* 1980). Regulation of the lysosomal system is dependent upon controlled fusion with other components of the vacuolar system such as phagosomes, primary lysosomes and the plasma membrane (Hawkins 1980). The digestive cells of molluscs are largely concerned with heterophagy and the digestion of food material (Owen 1972). Disturbances of the fusion processes involved could have marked consequences for the nutritional status of the organism by perturbing 'normal' intracellular digestion and the balance of autophagy to heterophagy (Moore 1980).

There are a number of indications that both experimental and field exposure to oil-derived PAHs induces profound alterations in the rate of fusion events in the lysosomal–vacuolar systems of molluscan digestive cells (see reviews by Moore (1985); Moore *et al.* (1987)). Ultrastructural studies show that the large secondary lysosomes (2–5 μm diameter approximately) in the digestive cells show marked increases in the presence of internalized membrane-bound vesicular components and that these secondary lysosomes become abnormally enlarged (up to 15 μm diameter) in mussels, oysters and periwinkles (Pipe & Moore 1986; Lowe *et al.* 1981; Moore *et al.* 1986; Couch 1984). Quantitative stereological analysis of these enlarged lysosomes (light microscopy) demonstrates that both lysosomal volume and surface area within the cells is significantly increased, while numerical density is decreased (Lowe *et al.* 1981). This is perhaps indicative of fusion of vacuolar components to produce these abnormally enlarged lysosomes. This type of response has also been observed to occur when mussels are exposed to an abrupt increase in salinity and has been linked to increased fusion of lysosomal vacuoles and autophagy (Pipe & Moore 1985*a*). These alterations are also associated with elevated intracellular protein catabolism and formation of amino acids as measured within the lysosomal cellular compartment (Bayne *et al.* 1981). The formation of these enlarged lysosomes has also been linked with atrophy of the digestive tubule epithelium which largely consists of digestive cells (Lowe *et al.* 1981) and this relationship will be discussed later in more detail.

The third category of lysosomal disturbance involves membrane permeability. This property can be investigated both biochemically and cytochemically. It is, however, more realistic to

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employ cytochemical procedures in molluscan digestive cells as the large secondary lysosomes do not readily lend themselves to the trauma of homogenization and subsequent fractionation (Bitensky *et al.* 1973). Cytochemical procedures are well established for a number of enzyme substrates in molluscs and these are all conceptually based on the Bitensky fragility test (see review by Bitensky *et al.* (1973)) for lysosomal stability as modified by Moore (1976, 1985). Lysosomal destabilization is measured as increased permeability to certain enzyme substrates whose products can be used to give a measurable final cytochemical reaction product (Moore 1976).

Destabilization of lysosomes has been demonstrated in molluscan digestive cells as a result of injury by 2-methyl naphthalene, 2,3-dimethyl naphthalene, anthracene, phenanthrene, diesel-oil emulsion, water accommodated fraction of crude oil (North Sea: Auk) and PAH-contaminated field samples from Shetland (table 6) (Moore 1985; Livingstone *et al.* 1985). Assessment of this type of injury has been confirmed as an extremely sensitive index of cellular condition and the destabilization of the lysosomal membrane appears to bear a quantitative relation to the magnitude of the stress response and this presumably contributes to the intensity of catabolic or degradative effects, as well as to the level of pathological change that results (Moore 1985).

TABLE 6. LYSOSOMAL STABILITY IN THE DIGESTIVE CELLS OF THE PERIWINKLE (*LITTORINA LITTOREA*) EXPOSED TO PAH IN AN EXPERIMENTAL MESOCOSM (SOLBERGSTRAND, OSLO FJORD) AND IN THE VICINITY OF THE SHETLAND OIL TERMINAL (SULLOM VOE, JULY 1982)

(Adapted from Moore *et al.* 1986*b* and Livingstone *et al.* 1985.)

experimental treatment or sample site		lysosomal membrane stability†
control		24 (20, 25)‡
ca. 30 μg l^{-1} total hydrocarbons	16 months exposure	2.6 (2, 5)§
ca. 130 μg l^{-1} total hydrocarbons	16 months exposure	2 (2, 2)§
Ronas Voe	(uncontaminated reference)	19.0 (15, 20)
Gluss Voe	(uncontaminated reference)	24.0 (20, 25)
Scatsta Voe		5.0 (5, 5)§
Tanker Jetty 4		3.8 (2, 5)§
Mavis Grind (contaminated reference)		8.0 (5, 10)§

† β-glucuronidase labilization period in minutes.
‡ Mean with data range in parentheses ($n = 5$).
§ $p \leqslant 0.01$, Mann–Whitney U-test comparing with either control data (experimental) or data for Ronas Voe (uncontaminated reference).

Further evidence of this type of effect has been obtained with cockles (*C. edule*) exposed to hydrocarbons in the Humber estuary following the *Sivand* oil spill. Contaminated cockles showed evidence of lysosomal destabilization (tables 4 and 7). Samples taken seven months later indicated that there was evidence of recovery at previously contaminated sites (tables 4 and 7); however, as all of these data represent small samples and two periods in time caution must be exercised in their interpretation.

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TABLE 7. LYSOSOMAL MEMBRANE STABILITY IN HEPATOPANCREATIC DIGESTIVE CELLS OF *CERASTODERMA EDULE* FROM THE HUMBER

site	lysosomal membrane stability Oct. 1983	May 1984
Horseshoe Point	20 (20, 25)†	24 (20, 25)
Humberstone Fitties	23 (20, 25)	
Cleethorpes	15 (15, 15)‡	23 (20, 25)
Grimsby	10 (10, 10)‡	
Spurn Bight	17 (10, 20)	23.3 (20, 25) $n = 3$

† Labilization period of latent lysosomal β-glucuronidase in minutes (range in parentheses) $n = 5$.
‡ $p \leqslant 0.01$, Mann–Whitney U-test, comparing with Horseshoe Point (uncontaminated reference).

The consequences of destabilization of the secondary lysosomal compartment have been investigated in several experimental studies. Subcellular fractions rich in destabilized secondary lysosomes have been shown to contain significantly increased concentrations of amino acids compared with stable lysosomes (Bayne *et al.* 1981). This is indicative of enhanced intralysosomal protein catabolism. Ultrastructural investigations of cells with destabilized lysosomes indicate increased secondary lysosomal volume with evidence of increased autophagy and possible heterophagy of apoptotic cell fragments, thus providing further indications of elevated catabolic activity (Pipe & Moore 1985*a*, 1986).

A quantitative cytochemical approach such as the measurement of lysosomal permeability or stability, based on substrate penetrability (hydrolase latency), may also provide insight into the mechanisms of PAH-induced cell injury in molluscs. Caution is required, however, in the interpretation of lysosomal damage as a primary event, when it may in fact be a secondary- or higher-order alteration. Recent investigations of lysosomal responses to specific PAHs have demonstrated that the lysosomal disturbances are complex and differ markedly for PAHs which are structurally dissimilar, such as the isomeric three-ring forms anthracene and phenanthrene (Moore & Farrar 1985). The biochemical evidence of relatively low activity for cytochrome(s) P-450 monooxygenase in molluscan digestive-gland cells, when considered together with the ability of these cells to accumulate and retain very high concentrations of PAH, indicates that their loss by metabolic transformation is limited (Livingstone 1985). The fact that the secondary lysosomes are often lipid-rich, particularly after exposure to PAHs (M. N. Moore, unpublished data) would tend to argue for a direct effect on the lysosomes by these xenobiotics, rather than a secondary effect. Aromatic hydrocarbons and PAHs have been shown to penetrate synthetic phospholipid membranes and alter their physical and chemical properties including membrane fluidity and permeability (Roubal & Collier 1975; Nott *et al.* 1985).

Further evidence of lysosomal destabilization comes from several ultrastructural studies of the effects of phenanthrene on secondary lysosomes in digestive cells (Pipe & Moore 1986; Nott *et al.* 1985). These have demonstrated the presence of corrugation of the bounding membrane with possible associated blebbing activity. Increased frequency of membrane breaks has also been described, and although these breaks may be artefacts of fixation and tissue processing, their relative infrequency in control lysosomes is indicative of the greater fragility of lysosomes from cells exposed to phenanthrene. Apparent leakage of lysosomal β-glucuronidase has been demonstrated in the case of lysosomes from the digestive cells of phenanthrene-exposed *L. littorea*; extracellular release of lysosomal β-glucuronidase was also observed in these cells

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(Pipe & Moore 1986). Szego (1975) described limited release of lysosomal enzymes into the cytosol and nucleoplasm after treatment of rat preputial-gland cells with 17β-oestradiol, which destabilizes the lysosomal membrane.

The consequences of such a release of lysosomal enzymes is uncertain but is believed to lead to enhanced cell damage and possibly cell death (Hawkins 1980; Ericsson & Brunk 1975). Evidence from rat preputial-gland cells indicates increased protein catabolism after oestrogen treatment, although enzyme release in these cells is non-injurious and precedes initiation of cell division (Szego 1975). This may represent a fundamental difference in the lysosomal response to physiological agonists as opposed to xenobiotics.

In summary, the evidence of both ultrastructural, quantitative cytochemical and morphometric approaches indicates that PAHs induce profound alterations in both structure and function. These involve all three categories of lysosomal response and there are some grounds for suggesting that membrane destabilization may represent the primary injury which could lead to the other events described above. Cytochemical demonstration of lysosomal membrane destabilization has proved to be a useful investigative tool both for PAH and other injurious agents. That this procedure does in fact measure membrane destabilization is further supported by evidence of reversibility and restabilization by treatment with hydrocortisone, an established membrane stabilizer (Moore 1976; Bayne *et al.* 1981).

Structural alterations in cells

Exposure of marine molluscs to single PAH and oil-derived hydrocarbon mixtures results in atrophy of the epithelium of the digestive tubules (Lowe *et al.* 1981; Couch 1984; Moore *et al.* 1987). This atrophy or epithelial 'thinning' involves structural changes in the digestive cells, the major component of the epithelium. These changes have been quantified in *M. edulis* (Lowe *et al.* 1981) by using image analysis of histological sections, and in the clam *Mercenaria mercenaria* by using morphometry (Tripp *et al.* 1984). There is evidence in both mussels and periwinkles that this may be a generalized response to toxic xenobiotics and other stressors such as starvation (Pipe & Moore 1985*a*; Moore *et al.* 1987).

Investigation of digestive cell atrophy has revealed that there is a significant increase in lysosomal volume as described in the previous section (Lowe *et al.* 1981; Moore & Clarke 1982). This increase in volume of the lysosomal compartment involved the formation of enlarged or 'giant' lysosomes and this alteration is associated with membrane destabilization and increased permeability (Moore & Clarke 1982). There is also evidence for increased lysosomal fusion events leading to the formation of the enlarged lysosomes (Pipe & Moore 1985*a*, 1986). As discussed in the previous section the consequences of these lysosomal disturbances would be increased autolytic and autophagic activity presumably leading to atrophy of the digestive cells.

Physiological investigation of mussels has shown that scope for growth is significantly reduced (and in some cases becomes negative) following exposure to PAH and oil-derived hydrocarbons (Widdows *et al.* 1982; Bayne *et al.* 1979). This situation is indicative of relatively enhanced tissue catabolism. Samples from these experiments demonstrate digestive cell atrophy and lysosomal disturbances as described above, arguing strongly for a mechanistic link from the lysosomal events through to the whole animal (Lowe *et al.* 1981; Moore & Clarke 1982; Widdows *et al.* 1982).

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Turning to consideration of effects on reproduction at the cellular level, mussels exposed to both *ca.* 30 and *ca.* 130 μg l^{-1} hydrocarbons showed a reduction in the volume of storage cells in the mantle tissue, a reduction in volume of ripe gametes and increased degeneration or atresia of oocytes (table 8) (Lowe & Pipe 1985). These data indicate a direct impairment of the reproductive processes and the implication is that reproductive capability would be reduced, both by degeneration of oocytes and reduction in ripe gametes, as well as by a reduction

TABLE 8. REPRODUCTIVE TISSUE IN THE MUSSEL: EFFECTS OF HYDROCARBON EXPOSURE AND A RECOVERY PERIOD ON TISSUE VOLUMES OF THE COMPONENT CELLS

(Adapted from Lowe & Pipe 1985.)

condition	developing gametes	ripe gametes	adipogranular cells	vesicular cells	degenerating oocytes
control	0.10±0.03†	0.77±0.27	0.11±0.03	0.53±0.07	0.05±0.01
ca. 30 p.p.b. by volume§	0.13±0.04	0.44±0.16‡	0.05±0.04†	0.38±0.07 ‡	0.35±0.13‡
ca. 130 p.p.b. by volume§	0.13±0.03	0.30±0.11‡	0.01±0.01‡	0.14±0.02‡	0.31±0.09‡
recovery 53 days	0.28±0.08‡	0.66±0.18	0.11±0.05	0.54±0.04	0.01±0.01‡

† Mean (cubic millimetres) ±standard error, $n = 10$.
‡ $p < 0.05$; one-way analysis of variance, comparing with control.
§ One billion is used to represent 10^9.

in the energy reserves available for gametogenesis as supplied by the connective tissue storage cells (Lowe *et al.* 1982). Ultrastructural investigations designed to explore the mechanisms of oocyte degeneration have revealed that degradative lysosomal enzymes are associated with yolk granules and with pinocytotic phenomena that occur along the basal membrane of developing oocytes (Pipe & Moore 1985*b*). Lysosomal enzymes are also associated with the degradation (atresia) and resorption of oocytes, as well as the resorption of adipogranular storage cells (Lowe *et al.* 1982; Pipe & Moore 1985*b*). Future experiments will test whether polycyclic aromatic hydrocarbons have a detrimental effect on oocyte lysosomes leading to enhanced lysosomal autolytic processes.

PHYSIOLOGICAL RESPONSES: EFFECTS OF OIL EXPOSURE AND RECOVERY

Production of matter (growth and reproduction) is a fundamental property of all living organisms and one that is necessary if a population is to persist in a given environment. The amount of production represents the difference between an individual's or a population's intake and output of matter or energy, and this will vary under different environmental conditions. The measurement of individual physiological responses, such as rates of feeding, digestion, respiration, excretion and growth, and their integration by means of physiological energetics, can provide insight into the overall growth process and how it might be disrupted by environmental stress and pollution; these have been extensively reviewed elsewhere in the literature (Bayne *et al.* 1985; Widdows 1985; Moore *et al.* 1987).

The ultimate effect of petroleum hydrocarbons on rates of feeding and energy metabolism is to markedly reduce the energy available for growth and reproduction, often termed 'scope

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for growth' (see review by Widdows (1985)). The effects of petroleum hydrocarbons observed in laboratory experiments are also apparent in mesocosm experiments and in the field. For example, *Mya arenaria* subjected to oil spills showed a reduction in the energy available for growth (Gilfillan *et al.* 1976, 1977) and a decline in tissue and shell growth (Gilfillan & Vandermeulen 1978; MacDonald & Thomas 1982) which persisted for up to five years after the spill owing to the continued presence of oil in the sediments.

Although there have been many studies of the effects of hydrocarbons on bivalves, until recently there has been relatively little information concerning the rate of recovery from oil exposure and the extent to which physiological recovery is related to the depuration of hydrocarbons from the body tissues. A recent study (Widdows *et al.* 1985) has shown a marked reduction in the feeding rate and scope for growth of *M. edulis* exposed to low concentrations of diesel-oil (*ca.* 30 and *ca.* 130 μg l^{-1}) for eight months (figure 2). The high-oil exposed mussels had a negative scope for growth, indicating the need to utilize body reserves in order to satisfy the animal's energy requirements. This was confirmed by the observed degrowth in body tissues which resulted in a high mortality in this group (27% over eight months, (Lowe & Pipe 1987)). During recovery from chronic oil exposure the depuration of hydrocarbons from the mussel's body tissues was concomitant with the recovery of physiological performance (i.e. feeding and growth), thus demonstrating that the effect of oil on the mussel's performance is related to the concentration of hydrocarbons within the body tissues and is not directly related to the hydrocarbon concentration in the water.

High-oil exposed mussels were found to recover more rapidly than the low-oil exposed mussels, both in terms of hydrocarbon depuration and scope for growth (figure 2) (Widdows *et al.* 1985). This led to an 'overshoot' in the feeding rate and consequently the scope for growth by high-oil exposed mussels and thus accounted for the observed 'catch up' growth in body tissues during the two months of recovery from high-oil exposure (*ca.* 130 μg l^{-1}). The rate of recovery by low-oil exposed mussels was slower both in terms of tissue depuration and physiological performance, but both groups showed complete recovery after 55 days (i.e. their growth rates were not significantly different from the control).

Several field and laboratory studies have demonstrated a significant negative correlation between scope for growth and the concentration of specifically aromatic hydrocarbons in the tissues of molluscs (see review by Widdows (1985)). Figure 3 provides a synthesis of data derived from a mesocosm experiment and a field study of mussel populations in the vicinity of the Sullom Voe oil terminal and illustrates the significant negative correlation ($r^2 = -0.95$) between scope for growth and log of the concentration of two- and three-ringed aromatic hydrocarbons in the body tissues of *M. edulis* (this simply reflects the nature of the analytical procedure and the dominant component of the accumulated aromatic hydrocarbons rather than identifying these aromatic hydrocarbons as the sole toxic components). Such a relation demonstrates that hydrocarbons affect scope for growth over a wide range of tissue concentrations without an apparent threshold concentration of effect. Moreover, it illustrates the degree of contamination in the 'control' mussels in the mesocosm experiment conducted at Solbergstrand on the Oslo fjord (Norway) relative to the Shetland Islands (U.K.).

It is generally very difficult to make an inter-study comparison of the relation between biological effects measurements and tissue hydrocarbon concentrations because of the lack of compatibility, particularly in the chemical data. This is primarily because of the different extraction, analytical and quantification procedures adopted by different laboratories, which

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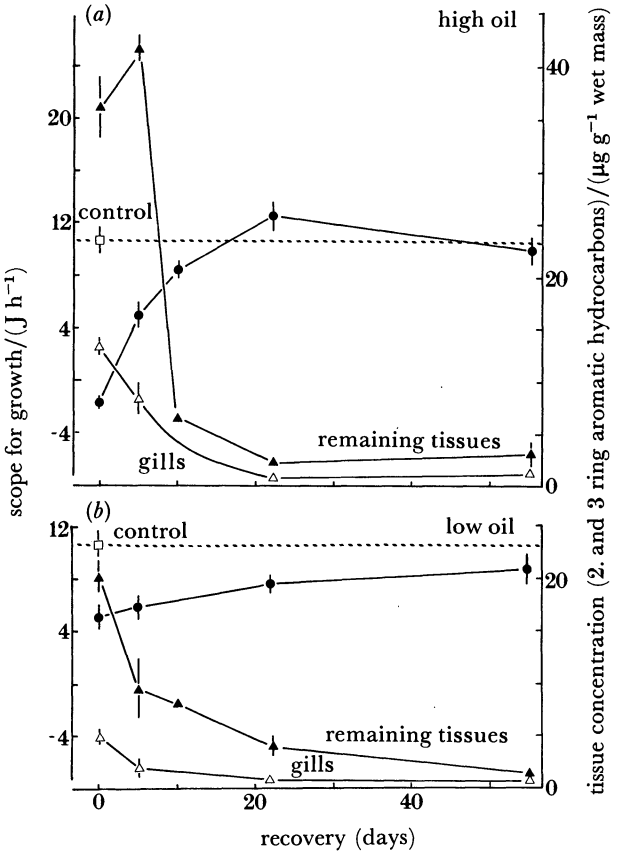

FIGURE 2. Scope for growth (● and □) (J h^{-1}; mean ± standard error) of *Mytilus edulis* (standard animal of 0.5 g dry mass) during recovery from eight months of exposure to *ca.* 130 μg l^{-1} (high-oil exposure) and *ca.* 30 μg l^{-1} (low-oil exposure). Short-term recovery (0, 5 and 10 days; May 1983) and long-term recovery (0, 22 and 55 days; May 1984) are expressed relative to control mussels (no significant differences between controls in 1983 and 1984). Depuration of two- and three-ringed aromatic hydrocarbons (μg g^{-1} wet mass) from gills (▲) and remaining tissues (△) (mean ± range of two pooled samples). Data from Widdows *et al.* 1985.

are usually semi-quantitative emphasizing either the lower or higher molecular mass fractions of aliphatic or aromatic hydrocarbons. A broad synthesis of data such as that achieved in figure 3, where there is consistency in both biological and chemical methodology, will not be possible until further standardization and intercalibration of procedures for the quantification of toxic hydrocarbons of low and high molecular mass has been agreed. However, to present the relation between scope for growth and the concentration of aromatic hydrocarbons in the tissues of mussels (figure 3) in the context of the degree of oil contamination in the North Sea we have inserted predicted tissue concentrations of two- and three-ring hydrocarbons based upon data for hydrocarbon concentrations in water (Massie *et al.* 1985). The conclusions drawn from these water and predicted tissue concentrations are that there is likely to be a very slight and probably measurable effect on the performance of mussels and other marine biota living in the water column in the vicinity of the North Sea oil platforms but that this degree of biological effect is not considered to result in a marked impact on the overall performance and survival of the mussel.

Reproductive processes provide the critical interface between the individual and the

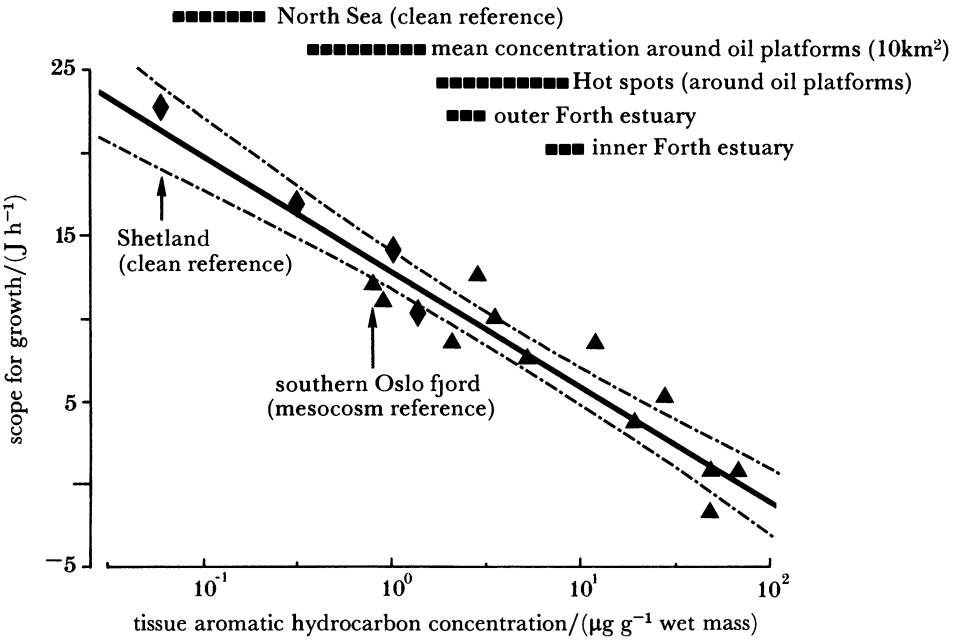

FIGURE 3. Relation between scope for growth (J h^{-1}; based upon a standard animal of 0.5 g dry mass, particulate organic matter of 0.5 mg l^{-1}, season of May–July) and the concentration of two- and three-ringed aromatic hydrocarbons (μg g^{-1} wet mass) in the body tissues of *Mytilus edulis*. (▲) Data from oil exposure and recovery experiments at Solbergstrand, Oslofjord, Norway (Widdows *et al.* 1985). (◆) Data from Sullom Voe, Shetland, U.K. (Widdows 1984). Horizontal bars represent an estimate of the tissue concentration based upon the observed concentration of hydrocarbons in the water at various sites in the North Sea (Massie *et al.* 1985).

population. Results of laboratory studies have indicated that stress, measured in terms such as scope for growth, has a significant effect on fecundity, egg size and subsequent growth rate of the larvae of *M. edulis* (Bayne *et al.* 1982). The reduction in energy available for growth and reproduction in response to hydrocarbon exposure (*ca.* 30 and *ca.* 130 μg l^{-1}) has been shown to result in a significant and concentration-dependent decline in both the mass of storage tissue in *M. edulis* and the mass of gametes produced (fecundity) (Lowe & Pipe 1985; Lowe & Pipe 1987). *Macoma balthica* exposed to 30 μg petroleum hydrocarbons per litre also showed gamete resorption and abnormal gamete development at 300 μg l^{-1} (Stekoll *et al.* 1980).

Therefore a decline in the overall energy available for production as a response to petroleum hydrocarbons results in a reduction in the energy allocated to reproduction (reproductive effort), which, when combined with an increased mortality, ultimately leads to a reduction in the residual reproductive value (Bayne *et al.* 1982) or the long-term prospects of producing successful offspring in future years.

POPULATION AND COMMUNITY EFFECTS

The damaging effects of petroleum hydrocarbons on cellular and physiological responses, which are ultimately reflected in the growth, reproduction and survival of the individual, suggests a major reduction in population fitness and the ability to maintain the population or colonize new habitats, as well as increased susceptibility to predation, parasitism and disease. Such population effects have been observed in both field (*Mya arenaria*: Gilfillan *et al.* (1977);

Gilfillan & Vandermeulen (1978)) and mesocosm studies (*Mytilus edulis*: Widdows *et al.* (1985); Bakke & Sorensen (1985)) in the form of reduced recruitment, increased mortality and reduced production.

The effects of oil on populations and communities have been studied in two major benthic mesocosm experiments (Grassle *et al.* 1981; Bakke & Sorensen 1987) with the aim of bridging the gap between laboratory based short-term toxicity experiments and field observations after oil pollution, and examining the effects of oil on trophic interactions in the ecosystem. The findings from these experiments have been reviewed by Moore *et al.* (1987). The results of mesocosm studies have largely confirmed the conclusions of earlier studies that bivalves, including mussels, are sensitive to petroleum hydrocarbons when chronically exposed.

The ecological consequences of changes in populations will be a shift in species composition and diversity within the community. The effects of petroleum hydrocarbons on community structure have been demonstrated in many field studies, e.g. effects of an oil spill (Sanders *et al.* 1980) industrial petroleum waste (McLusky 1982) and offshore oil platforms (Davies *et al.* 1984).

The community response to oil pollution is a classical successional response to a point-source of organic pollution (Pearson & Rosenberg 1978). There is a gradient of increasing species diversity, beginning with an abiotic zone in the immediate vicinity of a point-source of heavy contamination, followed by a zone characterized by a low number of species, low abundance and low biomass, and then a zone showing a progressive increase in the number of species and a dramatic increase in abundance and biomass of a few dominant species. Finally, with increasing distance from a discharge the community becomes more diverse but abundance and biomass declines.

In relation to North Sea oil activities there is significant offshore contamination of sediments close to platforms using oil-based drilling muds (Davies *et al.* 1981). A review of the environmental effects of these oil-contaminated sediments around oil platforms (Davies *et al.* 1984) has concluded that, beyond the immediate area of physical smothering by oil-based mud cuttings, the major deleterious effects on the benthic community (e.g. low species diversity and high numbers of opportunistic species such as *Capitella capitata*) occurred within 500 m of the platforms and beyond which there was a transition zone, generally within 400–1000 m, where 'subtle' biological effects (e.g. slight changes in species diversity and community structure) could be detected. Species diversity declined progressively with a logarithmic increase in oil concentration in the sediments and there was no evidence of a theshold of effect. Where there were quantitative gas chromatography–mass spectrometry data for two oil-fields (Beryl and Thistle), a linear relation between species diversity and log of naphthalene concentration in the sediment was also recorded. It is interesting to note that where hydrocarbon concentrations (regardless of units) span several orders of magnitude above background levels there is a parallel relation and good agreement between biological effects such as scope for growth, measured at the individual level of organization using an indicator species, and changes in species diversity at the community level (see review by Moore *et al.* 1987). This supports the notion that adverse effects measured at the cellular and individual levels ultimately manifest themselves at the population and community levels of organization. Cellular and physiological responses therefore serve to complement ecological surveys of community structure, not only by providing an early detection of effects, often before statistically significant changes occur at the community level, but also by providing some insight into the toxicological causes of changes in population and community structure. A particularly important attribute of sublethal cellular and

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physiological responses is that they are amenable to both laboratory and field measurement, unlike traditional toxicity testing based upon LC50 and ecological surveys based upon community structure. On the one hand they can be used to derive 'concentration–response' relations to help identify and predict the effects of potential pollutant levels; on the other hand they can be used to monitor the effects in the environment.

Conclusions

The application of an integrated approach in the use of indices of effect for the assessment of sublethal effects of petroleum-derived hydrocarbons in molluscs has been described. This rationale has been productive in enabling us to develop conceptual links between responses at various levels of biological organization, certainly from the molecular level to that of the individual. Study of the responses to oil-derived hydrocarbons has extended our understanding of certain aspects of physiological processes and has also highlighted the capacity for PAHs to disturb biochemical, cellular and physiological processes.

Although there is promising evidence of oil-induced effects on the cytochrome P-450 monooxygenase system, our understanding of its role in molluscs is still somewhat limited and considerable research will be required to characterize the enzymic components and patterns of hydrocarbon metabolism before we can assess the significance of this system in toxicity and detoxication. We have, perhaps, a better understanding of lysosomal function in molluscs and the consequences of disturbance by PAHs. Here, we see a logical progression from lysosomal dysfunction leading to enhanced catabolism resulting in epithelial atrophy manifesting in turn as a reduction in 'scope for growth' and perhaps a reduction in viable oocytes.

There is also a considerable requirement for a better understanding of the physiological and molecular processes involved in the uptake, retention, compartmentation and loss of oil-derived PAHs in molluscan tissues. Such an understanding should provide a toxicological interpretation of the chemical data for tissue residues.

Prediction of ecological consequences has been discussed, with some attempt at synthesis, and this is probably one of the most difficult problems facing those involved in biological effects measurements. This does not, however, invalidate the use of indices of biological effect as an 'early warning system' for detection of environmental deterioration. A further complicating factor arises from the fact that contaminant PAHs seldom occur in environmental isolation and this therefore poses the question of interactive biological effects resulting from multiple xenobiotic challenge (Malins & Collier 1981; Moore *et al.* 1984). Indices of biological effects must also be capable of resolving such a situation, particularly those indices that are regarded as being relatively specific for particular types of xenobiotic. Although subtle interactive effects have been observed in experimental studies there is no evidence as yet that they are obscuring measurement of environmental impact (Moore *et al.* 1984).

Appendix 1

Materials and methods relating to Sivand oil spill

Cockles, *Cerastoderma edule* L. (3–4 cm length) were collected within 6–8 days of the *Sivand* oil-spill in Immingham Dock (October 1983) from the sites shown in figure 1. Digestive glands (hepatopancreas) were dissected out, damp-dried, frozen in liquid nitrogen and stored at

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−70 °C before biochemical analysis. The pooled tissues of about six cockles per sample were used and, because of the limited number of cockles available, three or less samples only were taken per site. Microsomes were prepared and assayed for cytochrome P-450 and b_5 content, NADPH- and NADH-cytochrome *c* (NAD(P)H-CYTCRED) and NADH-ferricyanide (NADH-FERRIRED) reductase activities and benzo(*a*)pyrene hydroxylase (BPH) activity as described by Livingstone & Farrar (1984); microsomal protein was measured by the method of Lowry *et al.* (1951). Whole tissues of cockles were also taken for hydrocarbon analysis and two pools of five animals each were used per site. The shells of the cockles were cleaned with acetone, the tissues removed, damp-dried and similarly stored at −70 °C before chemical analysis. PAHs were extracted by steam distillation, analysed by high performance liquid chromatography and quantified in terms of two-ring (2,3-dimethyl-naphthalene equivalents) and three-ring (1-methylphenanthrene equivalents) aromatic hydrocarbon compounds as described by Donkin & Evans (1984).

For cytochemical determinations of NADPH-neotetrazolium reductase (NTR) and lysosomal stability (based on the latency of β-glucuronidase), digestive glands ($n = 5$) were excised and processed as described by Moore *et al.* (1982) and Lowe & Moore (1985).

A further sample of cockles was taken seven months after the spill occurred (May 1984) from Horseshoe Point (reference site), Cleethorpes and Spurn Bight. These were also processed for NTR, lysosomal stability and analysis of PAHs (as above).

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Discussion

R. E. Jones (*School of Ocean Sciences, University College of North Wales, Menai Bridge, U.K.*). The sites deemed to be affected by oil in the Humber estuary are situated near a very large discharge of acid waste (rich in metals) from a titanium dioxide processing installation; could such a discharge account at least in part for the observed changes? In addition, metals such as copper are associated with oil installations and may exert toxic effects at concentrations as low as 10 μg l^{-1} on *Mytilus edulis*. Can such effects be separated from those induced by oil?

M. N. Moore. The biological effects observed in cockles after the *Sivand* oil spill are essentially what we would have predicted based upon evidence from laboratory and field investigations in mussels and periwinkles. Although the lysosomal destabilization is a generalized stress response, and can be induced by metals such as copper, the stimulation of the components of the cytochrome P-450 monooxygenase system would be expected from exposure to oil-derived polycyclic aromatic hydrocarbons and not from metals. Moreover, there is evidence of recovery of lysosomal stability after depuration of hydrocarbons at the Cleethorpes site.

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