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Measurement of the Responses of Individuals to Environmental Stress and Pollution: Studies with Bivalve Molluscs [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1979 **286**, 563-581
doi: 10.1098/rstb.1979.0046

Phil. Trans. R. Soc. Lond. B. **286**, 563–581 (1979)
Printed in Great Britain

Measurement of the responses of individuals to environmental stress and pollution: studies with bivalve molluscs

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[Plate 1]

Certain physiological differences between individuals in different populations of the mussel, *Mytilus edulis*, are described. In particular, the scope for growth differs in space and time and may be used to assess the animals' physiological condition. When the required measurements are made in the field, the rates of growth predicted from the physiological data agree well with observed rates of growth. An alternative approach utilizes mussels transplanted to various waters, with indices of condition then measured in the laboratory under standard conditions; an example of this approach is illustrated. Laboratory experiments are used to equate various levels of physiological condition with fecundity, in an attempt to equate physiological effects on the individual with likely population damage. A cytochemical index of stress is described, based on the latency of lysosomal enzymes; spatial variability in this index, and its relation with the scope for growth, are discussed. Finally, the results of some experiments on the effects of petroleum hydrocarbons on mussels are described and the presence of inducible activity of NADPH-dependent tetrazolium reductase in the blood cells is demonstrated. Certain considerations that apply in adopting similar measurements of biological effects of pollution in environmental monitoring programmes are discussed.

Introduction

Although the vitality of populations may be of ultimate concern when assessing the effects of pollution, it is on the survival, growth and reproduction of individuals in a population that this vitality depends. In seeking criteria with which to measure the effects of environmental change, physiological responses should provide both an integration of the biochemical and cytological effects and an indication of the likely consequences of environmental change to a population. However, it is unlikely that analyses of physiological properties (behaviour, growth, reproduction) will provide information on the effects of specific environmental stressors, since changes in the biochemical targets of particular chemical contaminants, for example, will be integrated into more generalized physiological changes as part of a response syndrome (Jeffries 1972; Bayne 1975*a*).

In detecting and analysing the effects of pollution on organisms, therefore, the aim is to make a general assessment of physiological condition and to couple this to biochemical and cytological analyses for identification of the relevant environmental stressor(s) and to an ecological assessment of the consequences to the population of the measured physiological condition. The physiological measurements must be made under conditions that permit reasonable interpretation of the condition of the animals in nature.

In this paper we review some recent studies on the common estuarine bivalve mollusc *Mytilus edulis* L., which were designed to measure the physiological condition of individuals

from different populations and to equate these measurements with cytological observations made simultaneously. We describe alternative procedures for the assessment of the physiological condition of mussels, and relate the results of these studies to one aspect of ecological fitness, namely fecundity. Finally, by reference to work on the effects of petroleum hydrocarbons, we illustrate a rôle for biochemical and cytochemical measurements in evaluating the biological effects of an identified pollutant.

Two indices of physiological condition

Physiological measurements provide most information on an animal's condition when they can be integrated into indices of the effects of stress (Bayne 1975*a*). One such index is the 'scope for growth' (Warren & Davies 1967; Brett 1976), which is based on the balanced energy equation of Winberg (1960):

$$C = P+R+U+F,$$

where C is total consumption of food energy, P is production of both somatic tissue and gametes, R is respiratory heat loss, U is energy lost as excreta, and F is faecal energy loss. The absorbed ration, A, is the product of consumption, C, and the efficiency of absorption of energy from the food, e.† Production may then be expressed as

$$P = A-(R+U).$$

When P is estimated in this way it may be referred to as the scope for growth and the production of gametes.

Another potentially useful physiological index is the oxygen:nitrogen (O:N) ratio, which is the ratio by atomic equivalents of oxygen consumed to nitrogen excreted (Corner & Cowey 1968; Bayne & Scullard 1977). Values less than 30 signify a high proportion of protein catabolized relative to carbohydrate and lipid.

Physiological differences between populations

Populations of mussels at different estuarine sites within the U.K. were visited regularly between May 1976 and July 1977. At each visit, which lasted between two and four days, the physiological measurements (C, R, U and e) necessary to calculate the scope for growth were made on individuals covering a range of sizes typical of the population, with the use of equipment mounted in a minibus which had been modified as a mobile laboratory. The methods used (similar to those discussed by Bayne, Widdows & Newell (1977) and by Bayne & Widdows (1978) were designed to ensure that the measurements were taken under ambient conditions of water temperature, salinity and suspended particulate matter, with minimal disturbance to the animals. Samples of suspended particulates were collected for later analysis of the food available to the mussels, and individual animals were returned to the laboratory for cytological evaluation by methods described by Moore (1976). The statistical analysis of the physiological data was by regression and covariance techniques (Bayne & Widdows 1978).

The results of these studies will be recorded in full elsewhere; in this paper we discuss observations from three sites in order to assess the extent of physiological differences between

† In this study, e is considered as the efficiency of absorption of energy from all suspended material filtered by the animal, whether ingested or rejected as pseudofaeces (see Bayne & Widdows 1978).

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populations and, in a single population, the variability in the scope for growth over time. The populations of mussels are located at (1) the Lynher river, near Plymouth, (2) the Swale, in the Thames estuary, (3) the Kings Dock, in Swansea, South Wales. In order to exclude physiological differences that are attributable only to differences in the sizes of the individuals within each population, the results of the regression analyses were used to relate the physiological measurements for each population to a 'standard animal' of 1.0 g dry flesh mass.

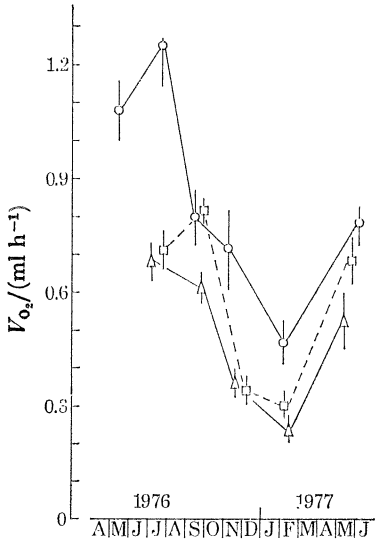

FIGURE 1. Rates of oxygen consumption (V_{O_2}) by *Mytilus edulis* of 1 g dry flesh mass from three populations during 1976 and 1977. o, Swale; □, Kings Dock, Swansea; △, Lynher. Results are mean values (±s.d.) taken from regression equations relating V_{O_2} to dry mass.

Two general relations have been established to facilitate the calculation of the scope for growth:

1. A relation between the energy available as food in the suspended particulate matter and the mass of particulate organic nitrogen. The energy value of the particulates was calculated from analyses of their protein, lipid and carbohydrate contents, with the use of methods described by Widdows, Worrall & Fieth (1978), and standard energy conversion constants (Crisp 1971). The particulate organic nitrogen was determined by Kjeldahl digestion. Data from six sites were used to establish the relation between energy, in joules per litre, available as suspended particles (E_s) and mass in milligrams of particulate organic nitrogen per litre (M_N):

$$E_s = 264\ M_N + 3.2;\ n = 26;\ r^2 = 0.59.$$

2. The relation between absorption efficiency and the proportion of organic matter present in the suspended particulates (O). The particulate organic matter was measured as the percentage loss of mass after ignition of total particulate material at 450 °C. The absorption efficiency (e) was measured as described by Bayne & Widdows (1978). The descriptive equation is:

$$e = 0.5 \log_{10} O - 0.32;\quad n = 26;\ r^2 = 0.76.$$

Rates of oxygen consumption

The rates of oxygen consumption (V_{O_2}) varied during the year, with high values in the spring and early summer and lower values in the winter (figure 1). Related studies (Bayne &

Widdows 1978) have shown that this seasonal variation is due largely to the annual gametogenic cycle of the mussels. Although the seasonal pattern was similar in all populations, there were differences in the intensity of metabolism.

Rates of clearance

The clearance rate (r_c; litres of water cleared of particles per hour) is a measure of the feeding of the mussels. It is dependent in part on the amounts of particulate material in suspension (Widdows *et al.* 1978; Bayne & Widdows 1978) but no consistent seasonal pattern was discernible in the present data. Consequently, a single equation may be written for each population relating r_c to dry flesh mass in grams (M):

1. Lynher: $r_c = 1.35 \pm 0.43\ M^{0.372 \pm 0.078}$; $n = 45$; $r^2 = 0.51$;
2. Swale: $r_c = 1.79 \pm 0.37\ M^{0.817 \pm 0.192}$; $n = 56$; $r^2 = 0.39$;
3. Kings Dock: $r_c = 1.15 \pm 0.118\ M^{0.443 \pm 0.103}$; $n = 55$; $r^2 = 0.49$;

There were significant differences between populations as to their clearance rates.

Absorption efficiency

Differences in absorption efficiency could be related to differences between sites in the organic content of the suspended particulate matter. In the Kings Dock, particulates were at low concentration (2.0–10.3 dry mg l^{-1}) but the proportion of organic material was high (25–57%) and absorption efficiencies were also high (0.36–0.68). In the Swale, however, the concentration of particulates was high and variable (18.4–76.0 dry mg l^{-1}), the proportion of organic material was low (11–15%) and absorption efficiencies were also low (0.12–0.34).

Rates of ammonia excretion

These were variable seasonally and between populations. However, the energy losses associated with ammonia excretion represented only between 3 and 12% of the equivalent respiratory heat losses (see also Bayne & Widdows 1978).

The scope for growth

An example of the calculation of the scope for growth for mussels of 1 g dry flesh mass is shown in table 1 and values for mussels from the Swale and Kings Dock populations during 1976 and 1977 are plotted in figure 2. Since each value is estimated from parameter values in the fitted regression equations, a direct estimate of the variance associated with each point in figure 2 is not possible. However, Bayne & Widdows (1978) calculated, by simulation, a variance:mean ratio of 0.55 for similar estimates of the scope for growth.

The seasonal pattern for the scope for growth was similar in both populations (figure 2) but mussels in the Kings Dock had less overall potential for growth than those from the Swale; integrated values for the 12 months were 32 kJ for Swale and 2.7 kJ for Kings Dock.

Estimates of the scope for growth for *Mytilus* in the Lynher estuary have been made since 1973 (figure 3). The data demonstrate a decline in physiological condition during 1976 and 1977, which is consistent with observations (D. Lowe, personal communication) of a depression of gametogenic activity during this period. The reasons for this decline in condition are probably complex but may include the high summer temperatures of 1976, an unusually high level of infestation by *Mytilicola intestinalis* over the same period (J. Davey, personal communication) and a gradual deterioration of water quality at this site (unpublished observations).

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TABLE 1. THE CALCULATION OF THE SCOPE FOR GROWTH FOR *MYTILUS EDULIS* OF 1 g DRY FLESH MASS FROM A POPULATION IN SWANSEA BAY, AS BASED ON PHYSIOLOGICAL AND ENVIRONMENTAL MEASUREMENTS MADE IN THE FIELD

	winter	summer
particulate organic nitrogen/(mg l^{-1})	0.11	0.15
particulate energy content†/(J l^{-1})	32.2	42.8
clearance rate‡/(l h^{-1})	1.59	1.59
cleared ration, C/(J h^{-1})	51.2	68.0
particulate organic matter as % of total	11.1	13.3
absorption efficiency, e§	0.20	0.24
absorbed ration, $A(=Ce)$ / J h^{-1}	10.2	16.3
rate of oxygen consumption‖ / ml h^{-1}	0.534	0.696
respiratory heat loss, R¶ / J h^{-1}	10.6	13.8
rate of ammonia excretion†† / (μg N h^{-1})	22.7	29.3
energy loss as excreta, U‡‡/(J h^{-1})	0.6	0.7
scope for growth, $A-(R+U)$ J h^{-1}	−1.0	1.8
scope for growth, $A-(R+U)$ J d^{-1}§§	−18	+32

† Calculated from particulate organic N(M_N) as $E_s = 264\ M_N + 3.2$.
‡ From equation relating clearance rate (r_c) to body mass (M, dry flesh in grams): $r_c = 1.59\ M^{0.406}$.
§ Calculated from particulate organic matter as percentage of total particulates (O) as $C = 0.5 \log_{10} O - 0.32$
‖ From equation relating oxygen consumption (V_{O_2}) to body mass (M): $V_{O_2} = aM^{0.616}$, where a is seasonally variable.
¶ Calculated as 19.9 V_{O_2}.
†† From equation relating ammonia excretion ($V_{NH_4.N}$) to body mass (M): $V_{NH_4.N} = nM^{0.617}$, where n is seasonally variable.
‡‡ Calculated as 0.025 $V_{NH_4.N}$.
§§ Calculated for total immersion of 18 h d^{-1}, and assuming no feeding, respiration or excretion during emersion in air for 6 h d^{-1}.

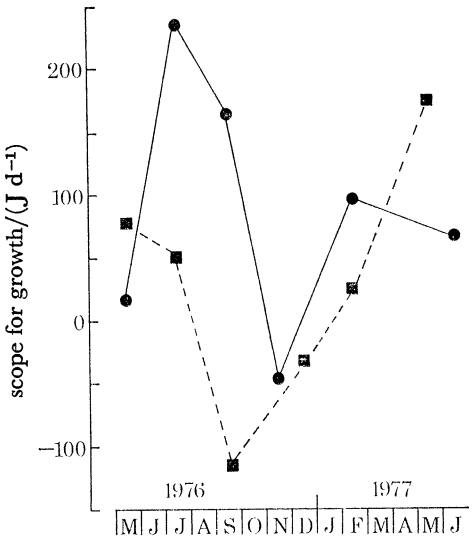

FIGURE 2. The scope for growth in *Mytilus edulis* of 1 g dry flesh mass from two populations during 1976 and 1977 ●, Swale; ■, Kings Dock, Swansea.

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These results illustrate spatial and temporal differences in the scope for growth in populations of *Mytilus*. A similar approach has been used by Gilfillan and his colleagues to demonstrate some effects of a spill of no. 6 fuel oil on the bivalve *Mya arenaria* in Casco Bay, Maine (Gilfillan *et al.* 1976, 1977). Gilfillan *et al.* (1976) concluded that *Mya* from a population exposed to the oil suffered a 50% reduction of growth potential compared with individuals from a site not subjected to pollution.

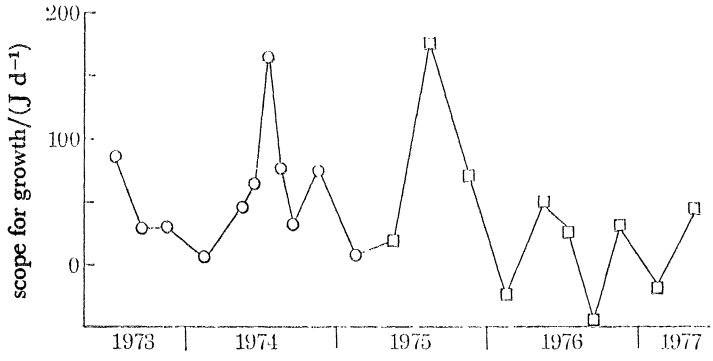

FIGURE 3. The scope for growth in *Mytilus edulis* of 1 g dry flesh mass from the Lynher population during five years. ○, data published by Bayne & Widdows (1978); □, previously unpublished data. Scope for growth was reduced in 1976 and 1977.

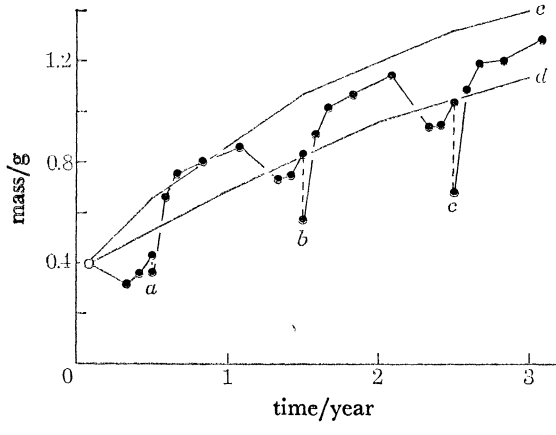

FIGURE 4. A growth curve for *Mytilus edulis* calculated from physiological estimation of the scope for growth in the Lynher population. An initial dry mass of flesh of 0.4 g is assumed at the beginning of year 1; *a*, *b* and *c* are predicted periods of mass loss due to spawning. Minimum (*d*) and maximum (*e*) curves of growth are also shown, as calculated from monthly sampling for year class and length–mass analysis.

In the experiments with mussels described in this section, physiological measurements have been made, in the field, under conditions as close as possible to ambient. The aim is to arrive at estimates for the scope for growth that are representative of real values in nature; the results should therefore reasonably predict actual production (somatic and germinal) in the population. Figure 4 shows a comparison between growth as observed and growth as predicted from physiological measurements on mussels from the Lynher population over three years. The agreement is good. However, when the objective is to compare the potential for growth at different sites without attempting a prediction of the true production, the experimental protocol may be simplified, as described in the next section.

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Transplant experiments

Narragansett Bay, in Rhode Island, U.S.A., receives substantial pollution, with greatest inputs in Providence river at the head of the Bay, originating from sewage effluents, land runoff and shipping operations in Providence Harbor (Boehm & Quinn 1977); a gradient of contamination results, from north to south. For example, Farrington & Quinn (1973) recorded 2.04 mg of petroleum hydrocarbons per gram dry mass of sediment at Sabin Point in the north, declining through 0.40 mg g^{-1} near Jamestown to 0.11 mg g^{-1} near the Rhode Island Graduate School of Oceanography at the mouth of the Bay in the south. Eisler *et al.* (1977) demonstrated a similar gradient in metals from the sediments at three stations. Jeffries (1972) described some of the effects of pollution in Narragansett Bay on the clam, *Mercenaria mercenaria*, and attributed most of these effects to petroleum hydrocarbons.

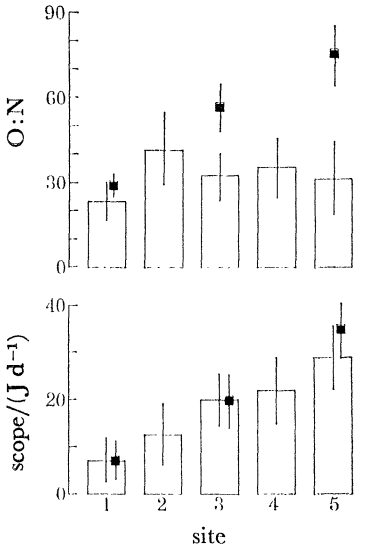

FIGURE 5. The scope for growth and oxygen:nitrogen ratios for *Mytilus edulis* transplanted from the mouth of Narragansett Bay to five sites within the Bay. See text for the designation of the sites, which ranged from a polluted situation at Sabin Point (site 1) to cleaner conditions in a laboratory seawater system at Narragansett (site 5). Values are means and standard deviations for measurements in August (■) and October 1977 (histograms).

Mussels (*Mytilus edulis*) were collected at the mouth of the Bay and transplanted in baskets to four sites: Sabin Point at the mouth of the Providence river (the most polluted site), Conanicut Point (Jamestown), the northern end of Jamestown island and at Dutch Island near the mouth of the Bay; a fifth sample was held in the aquarium at the laboratory of the Environmental Protection Agency at Narragansett (the least polluted site). After 30–40 days the mussels were taken to the laboratory for physiological measurements and the calculation of the scope for growth and the O:N ratio (Widdows, unpublished data). In the laboratory, field ambient temperatures and salinities were reproduced, but no attempt was made to simulate the naturally occurring suspended particulate material. Instead, a concentration of algal cells (*Platymonas suecica*) slightly greater than the maintenance requirement of the mussels (Bayne 1975*b*; Widdows 1978*b*) was maintained in the water supply to the animals; at this cell concentration *Mytilus* feeds maximally and absorption efficiency is maintained at high values

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(Widdows 1978*a*; Winter 1978). The scopes for growth and O:N ratios were therefore estimated for mussels from the various sites under standard conditions in the laboratory. The experiment was repeated in August and in October 1977.

The results (figure 5) demonstrate a gradient in the scope for growth that matches the pollution gradient in Narragansett Bay, with low values for mussels from Sabin Point and maximum values in the laboratory population. In August the O:N ratio was also correlated with the pollution gradient (lowest value at Sabin Point) but in October, after the spawning of mussels in the field, when there is a natural increase in reliance on protein catabolism for maintenance (Bayne & Scullard 1977), the differences in O:N were not as apparent.

This study demonstrates the potential for employing mussels as monitors of water quality, with the effects of exposure to the polluted environment measured under standard conditions in the laboratory. The relevance of this and the more field-orientated approach described earlier lies in the evaluation of physiological condition as a component in the syndrome of the effects of environmental stress. Two questions follow: what are the likely ecological consequences of a decline in physiological condition, and are there cytological processes that can be correlated with the physiological changes in order to elucidate specific causes for the observed decline in performance?

Fecundity and the physiological condition of the adult

The scope for growth measures the potential for both somatic growth and the production of gametes. We have recently completed experiments in which mussels were subjected to different conditions in order to induce levels of the scope for growth from 46 to -218 J d^{-1}; between 52 and 65 days later the mussels were stimulated to spawn and their fecundity (numbers of eggs produced) and the biochemical composition of their eggs was measured (Bayne *et al.* 1978). Fecundity declined from 7.6×10^6 eggs per female at the highest scope for growth to 3.4×10^6 at a scope of -218 J d^{-1}. The energy content of the eggs also declined at reduced scope for growth, from 2.5 mJ per egg at 46 J d^{-1} to 0.4 mJ per egg at the minimum scope. This result was in agreement with an earlier study by Helm, Holland & Stephenson (1973) on the oyster, *Ostrea edulis*, in which the lipid content of young larvae declined with a reduction in the physiological condition of the adult; the subsequent rates of growth of the larvae were also correlated with lipid levels.

In the mussel a decline in fecundity after stress imposed on the adult is caused in part by the resorption of eggs (Bayne *et al.* 1978) as a result of the release of lysosomal enzymes into the cytoplasm of the developing oocytes. At the same time, however, gametogenesis continues (see also Sastry 1970) although the oocytes that develop are smaller than normal. The gametes have reduced chances of survival through cleavage and embryogenesis before development to the feeding larval stage (Bayne 1972). The result is a deterioration in the ecological fitness of the population as a consequence of environmental stressors acting on the adults.

Cytochemical indices of the effects of stress

A negative scope for growth implies that reserves of energy within the body must be utilized for the animal to meet its metabolic requirements for maintenance. The controlled digestion of cytoplasmic components by autophagy, involving lysosomes and their associated hydrolytic enzymes, is a recognized property of many animal cells (Ericsson 1969). The association between the lysosomal hydrolases and the lysosomal membrane (Koenig 1969; Verity 1973) results in most of the enzyme activity's being normally latent; if for any reason the lysosomal membranes

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are rendered unstable, free hydrolases may be released into the cytoplasm with resultant autolytic cell damage (Miller & Wolfe 1968; Koenig 1969). The induction of free enzyme activity, as opposed to latent (or bound) activity, can be measured cytochemically (Moore 1976) as the 'labilization period', i.e. the length of time of preincubation at pH 4.5 that is required to give maximum staining activity for the lysosomal enzymes β-glucuronidase or *N*-acetyl-β-hexosaminidase.

TABLE 2. THE LABILIZATION PERIOD FOR *N*-ACETYL-β-HEXOSAMINIDASE IN THE DIGESTIVE CELLS OF *MYTILUS EDULIS* FROM SEVEN POPULATIONS

(All determinations cover a period from May 1976 to July 1977, except for the Lynher population for which more data are available. The labilization period is measured as the length of time at pH 4.5 required to give maximum staining activity for the enzyme in tissue sections. Numbers of determinations are given in parentheses.)

population	mean labilization period ± s.e./min
Lynher (1975)	20.8 ± 1.34 (3)
Lynher (1976)	12.3 ± 1.11 (6)
Lynher (1977)	18.5 ± 1.05 (4)
Mumbles	14.2 ± 2.0 (5)
Atlantic College	12.8 ± 1.8 (4)
Minehead	12.0 ± 4.0 (3)
Swale	11.0 ± 1.1 (5)
Kings Dock	7.1 ± 2.3 (6)
Teignmouth	5.0 ± 1.8 (6)

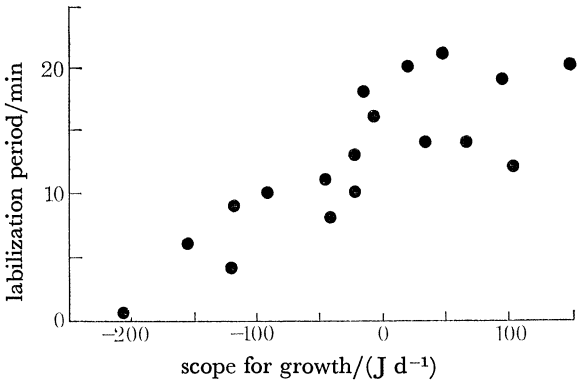

FIGURE 6. The labilization period (see text) of the lysosomal enzyme *N*-acetyl-β-hexosaminidase related to estimates of the scope for growth for *Mytilus edulis* from four different populations.

The labilization period for hexosaminidase from digestive gland cells of *Mytilus* from seven populations was measured on a number of occasions at the same time as the scope for growth was estimated. The results over a period of 12–18 months (table 2) demonstrated persistent differences between populations; they also indicated a significant correlation ($r = 0.86$ for 8 degrees of freedom; $p < 0.001$) between the labilization period in minutes and negative values for the scope for growth (figure 6). This agrees with earlier laboratory data (Bayne, Livingstone, Moore & Widdows 1976) and suggests that, when the scope for growth is markedly negative, cellular damage would occur due to the release of free hydrolases into the cytoplasm. When the scope for growth is only slightly negative, lysosomal destabilization is not acute and autophagy is probably better controlled.

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Lysosomal destabilization has now been demonstrated in *Mytilus edulis* as a result of high temperatures (Moore 1976), the combined action of nutritional and thermal stressors (Bayne *et al.* 1976), reduced salinity (Moore, unpublished data) and injection with low doses of certain aromatic hydrocarbons (Moore, Lowe & Fieth 1978). Reduced labilization periods for lysosomal enzymes are linked with increased cell damage and autolysis in the digestive gland of *Mytilus* (Moore, Lowe & Fieth 1978).

These results suggest that lysosomal destabilization is a general cytolytic or cytotoxic response in mussels. The generalized nature of the response does not allow the identification of specific stressors from data on lysosomal enzyme activity alone. Both the scope for growth and lysosomal destabilization are rather components of a general syndrome of stress effects. Lysosomes in mammals are known to sequester many potentially toxic chemicals (Allison 1969; Allison & Young 1969), and in bivalve molluscs certain metals have been shown to accumulate in membrane bound vesicles that resemble lysosomes in appearance (George, Pirie & Coombs 1976; Moore 1977; George *et al.* 1978; Lowe & Moore, unpublished data). Therefore a variety of pollutants may be expected to cause disturbance to normal lysosomal function. In order to arrive at indices that might measure the effects of single classes of pollutants, processes must be chosen for study that are linked biochemically with the particular contaminants. Some recent work with petroleum hydrocarbons may serve as an illustration.

Some effects of petroleum hydrocarbons on bivalve molluscs

The capacity of bivalves to accumulate hydrocarbons from solution in seawater and in the particulate phase is now well documented (Lee, Sauerheber & Benson 1972; Stegeman & Teal 1973; Farrington & Quinn 1973; Di Salvo, Guard & Hunter 1975; Neff *et al.* 1976; Boehm & Quinn 1976). However, studies on the loss of hydrocarbons from bivalves have yielded conflicting results. In a recent review of this literature, Boehm & Quinn (1977) have suggested that chronic exposure to hydrocarbons (i.e. sublethal levels of pollutant over one to two months) results in the hydrocarbons reaching stable compartments in the animal, whence they are only slowly released. Acute exposures, however (lasting a few hours to one or two days) do not result in the hydrocarbons reaching stability and, once the animals are transferred to clean water, depuration may occur rapidly (Neff *et al.* 1976).

Some of these studies argue that bivalve molluscs do not have the biochemical capacity to metabolize foreign compounds such as petroleum hydrocarbons (Carlson 1972; Lee *et al.* 1972; Stegeman 1974; Neff & Anderson 1975; Payne 1977); this is taken to explain the persistence of hydrocarbons within the tissues of mussels, clams and oysters that have been exposed to oil (Lee, Furlong & Singer 1977). However, Khan *et al.* (1972) recorded the epoxidation of aldrin to dieldrin *in vitro* by the freshwater bivalve *Anodonta* sp. More recently, R. I. Krieger (personal communication) has confirmed this finding both *in vitro* and *in vivo* for *Mytilus californianus* and P. Donkin (personal communication), working at Plymouth, has also found evidence of aldrin epoxidation in tissue homogenates of *Mytilus edulis*. Aldrin epoxidation models monooxygenase-catalysed epoxidation and has been used as an index of the activity of the mixed function oxygenase enzyme system in a variety of invertebrates (Krieger & Wilkinson 1969; Khan *et al.* 1972; Burns 1976). The mixed function oxygenase system comprises the enzymes responsible for the metabolism of foreign compounds, including hydrocarbons.

In recent experiments we have used a quantitative cytochemical technique to examine the

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cellular distribution of NADPH-dependent tetrazolium reductase in *M. edulis* and the effects on this enzyme of injected anthracene. NADPH-tetrazolium reductase (or 'diaphorase') has been shown to be linked with the electron transport system of the cytochrome P_{450}-linked mixed function oxygenases in mammals (Hardonk & Koudstaal 1976; Lindner & Beyhl 1978) where it is believed to be a reduced flavoprotein component acting as the neotetrazolium reductase (Koudstaal & Hardonk 1969). Microcrystalline suspensions of anthracene in sterile saline (100 µl) containing 1% acetone were injected into the mussels via the posterior adductor muscle sinus at four dose levels (10, 25, 50 and 100 µg anthracene per mussel). A 1% solution of acetone in sterile saline was used as the vehicle control (v.c.). The mussels were returned to seawater immediately after injection and sampled after 24 h; a separate group of mussels was injected with 100 µg anthracene per mussel and sampled, together with vehicle control animals, after 1, 2, 4 and 7 days.

TABLE 3. THE EFFECTS OF ANTHRACENE INJECTED *IN VIVO* ON THE STAINING INTENSITY OF NADPH-DEPENDENT TETRAZOLIUM REDUCTASE IN THE BLOOD CELLS OF *M. EDULIS* OVER A PERIOD OF 24 h

(Values are means ± s.e. for measurements on five animals in all cases. The results were tested for significance against vehicle control I by Mann–Whitney *U*-test.)

treatment	absorbance of NADPH-tetrazolium reductase as percentage of v.c. I	result of Mann–Whitney *U*-test
vehicle control I	100 ± 4.6	—
vehicle control II	101.8 ± 4.0	n.s.
10 µg anthracene	150.8 ± 13.5	$p < 0.01$
25 µg anthracene	155.2 ± 2.7	$p < 0.01$
50 µg anthracene	153.4 ± 8.2	$p < 0.01$
100 µg anthracene I	179.0 ± 11.9	$p < 0.01$
100 µg anthracene II	184.0 ± 11.9	$p < 0.01$

TABLE 4. THE EFFECTS OF 100 µg ANTHRACENE INJECTED *IN VIVO* ON THE STAINING INTENSITY OF NADPH-DEPENDENT TETRAZOLIUM REDUCTASE IN THE BLOOD CELLS OF *M. EDULIS* OVER A PERIOD OF 7 DAYS

(The results were tested for significance against the vehicle control for each day by Mann–Whitney *U*-test. Measurements were taken on five animals in each case.)

days	absorbance of NADPH-tetrazolium reductase (machine units, mean ± s.e.) vehicle control	100 µg anthracene	result of Mann–Whitney *U*-test
1	15.8 ± 1.1	29.0 ± 1.9	$p < 0.01$
2	16.5 ± 0.5	26.2 ± 1.5	$p < 0.01$
4	16.3 ± 1.2	22.5 ± 2.8	n.s.
7	17.4 ± 1.9	22.2 ± 1.1	$p < 0.05$

On sampling, a small piece (approx. 5 mm^3) of digestive gland was removed, frozen in hexane (−70 °C) and sectioned at 10 µm in a Bright cryostat at a cabinet temperature of −26 °C with a dry-ice cooled knife. NADPH-dependent tetrazolium reductase was demonstrated in the sections by a modification of the method described by Altman (1972) with HEPES buffer (pH 8.0) containing 20 mM $MgCl_2$. Incubation was for 30 min in a nitrogen

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atmosphere at 37 °C. Control sections omitted NADPH from the incubation medium. Microdensitometric measurements were made on individual cells (ten readings per section) by using a Vickers M85 scanning, integrating microdensitometer at a wavelength of 585 nm (Butcher & Altman 1973) with slit width setting at 60 and measuring spot size 0.5 μm diameter.

Reaction product for NADPH-dependent tetrazolium reductase was localized in the haemocytes (both basophilic and acidophilic types; figure 7*a*, plate 1). The injection of anthracene into the blood system induced an increase in the intensity of the staining reaction in the cytoplasm of the blood cells after 24 h (table 3; figure 7*b*). This increased staining reaction persisted for at least 2 days (table 4). We have also confirmed an increase in tetrazolium reductase activity in haemocytes of mussels which had been exposed for 2 days to water-soluble extracts of crude oil (3.0 μg l^{-1}, measured as napthalene equivalents).

TABLE 5. ACTIVITIES OF THREE ENZYMES FROM THE GILL AND KIDNEY TISSUES OF *MYTILUS EDULIS* KEPT FOR 7 DAYS IN CLEAN SEAWATER (THE 'CONTROL ANIMALS') AND IN SEAWATER CONTAINING WATER-SOLUBLE EXTRACTS OF NORTH SEA CRUDE OIL AT A CONCENTRATION OF 3 μg NAPTHALENE EQUIVALENTS PER LITRE (THE 'EXPERIMENTAL ANIMALS')

(Activities are recorded as micromoles of product formed per minute per gram fresh mass, at 25 °C.)

	specific activity:		experimental
enzyme	in control animals	in experimental animals	as percentage of control
G6PDH	1.950 ± 0.715	3.344 ± 1.127	172
6PGDH	0.857 ± 0.169	1.252 ± 0.447	146
ICDH	0.160 ± 0.067	0.379 ± 0.185	236

Assay conditions were: (i) glucose-6-phosphate dehydrogenase (G6PDH), 100 mM HEPES–NaOH (pH 8), 10 mM $MgCl_2$, 2 mM glucose-6-phosphate, 0.5 mM NADP; (ii) 6-phosphogluconate dehydrogenase (6PGDH), as for (i) but with 2 mM 6-phosphogluconate instead of glucose-6-phosphate; (iii) NADP-dependent isocitrate dehydrogenase (ICDH), 100 mM MOPS/NaOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 4 mM DL-isocitrate, 0.5 mM NADP.

Other enzymes which are associated with the smooth endoplasmic reticulum (or microsomal) compartment of the cell, such as glucose-6-phosphate dehydrogenase, have also been shown to increase in activity in mammalian liver cells, after treatment of the animal with foreign compounds (Hardonk & Koudstaal 1976). Indeed, the function of the mixed function oxygenase system is dependent upon a supply of NADPH via normal metabolic processes occurring within the cell. We have recently recorded increased activity of some NADPH-producing enzymes in tissue extracts of *Mytilus* exposed to the water soluble extracts of crude oil (between 0.8 and 3.0 μg l^{-1}, measured as napthalene equivalents) for seven days in flowing seawater (table 5). Mussels were sampled from oil-free and oil-contaminated experimental seawater systems and the gills with attached kidney tissue dissected and homogenized in 10 mM Tris-HCl buffer (pH 7.7) with 1 mM EDTA and 1 mM Cleland's reagent, at 4 °C. Following centrifugation at 12000 and 40000 *g* (30 min each), aliquots were passed through Sephadex G-25 columns equilibrated in the homogenization buffer. The enzyme assays were performed as indicated in table 5, and enzyme activities recorded as micromoles of product formed per minute per gram fresh mass of tissue. Enzyme activities in the mussels exposed to the water soluble oil extracts increased to between 136 and 236 % of values from the control mussels.

The recent data on aldrin epoxidation and the activity of NADPH-dependent tetrazolium reductase therefore indicate the presence, in *Mytilus*, of some elements of a mixed function

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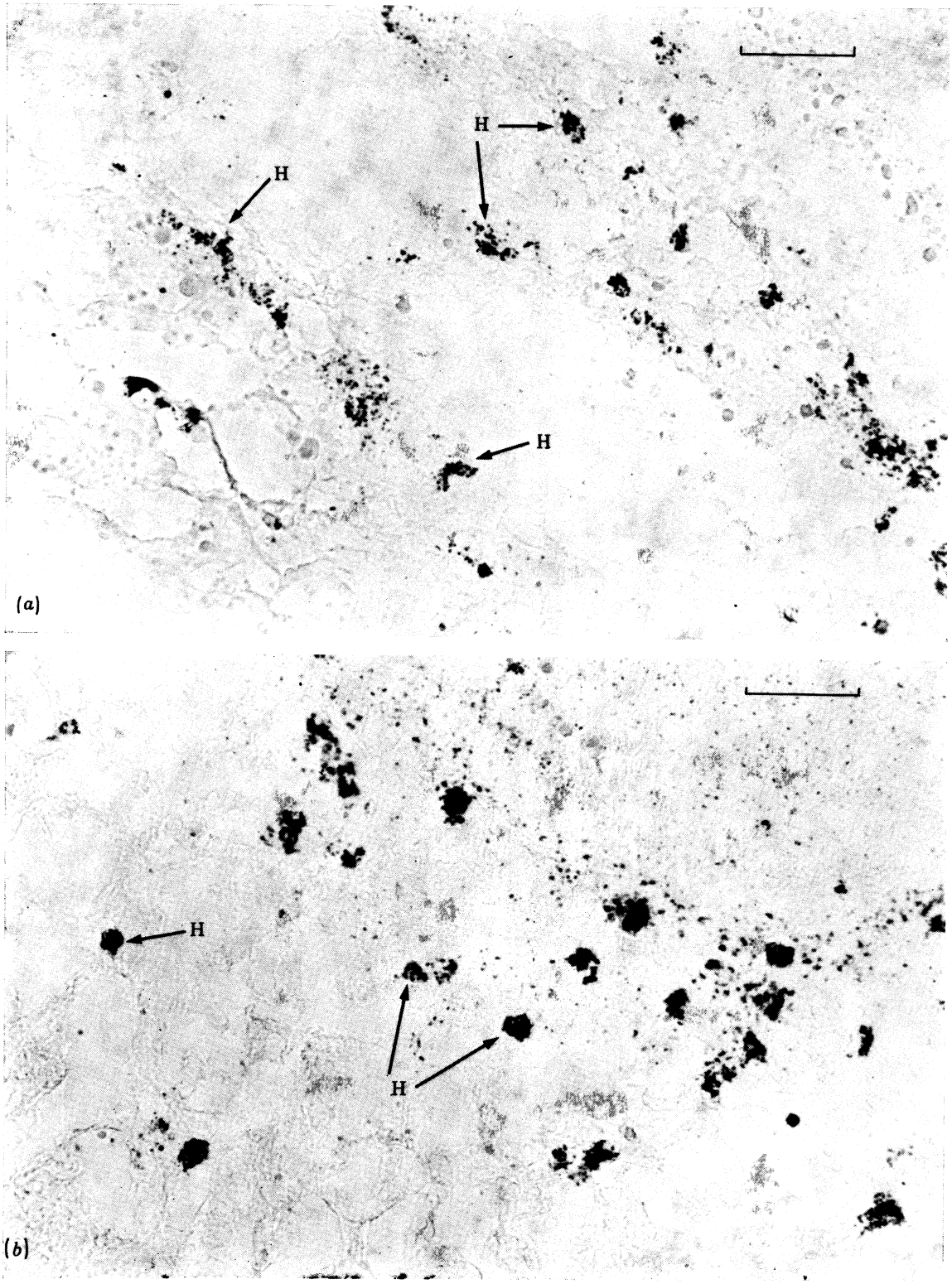

FIGURE 7. (*a*) Section through the connective tissue of the digestive gland of *Mytilus edulis*, injected with 100 µl of vehicle control solution (24 h), showing haemocytes (H) stained for NADPH-tetrazolium reductase activity. (*b*) Section as in (*a*) of *M. edulis*, injected with 100 µg of anthracene (24 h), showing increased staining reaction for NADPH-tetrazolium reductase in the haemocytes (H). Scale bar = 50 µm in both photographs.

(*Facing p.* 574)

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oxygenase system which, in other organisms, has been associated with the metabolism (or 'biotransformation') of foreign compounds. Our data also suggest that increased activities of some components of this system are inducible after exposure of the animals to hydrocarbons. However, the activity of the system is very low (approx. 0.2 pmol dieldrin produced from aldrin per hour per milligram tissue; Donkin, personal communication) and other studies have failed to detect either the metabolism of petroleum hydrocarbons *in vivo* with the use of radio-labelled substrates (Lee *et al.* 1972) or the presence of certain hydroxylating enzymes which are normally associated with these metabolic processes (Payne 1977). Possibly as a consequence of this, bivalve molluscs accumulate high concentrations of organic contaminants of various kinds (Anderson *et al.* 1974; Neff & Anderson 1975; Courtney & Denton 1976; Risebrough, de Lappe & Schmidt 1976; Dunn & Young 1976; Langston 1978) and are therefore increasingly in use as indicator organisms in chemical monitoring programmes.

Burns (1976) recently reported that the microsomal mixed function oxygenase system is present in the crab *Uca pugnax*, but its activity is too low to clear from the body, by biotransformation alone, the observed levels of hydrocarbons accumulated in crabs living in a polluted salt marsh. A similar interpretation may apply to mussels. Three properties of these animals may therefore be recognized as significant for measuring the effects of exposure to oil: (*a*) the persistence of the hydrocarbons in the body after chronic exposures (Boehm & Quinn 1977), (*b*) the prolonged physiological effects that probably result from this persistence (Gilfillan *et al.* 1976; Vandermeullen 1977), and (*c*) the presence of inducible aldrin epoxidation and tetrazolium reductase processes that may provide an index of pollution in the way postulated by Payne (1976) for fish and by Lee *et al.* (1977) for crustaceans.

Conclusions

Three main considerations apply when recommending the use of the biological responses of individuals for the assessment of the effects of pollution.

First, indices of response must be chosen that represent integrated measures of the physiological condition of the animal and that can be related quantitatively to environmental changes. The selection of suitable effects (stress) indices depends upon good understanding of the ways in which the animal responds both to natural and to man-induced stressors. Eventually, the relation between the index of response and the environmental change that has occasioned this response needs to be stated quantitatively, since this will be the most effective way for values of the biological effect to be assigned a wide environmental relevance. However, this relation is unlikely to be a simple one, and its quantitative expression may have to take the form of non-linear simulation models rather than linear 'dose–response' statements. In some situations knowledge of the biological response itself, and its likely ecological consequence, will be sufficient for effective action to be taken; in other situations the simulation model, with its various forcing functions, may provide the useful tool for environmental management.

The second condition follows, namely that any index of effect based on the response of the individual should be linked quantitatively with an effect on the ecological fitness either of the individual or of the population, or both. This relation is also complex, dependent upon seasonal changes in the physiology of the animal and upon spatial and temporal variability in the demands made on the animal by its ecological niche. Here also, mathematical simulations will be needed to contribute to the practical application of biological effects indices.

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The third consideration concerns requirements for sensitivity and for specificity. Cytological and biochemical responses are likely to prove more sensitive to environmental changes than some physiological (or 'whole animal' response) indices. For sessile organisms that are likely to accumulate contaminants within their tissues, early warnings of damage may best result from indices of cytotoxicity. Also, if the biological effects are to be linked with specific environmental contaminants, particular cytochemical targets should be identified and monitored. However, these cytochemical effects must also be linked with the more generalized physiological responses in order to demonstrate ecological relevance.

An effective programme for the measurement of the biological effects of pollution will, of course, be required to distinguish between natural and pollution-induced effects. Experience suggests that this requirement is met, in part at least, by ensuring that sampling is adequate to describe the normal seasonal cycle that exists in most physiological processes. A seasonal anomaly in a physiological index may be the first indication of potential damage to the population. Also, natural variability is smaller at the cytochemical than at the physiological level, so that cytochemical or biochemical indices should be employed where possible. A related problem concerns the discrimination between populations at risk from environmental deterioration and populations not so acutely threatened. Here also discrimination may reside in monitoring as many sites as possible in order to recognize the anomalous situation.

Recent studies with the bivalve *Mytilus edulis* have attempted to incorporate some of these considerations in a programme designed to formulate criteria for monitoring some biological effects of pollution. Measurements of physiological indices have been coupled with cytological and biochemical observations to provide information on the physiological condition of individuals and, by inference from laboratory experiments, on the likely ecological consequences of different degrees of physiological stress. Related studies with hydrocarbons suggest ways in which the effects of a specific class of potential pollutants may be assessed and possibly monitored. For a number of reasons, mussels are the animals of choice in many chemical monitoring programmes that may cover wide geographical areas. The sampling in space and time that results also provides a convenient opportunity for biologists to become involved in assessing the effects of the body burdens of the contaminants that are identified by the analytical chemists.

We are grateful to D. Lowe and P. Donkin (I.M.E.R.) for allowing us to quote from some of their unpublished data, to Ms E. Head for advice on the optimal assay conditions for ICDH, and to Mrs P. Fieth for skilled technical assistance. This work forms part of the experimental ecology programme of the Institute for Marine Environmental Research, a component of the Natural Environment Research Council. It was commissioned in part by the Department of the Environment (Contract no. DGR 480/288) and in part by the European Economic Community (Contract no. 279-77-4 ENV UK). One of us (J.W.) wishes to thank the Environmental Protection Agency, U.S.A. for funds and hospitality at their laboratory at Narragansett, Rhode Island. North Sea crude oil was kindly supplied to us by Shell (U.K.) Ltd.

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Discussion

D. J. Crisp, F.R.S. (*Marine Science Laboratories, Menai Bridge, Anglesey, U.K.*). The authors' work has developed a number of very useful indices which measure aspects of the 'health' of the population, to use a popular non-quantifiable concept. 'Stress' or 'pollution' are similar anthropomorphic concepts which presumably affect the indices adversely, but which cover many qualitatively different substances and effects. Thus when the authors correlate positively the fall in any index of performance with total pollution load they must implicitly or explicitly have given a weighting to the various pollutants making up the environmental load. Would Dr Bayne comment on how the weighting was done?

B. L. Bayne. The main objective of this work to date has been to establish the extent to which populations differ in measurable indices of physiological condition. We have seen that such differences do exist. In attempting a further objective, namely to equate these differences with pollution, we need more information on the total pollution load in the environment. To date, we have simply ranked the populations as regards physiological condition and compared this with a ranking of the sites in terms of the total discharge of anthropogenic inputs at each site. The rankings agree to an encouraging extent. However, as I indicated in my paper we must now concentrate on the derivation of indices that relate to the effects of specific pollutants. We have started by looking at hydrocarbons. When a variety of indices that relate specifically to classes of pollutants is available, we should be able to make firmer statements on the extent to which our observed physiological differences are, in fact, due to pollution stress.

R. J. Morris (C.U.E.P., *Department of the Environment, London, U.K.*). Would not changes in an organism's gross biochemical composition – relative levels of protein, lipid and carbohydrate – also be a measure of its ability to grow (i.e. scope for growth)?

B. L. Bayne. Early in this study we explored the extent to which gross changes in biochemical composition might reflect the physiological condition of the animal. However, it soon became clear that there is a good control over the proportionality between protein, lipid and carbohydrates in the tissues. Ratios between these do not seem to provide a measure of growth potential.

J. S. Gray (*University of Oslo, Norway*). Concerning the quantification of scope for growth, I am a little worried about summation of values over a whole year. For example, Gillfillan (1975 *Mar. Biol.*) quotes a 50 % reduction in scope for growth in a population of *Mya* from a polluted beach based on summation over a year. Yet a paired *t* test of the month-by-month data shows no significant difference. Does Dr Bayne think that summation of month-by-month data is the correct approach or that comparison of data monthly is best?

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B. L. Bayne. In his more recent work, Gillfillan has gone further in making quantitative comparisons between populations of *Mya*. In our work I prefer to compare measures of the scope for growth made simultaneously or near simultaneously at different sites, rather than by summation over a 12 month period. Seasonal variation in indices of stress is considerable and, to some extent, slight differences in the timing of particular seasonal events give most clue to the existence of the stress. For example, a major period with negative scope for growth at a time when gametes are normally produced will be much more stressful than a similar period of negative growth after spawning. This kind of difference in the seasonal cycle only becomes apparent when paired observations are made between populations.

A. V. Holden (*D.A.F.S., Pitlochry, U.K.*). In reply to a question regarding metal residues, Dr Bayne said that no effects were seen over the range found in the mussel surveys. In a recent survey of organochlorine residues in mussels in Scottish waters, the concentrations ranged over almost three orders of magnitude. Would Dr Bayne expect to find differences in physiological conditions resulting from such variations?

If a standard stock of mussels is used for planting in different areas, this will eliminate the influence of any adaptation to pollution, but might this not be a desirable characteristic?

B. L. Bayne. Unfortunately we have no data on the specific effects of organochlorine residues. However, judging from our work with hydrocarbons I would guess that a difference in body burden of three orders of magnitude in any pollutant would result in measurable physiological and biochemical effects. As regards the second question, I discussed in my paper an approach in which animals from a standard stock were transplanted along a pollution gradient; this is indeed a useful approach and one which we are developing presently.

R. C. Newell (*Department of Zoology, University of Capetown, South Africa*). I should like to pose two questions related to the spatial differences that the authors have noted between populations in relation to pollution load. It seems to me that these could be due to either (*a*) differences in the availability of energy in different areas (since planktonic organisms may be affected by pollution load), or (*b*) differences in the ability of organisms to utilize the energy available. Could Dr Bayne comment on whether the different areas have qualitatively or quantitatively comparable energy availabilities? My second question relates to the first. If differences in the scope for growth reflect differences in the ability of organisms to utilize the available energy, this might be due to either (*a*) a reduction in the components of energy input (e.g. feeding and assimilation) under polluted conditions or (*b*) an increase in the components of energy expenditure (e.g. respiration). Can Dr Bayne comment on whether there are any common patterns of adjustment in either of these components in relation to different pollutant substances?

B. L. Bayne. We measure the quantity of food energy available at each site and, of course, there are differences. These differences are reflected in almost all aspects of the energy budget and some of these relations I have discussed in the paper. It is true to say, however, that differences in physiological condition between populations exist that are greater than our expectations for such differences based only on the range of food energy available.

As regards the second part of the question, the main merit of the scope for growth is that it integrates the various components of the energy budget; in some populations there may be an increase in respiration leading to a depression in the scope; in other populations feeding may

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be impaired. A calculation of the scope for growth allows one to assess these differences and also to gauge their effects in an integrated way.

A. D. McIntyre (*Marine Laboratory, P.O. Box 101, Victoria Road, Aberdeen AB9 8DB, U.K.*). Dr Bayne has described a package of techniques for detecting and quantifying pollution effects. Could this package now be picked up and utilized generally, or is the level of sophistication and expertise such that only large and highly developed laboratories could use it?

B. L. Bayne. The package of techniques that Dr McIntyre refers to now comprises eight or nine different indices. In developing these indices we have sought specifically to simplify their measurement and the apparatus and techniques required. Sometimes interpretation of the data requires some expertise, but the measurement of the indices *per se* is, in most cases, simple enough for most laboratories.

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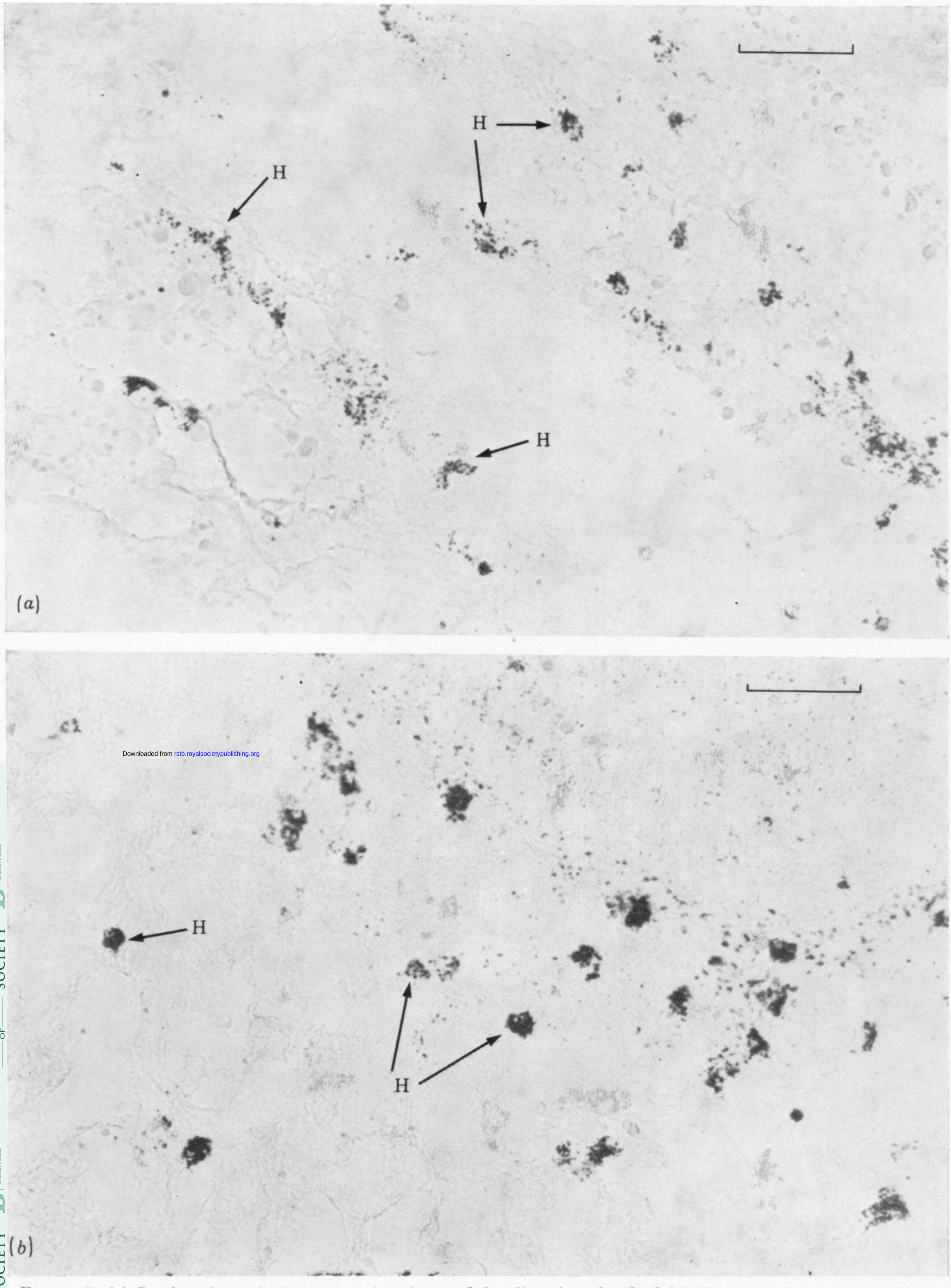

FIGURE 7. (*a*) Section through the connective tissue of the digestive gland of *Mytilus edulis*, injected with 100 µl of vehicle control solution (24 h), showing haemocytes (H) stained for NADPH-tetrazolium reductase activity. (*b*) Section as in (*a*) of *M. edulis*, injected with 100 µg of anthracene (24 h), showing increased staining reaction for NADPH-tetrazolium reductase in the haemocytes (H). Scale bar = 50 µm in both photographs.