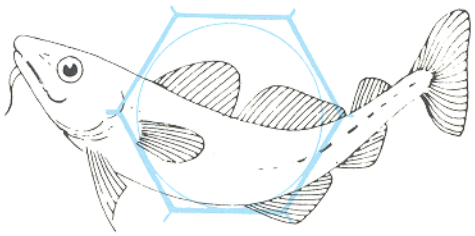
ISSN 0142-2499

AQUATIC ENVIRONMENT MONITORING REPORT

Number 24



Utility of experimental measures of biological effects for monitoring marine sewage-sludge disposal sites.

Prepared by the Biological Effects Task Team for the Marine Pollution Monitoring Management Group's Co-ordinating Group on Monitoring of Sewage-Sludge Disposal sites.

MAFF **Directorate of Fisheries Research**
Lowestoft, 1990

MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

DIRECTORATE OF FISHERIES RESEARCH

AQUATIC ENVIRONMENT MONITORING REPORT

NUMBER 24

Utility of Experimental Measures of Biological Effects for Monitoring Marine Sewage-Sludge Disposal Sites

Prepared by the Biological Effects Task Team for the Marine Pollution Monitoring Management Group's Co-ordinating Group on Monitoring of Sewage-Sludge Disposal Sites

Lowestoft 1990

This report has been compiled by H. L. Rees, MSc PhD (Chairman of the Biological Effects Task Team) of the MAFF Fisheries Laboratory, Burnham-on-Crouch, Essex CM0 8HA, from whom further copies can be obtained.

Aquat. Environ. Monit. Rep., MAFF Direct. Fish Res., Lowestoft, (24), 45pp.

CONTENTS

Page

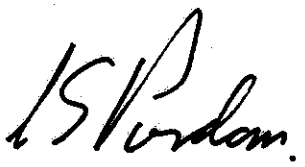
/Contents continued....

FOREWORD

As part of a continuing review of the suitability of methods for monitoring at UK sewage-sludge disposal sites, a Biological Effects Task Team met during 1989, under the auspices of the Marine Pollution Monitoring Management Group's Co-ordinating Group on Monitoring of Sewage-Sludge Disposal Sites. (The first Annual Report of this Group has been published: see MPMMG, 1989.)

The following report presents the outcome of the Task Team's work. It includes an outline of the legislative background and of the objectives of biological monitoring, along with an evaluation of the capacity of sewage sludge to induce biological effects following its disposal at sea. Detail is provided of a range of tests which were selected in accordance with the monitoring objectives.

Tests were scored against a set of criteria governing their utility, and these scores were used as the basis for recommendations concerning future applications. The practical implications for development of routine monitoring programmes using specified biological effects tests are discussed, and draft protocols for top-ranked tests are presented. Finally, a series of proposals are made for future research and development.



C.E. Purdom
Deputy Director
(Aquatic Environment Protection)
Ministry of Agriculture, Fisheries and Food,
Directorate of Fisheries Research.

SUMMARY

The background to the requirement for biological monitoring of sewage-sludge disposal sites is reviewed, and the attributes of biological effects techniques which are considered to be most important in meeting this requirement, are identified.

Consideration is given to the way in which the composition of sewage sludge (especially suspended particulate matter, nutrient and organic content, and potentially toxic trace elements) may affect the conduct of biological effects tests and the interpretation of results.

An outline is given of a range of tests suitable for assessment of water and sediment quality, both in the field and in the laboratory. These were selected partly on the basis of previous experience of their use at sewage-sludge disposal sites, but also following a general review of the relevant attributes of other available tests at the present stage of their development.

A list of criteria governing the utility of tests was prepared. Tests were then scored against these criteria, as an aid to ranking. The final outcome was as follows:

(i) Recommended for routine application

- oyster embryo test; and
- feeding rate in *Mytilus*.

(ii) Recommended for routine application, subject to further R and D and/or refinement of protocol (in ranked order of scores)

- zooplankton tests (copepods, mysids);
- 'Microtox' test;
- planktonic algal tests; and
- polychaete enzyme activity.

(iii) Not recommended for routine application at the current state of development/knowledge

- fish egg and fish larvae tests;
- other tests using infaunal invertebrates; and
- hydroid test.

Certain qualifying remarks appear against at least some of these tests; practical considerations arising from the implementation of these recommendations are reviewed, and draft protocols are presented for conducting the oyster embryo test, and for determining feeding rate in *Mytilus*. These tests are designed for use as part of an integrated monitoring strategy, as outlined by the MPMMG Co-ordinating Group on Monitoring of Sewage-Sludge Disposal Sites (MPMMG, 1989).

Future needs for research and development are highlighted. These concern the regular supply of test organisms, the extraction of contaminant concentrates from environmental samples prior to testing, and periodic intercalibrations accompanied where necessary by revisions of test procedures. It is also recommended that every encouragement should be given to further evaluation of a variety of measures (especially sediment bioassays) at sewage-sludge disposal sites, in an applied research context.

1. INTRODUCTION

In the UK, statutory control of the disposal of wastes from ships dates back to 1974 with implementation of the Dumping at Sea Act (Great Britain - Parliament, 1974). Implicit in both this Act and its successor (the Food and Environment Protection Act Part II, 1985 (Great Britain - Parliament, 1985), further details of which are given in Section 3) has been the requirement to monitor the effectiveness of licensing conditions through periodic field assessments of the environment at disposal sites.

Much of this work has been conducted by the statutory authorities, the Ministry of Agriculture, Fisheries and Food (MAFF), the Department of Agriculture and Fisheries for Scotland (DAFS) and the Department of the Environment for Northern Ireland (DOE (NI)) but, more recently, the concept of 'self-monitoring' has been introduced. This requires that licensees undertake an agreed component of the monitoring, usually with the emphasis on spatial studies, and is a reflection of the increasing level of commitment to the monitoring of sewage-sludge disposal sites, in order to meet both national and international obligations.

Recognising the importance of closer co-ordination and harmonisation of monitoring activities between the organisations involved, an initiative by the MPMMG (a non-statutory body with a membership drawn from Government Departments, the Water Industry and Research Institutes) led to the setting up in 1987 of a Co-ordinating Group on Monitoring of Sewage-Sludge Disposal Sites. The Terms of Reference of this Group are given in its first report (MPMMG, 1989), and include continued evaluation of the effectiveness of monitoring programmes, and the identification of areas of research which are necessary in support of this monitoring.

Much of the technical input to this Group has been via a series of expert Task Teams. This report gives the outcome of the activities of a Biological Effects Task Team (see Annex 1) which met during 1989, with the following Terms of Reference:

(i) to examine the utility of alternative measures of biological effects for use in monitoring UK sewage-sludge disposal sites;

(ii) to make recommendations on the suitability of techniques:

(a) for immediate application and, where appropriate, to present standard procedures; and

(b) for further development, and to allocate priorities for their evaluation.

A complementary summary of this report will appear in the Annual Report of the MPMMG's Co-ordinating Group for 1989 (MPMMG, in preparation).

The account begins by providing a rationale for the use of biological effects tests at sewage-sludge disposal sites (Section 2), and then outlines the legislative and 'customer' requirements (Section 3). A consideration of the gross physical and chemical properties of sludge in relation to test procedures (Section 4) is followed by a review of selected biological effects measures appropriate to the legislative requirements (Section 5). It should be noted that studies on fish pathology and disease have been dealt with separately (MPMMG, 1989) and so are not considered here.

A list of criteria is presented (Section 6), as the basis for scoring the utility of these and other tests from the standpoint of different monitoring objectives. Recommendations are made concerning the application of a limited suite of measures, in order of their suitability for present application (Section 7).

The practical implications for development of a monitoring routine are highlighted, notably in relation to the planning, design and execution of field surveys. Finally, a list of requirements for further research into appropriate measures, and their application, is also given (Section 8).

2. RATIONALE FOR THE USE OF BIOLOGICAL EFFECTS TECHNIQUES

It has long been recognised that an assessment of the effects of disposal of sewage sludge on the biological component of the marine environment cannot be achieved by the use of chemical analysis alone. Complementary studies of the biota, either in their natural assemblages or as transplanted groups, is clearly a more appropriate approach in determining a measure of the impact of the sludge on the environment.

Additionally, it is possible to bring samples into the laboratory and carry out toxicity tests on sludge, or bioassays on environmental samples. The samples may be of the sludge itself, the water into which the sludge has been released, or samples of the sediment in and around the disposal site. In all cases, the sampling strategy will be determined by the question being posed.

Study of the community structure of benthic organisms has been the traditional method for field assessment of biological effects of disposal of sewage sludge, and indeed of most other discharges to sea. Aside from the practical reasons for the choice of the benthic fauna (notably a sedentary habit), they have the merit of allowing assessments of the effects of a discharge

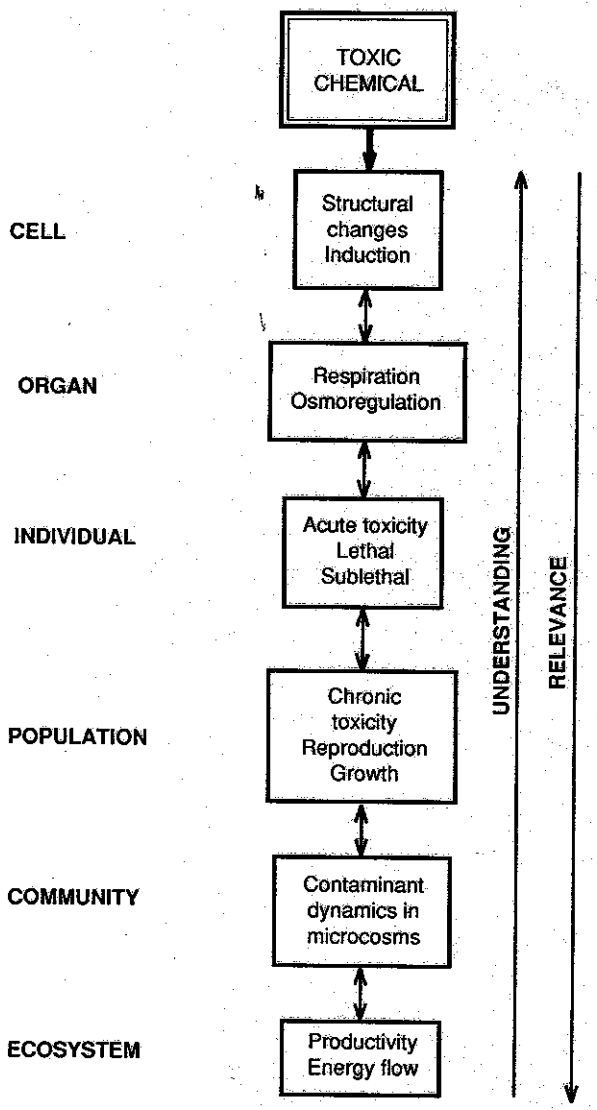


Figure 1. Effects of toxicants occur at different levels of biological organisation. Toxic effects are best known and understood at the cell and organ level,while the ecosystem and community levels are least understood although most relevant (from Haux and Forlin, 1988).

integrated over an appreciable time, and at a level of biological organisation which fits the usual conception of a 'relevant' environmental target - i.e. they show what is actually there, and what is occurring at the sea bed in response to the discharge.

However, there are costs attached to the effective conduct of field surveys of the benthos, not least because of the labour-intensive nature of the work and the difficulty, frequently encountered in dispersive areas where impact is less easily detected, of separating the effects of sewage sludge from high natural variability.

While major successional changes in the benthos in response to discharges are to a degree predictable, it is clear that a comprehensive understanding of all interactions which account for variability is unlikely to be feasible in most routine programmes. This gives rise to a dilemma (not peculiar to marine ecology) that the most relevant target for assessment of effects of pollution, namely the community in its entirety, is also frequently the least well understood. This is illustrated in Figure 1 from Haux and Forlin (1988).

In the face of such natural complexity, it is thus understandable that effort has been directed at the identification of 'alternative' measures which, as well as easing the burden of interpretation, might offer greater sensitivity and predictive capability. Clearly, the facility to accurately predict changes in populations and communities from a straightforward test on a single species is a most attractive, if regrettably an unrealistic, proposition. In this respect, some cautionary remarks from Bayne *et al.* (1988) are appropriate:

> '... there is no single biological measurement that will serve to indicate the effects of pollution'.
>
> 'The pressures to adopt biological measurements within environmental monitoring programmes are great and, as a consequence, so are the temptations to expect (and to claim) more of the available biological techniques than is scientifically reasonable'.

No ecotoxicologist would claim, therefore, that predictions of actual effects of complex wastes can be made from a few single-species tests. However, predictions can be made of the *likelihood* of significant effects, even though the nature of these effects is unpredictable.

A number of recent initiatives have helped to focus attention on the potential for application of a variety of biological effects measures, notable among these being meetings and workshops under the auspices of the International Council for the Exploration of the Sea (ICES) and the Intergovernmental Oceanographic Commission (IOC). These have served to highlight the potential advantages and limitations attached to the application of several novel techniques in 'routine' assessments of anthropogenic effects on various spatial and time scales. Such work has been widely reported in the literature, notable syntheses being given by McIntyre and Pearce (1980) and Bayne *et al.* (1988).

Of special relevance to the issue of point-source monitoring will be the publication of results from an ICES/IOC Workshop on Biological Effects of Contaminants, held at Bremerhaven in March 1990 (Stebbing, in preparation). This included the applica-

tion of a variety of measures along a gradient of contamination arising from a North Sea oil production platform, and in due course will provide a substantial amount of new information regarding the utility of these measures in monitoring programmes.

The purpose of this report is to consider only those techniques which on present evidence have shown, or are close to showing, the potential for specific application in routine surveys of sewage-sludge disposal sites. In selecting these techniques, priority was therefore given to those which had previously been deployed with some success at UK sites, and which appeared to be the most relevant in terms of assessments of field effects at the population level.

Of those selected, most involved tests on whole organisms. Regarding biochemical indices, determination of polychaete enzyme activity was chosen, because of the notable efforts that have been made to link observed changes near to waste disposal sites with those occurring in the benthos at the population and community levels. Lastly, the measurement of bacterial luminescence, as an indicator of water quality, was selected as an example of an established 'off the shelf' method, which appeared to have potential for monitoring effects of sewage-sludge disposal.

3. LEGISLATIVE/CUSTOMER REQUIREMENTS

In the UK, statutory control of disposal of sewage sludge to sea comes under the Food and Environment Protection Act (FEPA), Part II, 1985 (Great Britain - Parliament, 1985). This control is not 'static', since the Act explicitly recognises (*inter alia*) the continuing role of the international Oslo and London Dumping Conventions (Great Britain - Parliament, 1972a and b), in influencing the regulation of this sea disposal option. The relevant parts of the FEPA relating to the requirement for scientific monitoring include Section 8, item 3, which states:

"A licensing authority - (a) shall include such provisions in a licence as appear to the authority to be necessary or expedient -

(i) to protect the marine environment, the living resources which it supports and human health; and

(ii) to prevent interference with legitimate uses of the sea;..."

Also, Section 8, item 11:

" A licensing authority may vary or revoke a licence which the authority has issued if it appears to the authority that the licence ought to be varied or revoked -

(a) because of a change in circumstances relating to the marine environment, the living resources which it supports or human health; or

(b) because of increased scientific knowledge relating to any of those matters; ..."

Accordingly, the results from any monitoring programme should ultimately be capable of interpretation in these terms. The utility in monitoring programmes of any given measure of biological effect will depend on how it meets the various criteria for selection. These are reviewed in Section 6.

The 'customer' may be:

(i) the licensing authority, whose goal it is to identify and then specify appropriate test requirements, based on expert advice from a combination of 'in-house' and external sources; or

(ii) a department within the licensee's authority, which is delegated the task of conducting the work according to agreed protocols; or

(iii) an outside contractor, acting on behalf of (i) or (ii).

Discussion on this topic revealed the absence of any conflicting interests; such differences as there were rested on the emphasis placed on certain aspects within the broad framework of the target requirement. For example, if an outside organisation is sub-contracted by a licensee to carry out a particular test, it is important that an unambiguous protocol for the test procedure is provided.

The core monitoring programmes conducted by licensing authorities will ensure (*inter alia*) that the necessary expertise will be available in order to evaluate the outcome of surveys by licensees or their contractors.

A number of the issues raised in this section interface with Section 6, which deals with criteria for selection of tests. As will be seen, this recognises a pragmatic as well as a purely scientific dimension to test requirements. Accordingly, we have drawn out only those aspects which require special emphasis, the foremost of which is reflected in a MAFF statement of aims and principles for the monitoring of disposal grounds under FEPA (II), as follows:

(i) provision of information on which to assess licence applications (including 'check' monitoring by MAFF of the quality of information submitted by licensees);

(ii) assessment of compliance by licensees with licence conditions including, where appropriate, compliance with Environmental Quality Standards (EQSs); and

(iii) assessment of the effectiveness of licence conditions in complying with FEPA (II) section 8(1) (protection of human health, the marine environment and living resources).

Translated into working objectives, these are:

(i) limitation of the extent and intensity of the impact of disposal on the marine environment and its living resources, to levels as low as reasonably achievable, and which ensure compliance with the relevant EQSs; and

(ii) stabilisation of impact so that the input does not exceed the dispersive capacity; once the effect of dumping inputs have stabilised, the dispersive capacity should be sufficient to avoid any worsening trend.

'Customer' requirements which merit special attention include the conditions:

(i) that techniques should be suitable for one or more of the following purposes

- screening of sludge prior to discharge

- assessment of impact on the receiving environment

- monitoring to demonstrate compliance;

(ii) that proposed techniques to assess environmental effects should be applicable to all disposal sites, although this should not exclude those designed to study specific environmental compartments, or those of proven site-specific value; and

(iii) on purely practical grounds, that preference should be given to simple and reliable techniques. 'Complicated' techniques should still be included if they are sensitive to environmental impact, but excluded if their ecological relevance is questionable. Simple techniques which provide an indicator of an environmental effect should be considered, *provided* that they are combined with and complement other relevant techniques.

Further practical considerations arising from the above include the conditions:

(i) that a range of techniques will be required to cover each environmental compartment. Attention should be paid to obtaining a measure of effect in the water column, particularly in the context of 'far-field' effects;

(ii) that methods giving results which could provide the basis of a numerical standard should be investigated;

(iii) that priority should be given to methods which are amenable to standardisation and harmonisation. This would tend to favour the technically more simple tests;

(iv) that the results generated should allow interpretation of effects of discharge with a minimum of ambiguity; and

(v) that the results, and their interpretation, should be easily communicable to those responsible for waste-disposal management and, where necessary, to the wider public.

4. COMPOSITION AND TOXICITY OF SEWAGE SLUDGE

Sewage sludges disposed of to sea typically contain a very wide range of physical and chemical constituents at concentrations which will vary notably according to the balance between industrial and domestic wastes which have previously been directed to treatment works. Some commonly determined constituents are given in Table 1. It is not our intention to review sludge composition in detail, but rather to consider the medium as an entity from the standpoint of:

(a) the induction of biological effects following disposal to sea;

(b) the selection of appropriate test measures; and

(c) the interpretation of the outcome of these tests.

Table 1. Some physico-chemical properties of sewage sludge. (Unless otherwise specified, these are weighted averages of all UK sewage sludges disposed of to sea in 1987; source: MAFF, unpublished data)

Constituents	Dry	Wet
Dry solids (%)	-	3.2
Organic matter (%)	64.2	2.0
Organic carbon (%)*	28.2	0.9
Total nitrogen (%)+	5.6	0.2
Total phosphorus (%)+	1.5	0.1
Mercury (ppm)	4.8	0.2
Cadmium (ppm)	13.6	0.4
Chromium (ppm)	547.4	17.3
Copper (ppm)	601.5	19.0
Nickel (ppm)	86.6	2.7
Lead (ppm)	662.0	20.9
Zinc (ppm)	1816.7	57.4

*assuming that 44% of OM = OC
+not available for all arisings

4.1 Some properties of sewage sludge which affect the selection of biological techniques

Very little information exists on the toxicity of sewage sludges to marine organisms. A comprehensive study was carried out by Franklin (1983) using dilutions of sludges from 10 different sources (digested, primary, lagoon and primary/secondary) on adults of one species of fish, one crustacean (including larvae) and three species of molluscs.

All but two of the sludges exhibited low acute toxicity to adult animals (96 h LC_{50} greater than 10 000 µl l^{-1}), but newly hatched larval shrimps were found to be between 10 and 500 times more sensitive than adults of the same species. This study shows that acute toxicity studies provide little useful information on the environmental impact of sewage sludges, because they do not simulate the complex environmental events and the effects of chronic exposure which occur at disposal grounds.

The particulate matter in sewage sludge may induce stress by physically preventing normal feeding processes and apparently indicating toxic stress, which is actually due to nutritional stress. Some techniques may only be usable if particulate matter is removed. However, this is not meant to imply that the solids component can be ignored, since this is an integral part of the material released to the environment; indeed, a high proportion of many chemical constituents may be bound to the particulate fraction.

A mixture of sewage sludge in sea water is a biologically active medium in which the binding state of metals in particular, and therefore their biological availability, changes very rapidly. The nature of the material, its chemical instability and changing toxicity therefore indicate that short-term techniques to estimate its toxicity are preferable.

Sewage sludge may exert deleterious effects in different ways, due to particulate loading, nutrient concentrations (see sub-section 4.2) or the toxic constituents of sewage sludge. It is likely, therefore, that no one technique will provide an adequate indication of its likely biological effects in the environment.

The toxicological literature provides numerous examples where extrapolations from a single species to other individual species or assemblages are unreliable. Any application of biological effects techniques should not depend on one species.

4.2 Nutrients in sewage sludge: the potential ecological impact of marine disposal

In addition to trace contaminants (of industrial, agricultural and domestic origin), which pose problems of toxicity to the marine environment, sewage sludge contains large quantities of organic carbon, nitrogen and phosphorus.

Organic carbon represents an energy source which can readily be utilised by planktonic and benthic organisms. Organic enrichment of the receiving environment may lead to a substantial increase in the numbers and biomass of opportunistic species, possibly at the expense of others (i.e. secondary production can be enhanced and diversity decreased). Sub-lethal toxic effects of trace contaminants may, to some extent, be masked by the availability of abundant energy. An enriched energy supply can help to meet the energetic costs of detoxifying, metabolising and excreting ingested or absorbed contaminants, whilst minimising impact on normal growth and reproduction.

Nitrogen and phosphorus in sludge represent a nutrient resource for phytoplankton populations. The accumulation of these nutrients as a result of marine disposal can, especially in confined waters, lead to increased primary production, which may stimulate secondary production. However, blooms of atypical species have been observed to destabilise or damage the structure of marine communities by excluding other algal species normally used as food, or by releasing toxic metabolites. In nearshore areas, water and sediment quality may be impaired by the high oxygen demand associated with the decomposition of large algal populations.

Predictions of toxicity to animal species, where possible, should take into account the possible ameliorative effects (expressed at the population level) of an enhanced energy supply. The potential of a sludge for eutrophication should be assessed not only by the use of a standard algal assay, but perhaps also by an assay utilising a species known to represent a particular risk in confined waters and nearshore areas.

4.3 Hormesis

Hormesis is defined as the *stimulatory* effect of low levels of toxic substances and is a common phenomenon. Although it is commonly cited in the toxicological literature (see review by Stebbing, 1982), its occurrence is often not reported because stimulation is usually the reverse of the expected effect and data are therefore set aside as anomalous. However, for those processes, like growth, that offer scope for a supernormal response, it is a common feature of dose-response curves for a wide range of toxicants as a stimulatory peak at concentrations just below those that inhibit growth.

Studies of growth control indicate that hormesis is due to regulatory over-corrections to the inhibitory challenge presented by low levels of toxic agents. These agents only begin to inhibit growth when the capacity of control mechanisms to counteract an inhibitory challenge is exceeded (Stebbing, 1987). Hormesis therefore indicates the action of adaptive homeostatic mechanisms whose response is greater than that necessary to neutralise toxic inhibition.

5. REVIEW OF SELECTED LABORATORY-AND FIELD-BASED TECHNIQUES

5.1 Background

In the following account, distinction is made between bioassays and toxicity tests as follows:

(i) a bioassay quantifies the *biological potency of a water sample* of unknown composition, in terms of a chosen response of an organism; and

(ii) a toxicity test quantifies the concentration/effect relationship for known chemicals or complex mixtures (e.g. dilutions of sewage sludge) and the chosen response(s) of an organism. In other words, it measures the *sensitivity of an organism* to a chemical or complex mixture.

Most of the tests specified below may be used either as toxicity tests or bioassays depending on circumstances. The way in which they can be allocated is shown in Section 7. Here, for convenience, we have considered a selected range of tests according to the target medium (namely the water-column within which most tests are conducted) and sediment. Finally, consideration is given to the utility of mesocosm and microcosm studies for the experimental study of effects on the ecosystem.

Selection of tests for further consideration (below) was restricted to those which have previously been employed with some success at sewage-sludge disposal sites, or sewage outfalls, or which appear to show promise for routine application on the basis of current knowledge. Thus the coverage is not exhaustive.

5.2 Water-column tests

5.2.1 Dispersion of sewage sludge following release

When sewage sludge is released into the sea in the wake of a disposal vessel, there is (initially) a high concentration of sewage-sludge solids and liquor in the area around the vessel. The rate at which the slick subsequently disperses will depend on prevailing weather and tidal conditions, but some of the solids will eventually settle out and reach the sea bed. Since most of the licensed disposal sites are situated in highly dispersive environments, in the near-field, accumulation of material on the bed is small. The accumulation can be detected by the use of tracers, either those persistently occurring in the sludge (for example, faecal bacteria) or added tracers (usually radioactive tracer). These techniques are widely used in monitoring studies.

The sludge solids and liquor interact with organisms in the water column, and a variety of tests have been developed to investigate the effects of pollutants in this area. The exposure of test organisms may take place either in the field or in the laboratory.

5.2.2 Physiological indices of sub-lethal stress

In recent years, there has been a move towards using tests aimed at identifying sub-lethal effects of toxicants on indigenous organisms, or organisms transplanted into the sewage-sludge disposal area. Bayne *et al.* (1985) discuss the use of novel sensitive techniques for the assessment of pollution on marine organisms, and it was the success of some of the techniques given in that work that prompted their application to sewage-sludge disposal sites.

The technique which has received the most attention is the measurement of 'Scope for Growth' (SFG) in the common mussel *Mytilus edulis*, a technique developed at the Plymouth Marine Laboratory, and recently applied by the Water Research Centre (WRc) (e.g. Lack and Johnson, 1985). *Mytilus* is frequently used as a monitoring organism because of its well understood physiology, the ease by which it can be handled both in the laboratory and in the field, and its widespread distribution and hence relevance. However, it is not commonly found at sewage-sludge disposal sites, and so transplanted populations are required.

SFG is an energetic measure of the physiological fitness of an organism in response to changes in the environmental conditions in which the animal is living. It is measured in terms of the energy available for growth and/or reproduction; under normal circumstances, organisms have surplus energy after the basic metabolic requirements have been met and this shows as positive SFG. If, however, an animal is stressed by adverse environmental conditions, or is required to expend significant amounts of energy on detoxification or tissue repair, then the amount of energy available for growth is reduced. In particularly adverse conditions, SFG may be negative.

The techniques involved in the measurement of SFG are relatively simple: measurement of food intake (as clearance rate) and the efficiency of absorption, respiration rate (as oxygen consumption) and nitrogen excretion. All of these measurements can be readily converted to energy units and an energy balance can be calculated.

5.2.3 *Comments arising from the WRc's experience with the use of mussels in toxicological studies*

As a consequence of the difficulties inherent in studies involving field deployment (such as estimation of time-averaged local conditions of food quality and quantity), and on the basis of the results of 26 studies on sensitivity of components of the SFG equation , the WRc now measures only feeding and respiration rates. In all studies, feeding rate was the most sensitive variable; respiration rate made a significant contribution in less than 8% of studies.

The term 'Scope for Growth' is therefore not strictly appropriate; rather than estimating true, *in situ*, energy budgets, the WRc approach has been to use changes in physiological rates as direct indices of sub-lethal stress. In the majority of studies known to the WRc, change in feeding rate has been the main response observed.

When the aim of the study is to detect toxicological effect (rather than to predict effects on production and populations), measurements of feeding rate alone will generally be sufficient.

Further details of these techniques are given at Annex 2. A draft protocol for the measurement of feeding rate in *Mytilus* is given at Annex 3.

In studies requiring the long-term exposure of mussels to contaminants either in the field or in the laboratory, it is recommended that (where practicable) measurements also be made of net growth (length and flesh weight) and of accumulated tissue burden of contaminants. These measurements will provide complementary and additional information of value in drawing conclusions regarding toxicity.

In field studies, the following approach is recommended in order to achieve a degree of quality control:

(i) feeding (and respiration) rate in the experimental batch of mussels should be measured prior to deployment. This provides a degree of quality control in relation to both the source of experimental animals, and to stresses not associated with contaminants but imposed by the processes of deployment and recovery;

(ii) effects should be interpreted in terms of the relative, rather than absolute, values for rates of physiological or energy metabolism calculated for different sites; and

(iii) at intervals, the period of post-deployment measurement should be extended for selected groups of 'exposed' mussels, to establish the degree of persistence of response, and thus the presence of short-term variability.

5.2.4 *Hydroid test*

The hydroid bioassay was developed about 15 years ago as a technique sensitive to environmentally realistic concentrations of toxic contaminants (Karbe, 1972; Stebbing, 1976, 1979). It was hoped, in this way, to introduce greater experimental rigour to biological studies of environmental pollution. (For a more recent review and some papers on the use of hydroids in pollution studies, see Persoone *et al.*, 1984; also Stebbing *et al.*, 1987.)

Different techniques employ various indices such as colonial growth rate, tentacle shortening, gonozooid production and the curving of stolons during growth. The hydroid bioassay offers advantages such as the use

of a single genotype and considerable sensitivity, with species that can be easily cultured in the laboratory for indefinite periods. However, it is also labour-intensive to maintain, and responses may require over 10 days to develop.

Nevertheless, hydroids have been used effectively for pollution studies of various kinds, including those in Swansea Bay, the River Tamar, and in the North Sea on production water from oil drilling platforms (R. R. Stephenson, personal communication), and on concentrates of contaminants as eluates from XAD resin columns (L. Karbe, personal communication). However, it has to be assumed that the chemical stability of water samples remains the same for the duration of the bioassay.

In bioassays conducted on water samples, taken at intervals following the discharge of sewage sludge to sea, the weakness in this assumption was clearly demonstrated (A. R. D. Stebbing, J. J. Cleary, J. E. Thain and M. Brinsley, unpublished data). Significant changes in the complexation states of metals during the 11-day period of the hydroid bioassay indicated why the oyster embryo bioassay, conducted over 48 h on the same water samples, gave results that related much more closely to the data on metals.

5.2.5 *Fish egg and fish larvae tests*

The literature on the use of developmental abnormalities in fish embryos to monitor pollution is extensive and has recently been reviewed (Westernhagen, 1988). Significant correlations have been determined between a number of anthropogenic factors and observed abnormalities. However, physical factors such as low oxygen and salinity, and high temperature, have also been implicated in the elevation of abnormalities and mortalities in developing embryos, in a number of studies (Chang and Longwell, 1984; Westernhagen, 1974; Braum, 1973).

For the incidence of developmental abnormalities to be of use in monitoring the health of marine environments, the areas in question must be free from extreme hydrographic conditions and, together with the movements of spawning populations, well understood (Westernhagen *et al.*, 1988). The highly dispersive nature and coastal location of the majority of UK sewage-sludge disposal sites effectively precludes the use of these techniques in monitoring the biological effects of sludge disposal on embryos caught in the field. The use of similar techniques with invertebrates which carry their developing embryos may overcome some of these problems, but requires evaluation.

Embryonic abnormalities may have potential as a laboratory bioassay on transported materials but, whilst the relevance is undisputed, the seasonal availability of indigenous spawning stocks may necessitate further studies on other fish (e.g. turbot or sheepshead minnow), such as have been conducted by Hughes *et al.* (1989). Given that the basic parameters to be assessed in field populations and those exposed in the laboratory would be the same, then this work should be amenable to extrapolation and intercomparison.

5.2.6 *Bivalve embryo test*

The early life stages of organisms are often the most sensitive to the effects of pollutants. A test using developing embryos makes use of the fact that, immediately following fertilisation, the rate of cell division is very high, giving many opportunities for any pollutant to cause damage to DNA structures, and also to affect the surface charges on the ball of cells causing a breakdown in cell differentiation. Either of these effects will prevent the normal development of the embryo. Also, the duration of the test is limited, so that embryos with reduced rates of development contribute to the effect measured. The successful development of the embryonic stage of an organism is a measure of the potential for recruitment to succeeding generations, and hence the survival of the species in that locality.

The bivalve embryo test is one which is widely used, notably by MAFF, to monitor waste disposal areas in the coastal waters around the UK. The technique requires a supply of ripe male and female oysters or mussels to provide a source of embryos, whose development is measured after an incubation period of 24-48 h. Because the larvae are still very small, it is necessary to filter the samples prior to their addition, in order to ensure that they can be seen clearly at the end of the test.

The mussel embryo test has been used to investigate the toxicity of water samples collected in the wake of a sludge disposal vessel, and also of sea water extracts from sediments collected at sites known to have elevated levels of faecal bacteria (see sub-section 5.3). Water samples were collected at regular intervals from a patch of water identified by a drogue dropped in the wake of the sludge disposal vessel. The water samples were pressure-filtered immediately following collection and the water was subjected to the embryo toxicity test; the residue on the filter was dried and weighed. Toxicity decreased with time from release, and a similar pattern of suspended solids was observed, indicating that the reduction in toxicity was associated with dilution of the slick (Roddie *et al.*, 1989).

A draft protocol for conducting the oyster embryo test, along with some examples of results of surveys, is given at Annex 4.

5.2.7 Planktonic algal tests

Typically, these involve measurement of growth of cell cultures in water samples by means of cell counts, chlorophyll concentrations or C_{14} uptake, and are technically relatively straightforward to conduct. The techniques are widely-used and well-developed, frequently based on standard United States Environmental Protection Agency (US EPA) guidelines (Walsh, 1987). Walsh (1988) provides a useful statement of principles governing the conduct of tests employing unicellular algae.

Fairhurst *et al.* (1987) reported considerable enhancement of growth of *Phaeodactylum tricornutum* in extracts of sewage sludge. Growth at 1% sludge was twice that in controls after 96 h. This method, using a relatively insensitive alga, appears to be best suited to estimating possible effects of eutrophication. A range of more sensitive species is available. For example, Walsh *et al.* (1988) have recently recommended use of the marine diatom *Minutocellus polymorphus* as a standard test organism, following comparative experiments with a range of toxicants.

In the UK, an early example of the use of a planktonic algal test at a sewage-sludge disposal site is the work of Burrows and Sharples (1973), using *Skeletonema costatum* in Liverpool Bay. MAFF has used the alga *Tetraselmis suecica* in a test which is suitable for deployment in the laboratory and field. In the field, replicate samples are spiked with known concentrations of actively growing *Tetraselmis*. Culture flasks are maintained for 5 days on a platform shaker, under conditions of constant temperature and light. Cell densities are then determined using a Coulter Counter or haemocytometer.

The results are examined for four main effects relative to control cultures, namely:

(i) enhanced logarithmic growth, indicating enrichment;

(ii) normal logarithmic growth;

(iii) no increase in cell number, indicating lack of cell division; and

(iv) net decline in cell number, indicating cell death in some portion or all of the culture.

For treatments which show a response, follow-up culturing of extracted cells in clean medium for a period of 9 days, along with the appropriate controls, provides a means of estimating recovery, and hence the permanence of any toxic reaction.

5.2.8 Zooplankton tests

Zooplankton tests fall into two general categories: those conducted on small (c. 1 mm in length) copepods and those conducted on larger crustaceans such as decapods, peracarids and mysids. Copepods and mysids are generally more sensitive than other taxa, and a recent paper (Miller *et al.*, 1988) describes methods of assessing the toxicity of sewage sludge to both groups. The copepod species most widely used are of the genera *Acartia* and *Eurytemora*; tests are performed on adult animals, and mortality is the most common endpoint.

Both laboratory-cultured and wild-caught specimens have been used, and standard methods for the maintenance of laboratory cultures have been published (see, for example, Miller *et al.*, 1984). When laboratory-cultured specimens are used, it is usual to produce a uniformly-aged cohort from eggs collected within a restricted period of time. Tests with copepods may be conducted with filtered or unfiltered dilutions of sludge, and are generally of 48-96 h duration.

Culturing and testing procedures are simple and of relatively low cost, and could readily be adapted to a number of other indigenous UK species.

The use of mysids in toxicity testing has been reviewed by Nimmo and Hamaker (1982). An American species (*Mysidopsis bahia*) is the most widely-used mysid, and standard methods have been published for its culture and maintenance (e.g. US EPA, 1978; Nimmo *et al.*, 1977) and testing procedures. *Mysidopsis* breeds more continually than indigenous UK species, and also grows faster. At present, none of the latter species can yet be considered as available alternatives.

With an adult length of c. 10 mm (attained in less than 28 days), *Mysidopsis* is probably easier to handle than copepods. Mortality is a frequently-used endpoint, but tests involving studies of growth, development and full life-history are also widely applied. As with copepods, mysids may be tested in filtered or unfiltered samples. Tests involving estimates of acute mortality may last between 48-96 h, while the assessment of sub-lethal endpoints, such as fecundity, may take 7-14 days.

5.2.9 Microbiological tests

The use of faecal bacteria as indices of contamination of marine sediments by sewage sludge is well established, and protocols for conducting such work have recently been given (MPMMG, 1989). Methods have also been evolved which utilise properties of bacteria as indices of biological effects of waste disposal. Of these, the 'Microtox' test appears to be the most promising method presently available for application to monitoring sewage-sludge disposal sites.

This is a commercially available test which makes use of the light given out by a marine bacterium when incubated with suitable substrate. When the bacteria are stressed, the light output is reduced. The test has been widely used for assessing the toxicity of effluents and water samples, and has also been used to investigate sediment toxicity (see Bulich, 1979; Ribo and Kaiser, 1987; also Sub-section 6.3).

5.3 Sediment tests

The success in the development of techniques to measure toxicity of sediments has been less than that for measurement of toxicity in the water column. The approaches have been along three lines:

(i) examination of *in situ* benthic populations;

(ii) removal of natural populations by sieving followed by introduction of selected test species to the sediment; and

(iii) mixing of the sediment with clean sea water followed by measurement of the toxicity of the elutriate.

In the last case, the potential clearly exists to employ a number of water-column tests which have already been described.

5.3.1 In situ populations: polychaete enzyme activity

The relevance of using *in situ* testing has already been stressed. One such technique has been developed recently, based on assessing changes in the levels of enzyme activity in polychaetes collected from areas impacted by sewage sludge. The sensitivity of the activities of a number of key glycolytic enzymes, in a selection of infaunal polychaetes, to changes in sedimentary chemistry brought about by increasing organic enrichment, has been demonstrated (Blackstock, 1982, 1984; Blackstock and Filion-Myklebust, 1983), and procedures have been developed to assess the extent of changes in enzymatic activity in relation to effects of the increasing impact of sewage (Blackstock *et al.*, 1986).

The success of the technique is dependent on the careful selection of a target species in accordance with ecological criteria appropriate to the test area and the expected impact, and biological criteria which will permit the signals from externally induced changes to be identified against the background fluctuations in normal enzyme activities (Pearson and Blackstock, 1983). A group of potentially useful species, from soft sediments in Scottish and Scandinavian waters, has been identified using such criteria.

A drawback to the technique is the necessity to establish the range of levels of normal activity in those enzymes in the target species known to be sensitive to environmental change. This requires considerable development work prior to the routine use of any particular target species as a test organism. To date, four such species of polychaete have been assessed in some detail, and one (*Glycera alba*), a long-lived infaunal predator, has been used routinely in a number of areas impacted by organic wastes, including sewage sludge.

Once the background work has been undertaken, the measurement and assessment of levels of activity in an appropriate suite of enzymes is technically simple and relatively inexpensive. In parallel with *in situ* observations, such an approach should also have potential as a laboratory bioassay of contaminated samples of sediment.

5.3.2 Laboratory sediment tests

There are few reliable and robust toxicity tests available for sediments; examples of LC_{50} tests, which are currently available, are the US Army Corps of Engineers and the US EPA techniques for determining acute toxicity of dredged spoil and settleable sewage solids (United States Army Corps of Engineers, 1978; United States - Environmental Protection Agency, 1978). These are multi-species tests, the members of which are acclimatised in control sediment and then covered by a layer of the test material.

The most widely-reported test under this category is the phoxocephalid amphipod test described by Swartz *et al.* (1985). Swartz *et al.* (1984) describe the use of this test in assessing the toxicity of sewage sludge added to sieved sediments. Toxicity was related to the percentage of volatile solids added as sludge. A modification of this technique has been used by Southern Water to investigate the toxicity of intertidal sediments contaminated by industrial effluents (unpublished data).

The WRc has attempted to measure the toxicity of sediments collected from sewage-sludge disposal grounds, but at the present time has not been able to establish a reliable and sensitive methodology. It is currently developing techniques for culture and testing of the intertidal amphipod *Corophium*, with the aim of providing an acute test comparable to that on phoxocephalid amphipods.

5.3.3 *Elutriate tests*

This approach makes use of the assumption that, if a sample of the sediment is shaken with clean sea water, a proportion of the adsorbed contaminants will transfer to the aqueous phase, and that its toxicity can then be determined by conventional water-phase techniques. (Note that it will be necessary to determine this proportion, in order to make this a fully quantitative technique.)

The WRc has applied this test, using bivalve embryos, to sea-water extracts of sewage sludges from two sources, and to sediments from the zone of impact of three sewage-sludge disposal grounds. In one of the sediment studies, no toxicity was observed.

In the remaining two sediment tests, toxicity was observed, and the EC_{50} was estimated to be equivalent to approximately 2.5% sediment in sea water (Roddie *et al.*, 1989). Extraction of sediments was by agitation of a 1:9 mixture with sea water, followed by settling and filtration. The EC_{50}'s estimated from sea-water extracts of two sewage sludges were very similar to each other, at approximately 0.15%. One of these estimates was reported by Johnson *et al.* (1988), and was derived from exposure of test organisms to the same sludge as that used in a study of the 'Scope for Growth' of adult mussels.

Using the 'Microtox' test (see Sub-section 5.2.9), water extracts of sediments appear to cause little toxic response, but it is possible to produce a significant response if solvent extracts of the sediment are used.

The procedure has been used to investigate toxicity along transects either side of sewage outfalls. The sediment is shaken with dichloromethane (DCM), and a back-extraction made into ethanol which is then used in the toxicity test. The results show a decrease in extractable toxicity with distance away from the outfall. The significance of the toxicity measured in this way is not yet known; however, the method provides a potentially rapid indicator of dispersion away from the disposal point.

5.4 Mesocosm and microcosm studies

5.4.1 *Mesocosms*

The use of mesocosms to simulate field conditions during experimental examination of the fate and effects of various contaminants in the marine environment has become increasingly common (Underwood and Peterson, 1988). They have the advantage of allowing the assessment of simultaneous effects on pelagic and benthic biota of induced and natural perturbations under controlled conditions. In the larger and best designed of such systems, the simulations apparently approach the natural level of response (Lambert and Oviatt, 1986).

Mesocosm facilities have been used on at least two recent occasions, to investigate the impact and fate of sewage sludge dispersed to the marine environment. Experiments are currently in progress at the Department of Agriculture and Fisheries for Scotland (DAFS) mesocosms at Loch Ewe, but this work has yet to be reported. One extensive experiment into the impact of sewage sludge on the marine environment, using the Marine Ecosystems Research Laboratory (MERL) mesocosms at Rhode Island, has been recently reported (Keller *et al.*, 1987; Oviatt *et al.*, 1987). These mesocosms simulate the lower Narragansett Bay environment and were used to assess the fate and effects of sewage sludge from a local waste treatment facility over a 4-month summer period.

The experiment successfully quantified rates of settlement and degradation of sludge at different treatment levels and apportioned the differing rates of recycling to components of the water column and sediments at those levels. Toxic responses were not found, but the effects of hypoxia at the higher levels of treatment were well documented.

Such large-scale experiments are extremely useful tools for assessing the interactive effects of sewage sludge over all compartments of a system; indeed they may well be the only means of achieving this assessment. However, their expense in both time and capital-intensive facilities may preclude their deployment for routine monitoring purposes.

5.4.2 *Microcosms*

The Biological Effects Task Team is grateful to D. J. Murison, DAFS, Marine Laboratory, Aberdeen for preparing the following account.

Microcosms may be arbitrarily distinguished from mesocosms in that, unlike the latter, they are normally restricted to laboratory-scale designs, not exceeding 1 m^3 in volume, in which controlled experiments can be conducted. In practice, microcosms have ranged from vial-sized systems of a few millimetres in volume to larger units approaching the upper limit indicated above. Past studies have investigated a wide range of ecological relationships in simulations of both pelagic and benthic ecosystems.

A basic assumption in the use of microcosms has been that any experimental design which will successfully

maintain selected biotic components of a natural ecosystem for a prescribed period, must be simulating at least part of the relevant field environment. Recent reviews of experimental methods and results in microcosm-based ecological studies include those of Giesy (1980) and Lasserre (1990).

Microcosms have been employed in benthic ecological investigations for 25 years or more. An early study of energy flow in a sand ecosystem (McIntyre *et al.*, 1970), which focused on a natural meiobenthic assemblage, was successfully maintained in cylindrical chambers of 29 cm diameter for 1 year. In this instance, the experiment was designed to investigate pathways and relationships rather than to simulate a particular ecosystem. A similar study, investigating interstitial metabolism in exposed intertidal sand, was conducted over a period of 4 months using 18.5 cm diameter columns with simulated tidal inputs (McLachlan *et al.*, 1981).

Reports on the use of microcosms in studies related to sewage sludge, as in the case of mesocosms, are somewhat limited and have focused on effects in meiobenthic assemblages. The size of experimental units employed has reflected the more restricted basic requirements of this group.

The multi-microcosm, medium-term (12 weeks) experimental approach (described in Sandulli, 1987) has been applied by the DAFS Marine Laboratory, Aberdeen in studies of the effects of sewage sludge on selected meiobenthic taxa (unpublished). The principal advantages of this type of experimental design, incorporating small-scale (44 mm diameter) sediment columns, are as follows:

(i) large numbers of relatively undisturbed replicate samples can be easily obtained by coring in the natural environment;

(ii) following treatments, variability in abundance of target meiobenthic taxa, due to sub-sampling, can be eliminated by sacrificial sampling and analysis of complete replicates; and

(iii) experiments on the above scale can be maintained and monitored within the confines of a relatively small 'constant temperature' facility.

Small-scale experimental set-ups of this type may prove useful as a means of investigating responses in particular elements of an ecosystem, rather than as comprehensive simulations of the corresponding field environment. Thus, whilst experiments might be designed to investigate individual characteristic effects or pathways in simulated ecosystems set up to accommodate the selected target(s), it seems unlikely that a system on such a scale could realistically encompass a comprehensive spectrum of environmental parameters applicable to conditions in the field.

Principal advantages in the use of microcosms are, perhaps, reflected in the relative convenience of laboratory-based systems which can normally be set up, maintained and monitored within a significantly smaller budget than that required by mesocosmic and larger-scale installations.

6. CRITERIA FOR ASSESSING THE UTILITY OF BIOLOGICAL EFFECTS TECHNIQUES AT SEWAGE-SLUDGE DISPOSAL SITES

6.1 Objectives in using biological techniques

The main reason for using biological effects techniques is in support of the requirements of the Food and Environment Protection Act, Part II (Great Britain - Parliament, 1985), namely to 'protect the marine environment, the living resources it supports and human health' (see Section 3). This phraseology is very broad, and certain customer requirements may be more specific. For example:

(i) it may be important to use commercial species (or species shown to have similar sensitivity), because they represent a valued resource and are of public concern;

(ii) if it is intended to look for 'change' *per se* it can be as important to consider stimulatory as well as inhibitory effects. For example, microalgae are as likely to respond to the concentrations of nutrients in sludge as to the concentrations of toxicants. Therefore, it is obviously important to know whether a chosen technique is intended to detect the effects of particulates, nutrients or toxic constituents in sewage sludge; and

(iii) it may be important for the customer to use the same technique both as a method for carrying out toxicity tests on sewage sludge in the laboratory, and as a water quality bioassay for field samples. It is clearly an advantage to use the same technique where it is intended to correlate the results of laboratory and field investigations.

6.2 Types of approach relevant to determining the impact of the disposal of sewage sludge at sea

Criteria for judging techniques vary depending upon the kind of approach adopted, which may be:

(i) use of indigenous organisms
- water column
- sediment infauna;

(ii) deployment of transplanted organisms from single populations;

(iii) water quality bioassays
- with suspended particulate matter
- without suspended particulates;

(iv) sediment bioassay; and

(v) mesocosm experiments
- particularly for intercalibration.

6.3 List of criteria for assessing biological effects techniques

Several criteria are presented below, which together provide the basis for assessing the utility of biological effects techniques in monitoring sewage-sludge disposal sites. It should be noted that the relative importance of these criteria will vary according to whether a particular technique is being deployed as a laboratory toxicity test or as a bioassay of environmental quality. This matter will be dealt with in Section 7.

6.3.1 Availability of the test species

If the test species cannot be maintained in the laboratory continually, then it should be available from a convenient source such as an aquacultural operation, culture collection or in some dormant or suspended state, e.g. as cysts or deep-frozen specimens.

6.3.2 Importance of the test species in ecological or economic terms

As test species, MAFF has tended to prefer the use of commercial species or those closely related to them, largely because they are convenient to obtain or handle, and are available all the year round.

6.3.3 Cost effectiveness

This can be considered in terms of the cost of setting up a laboratory to perform a technique (i.e. training and capital equipment), but the most important criterion is the total cost per measurement.

Any technique used must also allow reporting of the results within a reasonable period of the completion of the study in the field and in the laboratory.

6.3.4 Ease of use

If considerable time and skill are required to learn a technique, few will end up using it. However, complex techniques are sometimes very robust, while simple techniques, requiring an element of 'green fingers', can prove difficult.

6.3.5 Rapidity of technique

As a general rule, the shorter the test the cheaper the cost, and hence more can be done for the same investment of effort. Sensitivity can increase with longer periods of exposure but, where the contaminant is likely to degrade or the water sample is chemically unstable, a rapid test may be essential.

6.3.6 Genetic uniformity

If it is important to minimise inherited variability, single genotypes can be used. However, over a long period (months with a cellular system and years with a metazoan), there is significant genetic drift in asexually reproducing organisms. The rationale for using inbred lines is well established in mammalian toxicology, but has not been established for ecotoxicology where genetic diversity may provide stability to population sensitivity.

6.3.7 Quality (precision) of the data

The form and quality of the data should satisfy the usual statistical criteria. Implicit in this is the need to give reproducible results between experiments, as well as precision within them.

6.3.8 Bioaccumulative potential

Chemists prefer to monitor species that are bioaccumu-

lators in order to simplify analysis, but also as integrating samplers. It is a short step to use the same species in biological effects techniques, with the added advantage of a data base for tissue burdens of contaminants, which can help in determining causality. For some organisms and contaminants, relationships between tissue burdens and effects are well enough established to be able to predict one from the other.

Ideally, the chosen test species will have some predictable relationship between concentrations of contaminants in tissues and environmental concentrations, whether between tissue burden and water concentrations, or between tissue burden and sediment concentrations, because it will provide a good index of bioavailable concentrations of contaminants.

One qualification to the above is that those substances with a high bioaccumulative potential are being increasingly controlled, and attention is now focusing on those substances which are more readily metabolised and excreted.

6.3.9 Integration of effects

One of the most important advantages of using biological systems is their capacity to integrate the effects of numerous contaminants (known and unknown) in terms of a single index. Whilst the non-specific nature of most of the indices available is a distinct advantage, nevertheless it should be noted that specific responses can play a significant role in pollution monitoring. For example, in the case of tributyltin (TBT), specific morphological indices of its effects have served a crucial role in pollution monitoring. It is improbable that such 'substance-specific' effects could be found for each of the numerous chemicals in sewage sludge.

6.3.10 Sensitivity

Whether deployed in the field or in the laboratory, organisms must be sensitive to the concentrations of contaminants likely to arise from waste disposal operations. However, it should be said that tolerance and sensitivity are not mutually exclusive traits; 'Scope for Growth' in mussels is an attempt to derive a sensitive index in a tolerant organism. Water quality or sediment bioassays need to be sensitive enough to respond directly to environmental samples; lack of sensitivity is the factor most likely to limit the choice of techniques for bioassays.

However, recent developments in pre-concentrating toxicants from large volumes of water on ion exchange columns and determining concentration-response relationships for the eluate, is one way now being explored of avoiding the limitations of sensitivity.

This criterion should be viewed in conjunction with the need to examine species present naturally within a given study area, and with an understanding of availability of materials in sediments.

6.3.11 Discrimination

Graduated (rather than 'all or nothing') responses tend to be both more precise and more sensitive. Also, the response should be measurable over a sufficiently wide range of concentrations which are actually encountered, so as to adequately discriminate between levels of effect.

6.3.12 Relevance of technique to populations

The relevance of a biological effects technique to populations in the environment is a function of the nature of the sub-lethal response and the level of biological organisation at which it is measured. Where a technique uses effects at low levels of organisation (e.g. sub-cellular indices) it is important in the first instance to establish their quantitative relationship to effects at the organismal level. This should then allow inferences to be made regarding effects on populations, (e.g. if the observed effects include reproductive impairment).

Clearly, it is also important to be able to extrapolate from data on one species to another, otherwise *sensu stricto* toxicity data are only applicable to the test species. As species are patently more similar at sub-organismal levels of organisation, extrapolation should in principle be more feasible at these levels. However, as noted above, it will still be necessary to quantify the relationship between observed changes occurring at the sub-organismal and organismal levels, both within and between species.

If the relationship is uncertain, then there is no alternative but to use 'test' species that are related as closely as possible to those from which the data are to be extrapolated, in the hope that their sensitivities are similar. This must also be done with due caution, as the linkage cannot be guaranteed.

Another widely used rationale is that, if most of the biota are less sensitive than the test organism, standards based on its responses can be used to ensure the well-being of other species. This is now linked with the rapidly expanding use of chemical 'quantitative structure-activity relationships' (QSAR) to establish common sensitivity-rankings of organisms to compounds of identical toxic action (Kaiser, 1987).

Note: It is clear that many of the criteria listed above are mutually exclusive, and that the more they are considered important, the less likely one is to find an appropriate biological effects technique for a particular application.

7. RECOMMENDED TESTS

Tests were scored on a scale of 1-5 against the various criteria which determine their utility in monitoring sewage-sludge disposal sites. These were identified in Section 6. Additionally, the criteria were weighted (also on a scale of 1-5) according to their perceived importance. In the case of four criteria, this weighting differed according to whether the technique was to be employed as a laboratory toxicity test or as a bioassay of environmental quality (Table 2).

Table 2. Weighting of criteria for selection of techniques according to their mode of deployment (1-5)

Criterion	Bioassay	Toxicity test
Availability of species	5	5
Importance of species (ecological/economic)	4	2
Cost-effectiveness	3	3
Ease of use	4	3
Rapidity of test	2	5
Genetic uniformity	1	1
Data quality (precision)	5	5
Bioaccumulative potential	3	3
Integration of effects	5	5
Sensitivity	5	5
'Discrimination'	5	5
Relevance of technique to populations	5	4

The products of the scores of individual tests and weighting factors are shown in Table 3. The tests have been allocated to five categories (A-E) in order to account for the major objectives and environmental targets, as shown in the flow-diagram below.

It can be seen that in a number of cases, a particular test can fulfil more than one role.

It should be emphasised that the scoring system is limited in scope to an assessment of utility in monitoring of sewage-sludge disposal sites, and so does not address the suitability of these tests to meet other environmental monitoring objectives.

Whilst this ranking provided a very useful aid to the short-listing of promising tests, the final decision inevitably took special account of the present state of development of the various test procedures, and hence the practical implications for immediate routine deployment. This led to the following choice:

(i) recommended for routine application

- oyster embryo test
- feeding rate in *Mytilus* (with qualifications on cost and difficulties in interpretation in some cases);

(ii) recommended for routine application, subject to further R and D and/or refinement of protocol (in ranked order of scores)

- zooplankton tests (copepods, mysids)
- 'Microtox' test (with qualification regarding its 'relevance' as presently understood)
- planktonic algal tests (with qualification regarding interpretation of stimulatory effects)
- polychaete enzyme activity;

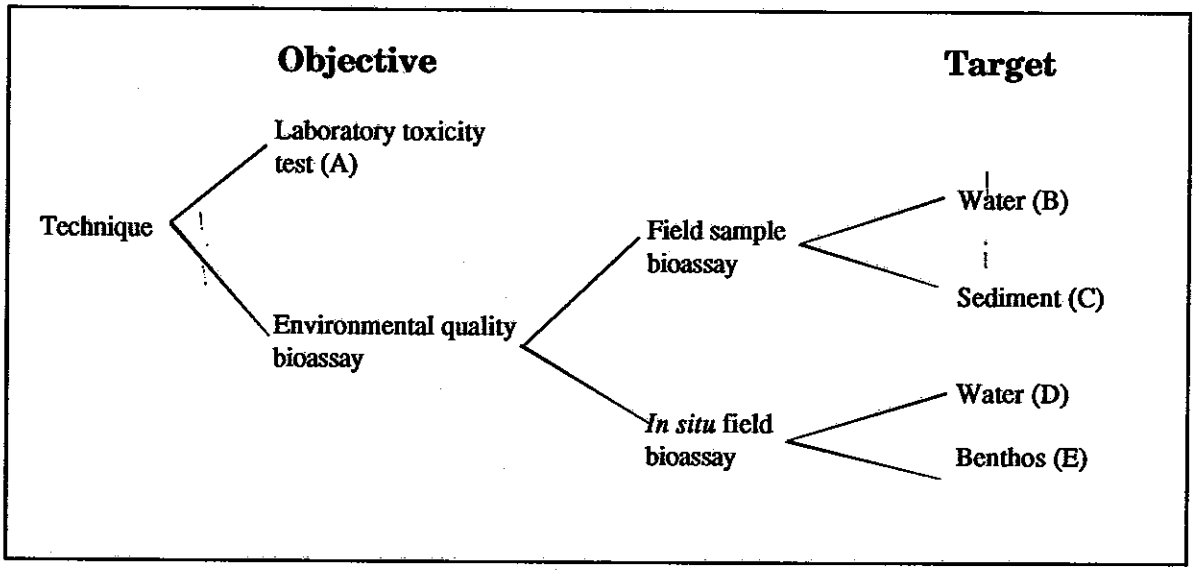


Table 3. Weighted scores for selected techniques against a range of criteria (see text)

Technique	Criterion: Availability of species	Importance of species (ecological/economic)	Cost-effectiveness	Ease of use	Rapidity of technique	Genetic uniformity	Data quality/precision	Bioaccumulative potential	Integration of effects	Sensitivity	Discrimination	Relevance of technique to populations	Total	% max score
A. LABORATORY TOXICITY TEST														
Physiological stress	25	8	9	12	10	3	20	15	25	20	20	16	183	80
Hydroid	25	2	6	9	10	5	20	3	20	20	20	8	148	64
Bivalve embryo	20	10	15	12	20	4	20	3	20	20	20	20	184	80
Algae	25	10	12	15	20	5	20	3	15	10	15	16	166	72
Zooplankton	25	10	9	9	15	3	20	9	15	20	20	16	171	74
Fish eggs and larvae	15	10	9	6	15	3	20	9	15	20	20	20	162	70
Infaunal invertebrates	15	6	6	9	10	3	15	12	20	15	15	20	146	63
Microtox	25	2	15	15	25	5	25	3	20	15	20	4	174	76
B. FIELD SAMPLE BIOASSAY - WATER														
Hydroid	25	4	6	8	4	5	15	3	20	10	20	10	130	55
Bivalve embryo	20	20	15	16	8	4	20	3	20	20	20	20	186	79
Algae	25	20	12	20	8	5	20	3	15	10	15	20	173	74
Zooplankton	25	20	9	12	6	3	20	9	15	20	20	20	179	76
Fish eggs and larvae	15	20	9	8	6	3	20	9	15	20	20	25	170	72
Microtox	25	4	15	20	10	5	25	3	20	15	20	5	167	71
C. FIELD SAMPLE BIOASSAY - SEDIMENT														
Polychaete enzyme activity	15	20	12	12	6	4	20	9	15	15	15	20	163	69
Infaunal invertebrates	15	12	6	12	4	3	15	12	20	15	15	25	154	66
(Sediment elutriate assays - see B)														
D. ***In situ FIELD BIOASSAY - WATER***														
Physiological stress	25	20	12	12	6	3	20	15	25	20	20	20	198	84
Algae	25	20	12	20	8	5	20	3	15	10	15	20	173	74
Fish eggs and larvae*	10	20	6	4	6	2	10	9	20	15	15	25	142	60
E. In situ FIELD BIOASSAY - BENTHOS														
Polychaete enzyme activity*	15	20	12	12	6	4	20	9	15	15	15	20	163	69

*Scored for specimens collected in the field, and not for those exposed experimentally at sea.

(iii) not recommended for routine application at current state of development/knowledge

- fish egg and fish larvae tests

- other tests using infaunal invertebrates

- hydroid test.

Regarding implementation of the recommendations, one aspect identified as being of critical importance was the transfer of expertise to allow routine application by presently inexperienced personnel. It is important to emphasise that the transition to routine application of most of the 'effects' measures discussed above has yet to be made in a regulatory context.

There will thus be a 'lead-in' time of varying duration depending on the nature of the test, and in a number of cases this may have significant implications for resources (including manpower). Given this, and the need for periodic intercalibration, there is every reason to expect that the tests will give reliable results.

Another important factor affecting the development of regional routines will be the establishment of a reliable supply of the test species. This is discussed further in Section 8.

The oyster embryo test has the advantage that it deals with a familiar and commercially important species. Moreover, the test is not complicated by the necessity to provide an external food source for the duration of the experiment. The wider significance of effects determined from the 'Microtox' test has yet to be established. Both of these tests are amenable to controlled experimentation in the laboratory, and hence the potential exists for calibrating responses in both field and laboratory.

However, it should be noted that when applied to water samples from the field, both the above tests will necessarily provide only 'snapshots' of the intensity and spatial extent of effects. Reproducibility in these terms would depend on identical environmental conditions (e.g. wind and wave action) prevailing from one sampling period to the next (an improbable occurrence).

Physiological stress tests as field bioassays have the advantage of being longer-term integrators, and therefore should be more amenable to temporal comparisons. However, an unambiguous outcome is at least partly dependent on the availability of suitable food, both in nature during field deployment, and in controlled conditions during testing procedures in the laboratory. Note that, in contrast to the oyster embryo test and the 'Microtox' test, the logistics and costs of deployment of caged mussels would normally dictate limitation of effort to a few key sites.

Therefore, regarding the potential for derivation of Environmental Quality Standards from field bioassays, physiological stress tests show promise, along with other methods involving *in situ* benthic species (e.g. polychaete enzyme activity) which have been exposed to the disposal operation for months or years. Laboratory tests of sediment toxicity also appear to have a future role in this respect. However, in all of these cases, practical experience of deployment of such tests at sewage-sludge disposal sites is too limited, and hence the data-base inadequate, to permit firm recommendations to be made at this stage.

Recognising this limited experience, the present account has necessarily concentrated on an assessment of the *potential* utility of a range of methods which are currently available, and this has led to a series of recommendations concerning future applications.

Critical to the success of any biological effects monitoring programme will be a precise formulation of the local objectives to be met, followed by an appraisal of the capability of the test(s) to meet these requirements. This appraisal will require consideration of a range of methodological and environmental factors, in order to ensure an unambiguous outcome. A summary of the practical issues to be considered prior to the development of a routine is given at Annex 5.

8. FUTURE NEEDS FOR RESEARCH AND DEVELOPMENT

8.1 Supply of test organisms

The reliable supply of test organisms is a key factor determining the success of regular monitoring programmes, and the development of stock-holding facilities is therefore essential. Additionally, support is also required for the development of techniques for cryogenically preserving gametes or embryos. If successful, this would greatly facilitate the application of a number of test procedures which depend on consistent supplies of these sensitive stages. Presently, much of the research in this area is carried out in connection with commercial shellfish farming.

8.2 Sediment tests

The high-scoring tests in Section 7 were water-column tests (though these can also be used for sediment elutriates). Accordingly, there is a need to give high

priority to future work aimed at development and comparative assessment of the utility of a wider range of invertebrate assays of direct sediment toxicity. This would be as a complement to traditional studies on benthic communities, which presently remain the most suitable way to assess sediment quality in the field.

The outcome of a Workshop in March 1990 under ICES/IOC auspices (Stebbing , in preparation), which included the conduct of sediment bioassays along a point-source gradient, will be of particular interest in this context (see Section 2).

8.3 Review of test protocols

For tests which have not yet been widely applied, the probable outcome of an evaluatory phase at disposal sites will be limited modifications of test procedures, and hence allowance must be made for future improvements in protocols for their conduct; this might, for example, take the form of a standing review. There will also be a need to ensure continued interaction between those engaged in research, on the one hand, and in routine application on the other.

8.4 Intercalibration of methods

Periodic intercalibration of methods between laboratories is important to maintain quality control, and is especially important where comparisons are to be made between results from different disposal site locations.

8.5 Pre-test manipulation of samples

Research into methods for concentrating contaminants found in water samples is of interest in connection with monitoring of sewage-sludge disposal sites. Such methods include XAD resins for organic compounds, C18 for organo-metallics, and 8-hydroxyquinoline for metals. These may permit the establishment of response thresholds, and could have particular significance at dispersive sewage-sludge disposal sites where, in practise, contaminant concentrations in most water samples will be relatively low. Such methodology would then allow the determination of the extent to which concentrations encountered in field samples would have to be increased in order to initiate a response.

Another potentially important application concerns the facility to take integrated water samples over periods of hours at and around disposal sites.

8.6 Other needs

In view of the existence of a number of potentially useful biological effects tests which, for a variety of reasons, are not presently considered to be suitable for routine application at UK sewage-sludge disposal sites, then their deployment at these sites in an applied research context deserves every encouragement.

9. REFERENCES

BAYNE, B. L., BROWN, D. A., BURNS, K., DIXON, D. R., IVANOVICI, A., LIVINGSTONE, D. R., MOORE, M. N., STEBBING, A. R. D. and WIDDOWS, J., 1985. 'The Effects of Stress and Pollution on Marine Animals'. Praeger Scientific, New York.

BAYNE, B. L., ADDISON, R. F., CAPUZZO, J. M., CLARKE, K. R., GRAY, J. S., MOORE, M. N. and WARWICK, R. M., 1988. An overview of the GEEP Workshop. Mar. Ecol. Progr. Ser., ***46***: 235-243.

BLACKSTOCK, J., 1982. Biochemical assessment of effects of pollution on marine invertebrates. Actes Colloq., Centre National pour l'Exploitation des Oceans, Brest, ***14***: 349-368.

BLACKSTOCK, J., 1984. Biochemical metabolic regulatory responses of marine invertebrates to natural environmental change and marine pollution. Oceanogr. Mar. Biol. Ann. Rev., ***22***: 263-313.

BLACKSTOCK, J. and FILION-MYKLEBUST, C., 1983. Environmental and biochemical investigation of some effects of organic pollution in inner Oslofjord, Norway. Mar. Biol., ***73***: 155-163.

BLACKSTOCK, J., JOHANNESSEN, P. J. and PEARSON, T. H., 1986. Use of a sensitive indicator species in the assessment of biological effects of sewage disposal in fjords near Bergen, Norway. Mar. Biol., ***93***: 315-322.

BRAUM, E., 1973. Einflusse chronischen exogenen Sauerstoffmangels auf die Embryogenese des Herrings (*Clupea harengus*). Neth. J. Sea. Res., ***7***: 363-375.

BULICH, A. A., 1979. Use of luminescent bacteria for determining toxicity in aquatic environments. In: Marking, L. L. and Kimerle, R. A. (eds.), 'Aquatic Toxicology'. Spec. Tech. Publ., Am. Soc. Test. Mater., (*667*): 98-106.

BURROWS, E. M. and SHARPLES, E. J., 1973. Bio-assay of sea water. pp.45-79. In: 'Out of Sight, Out of Mind. Report of a Working Party on the Disposal of Sludge in Liverpool Bay', *3*. Her Majesty's Stationery Office, London.

CHANG, S. and LONGWELL, A., 1984. Examining statistical associations of malformation, cyto-pathology and cytogenic abnormality of Atlantic mackerel embryos with indicator levels of environmental contaminants in the New York Bight. ICES C.M.1984/E:11, 15 pp (mimeo).

FAIRHURST, F. M., BUTLER, R. and WILLS, S., 1987. The biological assessment of Lothian sewage sludge extract using various sublethal toxicity tests. Water Research Centre, Medmenham, Report PRS 1507-M, 8 pp (restricted).

FRANKLIN, F. L., 1983. Laboratory tests as a basis for the control of sewage sludge dumping at sea. Mar. Pollut. Bull., *14*: 217-223.

GIESY, J. P., Jr. (ed.), 1980. 'Microcosms in Ecological Research'. National Technical Information Service, Springfield, Va., U.S. Department of Energy, Symposium Series 52, CONF-781101.

GREAT BRITAIN - PARLIAMENT, 1972(a). Convention for the Prevention of Marine Pollution by Dumping from Ships and Aircraft, Oslo, 15 February 1972. Her Majesty's Stationery Office, London, 12 pp. (Cmnd 4984).

GREAT BRITAIN - PARLIAMENT, 1972(b). Final Act of the Intergovernmental Conference on the Convention on the Dumping of Wastes at Sea, London, 13 November 1972. Her Majesty's Stationery Office, London, 20 pp. (Cmnd 5169).

GREAT BRITAIN - PARLIAMENT, 1974. Dumping at Sea Act, 1974. Chapter 20. Her Majesty's Stationery Office, London, 13 pp.

GREAT BRITAIN - PARLIAMENT, 1985. The Food and Environment Protection Act, 1985. Chapter 48. Her Majesty's Stationery Office, London, 38 pp.

HAUX, C. and FORLIN, L., 1988. Biochemical methods for detecting effects of contaminants on fish. Ambio, *17*: 376-380.

HUGHES, M. M., HEBER, M. A., MORRISON, G. E., SCHIMMEL, S. C. and BERRY, W. J., 1989. An evaluation of a short-term chronic effluent toxicity test using sheepshead minnow *Cyprinodon variegatus* larvae. Environ. Pollut., *60*: 1-14.

JOHNSON, D., MAINSTONE, C. and COSTELLO, M., 1988. Effects of sewage sludge on marine resources. Water Research Centre, Medmenham, Progress Report to the Department of the Environment, London, April 1988 to Sept. 1988; DOE 1948-M.

KAISER, K. L. E. (ed.), 1987. 'QSAR in Environmental Toxicology, II'. D. Reidel, Dordrecht, 465 pp.

KARBE, L., 1972. Marine Hydroiden als Testorganismen zur Prüfung der Toxizität von Abwasserstoffen. Die Wirkung von Schwermetallen auf Kolonien von *Eirene viridula*. Mar. Biol., *12*: 316-328.

KELLER, A. A., FRITHSEN, J. B., OVIATT, C. A., MAUGHAN, J. T., SULLIVAN, B. K., NIXON, S. W. and PILSON, M. E. Q., 1987. Marine ecosystem responses to sewage sludge and inorganic nutrient additions: a mesocosm experiment data report. University of Rhode Island, Kingston, Rhode Island, MERL Series, Report (*6*): 1-238.

LACK, T. J. and JOHNSON, D., 1985. Assessment of the biological effects of sewage sludge at a licensed site off Plymouth. Mar. Pollut. Bull., *16*: 147-152.

LAMBERT, C. E. and OVIATT, C. A. (eds.), 1986. Manual of biological and geochemical techniques in coastal areas. University of Rhode Island, Narragansett, Rhode Island, MERL Series, Report (*1*) (2nd ed.).

LASSERRE, P., 1990. Marine microcosms: small-scale controlled ecosystems. pp.20-60. In: Lalli, C. M. (ed.), 'Enclosed Experimental Ecosystems: A Review and Recommendations'. Coastal and Estuarine Studies, *37*. Springer Verlag, Berlin.

McINTYRE, A. D., MUNRO, A. L. S. and STEELE, J. H., 1970. Energy flow in a sand ecosystem. pp.19-31. In: Steele, J. H. (ed.), 'Marine Food Chains'. Oliver and Boyd, Edinburgh.

McINTYRE, A. D. and PEARCE, J. B. (eds.), 1980. Biological effects of marine pollution and the problems of monitoring. Rapp. P.-v. Réun. Cons. int. Explor. Mer, *179*: 1-346.

McLACHLAN, A., DYE, A. and HARTY, B., 1981. Simulation of the interstitial system of exposed sandy beaches. Estuar. Cstl. Shelf Sci., *12*: 267-278.

MILLER, D. C., MARCY, M., BERRY, W., DEACUTIS, C., LUSSIER, S., KUHN, A., HEBER, M., SCHIMMEL, S. C. and JACKIM, E., 1984. Final report: evaluation of methods to measure the acute toxicity of sewage sludge. United States Environmental Protection Agency, Environmental Research Laboratory, Narragansett, Rhode Island, 39 pp + appendix.

MILLER, D. C., MARCY, M., BERRY, W., DEACUTIS, C., LUSSIER, S., KUHN, A., HEBER, M., SCHIMMEL, S. C. and JACKIM, E., 1988. The acute toxicity of sewage sludge to marine fish, mysids and copepods. pp.103-113. In: Wolfe, D. A. and O'Connor, T. P. (eds.), 'Oceanic Processes in Marine Pollution', *5*. R. E. Kreiger, Florida.

MPMMG, 1989. First report of the Marine Pollution Monitoring Management Group's Co-ordinating Group on Monitoring of Sewage-Sludge Disposal Sites. Compiled by J. E. Portmann. Aquat. Environ. Monit. Rep., MAFF Direct. Fish. Res., Lowestoft, (*20*): 64 pp.

NIMMO, D. R. and HAMAKER, T. L.. 1982. Mysids in toxicity testing - a review. Hydrobiologia, *93*: 171-178.

NIMMO, D. R., BAHNER, L. H., RIGBY, R. A., SHEPPARD, J. M. and WILSON, A. J., Jr., 1977. *Mysidopsis bahia*, an estuarine species suitable for life-cycle tests to determine the effects of a pollutant. Spec. Tech. Publ., Am. Soc. Test. Mater., (*634*): 109-116.

OVIATT, C. A., QUINN, J. G., MAUGHAN, J. T., TAYLOR ELLIS, J., SULLIVAN, B. K., GEARING, J. N., GEARING, P. J., HUNT, C. D., SAMPOU, P. A. and LATIMER, J. S., 1987. Fate and effects of sewage sludge in the coastal marine environment: a mesocosm experiment. Mar. Ecol. Progr. Ser., *41*: 187-203.

PEARSON, T. H. and BLACKSTOCK, J., 1983. Selection of indicator species: a coordinated ecological and biochemical approach to the assessment of pollution. Proceedings 17th European Marine Biology Symposium, Brest, France, 27 September-1 October, 1982. Oceanol. Acta. (Spec. Vol.): 147-151.

PERSOONE, G., JASPERS, E. and CLAUS, J., 1984. 'Ecotoxicological Testing in the Marine Environment. Vols 1 and 2'. Institute for Marine Scientific Research, Ghent, 772 and 580 pp.

RIBO, J. M. and KAISER, K. L. E., 1987. *Photobacterium phosphoreum* toxicity bioassay. I. Test procedures and applications. Toxic. Assess., *2*: 305-323.

RODDIE, B., BUTLER, R. and MAINSTONE, C. P., 1989. Report on environmental stress and toxicity measurements in the laboratory and field using novel techniques. Water Research Centre, Medmenham, Report PRS 2034-M, 21 pp.

SANDULLI, R., 1987. Preliminary report of a laboratory experiment on the effects of organic pollution on meiofauna. Nova Thalass., *7*: 251-256.

STEBBING, A. R. D., 1976. The effects of low metal levels on a clonal hydroid. J. mar. biol. Ass. U.K., *56*: 977-994.

STEBBING, A. R. D., 1979. An experimental approach to the determinants of biological water quality. Phil. Trans. R. Soc. Lond. B, *286*: 465-481.

STEBBING, A. R. D., 1982. Hormesis - the stimulation of growth by low levels of inhibitors. Sci. Tot. Environ., *22*: 213-234.

STEBBING, A. R. D., 1987. Growth hormesis: a by-product of control. Hlth Phys., *52*: 543-547.

STEBBING, A. R. D., CLEARY, J. J., BRINSLEY, M., GOODCHILD, C. and SANTIAGO-FANDINO, V., 1987. Responses of a hydroid to surface water samples from the River Tamar and Plymouth Sound in relation to metal concentrations. J. mar. biol. Ass. U.K., *63*: 695-711.

SWARTZ, R. C., SCHULTZ, D. W., DITSWORTH, G. R. and WALDEMAR, A. D., 1984. Toxicity of sewage sludge to *Rhepoxynius abronius*, a marine benthic amphipod. Arch. Environ. Contam. Toxicol., *13*: 207-216.

SWARTZ, R. C., DEBEN, W. A., JONES, J. K. P., LAMBERSON, J. O. and COLE, F. A., 1985. Phoxocephalid amphipod bioassay for marine sediment toxicity. In: Cardwell, R. D., Purdy, R. and Bahner R. C. (eds.), 'Aquatic Toxicology and Hazard Assessment: Seventh Symposium'. Spec. Tech. Publ., Am. Soc. Test. Mater., (*854*): 284-307.

UNDERWOOD, A. J. and PETERSON, C. H., 1988. Towards an ecological framework for investigating pollution. Mar. Ecol. Progr. Ser., *46*: 227-234.

UNITED STATES ARMY CORPS OF ENGINEERS, 1978. Biological assessment methods to predict the impact of open-water disposal of dredged material. US Army Engineer Waterways Experiment Station, Environmental Laboratory, Vicksburg, Technical Report D-78-50.

UNITED STATES - ENVIRONMENTAL PROTECTION AGENCY, 1978. Bioassay procedures for the Ocean Disposal Permit Program. United States Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida, Publication No. 600/9-78-010, 121 pp.

WALSH, G. E., 1987. Methods for toxicity tests of single substances and liquid complex wastes with marine unicellular algae. United States, Environmental Protection Agency, Washington, Publication No. 600-8/87/043.

WALSH, G. E., 1988. Principles of toxicity testing with marine unicellular algae. Environ. Toxicol. Chem., *7*: 979-987.

WALSH, G. E., McLAUGHLIN, L. L., YODER, M. J., MOODY, P. H., LORES, E. M., FORESTER, J. and WESSINGER-DUVALL, P. B., 1988. *Minutocellus polymorphus*: a new marine diatom for use in algal toxicity tests. Environ. Toxicol. Chem., *7*: 925-929.

WESTERNHAGEN, H. von, 1974. Incubation of garpike eggs (*Belone belone* Linne) under controlled temperature and salinity conditions. J. mar. biol. Ass. U.K., *54*: 625-634.

WESTERNHAGEN, H. von, 1988. Sub-lethal effects of pollutants on fish eggs and larvae. pp.253-364. In: Hoar, W. S. and Randall, D. J. (eds.), 'Fish Physiology', *11A*. Academic Press, New York.

WESTERNHAGEN, H. von, DETHLEFSEN, V., CAMERON, P., BERG, J. and FURSTENBERG, G., 1988. Developmental defects in pelagic fish embryos from the western Baltic. Helgoland. Meeresunters., *42*: 13-36.

ANNEX 1. Membership of the Biological Effects Task Team

Mr N C D Craig
Imperial Chemical Industries plc
Brixham Laboratory,
Freshwater Quarry
Overgang
Brixham
Devon TQ5 8BA

Dr C J Redshaw
Clyde River Purification Board
Rivers House
Murray Road
East Kilbride
Glasgow G75 OLA
Scotland

Mr R Lloyd
Ministry of Agriculture, Fisheries and Food
Fisheries Laboratory
Remembrance Avenue
Burnham-on-Crouch
Essex CMO 8HA

Dr J G McHenery (Secretary)
Department of Agriculture and
Fisheries for Scotland
Marine Laboratory
PO Box 101
Victoria Road
Aberdeen AB9 8DB
Scotland

Mr R A Milne
National Rivers Authority
Welsh Region
Plas-yr-Afon
St Melons Business Park
Cardiff CF3 OLT

Dr T H Pearson
Scottish Environmental Advisory Services
c/o Scottish Marine Biological Association
Dunstaffnage Marine Research Laboratory
PO Box 3
Oban
Argyll PA34 4AD
Scotland

Dr H L Rees (Chairman)
Ministry of Agriculture, Fisheries and Food
Fisheries Laboratory
Remembrance Avenue
Burnham-on-Crouch
Essex CMO 8HA

Dr B D Roddie
Water Research Centre
Medmenham Laboratory
Henley Road
Medmenham
PO Box 16
Marlow
Buckinghamshire SL7 2HD

Dr M Service
Economic Development Department
Industrial Science Division
17 Antrim Road
Lisburn BT28 3AL
Northern Ireland

Dr A R D Stebbing
Plymouth Marine Laboratory
Prospect Place
The Hoe
Plymouth PL1 3DH
Devon

Mr J E Thain
Ministry of Agriculture, Fisheries and Food
Fisheries Laboratory
Remembrance Avenue
Burnham-on-Crouch
Essex CMO 8HA

ANNEX 2. Description of physiological measures of sub-lethal stress, with some examples of survey results

B. D. Roddie and D. Johnson (Water Research Centre (1989) plc., Medmenham)

'Scope for Growth' (SFG) is calculated from the balanced energy equation:

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

where P = production
A = energy assimilated
R = energy respired
U = energy excreted.

Feeding rate is estimated by measuring the removal of algal cells by individual mussels in feeding chambers supplied with a constant inflowing concentration. Absorption efficiency is estimated by comparing the organic content of the food with that of the faeces. The product of these two values gives the assimilated energy.

Pollutants can influence both feeding rate and absorption efficiency. Feeding (particle removal from the water) takes place by ciliary action on the gill surface, moving the particles towards the palps where they may be ingested or rejected. Pollutants may affect the efficiency of action of the cilia which are controlled by nervous impulses. Absorption of food takes place in the hepatopancreas, and damage of the cells in this organ by accumulated toxicants will reduce absorption efficiency.

Respiration rate is measured on individual mussels enclosed in sealed constant-temperature vessels, in which the reduction in oxygen concentration is measured by a micro-electrode connected to a chart recorder. Increases in respiration rate are brought about by elevated metabolic activity in response to exposure to pollutants; this increased activity is required for repair of tissue and for detoxification processes.

Excretion is measured by incubation of the mussels in filtered sea water for two hours, after which time the concentration of ammonia excreted in the water is measured. Elevated rates of excreted ammonia are indicative of increased protein turnover in response to cellular damage. Generally, the amount of energy lost via this route is around 5% of the total energy budget.

Over the last three years, the WRc has carried out a large number of exercises to evaluate the use of the measurement of SFG as a means of detecting sub-lethal effects on the marine environment. The results of these exercises can be summarised as follows:

Sewage-sludge disposal sites

Exercise	Insufficient evidence	No effect	Slight effect	Clear effect
A		*		
B1	*			
B2			*	
C1			*	
C2			*	
C3				*
C4				*

Sewage outfalls

Exercise	Insufficient evidence	No effect	Slight effect	Clear effect
D	*			
E1	*			
E2		*		
F1	*			
F2	*			
F3		*		

Industrial discharges

Exercise	Insufficient evidence	No effect	Slight effect	Clear effect
G1				*
G2				*
H1		*		
H2			*	
I		*		
J				*
Totals	5	5	4	5

While there are five exercises in which it was not possible to demonstrate an effect, there are five exercises in which a clear and statistically significant effect was observed. The latter indicates that the technique is capable of detecting effects in the field, and the former that there are areas where either sewage, sewage sludge or industrial effluents are being released to the marine environment and effects are not being detected.

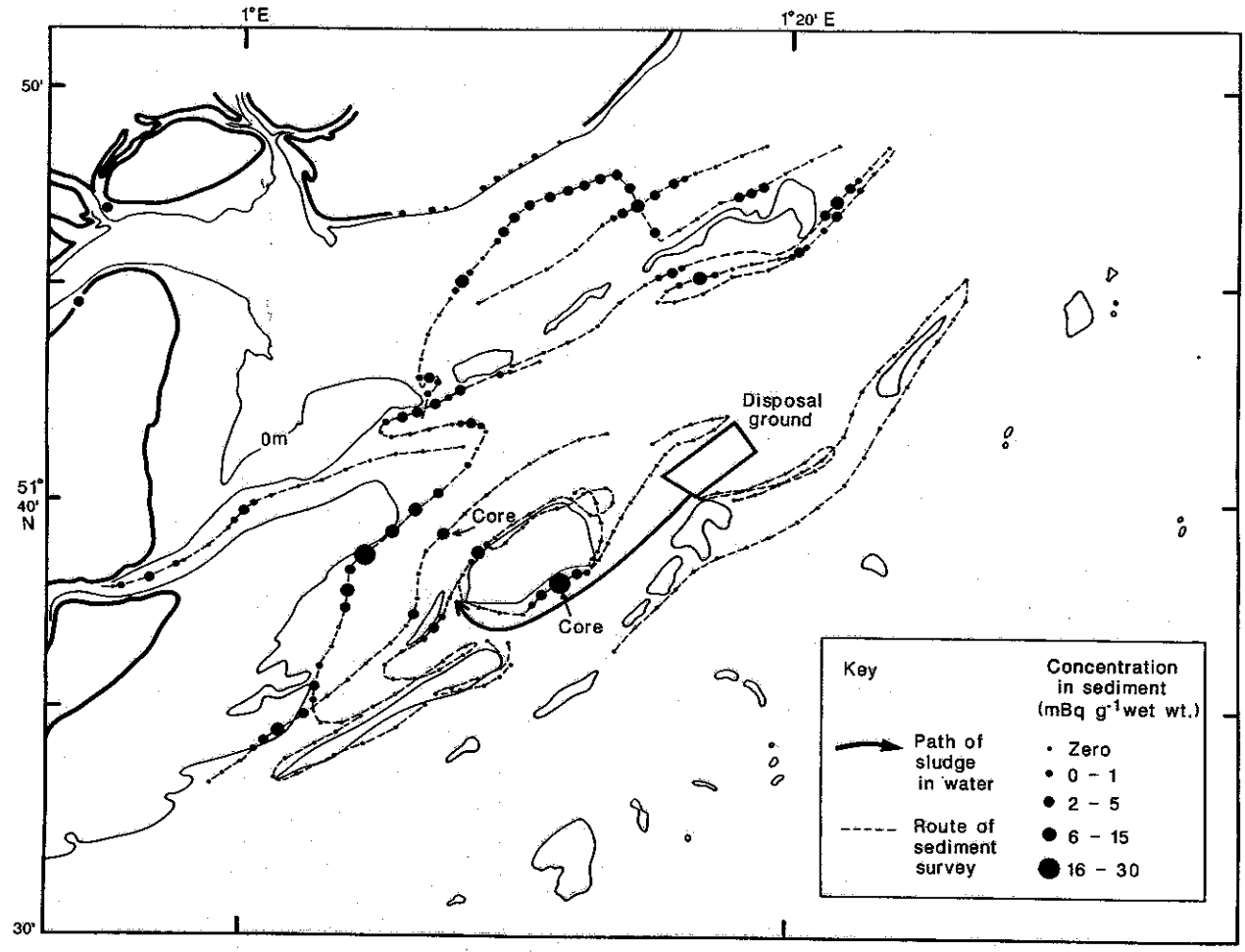


Figure A2.1. Pattern of dispersion and accumulation of radioactively-labelled sewage sludge, following release in the Barrow Deep disposal site.

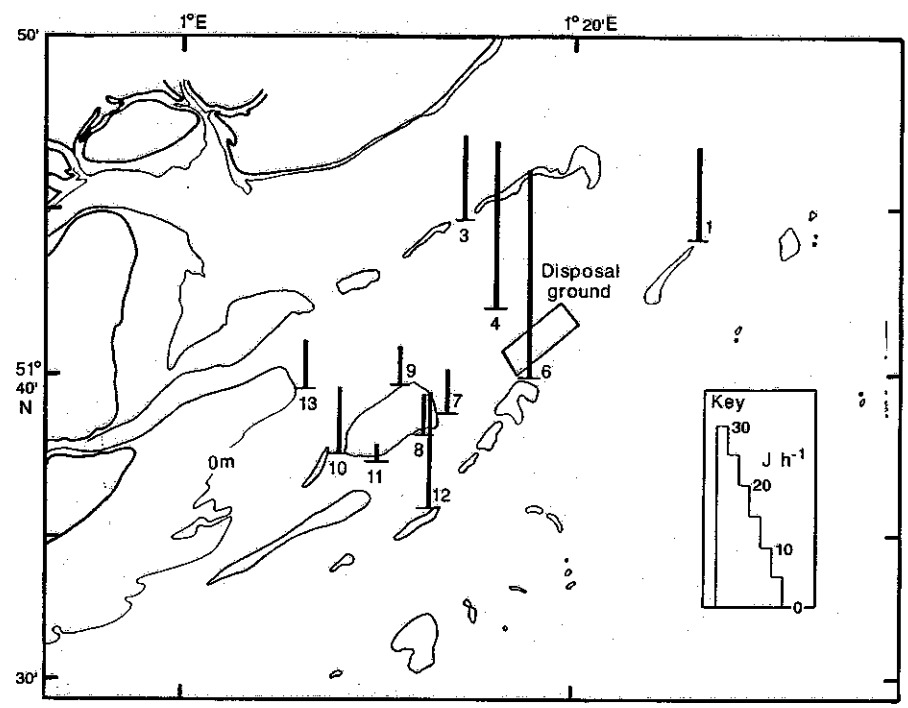


Figure A2.2. 'Scope for Growth' (J h^{-1}) in Mytilus edulis deployed in the vicinity of the Barrow Deep sewage-sludge disposal site.

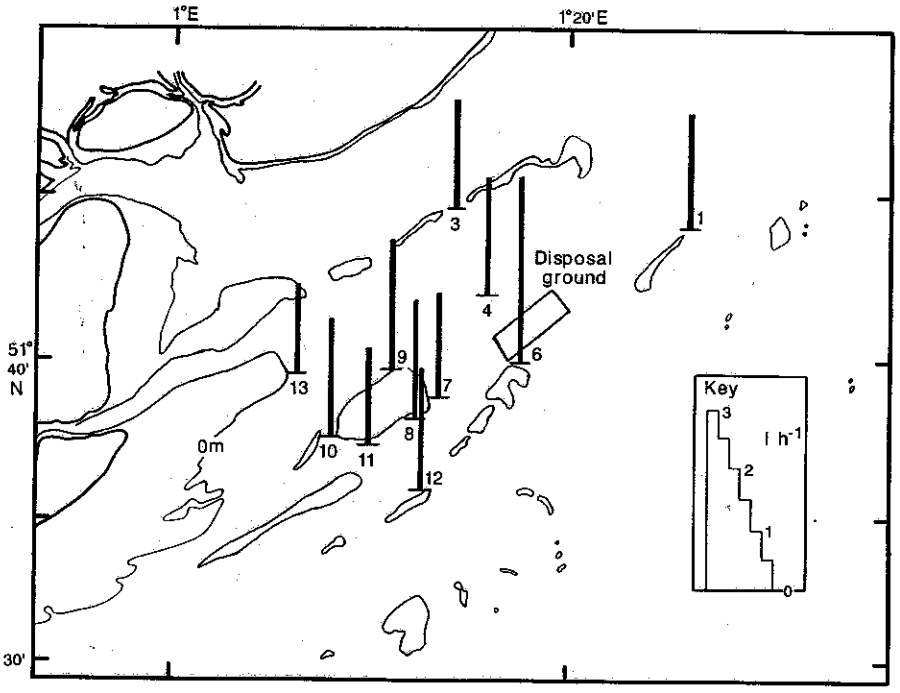


Figure A2.3. Feeding rate (l h^{-1}) in Mytilus edulis deployed in the vicinity of the Barrow Deep sewage-sludge disposal site.

The sewage-sludge disposal site which has been investigated most extensively using this technique is the Thames Estuary Barrow Deep disposal site used by Thames Water. Results of one of the studies, a collaborative exercise between the WRc and Thames Water have been reported by Whitelaw and Andrews (1987, 1988). At this site, conventional macrobenthic surveys have been carried out, together with studies of the dispersion of radioactively-labelled sludge (Figure A2.1) and SFG measurements on transplanted mussels (Figure A2.2).

There was no clear evidence of an effect of sludge disposal on the macrobenthic community, but some evidence of reduced SFG in mussels deployed in or near the sludge dispersal track (Figure A2.2). For comparison, and in the light of earlier comments on the use and interpretation of SFG, feeding rate has also been plotted (Figure A2.3). The differences between the two sets of data can be accounted for largely in terms of the effect of apparent variation in measured absorption efficiency on SFG values. It is not considered, however, that the estimates of absorption efficiency used were of sufficient accuracy and precision to permit changes in SFG to be interpreted with confidence in terms of the impact of disposal of sludge. Measurements on feeding rate are not subject to this bias. The relationship between feeding rate and SFG is illustrated in Figure A2.4.

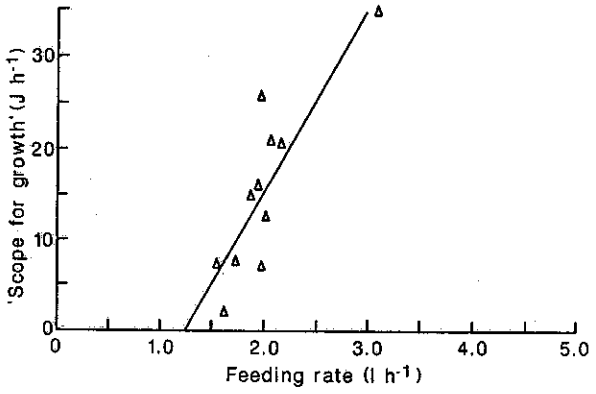


Figure A2.4. Relationship between feeding rate and 'Scope for Growth' in Mytilus edulis.

Physical growth during deployment was assessed in conjunction with physiological measurements. No clear, overall relationship between growth and SFG was observed, but a tendency was noted for higher body weight to be associated with low SFG (and feeding rate) and *vice versa*.

Evaluation of this technique has given rise to the conclusions and comments noted in Section 5 of the

main report. In particular, feeding rate is considered as the most robust and sensitive measure of response to the toxic effects of contaminants associated with sewage sludge. In respect of the study referred to above, the following points should be noted in relation to the interpretation of growth versus physiological stress, and in relation to the design and interpretation of field studies:

(i) it should not be expected, where toxicant impact has occurred, that actual (weight change) and instantaneous (SFG) growth estimates should agree. For instance, physical growth (and contaminant accumulation) may proceed at undiminished rates for the greater part of a deployment period; only when the tissue concentration exceeds a toxic threshold (of some substance or substances) will adverse physiological responses occur. Thus, low SFG or feeding rate at the end of a deployment period does not imply similarly low rates throughout the period;

(ii) it is rarely possible to estimate with confidence *actual* food availability and quality in the field. Consequently, unobserved restrictions on the availability of energy may result in low growth in mussels which are, physiologically, in good health. Where uncertainty exists, the direct interpretation of physiological rates (feeding and respiration) is to be preferred as being less ambiguous;

(iii) hydrographic heterogeneity within a study area may give rise to unacceptably large differences in the physical conditions to which animals are exposed. These differences may be entirely independent of the effects of a discharge, but may, for instance, affect feeding opportunity in a way which might bias exposure to contaminants. It is, therefore, important to ensure that sites within a study area differ as little as possible in respect of characteristics other than those associated with the discharge in question.

A2.1 References

WHITELAW, K. and ANDREWS, M. J., 1987. Quantifying the dispersion and impact of sludge disposal at sea. pp.65-73. In: 'The Handbook of the Institution of Water and Environmental Management, 1987-1988'. Business Information Limited, Norwich.

WHITELAW, K. and ANDREWS, M. J., 1988. The effects of sewage sludge disposal to sea - the outer Thames Estuary, UK. Wat. Sci. Technol., *20*: 183-191.

ANNEX 3. Protocol for measuring indices of physiological stress

B D Roddie (Water Research Centre (1989) plc., Medmenham)

A3.1 Introduction

The following protocol describes methods for measuring indices of physiological stress in *Mytilus edulis*. The primary index of response is change in feeding rate; for the sake of completeness, additional methods are described which allow a simple net energy budget to be calculated.

The calculation of a nominal net energy budget requires estimates of feeding rate and respiration rate. Feeding rate is measured as 'clearance rate', expressed as the volume of water cleared of food particles per unit of time. Respiration rate is expressed as volume of oxygen consumed per unit of time.

A3.2 General provisions

'Physiological Stress Index' (PSI) will be calculated using rates of acquisition and expenditure of energy in *Mytilus edulis*, estimated from the following measurements:

- clearance rate (feeding rate)	$l\ h^{-1}$
- respiration rate	ml oxygen h^{-1}
- dry flesh weight	g.

The 'source' population will be one free from significant industrial, domestic or natural contamination. Regulations governing the transplantation of molluscs require that they also be free from infestation by parasites.

The physiological and reproductive status of the population should be monitored at regular intervals of between 2 and 6 weeks throughout the year.

Any existing information on growth and reproduction in the population will be collated and archived.

The use of mussels for which no background information is available is strongly discouraged.

Each batch of mussels received will be designated with a unique batch number which will be cited in association with any measurement or treatment applied to mussels from that batch.

The size of the sample will be 15 for field studies and 8 for laboratory studies.

The size range will be restricted to 45-50 mm (shell length).

All measurements will be made on identified individuals, and PSI calculated on an individual basis.

The duration of deployments in the field will not be less than 4 weeks, and will preferably be 5 weeks or more.

The duration of exposure in the laboratory will be not less than 2 weeks, and preferably 4 weeks. In semi-static tests, aeration will be provided if the volume of medium per mussel is less than 0.5 litres.

All measurements will be made at the experimental or ambient field temperature ±2°C and under uniform lighting conditions. Consistent water quality will be maintained by aeration and filtration or by frequent replacement. The maximum permissible 'acute' temperature change prior to measurement of any variable is 2°C.

A standard order of measurement will be observed:

- clearance rate
- respiration rate
- dry weight.

Where mussels have been exposed to air for more than 2 h following removal from treatment, they will be held in flowing, air-saturated sea water from 2 h prior to commencing measurement.

Mussels will be carefully cleaned of all epibiotic growth prior to measurement, and indelibly marked using a decimal numbering system. Each treatment group should be uniquely identified and, to aid this objective, a register of treatment-group identifiers will be maintained.

The following records will be maintained in association with PSI measurements:

- mussel batch record
- pH of sea water in the laboratory) daily
- dissolved oxygen) obser-
- temperature) vations

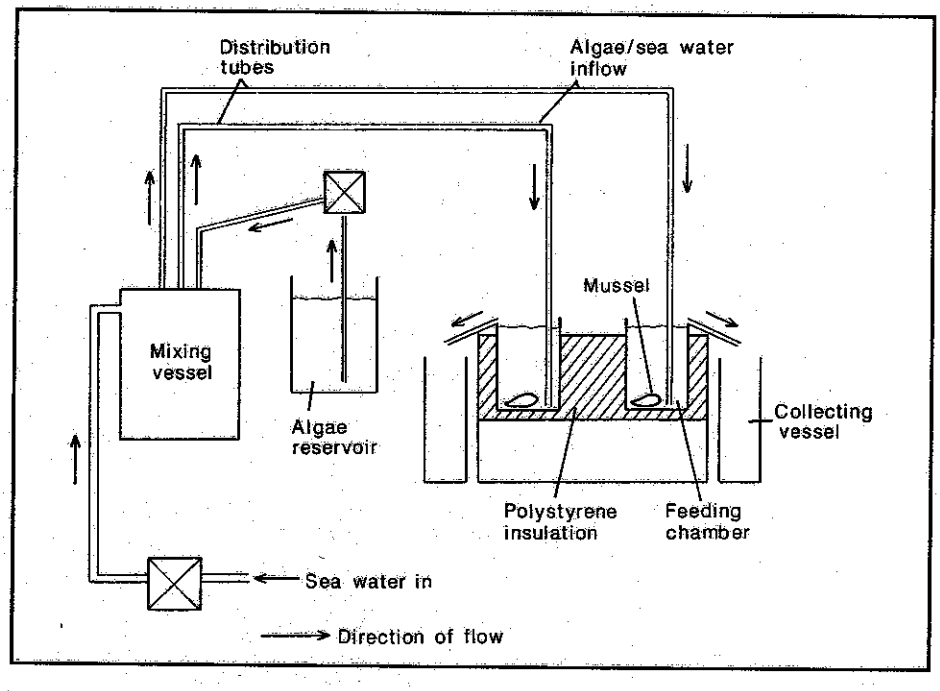


Figure A3.1. Diagrammatic representation of the apparatus used for measuring feeding rate in Mytilus edulis. (The feeding chamber rack is viewed end-on, and only 2 of 20 vessels and distribution tubes are shown.)

- log of 'treatment' groups processed
- record of progress with laboratory measurement
- record of feeding history of laboratory mussels
- clearance rate: raw data and controls
  : calculated values
- respiration rate
- physical measurements
- data sheet for calculation and statistical analysis.

The data will be entered on computer file and maintained as a permanent archive and for subsequent analysis.

A3.3 Measurement of clearance rates

Clearance rates will be measured at a constant temperature in a continuous-flow apparatus (Figure A3.1) supplied with air-saturated filtered sea water.

A3.3.1 Description and maintenance of apparatus

The apparatus consists of:

- a rack containing twenty 500 ml vessels embedded in expanded polystyrene
- a header tank, mixing vessel and (optionally) pump
- distribution tubes from the mixing vessel to the 500 ml plastic vessels
- a peristaltic pump which injects a suspension of algae from a reservoir into the mixing vessel.

The inflow to each plastic vessel is led to the base via 3 mm glass tubing, and the overflow from the top is positioned directly above a cylindrical 180 ml collecting vessel.

All surfaces in contact with the sea-water/algal suspension will be maintained free from contamination and obstruction by algal or bacterial growth. Components showing signs of such growth will be cleaned or replaced prior to any use being made of the apparatus.

A3.3.2 Operation of the apparatus

The apparatus is supplied with sea water from a temperature-controlled source via a constant-head tank. This may also be achieved by placing the main pump's intake pipe into a continuously overflowing container of at least 15 l capacity.

The procedure for operation is as follows:

(i) prime the main pump and switch on;

(ii) ensure that the mixing chamber fills and that all outflow tubes are unobstructed. Wait until the plastic vessels are filled and ensure that all overflows are unobstructed;

(iii) when all overflows are operating, collect 60 s samples simultaneously in the cylindrical collecting vessels. Measure the collected volumes and adjust the pump pressure to achieve flow rates in excess of 150 ml min^{-1}. Clean or replace tubing of any vessel in which the flow rate deviates by more than 10% from the mean value;

(iv) check that the constant-head tank is overflowing after adjustments have been made;

(v) determine the cell concentration* in the stock culture by adding a known volume (c. 1 ml) of culture to a known volume of 1.2 µm filtered sea water (c. 150 ml) and counting on a Coulter Counter with 100 µm-orifice tube and set to count all particles greater than 4 µm spherical equivalent diameter;

(vi) algae will be supplied to the apparatus from a 5-litre reservoir via a variable-speed peristaltic pump. The concentration of algae in the reservoir must be adjusted to give an inflow concentration at the plastic vessels of 7000-8000 cells ml^{-1}. The delivery rate of the peristaltic pump will depend on the pump's characteristics, the head of pressure in the reservoir, and the pressure generated in the mixing vessel by the sea-water pump. It is, therefore, necessary to determine the appropriate concentration in the reservoir by trial and error.

A3.3.3 Measurement of clearance rate

(i) Start the apparatus and set the vessel's flow rates > 150 ml min^{-1};

(ii) allocate the mussels from treatment groups randomly to the plastic vessels;

**Note: The marine diatom Phaeodactylum tricornutum will be used as the food source in all measurements of clearance rate.*

(iii) place the mussels in randomly designated vessels. Record the time of placement and the position of each mussel. The mussels will be placed with the exhalant siphon uppermost and the inhalant siphon adjacent to the end of the glass inflow tube;

(iv) maintain at least 2 vessels without mussels as controls;

(v) allow the mussels to settle for 30 min. Check and, if necessary, adjust the orientation of each mussel;

(vi) initiate a supply of algae. Record the time. Allow to equilibrate for 15 min and check the concentrations of cells in the control outflow. Adjust to achieve required concentration;

(vii) allow the mussels to acclimate for a further 30 min. Then re-check the orientation. Check the concentrations of cells in the control vessel. Simultaneous samples should not differ by more than ± 400 ml^{-1} prior to commencement of sampling;

(viii) sample all 20 outflows simultaneously for 60 ± 1 s, by moving the racks holding the collecting vessels to position the vessels directly under the outflows. TAKE CARE to avoid noise, shading or vibration in the vicinity of the apparatus at all times, but especially during sampling. Disturbance of the mussels during or immediately before sampling will invalidate the measurements. If it is suspected that disturbance has occurred, IT IS ESSENTIAL that the sample be abandoned and repeated after 10 min. Record the time of sampling. Three sets of samples will be taken, at 45-60 min intervals;

(ix) the concentrations of algal cells in each sample should be determined within 45 min of collection, using a Coulter Counter with 100 µm orifice tube set to count all particles > 4 µm (spherical equivalent) diameter in a 0.5 ml sub-sample. Three replicate counts should be made on each sample. Each sample should be decanted into a 200 ml measuring cylinder and the volume of the sample recorded ±1 ml prior to transfer to the Coulter Counter for counting;

(x) on completion of measurements, mussels should be removed from the apparatus within 15 min, and placed in flowing sea water. Failure to do this could invalidate subsequent measurement of other variables;

(xi) the plastic vessels should be rinsed and all faecal material removed prior to subsequent use of the apparatus for other purposes.

A3.3.4 Calculation of clearance rate

(i) Express all sample counts as mean number of cells per millilitre;

(ii) calculate the overall mean control count;

(iii) express flow rates as $l\ h^{-1}$;

(iv) clearance rate is calculated as ([in]-[out]/[in]) x flow rate ($l\ h^{-1}$) and is expressed to two decimal places,

where [in] = overall mean control count (cells ml^{-1})
[out] = individual outflow count (cells ml^{-1})
flow rate = vessel flow rate ($l\ h^{-1}$).

(v) transfer calculated values to a summary record sheet. The clearance rate for each individual will be based on the mean of the three observations.

A3.4 Measurement of respiration rates

A3.4.1 Operation and maintenance of the equipment

Respiration rates are measured in sealed polycarbonate Sartorius filter receivers. Oxygen concentration is monitored by Radiometer E5046 electrodes connected via Strathkelvin amplifiers to a multiple pen chart recorder.

Water in the vessels is stirred by means of magnetic followers retained by a PVC ring and covered by a perforated support on which the mussel is placed.

The electrode is inserted into the vessel via the top aperture, with the tip wrapped in Parafilm to provide an airtight seal. Vessels are maintained in an insulated

PVC tank mounted on an array of magnetic stirring units. Temperature-controlled water should be supplied from a 1.2 µm Sartorius capsule filter through the tank at approx. 2 lmin^{-1}.

A3.4.2 Electrodes

Careful assembly and maintenance of the electrodes is essential for reliable operation. The electrode consists of an inner stem, containing the anode and cathode, and an outer jacket with a polypropylene membrane over the open end and filled with KCl electrolyte. When the electrode is dismantled, the inner stem should be handled with caution, and touching of the silver/silver chloride anode should be avoided.

The majority of problems likely to arise in the use of Radiometer electrodes are associated with earth leakage - the establishment of an electrically conductive path between the electrolyte inside the electrode and the medium in which it is immersed.

There are three main paths of earth leakage:

(i) past the top 'o' ring and via the top of the electrode if this is wet;

(ii) via the pressure equilibration hole in the jacket and an external film of water;

(iii) behind the portion of the membrane held by the bottom 'o' ring.

The first two paths can be eliminated by carefully drying the exterior of the electrode. The third path may be eliminated by "bedding" the membrane onto a thin film of silicone grease applied to the groove of the 'o' ring.

The membrane and 'o' ring should be replaced after 10 separate measurements. 'O' rings stretch in use and should not be recycled.

The procedure for assembling the electrode is as follows:

(i) remove the electrode stem;

(ii) brush the electrode tip and clean with nitric acid if necessary;

(iii) remove the old membrane and 'o' ring;

(iv) carefully clean and dry the jacket;

(v) apply a thin film of grease to the groove of the 'o' ring;

(vi) remove ALL traces of grease from other parts of the jacket, especially from the inner and outer surfaces adjacent to the area on which the membrane is positioned;

(vii) lay a new membrane carefully on the end of the inverted jacket;

(viii) place a new 'o' ring onto the fitting sleeve and slip onto the jacket to retain membrane. Trim 'frill' to 1 mm;

(ix) check the membrane carefully to ensure that it is free from defects and contamination from silicone grease;

(x) fill the electrode jacket to 1 cm depth with electrolyte;

(xi) insert the electrode stem and screw the retaining cap down firmly;

(xii) carefully dry off any electrolyte on the external surfaces;

(xiii) plug the electrode into an amplifier and suspend the electrode with 1 cm of its tip in deionised water;

(xiv) set the polarising voltage on the amplifier to 650 mV. Refer to the Strathkelvin instruction manual for guidance if necessary. Leave with the amplifier switched on to stabilise for 6 h. Do not use an electrode which has been plugged in for less than 24 h.

A satisfactory electrode should show no transient response to dipping into earthed liquid or to the operation of nearby power switches.

A3.4.3 Measurement of respiration rates

All measurements must be conducted in a bath of flowing sea water supplied from the same temperature-controlled source as the apparatus for determining clearance rate, and at the same temperature (± 2°C) as the corresponding measurements of clearance rate.

The procedure is as follows:

(i) prepare the electrodes by wrapping a 1.5 cm wide strip of stretched Parafilm around the end of the jacket. Ensure that the membrane 'frill' is retained but avoid overlapping onto the membrane's active surface. Wrap the Parafilm up the jacket as far as the first 'shoulder'.

Check that there are no air bubbles within the electrode, especially around the anode and cathode;

(ii) set the polarising voltage on the amplifiers to 650 mV;

(iii) set the electrodes to zero by immersing their tips in a saturated solution of sodium sulphite in sodium tetraborate for 30 min. Adjust the amplifiers as necessary to give a reading of zero;

(iv) establish a flow of sea water of 2 l min^{-1} through the bath;

(v) fill one respirometry vessel with water from the inflow to the bath and aerate vigorously for 10 min;

(vi) remove the airstone from the vessel, and locate the vessel above a magnetic stirrer;

(vii) activate the stirrer and suspend the electrode tips in the vessel. It is important that calibration be carried out in a stirred vessel as all subsequent measurements will be conducted in stirred vessels;

(viii) wait 15-20 min, or until the amplifier's readings have stabilised, and then set all amplifiers to 9.95-9.98 (this convention sets 10 equal to 100% saturation and makes intercalibration with the chart recorder scale easier). Set the pens of the chart recorder to correspond with the readings of the amplifier and record on the chart that this has been done;

(ix) ensure that the respirometer vessels are clean. If necessary, clean with tapwater and mild bleach, rinsing thoroughly;

(x) fit the magnetic followers and glass supports for the mussels;

(xi) remove the mussels from the holding tanks and transfer them to the sea-water bath, where they can be held until needed in filtered water and at the correct temperature;

(xii) fill four respirometer vessels to the brim from the bath's inflow, ensuring that no air bubbles are trapped under the mussels' support;

(xiii) place one mussel from the bath into each vessel;

(xiv) check that all vessel lids have a silicone rubber seal in place, and fit the lids to the vessels;

(xv) top the vessels up carefully, avoiding the retention of any air bubbles;

(xvi) insert the electrode tip into the 'pool' in the top of the vessel, ensuring that no bubbles are trapped on the membrane's surface. Push the electrode downward, releasing one lid stopper to relieve pressure, and using a steady twisting action to ensure that it is firmly located. Secure the stopper. Check that there are no air bubbles within the vessel, or around the electrode's anode;

(xvii) position each vessel above a magnetic stirring unit and adjust until the follower rotates freely;

(xviii) start the chart recorder, and set its speed to 12-15 cm h^{-1}; record on the chart the date and time of day, the name of the exercise or experiment, the chart's speed, and the temperature of the bath (± 0.1°C). Record the oxygen consumption until at least 30 min of linear decline has occurred in all vessels. DO NOT allow the oxygen content of the water to fall below 65% saturation. Record on the chart the time of completion, the temperature of the bath (± 0.1°C)*, and the identity of the mussel against the corresponding trace;

(xix) replace the Parafilm on the electrode tips after each measurement;

(xx) run as many blank determinations as practicable, but at least one per electrode per day.

A3.4.4 Calculation of respiration rate

The following information is required in addition to that on the chart recorder:

- salinity
- tables of oxygen solubility
- mussel displacement volume.

Mussel displacement will be measured to within ± 1 ml in a measuring cylinder of a suitable diameter.

The procedure is as follows:

(i) for each trace, locate a straight line segment of

Note: The maximum permissible temperature drift during a single set of measurements is 0.3°C.

more than 20 min duration. Rule off a straight line along this segment. Mark convenient end-points, and note on the chart their value to 0.5 of a division;

(ii) using tables of oxygen solubility and the relevant salinity and temperature, convert these end-points into oxygen concentration in ml l^{-1}, expressed to two decimal places;

(iii) calculate respiration rate as:

$$R \text{ (ml oxygen } h^{-1}) = 60 \times (([\text{initial}] - [\text{final}])(vv - dv)/t)$$

where [initial] = initial oxygen concentration in ml l^{-1}
[final] = final oxygen concentration in ml l^{-1}
vv = vessel volume in l
dv = mussel displacement volume in l
t = time between end-points in min.

R should be expressed to 3 decimal places.

A3.5 Expression of results

Clearance rate is the primary, and generally the most sensitive, index of response. Statistical analysis should be performed independently on data for clearance rate and (if measured) on data for respiration rate. The greatest statistical rigour can be achieved if individual clearance rate measurements are replicated in the analysis.

Clearance and respiration rates should be allometrically corrected to a standard dry tissue weight (usually 1 g, or the mean weight of the treatment group).

Suitable exponents for allometric conversion are:

- clearance rate 0.4
- respiration rate 0.7.

A full explanation of the method of correction can be found in Bayne *et al.* (1985).

If respiration rate has been measured, then an overall nominal net energy budget may be calculated, after expressing both variables in common energy terms (i.e. J g^{-1} h^{-1}).

Clearance rate (CR) is translated into energy (J g^{-1} dry flesh wt h^{-1}) as follows:

$$\text{energy assimilated (A)} = CR \times 0.6 \times 16.0$$

where 0.6 = assumed absorption efficiency
16.0 = typical energy content (J l^{-1} sea water) of marine seston.

Respiration rate (RR) is translated into energy terms as follows:

$$\text{energy respired (R)} = RR \times 20.33$$

where 20.33 = the appropriate oxycalorific coefficient.

A simplified index of stress (PSI) can then be calculated as:

$$PSI = A\text{-}R \text{ J } g^{-1} h^{-1}.$$

This index is not an absolute measure of the energy status of mussels, but it does provide a relative indication of possible effects when changes in the two variables are of opposite sign.

A3.6 Reference

BAYNE, B. L., BROWN, D. A., BURNS, K., DIXON, D. R., IVANOVICI, A., LIVINGSTONE, D. R., MOORE, M. N., STEBBING, A. R. D. and WIDDOWS, J., 1985. 'The Effects of Stress and Pollution on Marine Animals'. Praeger Scientific, New York.

ANNEX 4. Protocol for conducting the oyster embryo test*

J. E. Thain (Ministry of Agriculture, Fisheries and Food, Burnham-on-Crouch)

A4.1 Introduction

The oyster embryo test was initially developed by Woelke (1972) and subsequently modified (Thain and Watts, 1987) to improve the accuracy of the test and allow its use on board research vessels. This paper describes the modified method, which has been used to obtain a measure of the deterioration of biological water quality in coastal areas receiving anthropogenic discharges.

The phrase 'deterioration in biological water quality' implies that a change in chemical composition has occurred which is potentially harmful to aquatic organisms. A bioassay to measure such a deterioration should be based on an organism's response which clearly represents a harmful effect at both the 'individual' and 'population' levels of organisation. The lowest level at which such responses can be measured with certainty are the three 'scopes' for activity, growth and reproduction.

The organism response used in this bioassay is the ability of the oyster embryo to develop normally and reach the 'D' shaped larval stage (at which the paired hinged shells can be seen within 24 h). Although the exposure time is short, it encompasses a period of intense cellular activity, so the impairment of a number of critical physiological and biochemical processes may result in poor growth and development. The response measured is, therefore, similar to that used in other tests on early life stages which record growth and development, and it has the advantage that exogenous feeding is not required, thus eliminating this source of variation in the test results.

A4.2 Test method

A4.2.1 'Reference' sea water

A large volume of sea water is taken during the winter months, filtered through Whatman GFC filter paper and stored frozen in acid-washed bottles for subsequent use in oyster gamete collection and control exposures.

A4.2.2 Water samples for bioassay

Sample volumes should be 200 ml; they are filtered through Whatman GFC filter paper to remove particulates and suspended solids which can affect embryo development (Davis and Hidu, 1969) and stored in sterilised bottles. They are bioassayed as soon as possible to avoid sample deterioration.

A4.2.3 Organism

The test described in this paper uses the Pacific oyster (*Crassostrea gigas*), but the method can be readily adapted for those other species of bivalve mollusc whose eggs are fertilised in the water column. Adult oysters are conditioned for spawning in the laboratory by maintaining them at 22°C in flowing sea water to which a mixed algal dietary supplement of *Isochrysis*, *Tetraselmis* and *Skeletonema* sp. is added. Maturation occurs within 1 week in summer and 8 weeks in winter.

A4.2.4 Collection of oyster gametes

For each assay, mature oysters are opened and the gametes stripped from the first 2 males and 3 females, using clean Pasteur pipettes for each oyster. The gametes from each oyster are then transferred to separate 1 litre volumes of reference sea water at 24°C. Sperm are identified by their milky appearance and eggs by their granular appearance, once they pair in the water. After filtration through a 90 µm filter to remove tissue debris, the three batches of eggs are pooled and the egg density adjusted with reference sea water to give a density of between 1000 and 4000 (and ideally between 3000 and 4000) eggs ml^{-1}. This density is then measured accurately using a Coulter Electronics particle counter. The two batches of sperm are also pooled but not filtered. All of the glassware used is sterilised in an autoclave.

---

*(Note: *this draft was submitted by R. Lloyd to the ICES Working Group on Biological Effects of Contaminants in 1989, and is intended for publication in an ICES series entitled 'Techniques in Marine Environmental Sciences'.)*

A4.2.5 Fertilisation

Mixing of gametes is made at a ratio of approximately 2 ml of sperm to 1 litre of egg suspension. After mixing, they are left for 1-2 h, during which time a sample is examined under a microscope to confirm that the early stages of cleavage are occurring. If cleavage is not observed within 2 h, the eggs should be discarded and further oysters stripped for gametes.

A4.2.6 Test sample

At least four 30 ml aliquots of each sample of filtered sea water from the survey area are placed in 50 ml polystyrene vials (stoppered or capped), and the temperature rated to 24°C.

A4.2.7 Control sample

At least eight 30 ml aliquots of 'reference' sea water at 24°C are placed in 50 ml polystyrene vials (stoppered or capped).

A4.2.8 Start of exposure

Using a sterilised Gilson (or similar model) automatic dispensing pipette, 1500 eggs at the early stage of cleavage are added to each 30 ml sample; the added volume of water containing the eggs is between about 0.37 and 1.50 ml (preferably between 0.37 and 0.50 ml), depending on the density of the eggs in suspension. The samples are incubated at 24°C for 24 h; no aeration is necessary unless the samples have a high oxidisable organic content. Measurements of dissolved oxygen and pH are necessary only if these are likely to deviate from natural values.

A4.2.9 End of exposure

At the end of the 24 h exposure period, a 2 ml aliquot of sample is removed and 2 drops of 8% formalin added. This sample, which should have originally contained 100 eggs, is transferred to a gridded shallow dish or slide, and the number of normal 'D' shaped larvae are counted. Where large numbers of samples have been taken, the contents of the vials can be preserved for future counting by the addition of 0.5 ml of buffered formalin.

A4.2.10 Calculation of results

The number of 'abnormal' embryos is calculated to be 100 minus the number of normal 'D', shaped larvae. 'Abnormal' includes those eggs which were not fertilised, and those which died at an early stage of development or became malformed. The additional abnormalities in the test samples, compared to those in the control, are expressed as the Percent Net Risk (PNR):

$$PNR = \frac{\%\text{ test abnormality} - \%\text{ control abnormality}}{100 - \%\text{ control abnormality}} \times 100$$

A4.3 Sources of error

A4.3.1 Number of exposed embryos

(a) The calculation of the results is based on the assumption that there were 100 embryos exposed in each 2 ml of test or control sample. This assumption may not be valid because of errors in:

(i) the measurement of the original egg density;

(ii) pipetting the aliquot containing the calculated 1500 eggs into each 30 ml in the sample vials;

(iii) the extra dilution which this aliquot gives to the 30 ml sample;

(iv) pipetting out the 2 ml aliquot for embryo examination.

(b) Errors in (i) and (iii) should be constant between the test and control samples and, therefore, lost to some extent in the calculation of the PNR. For example, if in practice only 95 eggs were present in each 2 ml sample, this would be equivalent to an extra 5% of non-fertilised eggs. In one series of tests, the mean number of eggs transferred to the sample vials was found to be 96 (n = 58, SD = 8.0, SE = 1.05); this slightly low value may reflect an error in the measurement of the original egg density. The variation between vials can be reduced by ensuring that the eggs are evenly distributed in the stock suspension. Errors in (ii) and (iv) are random; the overall error can be reduced by the use of replicate samples.

Table A4.1 Data from a survey of a transect between Plymouth and the Eddystone lighthouse. The sewage-sludge disposal site is at stations 5 and 6. This information is given to show the variation obtained between replicates

Station	Number of 'D' larvae in a 2 ml sample	$\bar{x}$	SD	PNR
Before disposal				
1 Plymouth	73, 75, 70, 74 77, 69, 69, 67	72	3.5	-4.3
4 Plymouth	69, 67, 66, 73 67, 74, 70, 70	69	2.9	0
6 Plymouth	67, 69, 72, 68 70, 70, 74, 68	70	2.3	-1.4
8 Eddystone	69, 70, 70, 72 76, 69, 65, 71	70	3.1	-1.4
Control	74, 68, 68, 70 65, 69, 68, 73 71, 66, 69, 66	69	2.7	-
Station 6 was further sampled at intervals during the disposal of sewage sludge:				
6.1 Plymouth	22, 18, 24, 20 28, 29, 19, 21	23	4.1	67.0
6.2 Plymouth	35, 40, 40, 32 33, 34, 38, 35	36	3.1	48.0
6.3 Plymouth	0, 0, 0, 0, 0, 0, 0, 0	0	-	100.0
6.4 Plymouth	46, 49, 53, 42 42, 47, 42, 53	47	4.6	32.0
6.5 Plymouth	76, 70, 71, 73 65, 69, 72, 71	71	3.2	-2.9

(c) While additional procedures could be introduced into the method which would help to achieve the nominal egg density, these would be time-consuming. Speed of operation is essential at the start of the exposure period, particularly when a large number of samples are being assayed, and the small errors which occur may be considered as acceptable.

(d) Table A4.1 shows the results of a typical experiment; it is clear that with experience in the techniques, the variation between replicates can be small. With a precision of this order, a statistically significant reduction in biological water quality can be shown when the PNR exceeds 5.

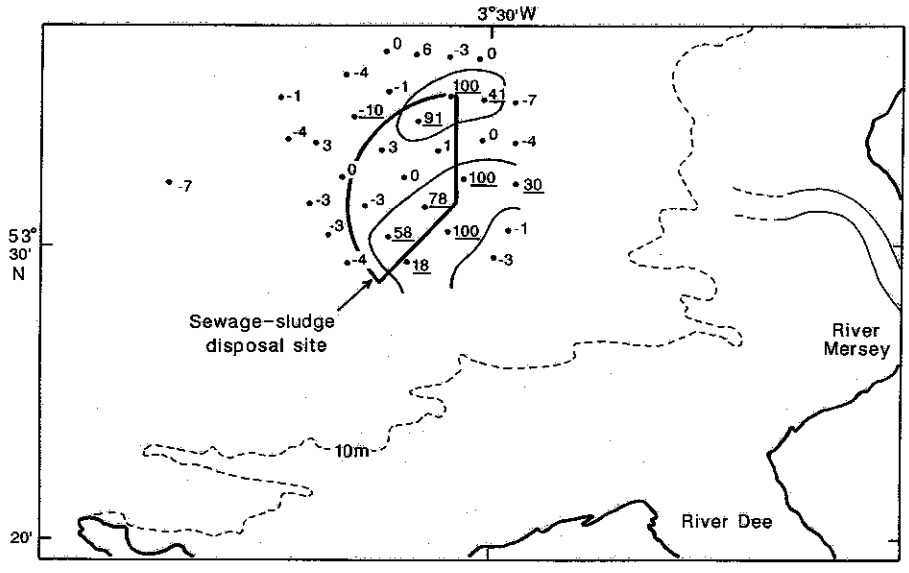


Figure A4.1 Oyster embryo bioassay, Liverpool Bay, August 1981. Percentage of net response for surface water samples. Underlined values are significantly different from the control values.

A4.3.2 Dilution of sample

The procedure described above will lead to a dilution of the test sample by up to 5%. Such a dilution effect may not be important in field surveys, where a variation between PNRs is looked for in relation to a known or suspected source of pollution (Figure A4.1). However, if the toxicity of a chemical is being measured, it will be necessary to take this additional dilution into account when calculating the nominal exposure concentrations. If the volume of egg inoculum is below 0.5 ml, the error from this source will be minimal.

A4.3.3 Acceptable control abnormality

In the original method of Woelke (1972), it is recommended that control abnormalities should not exceed 5%. However, these recorded abnormalities at the end of the exposure period do not include mortalities at the early stage of development, nor do they include non-fertilised eggs. It is common hatchery experience that, with good management practice, at least 80-85% of oyster eggs should develop successfully to the 'D' shaped stage, although sometimes this can fall to 50% (Loosanoff and Davis, 1963). Experience with the oyster embryo bioassay indicates that control 'abnormalities' of up to 20% is normal, and that up to 30 or even 40% is acceptable. Higher percentages of abnormalities may be caused by contaminated reference sea water, or the use of immature gametes from oysters which have not reached sexual maturity, or gametes from oysters in poor condition.

A4.4 Reproducibility of the test

In recent years, tributyltin has been used as a reference toxicant. Exposure of developing oyster embryos to a range of concentrations showed that 1.65 µg TBT l^{-1} would give a PNR of 50, and this concentration has been used in conjunction with subsequent field surveys. PNRs recorded for the reference toxicant in 8 successive surveys have ranged from 37 to 71 with a mean value of 51; using data from the initial calibration test, this range of PNRs is equivalent to a concentration range of 1.25 to 1.95 µg TBT l^{-1}. Although the control abnormality in the separate tests ranged from 13 to 44%, there was no apparent correlation between these levels and the calculated PNRs. Therefore, this bioassay has a reasonable reproducibility.

A4.5 Example of survey data

Table A4.1 gives data for the oyster embryo bioassay obtained in 1984 from a survey of the sewage-sludge disposal ground between Plymouth and the Eddystone lighthouse, Devon, UK. This shows the degree of reproducibility between replicate analyses of single samples.

Analyses of the toxicity of the sewage sludge to oyster embryos, showed that the 'field' PNRs correlated well with the concentration of sludge at the disposal site as calculated from concentrations of suspended solids (J. E. Thain and A. R. D. Stebbing, in preparation).

As an example of the type of information obtained, Figure A4.1 shows the variation in water quality at a sewage-sludge disposal site when discharges were being made.

The design of such surveys should follow the same pattern, and be conducted with the same rigour, as that used for chemical sampling in similar circumstances.

A4.6 Interpretation of data

The results of this bioassay cannot be used to predict the effects of a small measured deterioration in biological water quality on oyster populations, or on the general aquatic biota. This is especially the case where the natural biota are exposed to poor biological water quality for only a short period of time, whereas in the bioassay the embryos are exposed to the water sample for 24 h. The results can be used to measure the gradients and distribution of poor biological water quality in the vicinity of pollutant inputs; where a gradient is found, it can be assumed that the potential for harm to aquatic biota will increase with the degree of the bioassay response, but the nature and extent of the effect which may occur in practice cannot be predicted. The bioassay is sensitive to a wide range of chemicals; it is also sensitive to a deterioration in biological water quality caused by algal blooms (Thain and Watts, 1987)

A4.7 References

DAVIS, H. C. and HIDU, H., 1969. Effects of turbidity producing substances in sea water on eggs and larvae of three genera of bivalve molluscs. Veliger, *11*: 316-323.

LOOSANOFF, V. L. and DAVIS, H. C., 1963. Rearing of bivalve molluscs. Adv. Mar. Biol., *1*: 1-136.

THAIN, J. E. and WATTS, J., 1987. The use of a bioassay to measure changes in water quality associated with a bloom of *Gyrodinium aureolum* Hulburt. Rapp. P.-v Réun. Cons. int. Explor. Mer, *187*: 103-107.

WOELKE, C. E., 1972. Development of a receiving water quality bioassay criterion based on the 48-hour Pacific oyster (*Crassostrea gigas*) embryo. Tech. Rep. Dept. Fish. Wash., *9*: 1-93.

ANNEX 5. An inventory of practical issues relevant to the adoption of a programme for the monitoring of biological effects

A5.1 Desk study

A5.1.1 Environmental variables

Tidal and residual currents will determine the main pathways of sludge dispersal, and hence sampling design. Depth and turbidity of water, and the nature and distribution of sediments at the sea bed will be equally important in test selection and sampling design.

A5.1.2 Disposal practice/quantity and quality

This will notably influence the timing of sampling for the conduct of short-term tests, as well as determining site location.

A5.1.3 Other inputs/activities

These are important when interpreting test results, and may also influence the location of sampling sites. For example, with caged animals, it may be necessary to avoid areas of intense commercial fishing activity or shipping movements.

A5.1.4 Other studies of the locality

These may help in a number of ways in the identification of the most appropriate environmental target, and in enhancing the design of a sampling programme.

A5.1.5 General comments

It will be clear from the above points that the influence

of natural and man-made factors acting in combination may have a considerable bearing both on the choice of tests and the efficiency of their conduct. In some circumstances, it must be recognised that a desk-appraisal of cost-effectiveness might lead to the conclusion that there are no field tests presently available which can guarantee an unambiguous outcome.

A5.2 Planning and design of the sampling programme

A5.2.1 *Identify target: water/sediment*

This should become apparent from the desk study.

A5.2.2 *Clarify objectives: short-/long-term tests*

In a highly dispersive area, the main concern might be with the immediate impact in the water-column around the disposal site following a single discharge; in zones of known long-term accumulation, the concern might be with any biological effects integrated over an appreciable time period, probably at or near to the sea bed, but not necessarily near to the disposal site.

A5.2.3 *Select test*

This will be determined in part by the above considerations, and in part by the availability of tests which are presently suited for routine deployment.

A5.2.4 *Pre-survey calibration of response with dilutions of waste*

This will be easier for short-term tests (e.g. the oyster embryo test), but in principle should be feasible for all tests. In some circumstances, the outcome could have important implications for the detection of initial waste impact in the field, because of the variable but usually high rate of initial dilution following discharge from ships.

A5.2.5 *Define the acceptable level of change*

Such a task will be difficult in the absence of base-line data, or of a full understanding of the 'relevance' of test results to field populations. However, at an appropriate distance from a discharge, a management criterion might be 'no change' (acceptable or otherwise) relative to background. With careful experimental design, it should be possible to achieve a coefficient of variation of 20% of the mean for the two first-ranked tests at any given site, though the effort required is likely to vary appreciably from one location to another and will ultimately depend for confirmation on experience in the field. Clearly, a sound knowledge of dispersal pathways will be required to allow selection of appropriate sampling sites.

A5.2.6 *Grid/transect/profile in relation to disposal practice*

The selection of sampling locations (and the number) will be considerably aided by prior knowledge of the receiving area (see above). The choice of a grid or transect design will depend *inter alia* on the complexity of the dispersive process. In the simplest and best understood cases, sampling to meet the requirements for adequate comparisons, spatially and with time, may be achievable with very few stations. Profiles will be necessary to establish the rate of vertical as against horizontal mixing. For some tests (e.g. that for caged mussels) the number of stations will probably be limited by cost and logistical constraints.

A5.2.7 *Number of samples*

This will be dictated by the required precision of the results in order to meet specified objectives, and will inevitably vary from site to site. For long-term tests (e.g. using caged animals), the risks of accidental loss of samples, as a result of weather or human activities, must also be taken into account.

A5.2.8 *Supply of test organism(s)*

This may influence the timing of surveys; consistency of stock may be very important when it comes to 'year-on-year' comparisons.

A5.2.9 Adequate facilities on board vessels

Clearly, a pre-survey review of requirements for the conduct of sampling and test procedures at sea is essential.

A5.2.10 Adequately trained staff

This requirement is self-evident, and will have been costed at an early stage in the planning process.

A5.3 Execution of tests

A5.3.1 Method

For short-term tests, standard procedures for sampling of water or sediments in the field, and subsequent testing, should be followed.

For tests involving long-term exposure in the field (e.g. employing *in situ* caged animals), the appropriate organisations will already have been consulted regarding the survey plan, and advice as to the suitability of station locations sought where necessary. It will be essential to adequately buoy and mark all deployed cages in accordance with maritime requirements.

A5.3.2 Parallel measures

Especially in the case of short-term tests, it is most important that determination of an appropriate range of physico-chemical and biological variables is made on samples taken in parallel with those for the test. These will typically include measurements of salinity, temperature, turbidity, relevant contaminants and chlorophyll.

Clearly, the presence of a plankton bloom may induce a widespread reduction in water quality, even though the cause may be entirely natural.

A5.3.3 Weather

Weather conditions, preceding and during collection of samples, may have an important influence on the dispersion process for recently dumped wastes, and hence could account for much of the variability in intensity of effects between sampling occasions for short-term tests of water-column samples. For long-term tests (i.e. those spanning weeks or months of field exposure) effects of weather will tend to even out in accordance with natural seasonal expectations; however, even seasonal norms cannot be guaranteed, and hence accurate records should be kept.

A5.4 Frequency of sampling

This will depend on the objectives to be met (see subsection A5.2), but will be higher (typically once per year for long- term tests) for a new disposal operation. Other factors determining frequency will be the perceived sensitivity of the area in scientific or other terms, and changes to the quantity and/or quality of the waste discharged.

A5.5 Interpretation and reporting

A5.5.1 Trends

For short-term tests, assessment of trends will initially involve an examination of spatial pattern on grids or transects and, as monitoring data are accumulated, any changes in these patterns with time.

For long-term tests, a similar approach will be adopted, though in general fewer sites will have been sampled, and a more detailed comparison of differences between sites will be of greater interest, because the results will represent a time integral of any effects of waste disposal. Greater confidence may also be placed in examinations of 'year-on-year' trends at key sites, because of this.

A5.5.2 Correlation with other variables

This will involve correlation of results of tests with simultaneous measurements of water or sediment quality. The findings should also be examined in relation to those from other monitoring activities (e.g. those on sediments and benthos), and hydrographic data. Effort should continually be made to improve integration between the various monitoring actitivies.

A5.5.3 Intercalibration between surveys/locations

These exercises will be very important to ensure temporal consistency and, where appropriate, to allow comparisons between studies of different waste disposal activities. The results of such exercises should be annexed to monitoring reports.

Ministry of Agriculture, Fisheries and Food
Directorate of Fisheries Research
Fisheries Laboratory
Lowestoft
Suffolk
NR33 OHT
England

AQUATIC ENVIRONMENT MONITORING REPORT Number 24