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REGIONAL COORDINATING UNIT
EAST ASIAN SEAS ACTION PLAN

UNEP

UNITED NATIONS ENVIRONMENT PROGRAMME

PROCEEDINGS OF THE UNEP WORKSHOP ON
BIOLOGICAL EFFECTS OF POLLUTANTS

RCU/EAS TECHNICAL REPORTS SERIES NO. 3

Prepared in cooperation with:



PMBC



PML

UNEP

Bangkok, 1994

Note:

The Tenth Meeting of the Coordinating Body on the Seas of East Asia: COBSEA (Bangkok, 9-10 July 1993) approved the convening of a regional workshop on the Biological Effects of Pollutants in Phuket, Thailand. The workshop was organized and convened during 16-25 November 1993 by the Phuket Marine Biological Centre (PMBC) in close cooperation with the Plymouth Marine Laboratory (PML) under the UNEP project FP/ES/5101-93-04. This volume is published under the Regional Coordinating Unit for the East Asian Seas Action Plan's (EAS/RCU) Technical Reports Series, as one of the selected reports that are the result of various activities approved by the COBSEA and implemented within the framework of the Action Plan for the Protection and Management of the Marine and Coastal Environment in the East Asian Seas Region.

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PREFACE

This volume of the Regional Coordinating Unit for the East Asian Seas Action Plan (EAS/RCU) Technical Reports Series contains the proceedings of the Phuket Workshop on Biological Effects of Pollutants. The convening of the workshop was approved at the tenth meeting of the Coordinating Body on the Seas of East Asia (COBSEA) and it was organized by the Phuket Marine Biological Centre: PMBC (Thailand) with close collaboration with the Plymouth Marine Laboratory: PML (UK). The workshop was held at the Phuket Marine Biological Centre during 16-25 November 1993.

The main thrust of the Workshop, as perceived by the COBSEA, was essentially capacity-building in the form of training of the scientists of the countries of the region in the state of the art methodologies and techniques for assessing and monitoring the biological effects of pollutants in the marine environment. It was expected that these scientists will in turn impart their newly acquired skills to others back in their national laboratories and institutions.

Furthermore, the objectives of the Workshop were also selected in a manner so as to bring them close to those of the IOC/UNEP Group of Experts on the Effects of Pollutants (GEEP). Thus, ensuring that the results of latest methodologies in monitoring and assessment of the biological effects of pollutants in the marine environment, in as much as possible of a standardized manner in the region, be made available for the effective management of the region's coastal and marine areas.

The cooperation of the Plymouth Marine Laboratory (PML) was deemed important for the GEEP linkage and drawing on the PML's experience and input based on the results from workshops held under the auspices of Intergovernmental Oceanographic Commission (IOC) and International Council for Exploration of the Seas (ICES).

The scientific programme of the Workshop was devised by PML in consultation with PMBC and EAS/RCU, taking into consideration the limitations imposed by time and financial resources. The logistics for implementation of this programme was provided by PMBC and the programme was carried out by resource persons from PML with the assistance of the scientific staff of PMBC.

The assessment of the degree of the success of the Workshop by the participants as well as their recommendations on follow-up activities to be considered by the COBSEA, will be presented to the next intergovernmental meeting of the Action Plan.

The final editing and compilation of this volume was done by the staff of the Phuket Marine Biological Centre in consultation with the Regional Coordinating Unit for the East Asian Seas Action Plan.

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INTRODUCTORY AND WELCOMING ADDRESS

BY MR. PRAWIN LIMPSAICHOL
Workshop Organizer
Phuket Marine Biological Center, Thailand

The Deputy Governor

Colleagues from Plymouth Marine Laboratory

Distinguished Participants, Ladies and Gentlemen

In almost all of our countries many pollution episodes have occurred which have resulted in harmful and detrimental effects on marine flora and fauna, sometimes leading to large scale destruction of these species and with inevitable economic loss. The degree of success in remedial action has been mixed, but generally unsatisfactory. In addition to the identifying the causes and employment of preventative measure, an important task is to assess the degree of the damages and particularly on the detrimental effects of harmful pollutants on selected marine species.

Phuket Marine Biological Centre has for a few years been engaged in the assessment and evaluation of the detrimental effects of pollutants on marine flora and fauna. Among its research programmes are measuring specific stress responses in order to diagnose the harmful effects of pollutant to marine living organisms. In this type of research the experiences and methodologies of the GEEP's Bermuda Workshop in 1972, in which some of the personnel of Plymouth Marine Laboratory were involved, have been applied as far as possible.

In view of the experience and research advances in the state of the art technologies, Plymouth Marine Laboratory was contacted to assist in devising the scientific programme of the Phuket workshop.

The development of the Phuket workshop project and allocation of US\$ 60,000 towards its convening was decided at the Tenth Meeting of the Coordinating Body on the Seas of East Asia (Bangkok, 9-10 July 1993). The Phuket Marine Biological Center and other national institutions also had an input of approximately US\$ 30,000 as in kind contribution.

Thus, the present Workshop is the result of the combined efforts of the Phuket Marine Biological Center in cooperation with Plymouth Marine Laboratory and national institutions of the participating. The Regional Coordination Unit for the East Asian Seas Action Plan (EAS/RCU) has also played a major role in coordinating all the activities.

I am glad to report that the Workshop is attended by scientists from seven countries in the region, namely the participating countries of the East Asian Seas Action Plan: Indonesia, Malaysia, the Philippines, Singapore, and Thailand as well as those of People Republic of China and Socialist Republic of Vietnam.

I would like to take this opportunity and express my gratitude to all those who have rendered their cooperation and assistance in the convening of this Workshop. Specially all the colleagues who have assisted, encouraged and shared the work with Phuket Marine Biological Centre.

I hope that based on the experience of the next few days we come up with recommendations for follow-up activities after this Workshop, specifically a feasible programme for the next 2-3 years to sustain and coordinate an assessment and monitoring programme in the countries of the region.

Finally, I wish you all success for this Workshop and hope that the knowledge learned and shared here will contribute to your endeavours in mitigating the marine environmental problems of your countries.

Wishing you an enjoyable stay in Phuket.

Thank you.

INTRODUCTORY ADDRESS

By **K. R. CLARKE**
Representative of Plymouth Marine Laboratory, UK.

The Deputy Governor of Phuket

The Director of Phuket Marine Biological Center

Distinguished participants, Observers, Ladies and Gentlemen.

It is a pleasure for me and my colleagues to come to Phuket, an island renowned for its natural beauty. It is also our great privilege to collaborate with Phuket Marine Biological Center in implementing the Phuket Workshop on Biological Effects of Pollutants under the auspices of UNEP.

When first approached by Mr. Prawin Limpsaichol, Workshop Organizer, to provide the scientific programme for this workshop, I confess that we were a little skeptical that the substantial technical and administrative requirement necessary for such a wide-ranging workshop could be accomplished in the short time available. We need not have worried; the facilities and arrangements have been excellent and we look forward to a most successful workshop. The objectives of the Phuket Workshop match with the aims of the Intergovernmental Oceanographic Commission (IOC) Group of Experts on the Effects of Pollutants (GEEP), to provide linkage between results of the latest research on the effects of pollutants and effective management of environmental issue. One important strand is the involvement of IOC and UNEP regional programmes both as a means of capacity building in expertise with the latest methodologies and in promoting further research insight into applicability to tropical areas of technique conceived for temperate regions. Therefore, the UNEP-Phuket workshop will provide firstly an introduction to the development, testing, application and interpretation of biological effect techniques, including biochemical, physiological, cellular measurements, water quality bioassay and benthic community disturbance. The demonstrations and lectures will be practically based, many of them using local species (fish, bivalves, echinoderms, etc.) sample at control and potentially impacted locations. Secondly, there will be discussion on the use of biological effect techniques within monitoring programmes to maintain environmental quality and underpin managerial action on pollution issues. In the later stages of the Workshop there will be discussions on how best to follow up this initiative in the East Asian Seas region.

The UNEP-Phuket Workshop, as a whole will draw heavily on the results of a series of practical research workshops held under the auspices of IOC and ICES on pollution gradients in Norway (MEPS Vol.46 1988), Germany (MEPS Vol. 91,1992) and Bermuda (JEMBE, Vol. 138,1990) and the North Sea (MEPS Vol. 91, 1992) over the last 6 years. The workshops were designed to calibrate a wide range of biological effect techniques, deployed simultaneously on known pollution gradients, relating results to appropriate chemical data. They succeeded in reflecting the advances that have been made over the last decade in improving sensitivity, diagnostic capability and relevance of biological monitoring of marine environments.

I am hoping that the Phuket Workshop will initiate a follow up programme to make advantage of techniques. The findings will response specifically to stressors which may be elucidated by group or individual of pollutants and would allow us to determine both the extent

of biological stress in native organisms and identifying the causative environmental factors responsible for the observed effects.

Finally, I would like to thank Mr Limpsaichol and the other local organizers, together with the Director of PMBC, for their kind hospitality and excellence of the arrangements and also formally thank UNEP for their sponsorship, with the host institute, of our participation. We are looking forward to what is certain to be most interesting and exciting 10 days. Thank you.

WELCOMING ADDRESS

**BY THE DEPUTY GOVERNOR
Phuket Province, Thailand**

Resource persons from Plymouth Marine Laboratory,

Director of Phuket Marine Biological Center

Distinguished participants, Observers, Ladies and Gentlemen

It is my honour to welcome you all to visit Phuket, an island blessed with natural beauty. However, many recent development projects have led to detrimental impacts on Phuket's coastal resources in some areas. In the foreseeable future an increasing pressure appears to be unavoidable. All such impacts are attributed to anthropogenic activities and reflects careless planning and managements.

In certain cases we had little knowledge of the detrimental effects of the pollution or the pollutants that we introduced in our marine environment. I can say with confidence that in our rush for development, many of the countries of the East Asian Seas region have faced similar experiences.

Phuket has been campaigning to raise public awareness in order to address the problem and alleviate such impacts. However, the solution to the problems are many-faceted and one does not expect that much can be achieved within a short timescale. The efforts require a continuous long-term plan, sound and scientifically based building up public awareness and participation, supporting the necessary infrastructure that will contribute to the effective management and wise utilization of our resources. Within this context I would like to express our thanks to the Phuket Marine Biological Centre for continuing to contribute to this aim in the fruitful manner they have and directing their efforts and research of the Phuket coastal and marine environmental problems.

It is with great pleasure to learn that Plymouth Marine Laboratory, who are one of the most famous research institutes are assisting this Workshop with the most recent and the state of the arts methodologies on biological diagnosis towards stresses of pollutants. I do hope that these technologies would allow us to determine both the extent of biological stress in our native organisms and identify the causative agents.

On behalf of Phuket, I would like to extend my warm welcome to you all once again, and thank the resource persons who have been travelling for a long distance in order to contribute to this Workshop. Cooperation of all participants and observers are acknowledged. Thanks are also extended to PMBC for their contribution in being instrumental in introducing these latest methodologies to Thailand and the other East Asian Seas countries in order to assist addressing the marine environmental pollution problems which ultimately will benefit all of us.

I look forward to a very successful Workshop and your continued cooperation in the future.

I wish you all a joyous stay in Phuket.

Thank you.

PROCEEDINGS OF THE UNEP WORKSHOP ON BIOLOGICAL EFFECTS OF POLLUTANTS

Phuket Marine Biological Center (PMBC), November 16-25, 1993, Phuket, Thailand

1. OBJECTIVES OF THE WORKSHOP

1.1 Short-term objectives

- to assess the applicability of and requirement for modification, where necessary, of state of the art methods for assessing the harmful biological effects of pollutants in a tropical environment;
- improve the comparability of the results of scientific studies and assessments of the biological effects of pollutants in the participating countries of the region;
- building up a network of scientists and institutions in the countries of the region.

1.2 Long-term objectives

- to develop the scientific skills of the personnel that are engaged in studies and assessment of biological effects of pollutants in the East Asian Seas region; and
- to improve the knowledge and the ability of the middle and upper level administrators and policy/decision-makers in the management of coastal and marine resources through enhanced quality and quantity of relevant data and the analysis of these data.

2. BACKGROUND TO THE WORKSHOP

The pace of development and industrialization in the countries of the East Asian Seas region has continued at rapid rate over the past decade. All the indications are that this will continue for the foreseeable future. Some of the monitoring and assessment studies carried out within the framework of the East Asian Seas Action Plan have indicated that in certain areas such development has led to the environmental deterioration of the coastal and marine flora and fauna.

This fact was highlighted during the Ninth Meeting of the Coordinating Body on the Seas of East Asia: COBSEA (Kuala Lumpur, 13-15 November 1991) which approved the implementation of a follow-up project to an already completed one, aimed at the development of management plans for endangered coastal and marine resources in East Asia: Phase II (EAS-25). The first phase of the EAS -25 was completed under the EAS-19 project entitled "Development of Management Plans for Endangered Coastal and Marine Living Resources in East Asia: Phase I (EAS-19)".

However, the same meeting of the COBSEA also noted that in order to have a realistic and cost-effective foundation the management-orientated projects within the framework of the East Asian Seas Action Plan additionally required scientifically based assessment projects. Therefore, the meeting decided on the approval of convening a "training" workshop on the

Biological Effects of Pollutants as a first step and selected the Phuket Marine Biological Centre of the Royal Thai Government to organize and convene the workshop.

The reasons for approving the convening of the workshop were based on the following considerations:

- the scientists of the participating countries could benefit from being familiar and utilizing the state of the art methodologies on the studies of the biological effects of pollutants;
- the most up-to-date studies of the effects of pollutants on marine flora and fauna were usually conducted in temperate and not tropical countries. The effectiveness and efficiency of these methodologies needed to be discussed, tested and ascertained for the tropical conditions: and
- the necessity for comparable studies and results in the relevant institutions of the participating countries of the region.

Due to some unavoidable reasons, outlined in the report of the Executive Director of UNEP to the Tenth Meeting of the COBSEA [UNEP (OCA)/EAS IG.4/3], the organising and convening of the Phuket workshop was delayed. The Tenth Meeting of the COBSEA having noted the importance of the workshop reiterated the need for it and confirmed the necessity of the convening of the workshop in 1993.

The Phuket workshop is, therefore, planned to emphasize the above needs as well as to concentrate on:

- applying indices for assessing the biological impact of pollutants and to evaluate and extend the range of techniques which have proved successful in regions other than the East Asians seas region;
- offer advanced training to selected scientists of the region in these approaches, utilizing as far as possible the available standard equipment;
- encourage and ensure further in-country training by the scientists who participated in the workshop; and
- standardize the methodologies in the countries of the region and build up closer collaboration among the participating countries of the region in the East Asian Seas.

3. STRUCTURE OF THE WORKSHOP

The workshop ran from 16-25 November 1993, hosted by the Phuket Marine Biological Center (PMBC), Thailand, and with resource persons from the Plymouth Marine Laboratory (PML), U.K. The scientific programme of the workshop was developed by the staff of PML with the cooperation of PMBC. The work programme is given in Appendix 1.

The overall coordination of the workshop was carried out at PMBC by Mr.P. Limpsaichol, assisted by the staff of PMBC. On the PML side the coordination of the PML's input and activities was performed by Mr.A.R.D. Stebbing. The Course Director at PMBC was Mr.M. N. Moore of the PML.

The 19 participants came from 7 countries of the East Asian region: Philippines, Malaysia, Singapore, Indonesia, Thailand, People's Republic of China and Socialist Republic of Vietnam. A full list of the participants is given in Appendix 2.

In the opening session, held in the presence of the Deputy Governor of Phuket and the Assistant Director of PMBC, Mr.P. Limpsaichol referred to the background of the workshop, and joined the Deputy Governor, in welcoming the participants to the PMBC and Phuket.

Mr.K.R. Clarke (PML) introduced the resource persons team and in relation to the COBSEA-approved Phuket workshop, outlined the rationale for the series of related training workshops which had been spawned by the research activities of the United Nations Environment Programme and Intergovernmental Oceanographic Commission's Group of Experts on the Effects of Pollutants (GEEP).

The workshop itself covered three main disciplinary areas, each being led by two resource persons from PML:

- A. Cellular/Biochemical approaches covered by D.M. Lowe and L.D. Peters;
- B. Physiological/Bioassay techniques covered by J. Widdows and I.R.B. McFadzen;
and
- C. Community studies covered by K.R. Clarke and R.M. Warwick.

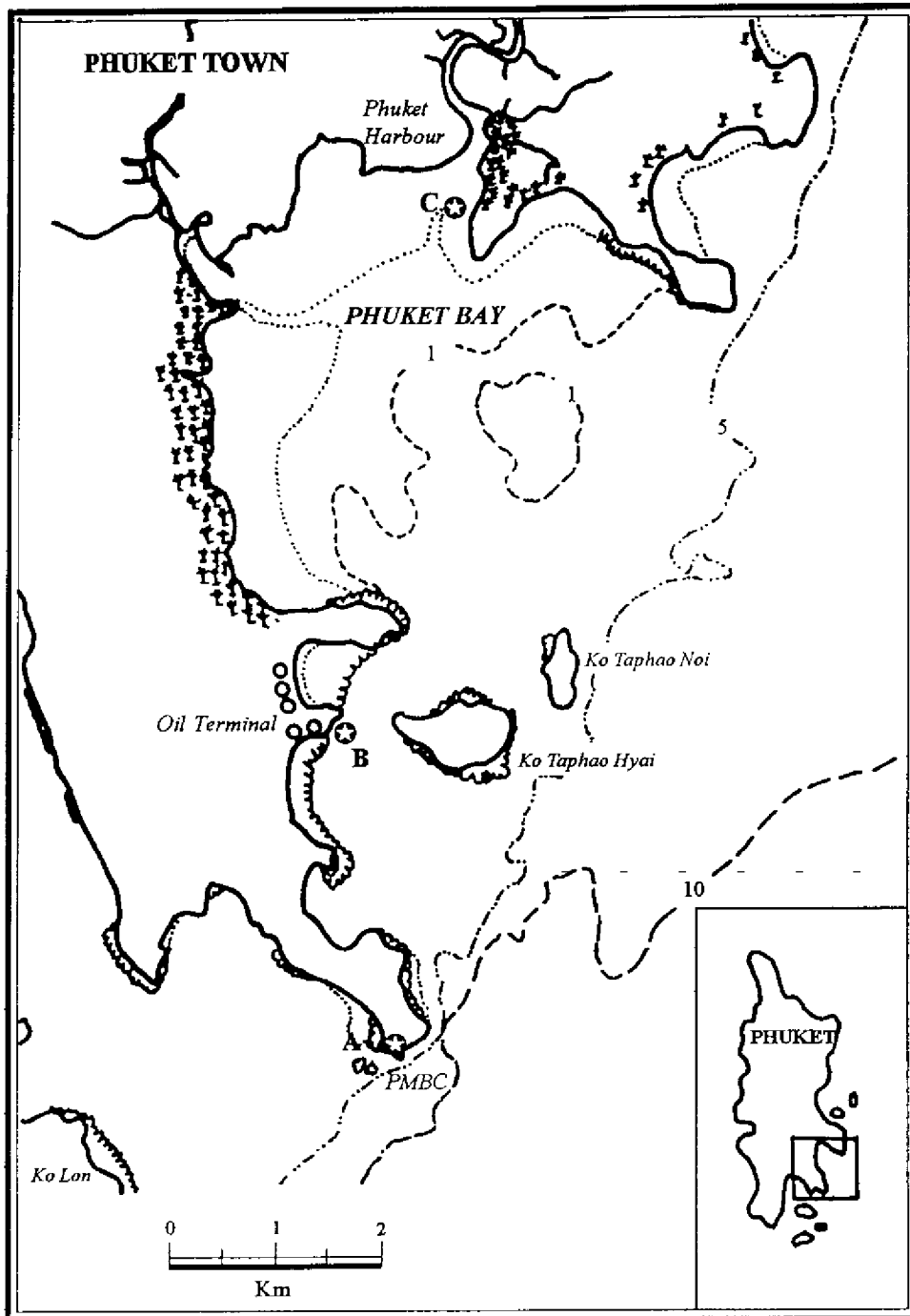
It was unrealistic and impractical to schedule all activities for every participant in full detail, both on the grounds of time and the prior expertise and/or areas of interest of participants. They participants were, therefore, invited to chose two areas between **A**, **B** and **C**, with the size of the **A + B** group being approximately the same as the size of the **B + C** group.

Space and facilities were made available to hold parallel sessions on most days, while all the participants took part in B area.

Practical sessions associated with the activities A and B involved both field collection (see Fig.1) and laboratory experimentation on local species of shallow-water invertebrates and fish, from control and polluted locations (including organisms transplanted to these locations). The benthic community component comprised lectures and computer practicals, since it was not logistically possible to complete a field programme in the time available.

In addition, lectures setting these techniques in the background of previous biological effects calibration exercises were given by Mr.M.N.Moore (Course Director, PML), who also led the closing discussion of future plans for follow-up activities and monitoring schemes in the region.

Figure 1 Indicating location of sampling sites: (A) reference site, at PMBC Jetty, (B) oil terminal jetty, and (C) Phuket harbour.



4. THE WORKSHOP COMPONENTS AND FINDINGS

4.1 Biochemistry

An initial lecture explained some primary fundamental biochemistry in order to allow common ground to be laid. This started with an explanation of bio-markers and categorising them into bio-markers of exposure or damage as well as higher order responses. Detoxication was then explained, by the use of diagrams illustrating uptake, biotransformation and excretion, specifically relating the liver as the primary site of metabolism. The mixed function oxygenase (MFO) system was then described as part of Phase 1 metabolism and cytochrome P450 was considered as a system of xenobiotic metabolism and bio-activation, using benzo[a]pyrene (B[a]P) as a model. The advantages and disadvantages of cytochrome P450 1 A CYP1A was explained as a bio-marker of exposure. Recent laboratory studies and field applications were presented including current research studies.

A second lecture explained the phenomena of free radicals and they were described as harmful using examples from human medical disorders eg Parkinsons' disease. The formation of reactive oxygen species (ROS) was demonstrated via the sequential reduction of molecular oxygen. Redox cycling was also covered and used to demonstrate the formation of the superoxide (O₂⁻) radical. Antioxidant processes were covered including scavengers (cytosolic and membrane) as well as the enzymes superoxide dismutase, catalase, glutathione peroxidase and DT diaphorase. Field and laboratory studies were presented showing small but significant changes in the levels of the enzymes. It was clearly explained that more research was necessary before antioxidant enzyme activities could be adopted as bio-markers of xenobiotic exposure.

The practical demonstration was split into two half day sessions. In the first, students were shown the gross anatomy of the dissected fish and then proceeded to dissect their own specimens. They then homogenised and centrifuged the liver preparations. Whilst the samples were being centrifuged, the students saw demonstrations of (and then practised) intra-peritoneal (ip) injections on living fish. On the second half day session, students determined EROD activity in fish injected (ip) with B[a]P and beta-naphthoflavone and expressed the activity as specific activity per g wet weight as well as per mg protein. Total protein was measured by the method of Lowry.

Table 1. shows the results of this laboratory study, on elevation of EROD activity in immature grouper (*Amyperodon lencogrammicus*) after 24 hour exposure to organic xenobiotics (20 mg kg⁻¹ ip injection).

Table 1 EROD activity in immature grouper (n = 2).

Treatment	Specific activity (pmol min ⁻¹ g ⁻¹ wet weight)
Control	0*
B[a]P	1043 ± 262
Bata-naphthoflavone	450 ± 367

* below detection limits of assay, as performed at PMBC

The demonstrations and lectures appeared to be well-received though the participants' command of English was variable and may have limited understanding in some cases; also the generally low level of background knowledge of biochemistry was restricting. There was a high level of support from PMBC staff throughout the workshop; and when the laboratory receives the new centrifuge and spectrophotometer, they will be well equipped for future biochemical studies.

4.2 Cellular methods

An initial session concentrated on quantitative histopathology in marine animals: this was an overview of methods including stereology, image analysis, densitometry and confocal laser scanning microscopy. It examined the necessity for objective quantification of histopathological and immunocytochemical bio-markers in the context of multi-disciplinary research on marine animals. The objective of the lecture was to demonstrate that sound quantification could be achieved for a range of parameters using simple equipment and was targeted at laboratories that cannot afford sophisticated computer-aided image analyses. The lecture was structured to introduce the techniques in such a way that the range of parameters that could be determined increased with each type of method as did the sampling resolution. There was no practical session associated with this lecture.

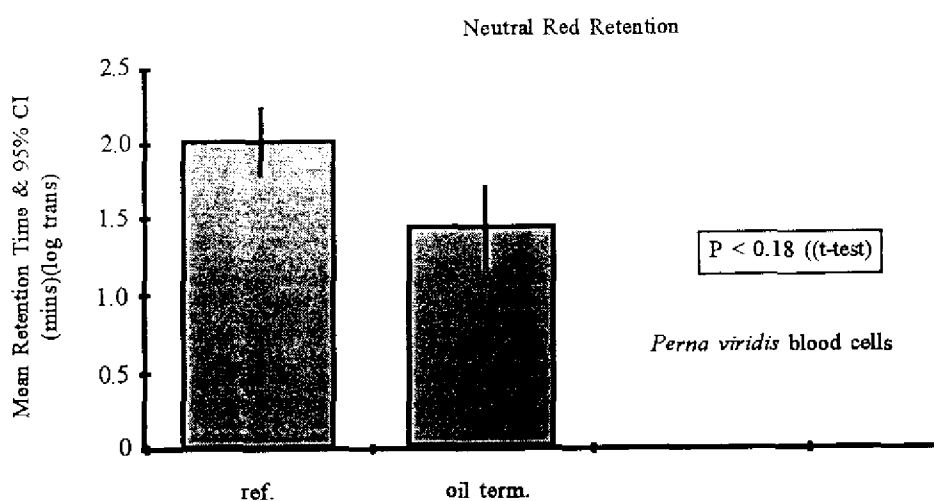


Figure 2 Lysosomal integrity (neutral red retention time) in blood cells of mussels (mean (log transformed) \pm 95% CI, $n = 10$). (Key: ref, cultivated mussel transplanted to clean reference site 6 days; oil term, transplanted to Oil Terminal Jetty, 4 days.)

A second session concerned lysosomal membrane damage in molluscs as a bio-marker of contaminant exposure and effect. The new *in vitro* assessment of lysosomal membrane damage was discussed and compared with the now well established histochemical method of lysosomal latency. Examples of the use of the method under field and experimental studies were presented and the results discussed in the context of a range of histological, histochemical and immunocytochemical bio-markers from the same studies.

This lecture was supported by a demonstration of the method, including a brief discussion on the anatomy of the mussel. Students were given an opportunity to take blood samples from mussels and set up the assay for study. During a practical session the students were given cultivated mussels that had been transplanted to a field site and had to set up the

assay, analyse the material and determine the retention period of neutral red for themselves. During the course of the practical session the students were able to see almost all the stages associated with the breakdown of lysosomal function. Figure 1 shows some of the results from this transplant study, for lysosomal membrane damage in *Perna viridis* blood cells expressed in terms of a reduction in the retention time of the cationic probe neutral red.

These results clearly show that lysosomes in the blood cells of mussels transplanted to the Oil Terminal Jetty for 4 days had been damaged by probable exposure to xenobiotics. This once again confirms the sensitivity of lysosomal injury as an indicator of pollutant induced damage to the health of the animal.

4.3 Physiology

Two main introductory lectures were presented to all participants. These outlined the role of physiological stress responses in environmental toxicology and pollution assessment. The first lecture began by examining the broad range of methods of pollution assessment and briefly reviewing the advantages and limitations of each in the context of the overall objectives of Environmental Protection Agencies. It emphasized that the current descriptive methods need to be complemented by more sensitive techniques that are able to provide environmental managers with an early detection and quantification of changes in environmental quality, as well as identification of the cause. The role of physiological energetic measurements (scope for growth) combined with chemical analyses of contaminants in body tissues of mussels in toxicological studies and pollution monitoring programmes was outlined. The main features of this approach were reviewed, including aspects of bio-accumulation, sensitivity, concentration - response relationships, quantitative structure - activity relationships (QSARs), mechanistic interpretation and integration, ecological relevance and its cost-effective application in both laboratory and field studies.

The second lecture focused on the field application of closely coupled measurements of scope for growth and chemical contaminant levels in mussels with reference to various case studies. The procedures for measuring scope for growth (SFG) were outlined. The results of field studies show that the combined measurement of SFG and the chemical contaminants in body tissues of mussels not only provide a means of detecting and quantifying effects of pollution, but also a means of identifying the causes. Identification of the 'cause' is achieved using QSARs and established concentration-response relationships between the concentration of contaminants in mussel tissues and the scope for growth response. Case studies included those assessing pollution impact along contaminant gradients in bays, estuaries and fjords (e.g. North Sea oil terminal, Bermuda and Oslofjord GEEP Workshops). In a recent study, the approach has been extended and successfully applied over a larger spatial scale involving > 1000km of North Sea coastline. The results of this programme were reviewed.

Before the start of the workshop, the basic equipment for measurement of physiological responses was set up and established procedures were checked using mussels (*Perna viridis*). PMBC were able to provide the basic facilities for physiological measurements and SFG (although it was necessary to adapt some of the usual procedures to the prevailing conditions at the laboratory). This was particularly important concerning the use of a spectrofluorometer to measure the concentration of algal cells in seawater for the determination of clearance rate. Excitation and emission wavelengths were scanned to establish the optimal wavelengths (Ex. 340nm; Em. 682nm). The readings were found to be stable following temperature equilibration of samples, although there was a limited range over which the fluorescence was linear. A calibration curve was established on each occasion by dilution of natural seawater with filtered seawater. Due to the more limited range of detection for the fluorometer compared to the electronic particle counter (e.g. Coulter Counter), the duration of the clearance rate

measurement was reduced from 2h to 1h. Mussels (*Perna viridis*) were collected from Phuket fish market and recovered in the laboratory for 2 days before being placed in cages (c. 70 per cage) and suspended at 2m depth at 3 sites (PMBC jetty (reference), Phuket Harbour, and Oil tanker jetty) on 16th November 1993.

The practical work started with demonstration of physiological procedures to all participants (throughout the practical work, the participants were split into two groups to provide a manageable number of people for bench-work).

Genuine fieldwork began at the end of the first week with collection of mussels from the reference site (PMBC jetty - c. 13% mortality) and measurement of clearance rate and respiration rate by group B/C. The clearance rate measurement had to be repeated (pm) due to problem of contamination of samples showing an increase in fluorescence rather than a decline. Detailed investigations established that this was due to the handling and mixing of samples by two participants using hand lotion containing fluorescing oils. This proved to be a very instructive exercise in careful methodology and detective work. Faeces and seston samples were collected overnight and filtered the following morning for measurement of food availability and absorption efficiency. Finally, there was a demonstration of computation of data using designed spreadsheets.

Table 2 A summary is given below of the physiological responses and the integrated scope for growth of mussels (*Perna viridis*) after transplantation in cages to the two sites (data are means and 95% CI).

	Site Phuket Reference (PMBC Jetty)	Phuket Harbour
Clearance Rate ($l\ g^{-1}\ h^{-1}$)	4.6 (5.5-3.7)	3.2 (3.6-2.7)
Respiration Rate ($m\ mol\ g^{-1}\ h^{-1}$)	58 (68-48)	58 (66-50)
Scope for Growth ($J\ g^{-1}\ h^{-1}$)	48 (64-33)	26 (33-17)

The second site collection of mussels came from both Phuket harbour (c. 23% mortality) and the oil tanker jetty. However, there was only time available for one site to be measured (Phuket Harbour) and these samples were analysed with the A/B group towards the end of the workshop. In general the physiological responses of these mussels proved to be more consistent due to the higher quality of data obtained as a result of previous experience gained using the fluorometric technique. Again these measurements were completed over the course of two successive days, ending with computation of physiological data using spreadsheets.

All participants successfully completed measurement of SFG on one of the two sites. The final results indicated that there was a statistically significant decline (50%) in the SFG of mussels collected from Phuket Harbour, due primarily to an inhibition of the feeding rate. However, mussels at both sites had positive SFG values. The ability to discriminate between sites would probably have been enhanced by a longer period of exposure (i.e. > 6d).

A summary is given above of the physiological responses (Table 2) and the integrated Scope for Growth of mussels (*Perna viridis*) after transplantation in cages to the two sites (data are means and 95% CI)

The participants at the workshop were very enthusiastic concerning the use of SFG for environmental monitoring, asking many relevant questions about its application in their particular region and in relation to specific environmental problems. The workshop provided an excellent opportunity to demonstrate the power of the technique in detecting pollution effects and the informative nature of the results.

While the measurement of feeding rate by means of a spectrofluorometer was found to be feasible, it was emphasised that the use of an electronic particle counter (e.g. Coulter Counter) is recommended as it offers many advantages (e.g. direct measurement of particles, high sensitivity over a wide range of concentrations (3-4 orders of magnitude), and high signal:noise ratio). PMBC have plans to purchase a Coulter Counter in the near future.

4.4 Water-quality bioassays

A short series of lectures outlined the role of acute water quality bioassays in biological effects monitoring, with specific examples from field studies. The main features of the lectures covered the requirement to address comparative sensitivities among life stages and different species, with an attempt to identify a range of potential target species associated with tropical coral reefs.

Table 3 Effect of temperature on the development of scallop embryos *Chlamys senatoria*.

Temperature	Time		
	t = 0 min	t = 70 min	t = 120 min
26°C	100% UF	10% UF 10% PB 80% 2 cell	10% UF 2% PB 0% 2 cell 60% 4 cell 28% 8 cell
18°C	100% UF	95% UF 5% PB	85% UF 15% PB

(UF unfertilized; PB polar body; 2 cell, 4 cell, 8 cell stage embryos.)

Criteria for test species selection were based on availability of test organisms, ease of rearing, cost effectiveness of conducting the tests and the ability to detect a clearly defined end-point within the limited time available.

Practical sessions were designed to allow each participant 'hands on' experience of spawning a range of local marine invertebrates (sea-urchins, mussels and scallops) by several methods for induction (thermal shock, KCl injection into the body cavity and the use of neurotransmitters for gamete release).

Each participant assessed fertilization success and subsequent development rates for scallops (*Chlamys senatoria*) and sea-urchins (*Diadema setosum*), in relation to temperature effects and xenobiotic exposure. The introduction of sub-lethal measurements to acute toxicity

tests was emphasized practically, to demonstrate the need to improve and maximise the sensitivity of water quality tests to integrate the environmental variables observed in field sampling.

Demonstrations assessing the sublethal effects of temperature on embryonic development enabled participants to practice their observational skills, utilising light microscopic techniques. This demonstration helped to emphasize the importance of regulating temperature whilst conducting bioassays as well as to make the participants aware that biological effects are not only attributable to xenobiotics.

The results in Table 3 show that the lower temperature suppressed embryonic development, whilst the ambient water temperature enabled embryos to proceed through the normal developmental stages.

Technology transfer sessions were restricted by time and equipment availability, but all participants had practical experience of how to assess lethal and sublethal end-points for invertebrates and marine fish. Samples preserved from field studies of temperate pollution gradients were used during these practical sessions.

Participants are now aware of how to select a test species, based on economic and scientific criteria, whilst considering the role of bioassays in an integrated approach to biological effects monitoring.

4.5 Community analysis

The lectures expounded a framework for the analysis and interpretation of community data, typically large matrices of counts (or biomass) of many species from samples taken at different sites or times. The material concentrated on a suite of graphical and multivariate statistical techniques exploited and developed at the Plymouth Marine Laboratory (PML), UK, over the last decade. Their utility in describing marine communities and identifying patterns of change, eg in pollution impact situations, has been successfully demonstrated in a number of published papers. The emphasis in the applications was on benthic community studies in soft sediments, though corals and other marine faunal components were covered, and the statistical methods have much wider applicability. Detailed notes for all these lectures were made available to the participants, together with reprints of a selection of relevant papers.

The workshop supplemented the lectures by practical sessions, involving the analysis of example community data sets from the published literature and using the suite of IBM PC programs developed at the Plymouth Marine Laboratory, the PRIMER package (Plymouth Routines In Multivariate Ecological Research). The UNEP funding for the workshop covered purchase of single-user licences of this software for all participants taking part in this component. This included full documentation on how to run the 24 component programs in this package.

The exposition throughout was directed at biologists (and, to a lesser extent, chemists) who collect and need to analyse community and environmental data in monitoring or field impact studies. It assumed a rudimentary knowledge of standard statistical concepts of variation and hypothesis testing but no formal statistical background was necessary: the multivariate methods were described from first principles and the advocated non-parametric approach lent itself to straightforward, non-technical explanations of how and why the methods work.

The following lectures were presented to Group C, comprising half the total number of participants:

- 1: A framework for studying changes in community structure.
- 2: Measures of similarity of species abundance/biomass between samples.
- 3: Hierarchical clustering.
- 4: Ordination of samples by Principal Components Analysis (PCA).
- 5: Ordination of samples by Multi-Dimensional Scaling (MDS).
- 6: Testing for differences between groups of samples.
- 7: Species analysis.
- 8: Diversity measures, dominance curves and other graphical analysis.
- 9: Transformations.
- 10: Species removal and aggregation.
- 11: Linking community analysis to environmental variables.
- 12: Causality: community experiments in the field and laboratory.
- 13: Data requirements for biological effects studies.
- 14: Relative merits of univariate, graphical and multivariate techniques.
- 15: The installation of PRIMER software and conversion of data file formats to standard PRIMER format.
- 16: Multivariate approaches to the determination of community stress.

In addition, a total of 6 practical sessions were held on IBM-compatible PCs, analysing 7 literature data sets using the techniques described in lectures 2 to 11. Four PCs were available for the practical exercises, with two participants per machine.

The following lectures were presented to Group A, comprising the other half of the participants who did not specialise in community studies:

- 17: Multivariate methods for community studies.
- 18: Applications of statistical methods to environmental impact studies.

Overall, the lecturers were very pleased with the enthusiasm and perception that the participants displayed. The standard of English comprehension was outstanding, allowing the lectures to proceed at a normal speaking pace, and thus facilitating full coverage of the difficult concepts involved. Most participants had no previous experience of most of the community analysis advocated but nonetheless were able to get to grips with the practical sessions very quickly, and produce analysis and interpretations of the full range of literature data studies. Widespread familiarity with PC computers and simple DOS operating system commands were a key factor here (in contrast to several previous UNEP or IOC training workshops) - also significant were the improvements that have been made to the PRIMER software fairly recently, to improve its user-friendliness.

5. DISCUSSION ON EAST ASIAN SEAS REGIONAL REQUIREMENTS

5.1 Regional Problems

With the aid of the questionnaire and a general discussion the perceived major regional problems were identified by the participants of the workshop. These included the following:-

- Coral Reef Degradation (by sewage, sedimentation, and chemicals);
- Eutrophication;
- Chemical Pollution;

- Aquaculture problems (toxic algal blooms, use of pesticides, contamination by bacteria and virus which are human pathogens); and
- Mangrove Forest Erosion.

Two separate problems were also identified which relate to environmental protection and management. These were:-

- Environmental impact assessment; and
- Use of toxicity tests in framing environmental legislation.

5.2 Requirements for Problem Solving

The need for a formal interactive network of laboratories emerged as the best probable solution for dealing with the range of regional environmental problems identified in 5.1 above. Such a network would be responsible for organising specialist training through personnel exchanges between laboratories for purposes of technology transfer and capacity building. It was felt that a research workshop of the type organised by Group of Experts on Effects of Pollutants (GEEP) in the past would help to strengthen the linkages in such a network and also in identifying laboratories that could form the nuclei for regional centres of excellence and specialization. Such centres would greatly facilitate the effective use of both material and human resources within the region by creating the infrastructure necessary for an integrated multidisciplinary approach to problems on a regional and sub-regional scale.

A formal network will also provide a focus for funding proposals to both governmental and international agencies.

The immediate requirements for the establishment of a Laboratory Network is the identification of a Regional Coordinator and National Coordinators within the region. It was recommended that these coordinators would be most effective if they are active scientists but with relatively senior management experience.

The workshop participants suggested that Mr Prawin Limpsaichol (PMBC) be invited to accept the role of Regional Coordinator given his background and successful organisation of this workshop. He agreed to act as an interim coordinator in order to not allow the network idea to lose momentum following the workshop.

Provisional proposals for national representatives were provided by the participating scientists for Indonesia, Malaysia, Philippines, Thailand, and Singapore. These were as follows:

Suggested Interim Co-ordinators

Regional and Thailand:

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6. CONCLUDING REMARKS

The Workshop was effective in achieving the objectives of the project. This was confirmed by the highly positive feedback from the participants. The success of the Workshop was due in a large measure to the unstinting efforts of the PMBC staff and in particular to Mr Prawin Limpsaichol, Mr Somkiat Khokiattiwong, and Mrs Nipawan Bussarawit. The participants thanks were also extended to other staff of PMBC worked very long hours and were also present during the weekends in order to ensure that support for the running of the Workshop was maintained without any significant problems.

It emerged during the course of this Workshop that PMBC is an eminently suitable site for any future Workshops. The Center has well equipped laboratories, very good technical staff and the facilities will shortly be extended with the acquisition of a new 38 metre research vessel (fully equipped), which is being donated by the Danish Government.

These excellent facilities would also make PMBC an ideal choice for any future Research Workshop of the type organised in the past by GEEP. There is obvious scope for Research Workshops related to the regional problems of coral reef degradation and mangrove forest erosion.

7. RECOMMENDATIONS FOR FUTURE UNEP ACTION IN THE REGION.

After due consideration of the discussions with the participants and with the PMBC Coordinator, Mr Prawin Limpsaichol, the following recommendations were felt to be appropriate.

7.1 Improve effect and impact of similar projects in the future

7.1.1 This was a broadly-based training workshop intended to make the participants aware of the range of biological effects techniques. For this purpose it was

would have been possible to direct some of the lectures towards specific regional problems.

7.2 Further action needed to meet the project objectives

- 7.2.1 Special training workshops related to specific regional problems.
- 7.2.2 Establishment of a regional network of laboratories and institutions.
- 7.2.3 Confirmation of a regional co-ordinator and national co-ordinators for the laboratory network.
- 7.2.4 Exchange of personnel between the laboratories in the network and the encouragement of local experts in extending training.
- 7.2.5 Intercalibration exercises to ensure standardization of methods and interpretation.
- 7.2.6 A research workshop of the type organised by GEEP in the past. This would heighten regional and international awareness of problems in the East Asian Seas Region.
- 7.2.7 Encourage the establishment of regional resource centres with a particular expertise. These should develop naturally out of the laboratory network.

7.3 Provisional proposal for a 3-year follow-up programme in the EAS region

PROVISIONAL PROPOSAL

Biological Effect Auditing and Monitoring Programme in the East Asian Seas Region

Rationale : Pollution problems are increasingly becoming an important issue in the EAS region and frequently caused economic loss. Keeping such issue in mind, future pollution studies that are relating to mitigation of adverse impact and damage to marine lives have to involve early detection through biological diagnosis. Biological techniques provides an integrated response to the totality of outer stressors which may further develop to be toxic environment. However, it is known that the general biological techniques are inadequate to accomplish the early detection objective. Therefore, the following proposal is to focus on specific response protein built up to counteract with outer stressors at the biochemical, physiological, cellular levels and bioassay testings as well as benthic community response. These techniques are to follow the outcome of the ICES/IOC Brumerhaven Workshop and The EAS regional UNEP Phuket Workshop. The advantage of specific biological techniques are the most cost - effective way to monitor pollutants, However, the causality can not be established without appropriate spatially and temporally related to chemical data (Stebbing & Dethlefsen, 1992, Mar. Ecol. Prog. Ser., vol. 91 : 1-8.)

Objective

- to develop the scientific skills of specific field of biological techniques for personnel that are engaged in studies and assessment of biological effects of pollutants in the East Asian Seas region.
- to intercalibrate and to ensure standardization of methods and interpretation.
- to feedback into and collaborate with existing regional action plan activities of EAS 23 (monitoring oil pollution) and EAS 24 (monitoring non-oil pollution).

Implementation

Base on the results of present regional UNEP Phuket Workshop, seeing that five individual countries in the region namely, Philippines, Indonesia, Singapore, Malaysia and Thailand have generally established her common research and monitoring activities in the field of oil and non-oil pollution on the ground of general outlook of pollution effects. The present programme is to support the existing activities into a more specific biological findings. Therefore the biological techniques can be implemented through a few training workshop and a follow up regional effective monitoring and meetings as below:

- Workshop 1 : Biochemical - Cellular Responses
- Workshop 2 : Physiological - Bioassay Responses
- Workshop 3 : Benthic Community Analysis

In these workshops, intercalibration in methodologies will also be established.

Pollutants of interest are oil and non-oil species, marinelives will be selected during Workshop 1, 2 & 3

Individual country will perform her auditing & monitoring in the collaboration and agreement of UNEP and Regional Sea Programme of EAS.

Budget:

Total budget estimated US\$ 854,000 would be partly contributed from Trust Fund (TF) and Environmental Fund (EF). Outside funding will be sought by the Secretariat after project formulation.

Activities	Year			Appr.* Budget
	1	2	3	
Consultancy				90
Training workshop 1,2&3				144
Laboratories set up				300
Auditing&monitoring				120
First meeting (evaluation)				40
Auditing & Monitoring				120
Final meeting (evaluation)				40
	Total			854

* Budget indicated : US\$x1000

APPENDIX 1

PROGRAMME FOR UNEP WORKSHOP ON BIOLOGICAL EFFECTS OF POLLUTANTS

Phuket, Thailand, November 16-25, 1993

Participants were divided into 2 groups, the first (A+B group) studying both biochemical/cellular (A) and bioassay/physiological (B) methods, with the second (B+C group) studying both bioassay/physiological and community analysis (C) methods.

Tuesday 16 November

- 09.00-09.45 Opening session. Formal welcome and opening; description of the background to the workshop in the context of previous UNEP and IOC/GEEP activities (Clarke, Limpsaichol)
- 10.00-10.30 Participants and lecturers introduce themselves and discuss their backgrounds and specialist areas of work
- 10.30-11.00 Introduction to the series of IOC/ICES (GEEP) research and training workshops on the biological effects of contaminants (Clarke)
- 11.00-12.00 Lecture: Physiological energetics (Widdows)
- 14.00-15.15 Lecture: Field application of 'Scope for Growth' (Widdows)
- 15.30-17.00 Lecture: Introduction to water quality bioassays (McFadzen)

Wednesday 17 November

	A + B group		B + C group
08.30-10.15	Lecture: Quantitative histopathology (Lowe)	08.30-09.30	Lecture: Framework (Warwick)
		09.30-10.15	Lecture: Similarity (Clarke)
10.30-12.00	Lecture: MFO system (Peters)	10.30-11.15	Lecture: Clustering (Clarke)
		11.15-12.00	Lecture: PRIMER programs (Clarke)
14.00-15.30	Lecture: Lysosomal damage (Lowe)	14.00-16.00	Practical: Clustering
15.45-17.30	Lecture: Oxidative stress (Peters)	16.15-17.30	Lecture: Ordination by PCA (Clarke)

Thursday 18 November

08.30-12.00	Demo: Bioassay (McFadzen)	08.30-12.00	Demo: Physiology (Widdows)
13.30-14.15	Lecture: Community methods (Clarke)	13.30-14.15	Lecture: Biochemical approaches (Peters)
14.30-18.00	Demo: Physiology (Widdows)	14.30-18.00	Demo: Bioassay (McFadzen)

Friday 19 November

08.30-12.00	Demo: Lysosomal damage(Lowe)	08.30-09.45	Lecture: Ordination by MDS (Clarke)
		10.00-12.00	Practical on MDS
13.30-14.15	Lecture: Community applications (Warwick)	13.30-14.15	Lecture: Cellular approaches (Lowe)
14.30-18.00	Demo: EROD activity (Peters)	14.30-15.15	Lecture: Testing (Clarke)
		15.15-16.00	Lecture: Species analyses (Clarke)
		16.15-18.00	Practical on testing and species analyses

Saturday 20 November

09.00-12.00	Practical: Bioassay (inoculation) on Site 1 samples (McFadzen)	09.00-12.00	Practical: Physiology (clearance rates) on Site 1 samples (Widdows)
13.30-16.30	Practical: Bioassay (development) (McFadzen)	13.30-15.15	Practical: Physiology (respiration) (Widdows)
		15.30-16.30	Lecture: Diversity etc. (Warwick)
		16.30-17.30	Practical: Diversity

Sunday 21 November

08.30-12.00	Practical: Bioassay (counts) on Site 1 samples (McFadzen)	08.30-10.45	Practical: Physiology (absorption) in Site 1 samples (Widdows)
		11.00-11.30	Lecture: Transforms (Clarke)
		11.30-12.00	Lecture: Aggregation (Warwick)

13.30-17.00	Practical: Cellular methods on Site 1 samples (Lowe)	13.30-14.45	Lecture: Linking to environment (Clarke)
		15.00-16.15	Practical: Linking
		16.15-17.30	Lecture: Causality (Warwick)

Monday 22 November

Free day (organised trip)

Tuesday 23 November

09.00-09.45	Lecture: Role of IOC/GEEP (Moore)
09.45-10.45	Lecture: Cellular bio-markers (Moore)
11.00-12.00	Lecture: Case studies in bioassay (McFadzen)

13.30-17.00	Practical: Physiology (clearance rate and respiration) on Site 2 samples (McFadzen)	13.30-17.00	Practical: Bioassay (inoculation/developm.) on Site 2 samples (Widdows)
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Wednesday 24 November

08.30-12.00	Practical: Biochemical methods on Site 2 samples (Peters)	08.30-09.00	Lecture: Data requirements (Warwick)
		09.00-10.00	Lecture: Relative sensitivities (Warwick)
		10.15-11.00	Lecture: Case studies on corals (Warwick)
		11.00-12.00	Q & A session (incl. PRIMER installation)
13.30-16.00	Practical: Physiology (absorption) on Site 2 samples (Widdows)	13.30-16.00	Practical: Bioassay (counts) on test samples (McFadzen)
	16.00-17.30	Initial discussion of lessons learnt and plans for the region (Moore)	
	20.00-21.00	Lecture: Chemical interactions in lysosomal pathology (Moore)	

Thursday 25 November

08.30-10.15	Practical: Data analysis for physiology (Widdows)	08.30-10.15	Practical: Bioassay (counts) on Site 2 samples (McFadzen)
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10.30-12.00 Practical: Bioassay
(counts) on Site 2 samples
(McFadzen)

10.30-12.00 Practical: Data
analysis for
physiology (Widdows)

14.00-15.00 Presentation of results by lecturers (Moore)

15.00-17.30 Discussion of future activities and draft report (Moore)

APPENDIX 2

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APPENDIX 3

REGIONAL RESEARCH CAPABILITIES

INDONESIA

**Research and Development Center for Oceanology
Indonesian Institute of Sciences**

Research Capabilities

Benthic community studies (coral reef, soft-bottom, mangraves, seagrass)
Pollution studies (trace elements, organic contaminants)

Relevant Existing Activites

Chemical pollutant monitoring
Bioassay (phenol, chrome, temperature)
Red tide monitoring

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MALAYSIA

**Fishery Research Institute
Fisheries Department**

**Department of Chemistry
University of Malaya**

Research Capabilities

Pesticides
Trace metals
HAC's and oil dispersants
Bioassay

Relevant Existing Research

Bioassay (LC50) : pesticides, HAC's and oil dispersants
Contamination of trace metals in fishes, bivalves and sediment which relate to minings
Water quality monitoring
Influence of environment induce fish disease
Impact of shrimp farm and coastal zone development on eco-system

Comments

There are high level of experience and expertise on basic study as indicated above, especially for the fisheries people. As a follow up from this workshop, there is a need to create working groups for bioassay, scope of growth, statistical study, cellular and molecular approaches. The fisheries people and people from University are able to form individual working group for bioassay, SFG, statistic and cellular, molecular studies respectively. This individual working group can then interact with similar working groups in the ASEAN region, subsequently forming the NETWORK!

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PHILIPPINES

**Southeast Asian Fisheries Development Center
Aquaculture Department**

**Environmental Management Bureau
Department of Environment & Nature Resources**

Research Capabilities and Relevant Existing Activities

Ecological indicators of pollution
Toxicity test, bioassays on different aquatic organisms (invertebrates, fish)
Monitoring for toxic algal blooms (*e.g.* red tide)
Pesticides and mussel watch
Ecology of seagrasses, seaweeds
Effects of aquaculture activities on coastal and freshwater ecosystems
Pollution monitoring of heavily impacted sites (*e.g.* coastal areas with problems on mining discharges)
Rehabilitation of mangroves which had been converted into fish or shellfish ponds for aquaculture
Resource management and sustainable development of marine and freshwater resources

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SINGAPORE

**Department of Zoology
National University of Singapore**

Research Capabilities

Benthic community studies (coral reef, soft-bottom, mangroves)
Environmental impact studies
Bioassay
Biochemical
Physiology
Cellular
Rad tide studies
Water quality : nutrients, hydrocarbons

Relevant Existing Activities

Benthic Community studies
Environment impact studies : consultancies for specific developmental projects
Bioassay : Graduate student research project
Rad tide studies
Water quality : carried out by Ministry of the Environment

Contact Person

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THAILAND

Phuket Marine Biological Center
Department of Fisheries

Pollution Control Department
Ministry of Science, Technology and Environment

Department of Marine Science
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Research Capabilities and Relevant Existing Activities

Pollution control and legislation
Water Quality auditing and monitoring
Biochemical, Physiological and Cellular Studies to eco-stressors on marinelives
Red tide and eutrophication studies
Benthic Community studies (coral reef, soft-bottom, seagrasses)

Comments

Pollution problems are increasingly becoming an important issue in the EAS region and frequently causing economic loss. Keeping such problems in mind, future pollution studies relating to mitigation of adverse impact and damage arising have to involve with early detection on biological responses to outer stressors. However, an individual field of response (biochemical or physiological or cellular, *etc.*) may not provide an adequate tool, but may require integrated field of responses to high-light the causative agents. Hoping that all participants from the region would aware such existing pollutant problems are deploy such new technologies to deal with pollution issues in individual country.

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APPENDIX 4

MIXED FUNCTION OXYGENASE (MFO) SYSTEM IN FISH : LABORATORY STUDIES

by Lon Peter
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INTRODUCTION

The detection and assessment the impact of pollution, particularly low concentrations of increasingly complex mixtures of contaminants, on environmental quality has led to study and development of molecular markers of the biological effects of contaminants on organisms called "bio-markers"

The two most important features of bio-markers are that they indemnify that interactions have taken place between contaminant and the organisms, and the measure sublethal effects. Bio-markers can detect the presence of both known and unknown contaminants. Sublethality and early detection of effects allow remedial or preventative action to be taken

All organism possess biotransformation or detoxication enzymes which convert lipophilic organic xenobiotics to water-soluble and excretable metabolites, Such enzymes are present in most or all tissues but in highest levels in the liver (vertebrates), or tissues involved in the processing of food. Enzymes of phase I metabolism (oxidase, reductase, hydrolases, hydrases) introduce a functional group into the xenobiotic, to which the enzymes of phase II metabolism attach a large water-soluble moiety, Cytochrome P4501A1 is an oxidative enzyme of central importance in phase I metabolism and represents the first step of metabolism for many organic xenobiotics including PAHs and certain PCB congeners Cytochrome P450s are the terminal components of the mixed function oxygenase (MFO) enzyme system. Cytochrome P4501A1 in mammal is induced by many organic xenobiotics, e.g. certain PAHs, PCBs and PCDDs, via binding of the xenobiotic to a soluble protein know as the aromatic hydrocarbon receptor. This induction forms the basis of its use as a bio-marker for impact by organic pollution

Additionally, cytochrome P4501A1 can activate certain xenobiotics such as particular PAHs to mutagenic metabolites, and therefore its increased synthesis has consequences for carcinogenicity.

P4501A levels may be determined by several biochemical procedures. The enzyme activity 7-ethoxyresorufin O-deethylase (EROD) is used to determine the activity of P4501A protein in liver homogenates by the use of specific antibodies or the levels of P4501A mRNA

PROCEDURES FOR PREPARATION OF THE MICROSOMAL FRACTION

- dissected the fish to bring the liver tissue, then weight the tissue
- Homogenised the liver tissue and centrifuged in low spin at 500 g. for 25 minutes, then transfer the supernatant
- Centrifuge the supernatant in high spin at 100,000 g. for 45 min. The pellet is the microsomal fraction

DETERMINATION OF ETHOXYRESORUFIN O-DEETHYLASE (EROD) IN MICROSOMAL SPENSIONS

Burke M.D. and Meyer R.T. (1974). Ethoxyresorufin : direct fluorometric assay of a microsomal O-deethylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos., 2, 583-588.

REAGENTS

EROD Buffer : A: Na₂(K₂)HPO₄ 0.066 M
 B: KH₂PO₄ 0.066 M

100ml buffer: 80.8 ml **A** + 19.2 ml **B** (approx.) to pH 7.4
Store at 4oC

Substrate: 7- Ethoxyresorufin (7-ER) 0.1 mg ml⁻¹ (415 mM) in DMSO.
Store in vials in freezer.

Reaction mixture:

Stock NADPH 8.33 mg ml⁻¹ in EROD buffer. (10 mM)
Take: 250 μ l NADPH solution
100 ul 7-ER solution
Make up to 10 ml with EROD buffer.

Resorufin standards:

Stock solution (2 mM) : Dissolve 11.8 mg in 25 ml H₂O.
Store at 4oC.

1. Dilute 50 μ l stock solution to 1 ml with EROD buffer (A) = 0.1mM
2. Dilute 30 μ l (A) to 3 ml with buffer (1 μ M)
3. Dilute 10 μ l (A) to 3 ml with buffer (0.33 μ M)
4. Dilute 5 μ l (A) to 3 ml with buffer (0.165 μ M)

METHODS

1. Pipette 0.1 ml microsomes (or S9) into test tubes (in duplicate) and 1 for to.
2. Add 2 ml acetone to each t₀ tube.
3. Equilibrate @ 30°C for approx 5 min. in a shaking water bath.
4. Add 0.9 ml reaction mixture to each tube, including t₀.
5. Incubate @ 30°C for 30 min., or longer if activity likely to be low.
6. Stop the reaction with 2 ml cold acetone and centrifuge @ 2,000 rpm for 10 min.
7. Set fluorometer Ex 537 nm, Em 583 nm and set zero using EROD buffer.
8. By varying the slit widths, use 0.33 μ M standard to set 100%.
9. Read samples, including t₀. without altering any controls.
10. If sample readings are very low, use 0.165 μ M standard to set 100% and reread samples.
11. Subtract blank values from sample reading before calculating activity.

Calculation:

$$\text{Protein} = \frac{1F_s \times S_c \times V_c}{1FR \times V_s \times t \times P} \quad \text{pmol min}^{-1} \text{ mg}^{-1}$$

Where IFs	= Fluorometric intensity sample
Sc	= Concentration standard (μ M)
IFR	= Fluorometric intensity reference
Vc	= Cuvette volume (ml)
Vs	= sample volume (ml)
t	= Incubation time (min)
p	= Protein conc. (mg ml ⁻¹)

LOWRY PROTEIN DETERMINATION - MODIFICATION OF LOWRY,

Rosenbrough, Farr & Randall (J.biol. Chem. (1951) **193**. 265-275).

Solutions:

1. Solution A. Dissolve 2 g potassium sodium (+) tartrate (COOK). (CHOH)₂. COONa.4H₂O - mol. wt. 282.2). and 10 g sodium carbonate (Na₂CO₃. 10H₂O - mol. wt. 286.1) in 500 ml of 1M NaOH (40 gms per litre - mol. wt. 40). Add water and make to 1 litre. Store at 4°C.
2. Solution B. Dissolve 2 g of potassium sodium (+) tartrate and 1 g of copper sulphate (CuSO₄.5H₂O - mol. wt. 249.7) in 90 ml of water and make up to 100 ml with 1 M NaOH. Store at 4°C.
3. Solution C. Dilute Folin & Ciocalteu's phenol reagent 1:15 (v/v) with water. Prepare fresh for each set of tests.
4. Standards. Dissolve 10 mg bovine serum albumen (fraction v) (B.S.A.) in 10 ml water (= 1 mg ml⁻¹) and dilute to give concentration range of (in mg ml⁻¹) 0.4, 0.2, 0.1, 0.06, 0.04, 0.02, 0.01.

Methods:

Pipette the following into duplicate tubes (disposable plastic):

1. water blank - 250 μ l water
2. buffer blank - 250 μ l of sample buffer diluted as for sample
3. sample(s) - 250 μ l of sample (diluted as appropriate with water)
4. standards - 250 μ l of each standard in separate tubes.

Add 230 μ l of solution A to each tube, whirlmix & heat at 50°C for 10 min. Dilute an aliquot of solution B 1:1 with water and add 50 μ l to each tube. Leave tubes for 10 min at room temperature. Add 750 μ l of solution C to each tube & heat at 50°C for 10 mins. Observe to see if colour development is sufficient. If not, reheat, for 2-3 min. Measure absorbance of each tube at 650 nm using 1 ml semi-micro cuvette. Plot standards on log-log paper (3 cycles) and read off samples (corrected for buffer reaction) Adjust for dilution to obtain protein conc. in mg ml⁻¹.

APPENDIX 5

BIOCHEMISTRY

by David Lowe

Plymouth Marine Laboratory

PART I: Quantitative histopathology and image analysis in aquatic toxicology.

The current method of choice for measuring cells and tissues for histological/morphological determinants of change is digital image analysis. The alternative technique which measure the same parameters is an appreciation of the roots of image analysis, not in computing or electronic terms, but from a methodological viewpoint. The image analyser may be the piece of equipment that everyone in the field desires , but not necessarily the only option.

1. Direct methods of quantitative analysis

Linear dimensions

Measurements taken from photographs using a ruler or directly down the optical microscope using a measuring eyepiece graticule can yield, following calculation, information about surface areas and volumes of cellular components which have well defined shapes such as spheres, cylinders or ellipsoids. The structure that has been sectioned should be in a truly representative plane.

Another parameter that comes under the wing of linear dimensions is of course number of objects i.e counts. If you are interested in say alternations in immunocompetence then a simple count of cell numbers or ratios is a good starting point.

Grid methods

An alternative method to measure the area of a cellular component is to place a squared graticule over the image, with squares of known area, and to count the number of squares that lie over the objects of interest. Part squares that fall on the edges of the object will need to be totalled up or alternatively make a sampling rule that any part square that is occupied 50% or above is counted as a whole square and any that is less than 50% occupied is not counted at all.

Point methods

An extension of the squared matrix graticule system for the determination of projected area that we have just looked at is to replace each square with a point that represents the centre of square.

However, few things in cells and tissues are regular shapes and the relationship between the volume of cells or cellular inclusions and their surface area is a very powerful measure. The techniques we will look at now really revolutionised quantitative microscopy in that they have the capacity to determine length, number, area and volume of an object or group of objects of any shape as well as ratios of these parameters such as surface to volume.

2. Indirect methods of quantitative analysis.

Stereology

Stereology is a branch of applied mathematics and has been defined as the extrapolation from two dimensional to three dimensional space. It would be equally true to say from one dimensional to two dimensional space. Stereology is based on what is called the Delesse principle.

All the methods we have looked at, so far have been able to measure morphometric parameters but have lacked the ability to measure the colour density of the subject area.

Colour density can be measured using a simple colour scale which could be either made up or purchased from Kodak or Fuji. The colour of the reaction can then be compared to the chart which will have values ascribed to individual densities by the manufacturer.

Alternatively the density could be determined using a densitometer available from most of the major microscope suppliers.

PART II: The *In Vitro* Assessment of Lysosomal Damage in Molluscs.

What are lysosomes?

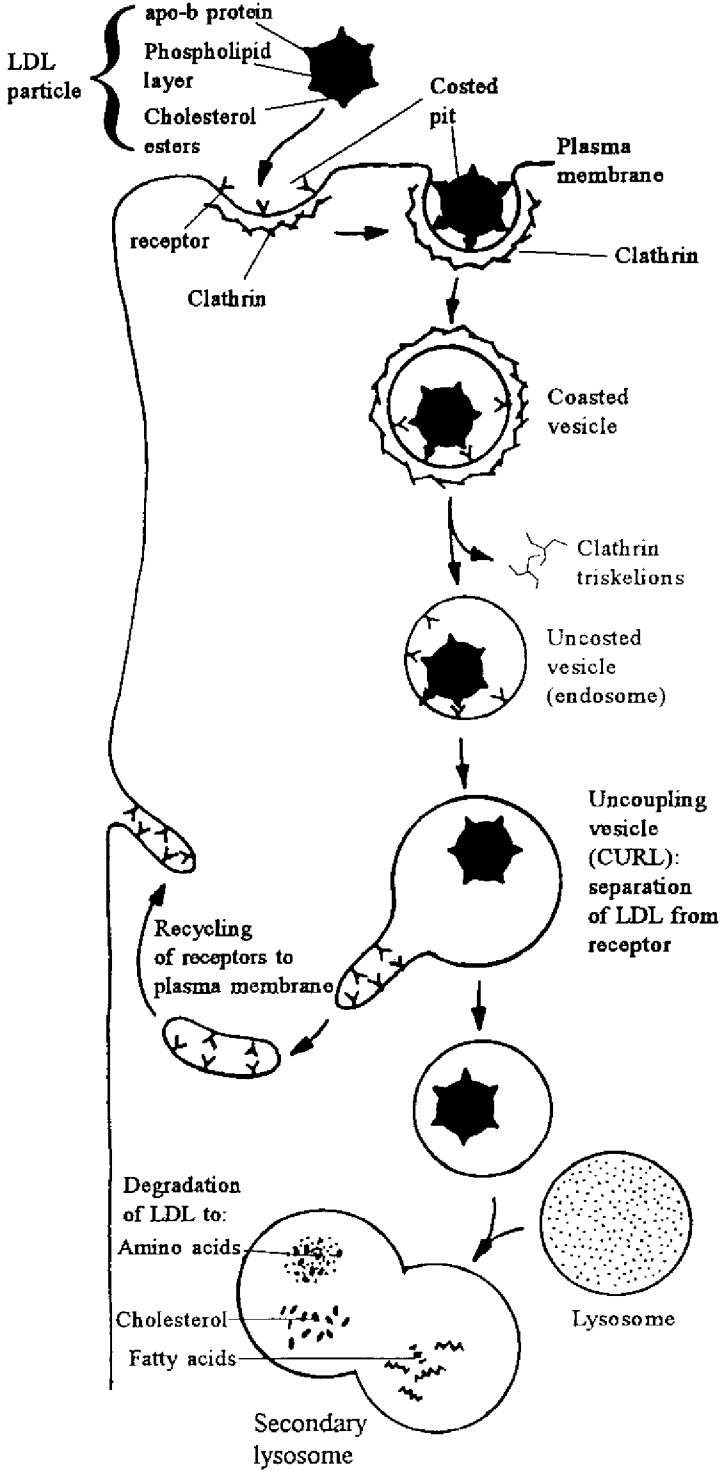
Lysosomes are acidophilic compartments within a cell that are involved in the breakdown of ingested materials and endogenous proteins. Lysosomal function can be demonstrated by considering a cellular process called receptor mediated endocytosis. By this process cells can for example selectively take macromolecules for such things as maintenance metabolism (Fig. 3).

Lysosomes are known to be linked to pathological changes in both plants and animals and these changes have been shown to be associated with a variety of degenerative disease conditions as well as to diseases induced by environmental pollutants. A remarkable feature of lysosomes is their ability to accumulate a diverse range of toxic metals and organic chemicals including heterocyclic compounds and PCBs. The presence of these toxins is believed to contribute to the process of cell injury. Since lysosomes are such good indicators of contaminant induced cellular damage, it was considered desirable to develop sensitive *in vitro* methods that could be used in parallel with the membrane permeability test that would permit further manipulation of the samples for experimental studies. In addition, as much of the work associated with environmental research is carried out in less than ideal conditions, for example on board or in small field stations, the techniques should be simple requiring the minimum of equipment and training so that they could be used in a field laboratory.

Until recently the chosen method for the assessment of lysosomal damage was to measure the enhanced permeability of the lysosomal membrane histochemically (the so called lysosomal latency technique) and associated changes including lysosomal enlargement and increases in numerical density. This technique has proven to be extremely reliable for use in a well equipped laboratory and tests for contaminant induced as well as non-xenobiotic stressors alterations in the permeability of the lysosomal membrane.

One way in which *in vitro* tests are used is to assess cell survival following exposure the some xenobiotic agent. Such lethality tests have been used for many years and are in essence, equivalent to an LC 50 assay used on whole animals. One of the most common of

Figure 3 Lysosomal function of cellular system as receptor mediated endocytosis to maintain metabolism.



these assays is the Eosin Y exclusion test. Cell plasma membranes are differentially permeable, i.e. substances may be excluded from the cell or actively absorbed, this may happen against the concentration gradient. When a cell dies the integrity of the plasma membrane is lost and substances may freely diffuse across it. The eosin Y will be excluded from viable cells but will be taken up by the dead cells colouring them pink. It is then simply a question of counting the numbers of live and dead cells to assess lethality of a test compound.

Material and Methods

Neutral Red Retention Protocol for Mussel Blood Cells

The basic procedure used does not change very much between the various cell types studied so we can use as a guide (the method use in the practical session on molluscan blood cells).

Mussel (*Perna viridis*) blood cell in vivo exposure from Ao Makham Bay was used for this method

Procedure : - (RR Method)

1. Withdraw 0.5 ml of blood from the posterior adductor muscle into a 2 ml hypodermic syringe containing 0.5 ml of Physiological saline
2. Remove the needle from the syringe and eject the blood cell solution into a siliconised 2 ml Eppendorf tube and store on water ice (or in a refrigerator) unit required.
3. Plate out 3 drops (using a 2.5 ml disposable pipette) of the blood cell solution onto a 3x1 microscope slide and allow cells to attach for approximately 15 mins in a humidity chamber.
4. Tip off excess fluid and replace with 2 drops of Neutral red solution and apply coverslip (18x18 mm size) and leave to incubate for 15 minutes in a humidity chamber.
5. Examine under the microscope (at x 40 mags) following the 15 minutes incubation and at 30 minute intervals thereafter to determine at what point in time the dye is lost from the lysosomes into the cytosol.

APPENDIX 6

BIOASSAYS

by I.R.B. McFadzen
Plymouth Marine Laboratory

INTRODUCTION

Many marine invertebrate species and biological responses have been proposed as "toxicity tests", but relatively few have been rigorously developed and evaluated in both laboratory and field. Techniques are required for detecting environmental pollution, and they should fulfil the following criteria:

- They should be sensitive to a wide range of chemical contaminants and be sensitive relative to other biological responses and species.
- They should be robust, easily and cost-effectively measured by trained personnel and respectable.
- They should be readily interpretable in terms of the biological consequences of pollution, ideally with mechanistic understanding, and have ecological relevance (Widdows, 1993).

The organisms response used in this bioassay are the ability of scallop and sea urchin embryos to develop normally through the developmental stages (polar body extrusion, cleavage, blastulation, gastrulation, trochophore stage) and reach the "D" - shaped prodissoconch larva (at which the paired hinge shells can be seen) for the scallop (Fig. 4) and reach the veliger larvae for the sea urchin (Fig. 5) within 48 hours (26 °C). Although the exposure period is relatively short, it encompasses a period of rapid, intense cellular activity during which 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 early life stage tests which record growth and development, with the advantage that exogenous feeding is not required, thus eliminating this potential source of variation in the test result.

The success of the bioassay depends on the quality of the test material prior to exposure to environmental stressors.

The purpose of this manual is to provide researchers with the ability to conduct a sensitive, reproducible bivalve embryo bioassay, whilst high-lighting potential sources of error.

MATERIAL AND METHODS

Artificial induction of spawning

Scallop (*Chlamys senatoria*) : - Injection of 1 ml of Serotonin solution into the adductor muscle can stimulate the release of gamete (Fig. 6).

Sea urchin (*Diadema setosum*) : - Injection of 0.5 M KCl solution total 1 ml. into each lobe of body cavity can also stimulate the release of gamete (Fig. 7).

Figure 4 Developmental stages of a typical bivalve (e.g. *Tapes philippinarum*) total time to reach the D-shell stage from fertilization is around 24-48 hours depending on temperature.

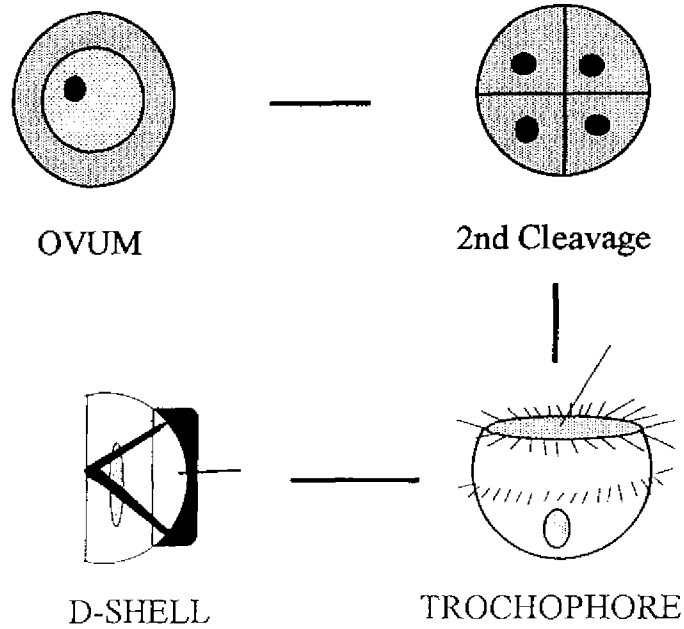


Figure 5 Diagram of veliger stage of sea urchin.

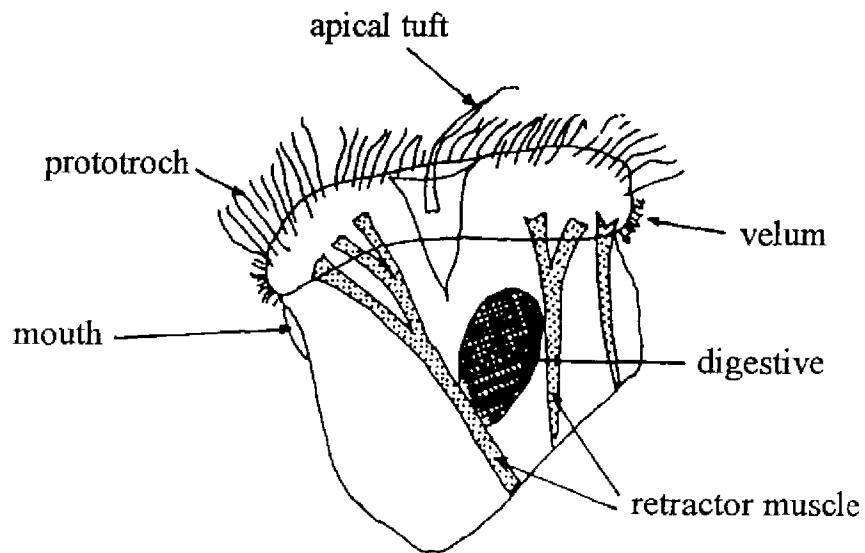


Figure 6 Diagram of vascular system of scallop

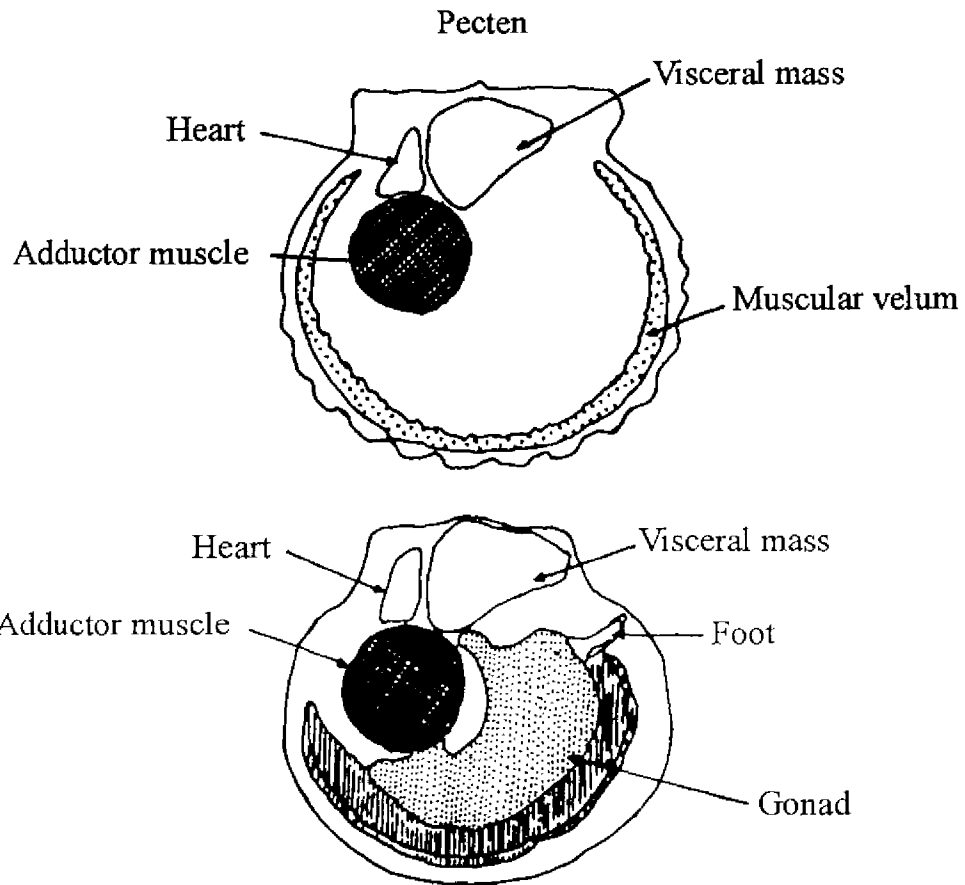
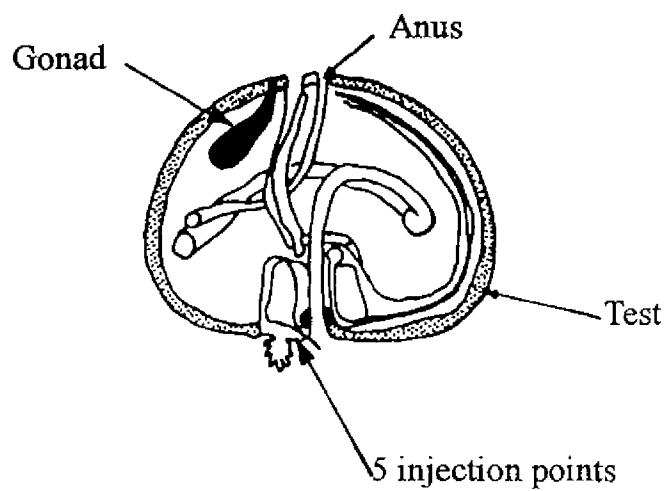


Figure 7 Diagram of vascular system of sea urchin.



When inducing gamete release there is the chance of adults releasing gametes which are not mature or have not completed meiosis. Immature gametes can be recognised by the presence of nutritive cells in sperm solutions and the presence of stalks on the oocytes. When the gametes are close to maturation they assume the morphology of ripe gametes (ova are spherical with distinct germinal vesicle; sperm are separate with single flagella), but require a period of maturation. Care must be taken to avoid physiologically immature gametes.

Gametes should be examined under a microscope to establish their quality prior to fertilization.

Fertilization

A ratio of 100 sperms to one egg is standard. Pooling of eggs from several females and sperm from several males is also a standard practice. Ten adults from each sex is the norm, but if greater numbers are required then clearly use more adults. Check the motility of sperm prior to use; poor motility of sperm will result in fertilized eggs but subsequent development will be poor.

Wait an hour after fertilization (26°C) to allow sperm penetration, then filter the eggs to rinse off excess sperm (Fig. 8). This reduces the chance of polyspermy and possible bacterial infection. At this stage (polar body or first cleavage), an assessment of fertilization success can be made.

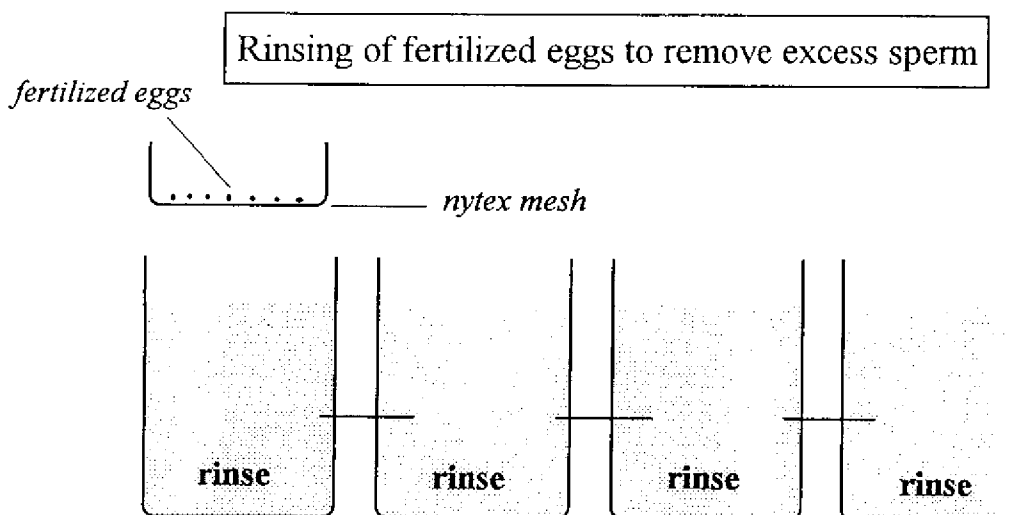


Figure 8 Rinsing of the fertilized eggs after 30-60 minutes to remove excess sperm. Nytex mesh should 40-53 μm aperture, with a minimum of 3 rinses.

The Test Procedure

Embryo development

This stage in the culture of any bivalve larvae is critical. Water quality, stock density, temperature salinity and nutritional requirements are variables which can effect the larval developmental rate and quality. We shall define the embryonic stage as that part of the life cycle from fertilized egg to D-shell larva (Fig. 4).

Eggs should be rinsed three times (minimum) and egg concentration should be established by means of an electronic particle counter or a Sedgewick-rafter cell under a microscope. Before sampling ensure the eggs are in a homogenous suspension by rapid mixing. Precise sperm counts are not possible but an egg : sperm ratio of c, 1:100 is advised.

The desired concentration of embryos in the test solution should be between 15-50 embryos/ml. Within 1 hour of fertilization, equal volumes of the mixed embryo suspension should be pipetted out into the test chamber, containing the test solution. The initial number of embryos should be established by Sedgewick-rafter cell under a microscope or by estimation from a precise count of the density in the stock suspension and the subsequent dilution rate.

A minimum of 3 replicate (5 or more would be ideal) of each control and treatment should be carried out. There is no need to feed the embryos during the 48 hours.

Every hour, 1 ml of test media containing embryo development should be taken up and fix with one drop of 4% formalin for preservation.

In this demonstration, sublethal effect of temperature on embryonic development of scallop was only done since the induce spawning of sea urchin on the time was not successful.

End of exposure period

At the end of the 48 hour exposure period, each vial is gently inverted 3 times and 1 drop of 4% buffered formalin is added. Embryos and larvae will settle to the bottom of the chamber, which will make collection of larvae for counting easier. The total number of shelled larvae containing tissue, without obvious deformity, at the end of the test are counted as normal.

The percentage of introduced live embryos that did not result in live larvae with completely developed shells are adjusted for the controls as follow :

$$\text{Percentage of abnormalities in test} = 100(\%AT - \%AC) / (100 - \%AC)$$

Where %AT = percentage of abnormal embryos in the test that did not result in live larvae with shell;

%AC = percentage of abnormal embryos in the controls that did not result in live larvae with shells.

The test is unacceptable if less than 70% of the embryos introduced into the control chambers develop into live larvae with completely developed shells at the end of the exposure period.

APPENDIX 7

PRACTICAL PROCEDURES FOR THE MEASUREMENT OF SCOPE FOR GROWTH

by John Widdows & Peter Salkeld
Plymouth Marine Laboratory

INTRODUCTION

Test Organisms: Bivalves/Mussels (*Mytilus edulis*).

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

GENERAL TEST PROCEDURES

Source of animals

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

Native or Transplanted Animals

Mussels can be collected from:-

Native populations, or

Transplanted from 'clean' reference sites and caged at particular sites of interest.

Laboratory or Field Measurement

Physiological energetic responses of mussels can either be measured in the field using a mobile laboratory (e.g. Widdows et al. 1987) or in the laboratory under 'standardised' conditions (e.g. Widdows and Johnson, 1988). Comparative studies (Widdows, 1983; Salkeld & Widdows -unpublished data) have found no significant difference between laboratory and field measurements of SFG if the former are made in high quality seawater and within 24 h of collection from the field (i.e. before they recover from pollution induced stress). When the objective is primarily to quantify the decline in growth potential or the degree of stress induced by environmental pollution, then measurement under 'standardised' laboratory conditions is recommended on the basis of convenience, cost and efficiency. Under controlled laboratory

conditions, natural environmental variables (such as food availability, temperature, salinity and dissolved oxygen) are held constant, so that the physiological responses reflect the underlying effects of toxic contaminants accumulated in the tissues. The basic physiological responses of mussels (such as feeding and respiration rate) are maintained relatively independent of short-term changes in natural environmental variables over a wide range of conditions; for example food/seston concentration (0.1- 10 mg seston L⁻¹. Widdows et al., 1979; Kiorboe et al., 1980), temperature (6-20°C; Widdows, 1976) and salinity (20-33 ppt; Widdows, 1986). In addition, transplantation experiments over >1000 km have shown that any measurable differences in physiological responses and growth rates of different populations reflect environmental factors rather than genetic differences (Kautsky et al., 1990; Widdows and Salkeld, unpublished data), thus enabling the direct comparison of mussels over a wide geographical area.

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

Collection of animals

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

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

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

Physiological Measurements: Static vs. Flow-through

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

Static

The main advantages of using a static (closed) system are:- (1) It generally requires less equipment; (2) Large changes in food or O₂ are recorded; and (3) Smaller quantities of seawater and food are utilised (also less toxicant is required in toxicological studies). The disadvantages

are:- (1) Experimental conditions (e.g. food, oxygen, toxicant concentration) are not held constant, but decline with time and may therefore affect the physiological rate if they fall below a specific concentration, and (2) Faeces are generally not produced in sufficient quantities and are not the result of a steady-state food concentration.

Flow-through

The main advantages of using an open flow -through system are:- (1) Experimental conditions are held constant; (2) Continuous and simultaneous monitoring of physiological responses is possible; and (3) Less physical disturbance of animals and more natural conditions. The disadvantages are:- (1) More complex experimental systems requiring pumps and tubing/plumbing; (2) Precise control of flow rates and inflow concentrations (of food, O₂, and toxicant) are required; (3) Large quantities of seawater are required (recirculated or to waste); and (4) Accuracy of measurement and the detection limit is dependent on the flow rate because there is an inverse relationship between the flow rate and the difference between inflow and outflow concentration. In the procedures outlined below both static and flow-through techniques will be described where appropriate.

MEASUREMENT OF FEEDING RATE (CLEARANCE RATE)

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

Flow-through approach

The clearance rate measuring system (Fig. 9A) consists of a small centrifugal pump (or sufficient pressure from a reservoir) discharging FSW into a mixing chamber (1.5 L volume) with a magnetic stirrer and thence via narrow (i.e. 2.5 mm) bore tubing through 18 identical chambers in parallel (16 experimental chambers with individual mussels and 2 control chambers without mussels).

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

Water samples from all chambers are collected simultaneously by moving a rack of measuring cylinders (> 200 mL volume) directly under the outflows and removing them after a period of 60 seconds. It is important to avoid disturbing the animals by shading or vibration in the vicinity of the apparatus. The flow rate through each chamber is recorded. The concentration of algal cells in each water sample is then measured using an electronic particle counter (e.g. Coulter Counter) with a 100 or 140 μm orifice tube and set to count all particles > 3 μm diameter (spherical equivalent) in a 0.5 mL sub-sample. Four replicate counts are made on each sample and the mean calculated. Clearance rate is then calculated as follows:-

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

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

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

Static approach

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

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

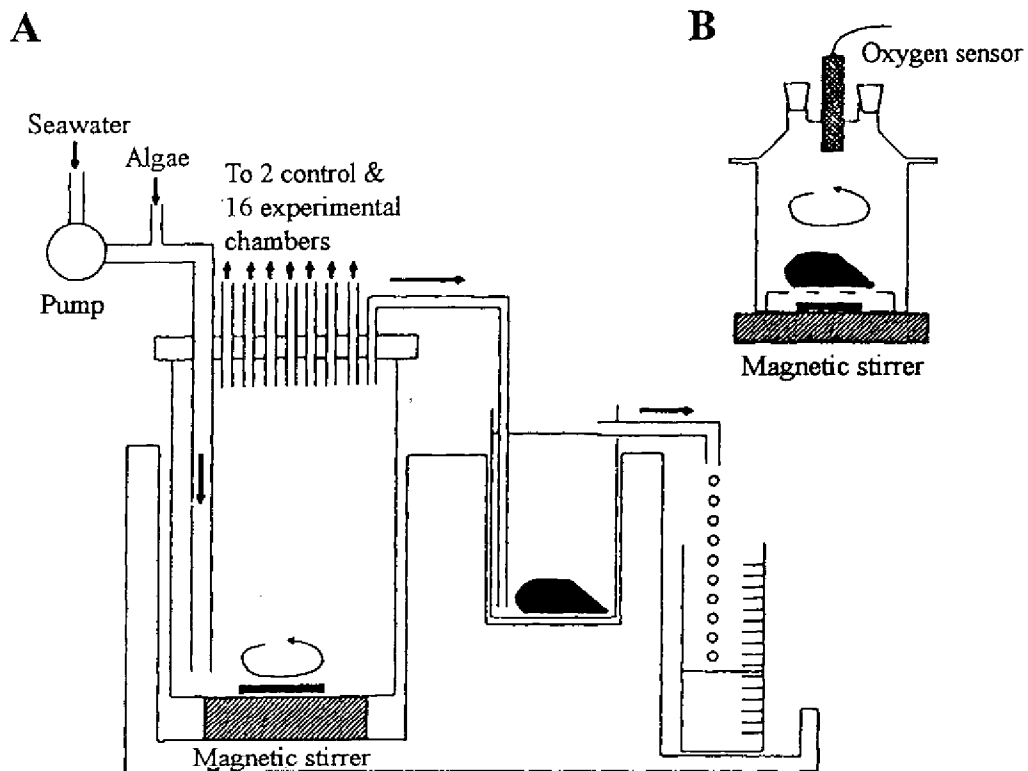
$$\text{CR (L h}^{-1}\text{)} = (\text{Volume of water e.g. 2 L}) \times (\text{Log}_e C_1 - \text{Log}_e C_2) / \text{time interval in h}$$

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

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

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

Figure 9 A: Flow-through apparatus for measurement of clearance rate by bivalves.
B: Glass respirometer for measurement of oxygen consumption.



MEASUREMENT OF FOOD ABSORPTION EFFICIENCY

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

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

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

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

Algal culture of known cell concentration is filtered through washed, ashed and pre-weighed 4.5 cm glass fibre (Whatman GF/C) filters (a minimum of five 'food' samples should be collected). The filters should not be 'over-loaded' so that salts can be readily washed out with 0.5 M ammonium formate (3x10 mL). Care should also be taken to wash salts out of the edges of the filters. Ammonium formate at 0.5 M is approximately iso-osmotic with seawater and provides a means of washing algal cells and faeces without inducing osmotic stress. It has a low melting point and can be sublimated at 100°C.

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

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

MEASUREMENT OF RESPIRATION RATE

Static approach

Rates of oxygen consumption by individual mussels are measured in 'closed' glass respirometers (e.g. 500 mL Quickfit flasks) held in a temperature controlled water bath mounted on a multi-plate magnetic stirrer. Air-saturated seawater is added to each respirometer and stirred by means of a magnetic stirrer bar beneath a perforated glass plate supporting a mussel (Fig. 9B). The rate of decline in oxygen partial pressure (P_{O_2}) in each chamber is measured by a calibrated oxygen electrode (e.g. Radiometer E5046 or Strathkelvin 1302) connected to an oxygen meter (e.g. Strathkelvin Model 781b). Eight respirometers are usually run simultaneously and each oxygen meter is coupled to a multichannel chart recorder. Twenty minutes are allowed for the mussels to open and to resume pumping, then oxygen uptake is measured over the next hour. The rate of oxygen consumption should not be measured below a partial pressure of c. 100 mmHg (13 kPa) because the rate then become dependent on the external P_{O_2} .

The preferred method is a continuous monitoring of oxygen uptake by means of an oxygen sensor in each respirometer (Fig. 9B). However, a less convenient but less costly alternative utilizes a single oxygen meter and sensor mounted in a thermostated cell (e.g. Strathkelvin 1302 electrode and MC 100 microcell). At c.40 min intervals, small volumes (c. 1 mL) are sampled from each respirometer by means of a glass (i.e. gas impermeable) syringe coupled to a stainless steel needle passing through a silicone stopper in the top of each respirometer. Each sample is then slowly injected into the thermostated microcell and a steady PO₂ reading is obtained her c. 4 min. The decline in PO₂ is measured by sampling from each respirometer on at least two, ideally three occasions. Ensure that the animals are not disturbed when taking the samples.

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

$$PO_2 \text{ (mmHg)} = [\text{Barometric press} - (5.7 + 0.03 \times (\text{Temp}^\circ\text{C})^2)] \times 0.20946$$

e.g. 160 mmHg = (776 mmHg - (5.7 + 0.03x15²)) x 0.20946
 [Conversion factors for pressure: 1 atm = 101.325 kPa; 1 mmHg = 0.133322 kPa]

Oxygen solubility values are used to convert PO₂ (mmHg) values to oxygen concentration in μmoles O₂ L⁻¹ as follows:

$$C(t) = [(\text{Exptl. } PO_2 \text{ in mmHg}) / (PO_2 \text{ at air saturation})] \times 259.6 \mu\text{moles } O_2 \text{ L}^{-1}$$

(e.g. 259.6 μmoles O₂ L⁻¹ is concentration and 156.6 mmHg is PO₂ at air saturation; when 15°C, 32 ppt & 760 mmHg or 101.325 kPa).

The rate of oxygen consumption is then calculated as follows:

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

where t_0, t_1 = start and finish times (min) of the measurement period;
 $C(t)$ = concentration of oxygen in the water (μmoles O₂L⁻¹) at time t;
 V_r = volume of respirometer minus the animal

Flow-through approach

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

should be maintained by a multichannel peristaltic pump. The rate of oxygen consumption is calculated as follows:

$$\text{Rate of O}_2 \text{ uptake (} \mu\text{moles of O}_2 \text{ h}^{-1}\text{)} = [C(t)_{\text{in}} - C(t)_{\text{out}}] \times \text{flow rate (L h}^{-1}\text{)}$$

Use of oxygen sensors

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

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

AMMONIA EXCRETION

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

CALCULATION OF SCOPE FOR GROWTH

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

Energy consumed or ingested (C)-

$$C = \text{clearance rate (L g}^{-1} \text{ h}^{-1}\text{)} \times (\text{mg POM L}^{-1}\text{)} \times (23 \text{ J mg}^{-1} \text{ POM)}$$

Where the energy content of POM or algal food is c. 23 J mg^{-1} ash-free dry weight (Slobodkin & Richman 1961, Widdows *et al.* 1979).

Energy absorbed (A)

$$A = (C) \times \text{absorption efficiency}$$

Energy respired (R)

$$R = (\mu\text{moles O}_2 \text{ g}^{-1} \text{ h}^{-1}\text{)} \times 0.456$$

where the heat equivalent of oxygen uptake is $0.456 \text{ J } \mu\text{mole}^{-1} \text{ O}_2$ (Gnaiger 1983).

Energy excreted (U)

$$U = (\mu\text{moles NH}_4\text{- N h}^{-1}) \times 0.349$$

where the excretion of $1 \mu\text{mole NH}_4\text{- N h}^{-1}$ is equivalent to an energy loss of 0.349 J h^{-1} .

PROCEDURES FOR MEASURING AMMONIA EXCRETION INTO SEAWATER

Five litres of sea water are filtered through a membrane filter ($0.45 \mu\text{m}$). The batch of filtered sea water (FSW) is then returned to full air saturation by aeration at the experimental temperature.

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

Elevated ammonia concentrations (above c. $100 \mu\text{M NH}_4\text{- N L}^{-1}$) can inhibit the rate of NH_4 excretion. The incubation time and volume of water should therefore be adjusted when necessary.

Ammonia Test

Into each tube place:

5 mL sea water sample

0.2 mL phenol solution

Mix well and add 0.2 mL nitroprusside solution

Mix and add 0.5 mL oxidising solution (made fresh)

Mix well; cover tubes and place in dark.

For standards, use distilled water as blank, and make up:

$1 \mu\text{M}$ ($5 \mu\text{L}$ of stock in 5 mL DW)

$5 \mu\text{M}$ ($25 \mu\text{L}$ of stock in 5 mL DW)

$10 \mu\text{M}$ ($50 \mu\text{L}$ of stock in 5 mL DW)

$20 \mu\text{M}$ ($100 \mu\text{L}$ of stock in 5 mL DW)

$40 \mu\text{M}$ ($200 \mu\text{L}$ of stock in 5 mL DW)

Use 5 mL of standards and treat same as samples.

Reagents

1. Phenol solution:
Dissolve 10 g of phenol in 100 mL of 96% v/v ethyl alcohol. Store at 5°C .
2. Sodium nitroprusside:
Dissolve 1 g of sodium nitroprusside in 200 mL DW. Store in amber bottle at 5°C for not more than 1 month

3. Oxidising solutions
Mix 25 mL, of fresh domestic bleach (commercial hypochlorite) with 100 mL alkaline solution. This solution is only stable for c. 12 h and has to be made up fresh each day.
4. Alkaline solution:
Dissolve 100 g trisodium citrate and 5 g NaOH in 500 mL DW. Store at 5°C.
5. Standard stock solution :
0.05349 g NH₄OH per litre = 1 mM NH₄ - N. Add a few drops of chloroform to preserve.

Calculation of Ammonia Excretion

Construct a standard curve and convert optical density (O.D.) readings for samples and control to μM NH₄ - N. Subtract controls from the samples and express rate of excretion as $\mu\text{moles NH}_4 - \text{N h}^{-1}$.

$$\mu\text{M NH}_4 - \text{N excreted h}^{-1} = (\text{Test } \mu\text{M} - \text{Control } \mu\text{M}) \times (V/1000) \times 1/t$$

CORRECTION FOR BODY SIZE

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

$$\begin{aligned} \text{or} \quad y &= aX^b & (1) \\ \log Y &= \log a + b \log X & (2) \end{aligned}$$

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

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

For example:-

The slope (b = 0.65) describing the relationship between oxygen uptake and dry body mass is substituted in equation 2. Therefore, if an individual has an oxygen uptake of 12.54 $\mu\text{moles O}_2\text{h}^{-1}$ and a dry mass of 0.83 g then :

$$\begin{aligned} \log a &= \log Y - b \log X \\ \log a &= \log 12.54 - 0.65 (\log 0.83) \\ a &= 14.16 \mu\text{moles O}_2\text{g}^{-1}\text{h}^{-1} \end{aligned}$$

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

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

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

PRINCIPLE OF PARTICLE ANALYSIS USING A COULTER COUNTER

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

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

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

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

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APPENDIX 8

A FRAMEWORK FOR STUDYING CHANGES IN COMMUNITY STRUCTURE

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STAGES

1. REPRESENTING COMMUNITIES (graphical description of faunal relations).
2. DISCRIMINATING SITES on the basis of faunal composition (*e.g.* spatial: control v. impacted, temporal: before v. after impact.)
3. DETERMINING LEVELS OF "STRESS" or disturbance in communities.
4. LINKING WITH ENVIRONMENTAL VARIABLES (*e.g.* correlating to contaminants).
5. ESTABLISHING CAUSALITY of link to contaminants.

TECHNIQUES

UNIVARIATE	- diversity indices - indicator species abundance
DISTRIBUTIONAL	- "ABC" curves (k-dominance) - distribution of individuals amongst species
MULTIVARIATE	- triangular matrix of similarities between samples, leading to: - hierarchical classification (CLUSTER) - multidimensional scaling (MDS) - principal component analysis (PCA)

UNIVARIATE TECHNIQUES

	EXAMPLE
Diversity indices	Indicator species

STAGES

1.	REPRESENTING COMMUNITIES	Mean \pm confidence intervals (CIs) for each site
2.	DISCRIMINATING SITE	One-way analysis of variance (ANOVA)
3.	DETERMINING STRESS LEVELS	By reference to historical data, <i>e.g.</i> Ultimately a decrease in diversity initial increase in "opportunistic species"
4.	LINKING TO ENVIRONMENT	Regression techniques
5.	ESTABLISHING CAUSALITY	Mesocosm or field <u>experiments</u> with controlled dosing of contaminants. All entries above apply, <i>e.g.</i> now significant discrimination of "site" (-treatments) demonstrates that contaminant <u>causes</u> biological effect.

DISTRIBUTIONAL TECHNIQUES

	EXAMPLE
"ABC" curves (k-dominance curves)	Distribution of individuals amongst species

STAGES

- | | | | | | |
|---|---------------------------|--|---|--|--|
| 1. | REPRESENTING COMMUNITIES | Curves for each site (or preferably replicate) | | | |
| 2. | DISCRIMINATING SITE | ANOSIM (Analysis of Similarities) test on "distances" between every pair of curves | | | |
| 3. | DETERMINING STRESS LEVELS | <table border="0" style="width: 100%;"> <tr> <td style="width: 50%;">Biomass curve drop below number curve when subject to disturbance</td> <td style="width: 10%; border-left: 1px solid black; border-right: 1px solid black;"> </td> <td style="width: 40%;">Species abundance distribution is less "smooth" with disturbance</td> </tr> </table> | Biomass curve drop below number curve when subject to disturbance | | Species abundance distribution is less "smooth" with disturbance |
| Biomass curve drop below number curve when subject to disturbance | | Species abundance distribution is less "smooth" with disturbance | | | |
| 4. | LINKING TO ENVIRONMENT | Possible for univariate summary statistics by Regression | | | |
| 5. | ESTABLISHING CAUSALITY | Mesocosm or field dosing experiments. Entries above apply. | | | |

MULTIVARIATE TECHNIQUES

EXAMPLES		
Hierarchical clustering	MDS ordination	PCA ordination

STAGES

Dendrogram of replicates		Configuration of replicates (often 2-D)
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DISCRIMINATING SITES

ANOSIM test on triangular matrix of similarities. Similarities percentage breakdown (SIMPER) give responsible.		Multinormal test (<i>e.g.</i> wilk' Λ invalid.
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DATA TRANSFORMATION AND SPECIES SELECTION/AGGREGATION

Some techniques may need TRANSFORMATION of the raw abundances/biomass (or derived statistics) for :

- a) validity of assumptions for statistical analysis (*e.g.* normality, constant variance);
- b) balancing contributions of rare/abundant species.

Some techniques may be possible with data on SELECTED (more dominant) species or data AGGREGATED to higher taxonomic levels, thus minimising identification time.

TECHNIQUE	EXAMPLES	TRANSFORMATION	SELECTION/ AGGREGATION
UNIVARIATE	Diversity indices	Counts:No Index: Possible	No
	Indicator species	Yes (on count/biomass)	Yes
DISTRIBUTIONAL	ABC curve	Possible but not usual	Possible
	Ind. among species	No	No
MULTIVARIATE	Cluster	Usual (log or 4th root) on count/biomass	Possible
	MDS	MDS transforms similarities also, to rank	No Possible
	PCA		Needed