



MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME



FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

**PROCEEDINGS OF THE FAO/UNEP/IOC WORKSHOP ON THE BIOLOGICAL
EFFECTS OF POLLUTANTS ON MARINE ORGANISMS**
(Malta, 10-14 September 1991)

Organised jointly with the Euro-Mediterranean Centre
on Marine Contamination Hazards (Council of Europe)

Edited by G.P. Gabrielides

MAP Technical Reports Series No. 69

In cooperation with



IOC

UNEP
Athens, 1992

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This volume is the sixty-ninth issue of the Mediterranean Action Plan Technical Reports Series.

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

Ce volume constitue le soixante-neuvième numéro de la série des Rapports techniques du Plan d'action pour la Méditerranée.

Cette série comprend certains rapports élaborés au cours de diverses activités menées dans le cadre des composantes du Plan d'action pour la Méditerranée: Programme de surveillance continue et de recherche en matière de pollution (MED POL), Plan Bleu, Programme d'actions prioritaires, Aires spécialement protégées et Centre régional méditerranéen pour l'intervention d'urgence contre la pollution marine accidentelle.

P R E F A C E

The present volume of the MAP Technical Reports Series contains the proceedings of the Workshop on the Biological Effects of Pollutants on Marine Organisms which was convened by FAO, UNEP and IOC and organised jointly with the Euro-Mediterranean Centre on Marine Contamination Hazards (Council of Europe) in the framework of the Long-term Programme for Pollution Monitoring and Research in the Mediterranean (MED POL - Phase II) which constitutes the scientific and technical component of the Mediterranean Action Plan. The Workshop was hosted by the Foundation for International Studies, Valletta, Malta, from 10-14 September 1991.

Two key-note papers and 19 other papers were presented at the Workshop. As decided, the papers were reviewed by other participants of the Workshop namely, Dr. John Widdows, Plymouth Marine Laboratory, U.K., Dr. Ken Renton, Dalhousie University, Halifax, N.S., Canada, Dr. David Abel, University of Sunderland, U.K. and Mr. Richard Lloyd, Chelmsford, U.K. The authors normally responded to the referee's comments; in case of disagreement, the referee's comments as well as the author's reply appear at the end of the paper. In two cases, the referees had strong objections to the papers and as a result they do not appear in the present publication. All other papers appear in full (in alphabetical order of the senior author's name) as Annex IV to the report while the discussions which took place and the recommendations appear in the main body of the report. The views expressed in the papers are those of the authors and do not necessarily represent the views of either FAO, UNEP or IOC.

The discussions concentrated primarily on the applicability of various biological effects techniques, in field studies, on a routine basis, and on their significance and interpretation vis-à-vis marine pollution risk assessment. In order to make the most rapid progress towards routine applications, the choice of techniques (test species, experimental and monitoring procedures) should be based on a few selected approaches which are well established and documented. A major research activity should therefore be to develop and apply these techniques within the Mediterranean, bearing in mind both the oceanographic and the biotic peculiarities of the area as well as laboratory organisation and availability of resources.

The Workshop recommended, among other things, the establishment of a Working Group to formulate a pilot biomonitoring exercise for implementation by selected Mediterranean institutions in their respective areas. Such a Working Group was set up and will have its first meeting in Malta, in November 1992. Training was also recommended and the FAO/UNEP/IOC Training Workshop on the Techniques for Monitoring Biological Effects of Pollutants in Marine Organisms was organised and took place in Nice, France from 14-25 September 1992.

Final editing and compilation of this volume was done by Mr. G.P. Gabrielides, FAO Senior Fishery Officer (Marine Pollution) at the Co-ordinating Unit for the Mediterranean Action Plan, while Ms Vanta Papapanagiotou was responsible for the typing.

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REPORT OF THE MEETING

INTRODUCTION

The present Workshop on the Biological Effects of Pollutants on Marine Organisms was convened by FAO, UNEP and IOC and organised jointly with the Euro-Mediterranean Centre on Marine Contamination Hazards (Council of Europe) in the framework of the Long-term Programme for Pollution Monitoring and Research in the Mediterranean (MED POL - Phase II) which constitutes the scientific and technical component of the Mediterranean Action Plan. One of the objectives of the MED POL programme is to generate information that can be used for the development of environmental quality objectives in the context of the technical implementation of the Protocol for the Protection of the Mediterranean Sea against Pollution from Land-Based Sources, which has already been ratified by nearly all the Mediterranean States. The study of the biological effects of pollutants on marine organisms is essential in assessing marine pollution risks and establishing water criteria.

The Workshop was hosted by the Foundation for International Studies, Valletta, Malta, which is an autonomous self-governing organization devoted to the pursuit of research and training at international level in a number of fields including environmental matters. It took place from 10 - 14 September 1991 and was attended by 50 participants from Algeria, Bulgaria, Canada, Egypt, France, Greece, Italy, Malta, Romania, Spain, Tunisia, Turkey, U.K. and Yugoslavia, as well as by representatives of FAO, IOC/GEEP and the Foundation. A number of participants was also attending the Second Intensive Training course on the Applications of Ecotoxicology in the Monitoring, Regulation and Control of Marine Pollution in the Mediterranean, which took place in Malta from 3 - 14 September 1991. A list of all participants appears as Annex I.

1. OPENING OF THE MEETING (Agenda item 1)

The Workshop was opened on Tuesday morning by Mr. G.P. Gabrielides, FAO Senior Fishery Officer (Marine Pollution), on behalf of the Food and Agriculture Organization of the United Nations and by Prof. S. Busuttìl, Director-General of the Foundation for International Studies, Malta, and Executive Chairman of the Euro-Mediterranean Centre. Mr. Gabrielides welcomed the participants to the Workshop, thanked the Foundation for accepting to host it and pointed out the excellent co-operation which exists between the Co-ordinating Unit for the Mediterranean Action Plan and the Euro-Mediterranean Centre on Marine Contamination Hazards. He also expressed the wish that the Workshop would motivate and encourage Mediterranean scientists interested in ecotoxicology to experiment with some promising biochemical and physiological response techniques being used in northern Europe for assessing marine pollution effects. He reiterated the intention of his Organization to suggest, in the future, the introduction of biological effects monitoring in the framework of the MED POL programme.

Prof. Busuttìl welcomed the participants to Malta and expressed his pleasure that his Foundation was able to host the Workshop and co-operate with the Mediterranean Action Plan. Mr. V. Axiak, Professor of Biology at the Malta University and Chairman of the Programme Co-ordinating Board of the Euro-Mediterranean Centre on Marine Contamination Hazards, and Mr. A. Micallef, Director of the Centre, associated themselves with the welcome remarks and briefly reviewed some of the activities of the Centre.

2. BACKGROUND AND SCOPE (Agenda item 2)

Mr. Gabrielides outlined the background and scope of the Workshop. In doing so, he referred to the previous MED POL activities in this field and specifically to the two pilot studies which took place in Phase I concerning the effect of pollutants on organisms, communities and ecosystems and the two research activities in Phase II, namely, activity G dealing with the toxicity, persistence, bioaccumulation, carcinogenicity and mutagenicity of chemical contaminants and activity I, dealing with ecosystem modifications in areas influenced by pollutants. Recently, the research component was revised and these activities now take place in the framework of Research Area III - "Effects".

The present Workshop would concentrate on techniques to study the sublethal toxic effects at the "individual" level of organisation or below. There exist a multitude of toxicological tests at the sublethal level which consider the pollutant stress on the biochemistry, physiology, behaviour, genetics and other aspects of biological activities of the individual organism. However, the utility and relevance of many of these techniques is still being assessed. The IOC/UNEP/IMO Group of Experts on the Effects of Pollutants (GEEP) has so far organized three practical workshops to compare and evaluate a number of techniques currently available or proposed for measuring the biological effects of pollutants at levels from the cell to the community.

The aim of the present Workshop is to provide a platform for Mediterranean scientists working in this field:

- a) to present their work relevant to biological effects;
- b) to exchange views and information on the various problems involved;
- c) to discuss the applicability of the techniques in field studies on a routine basis and their significance and interpretation vis-à-vis marine pollution risk assessment;
- d) to make recommendations on research needs and other matters.

3. ELECTION OF OFFICERS (Agenda item 3)

The Workshop unanimously elected Mr. Victor Axiak, Professor of Biology at the University of Malta, as Chairperson, Dr. Marc Lafaurie, Maître de Conférence at the University of Nice, as Vice-Chairperson and Dr. Giovanni Pagano, Senior Research Officer at the Istituto Nazionale Tumori, Naples, as Rapporteur. Mr. Gabriel P. Gabrielides, FAO Senior Fishery Officer (Marine Pollution) at the Co-ordinating Unit for the Mediterranean Action Plan, Athens, acted as Technical Secretary of the Workshop.

4. ADOPTION OF THE AGENDA (Agenda item 4)

The provisional agenda as proposed by the Technical Secretariat was accepted without modifications. It appears as Annex II.

5. ORGANIZATION OF THE WORK (Agenda item 5)

The Workshop agreed to devote the first two days for the presentation of papers. It would work in plenary only but, if necessary, drafting groups could be formed for specific tasks assigned by the Chairperson. The working hours would be 09.00 - 12.30 hours for the morning session and 14.30 - 18.00 hours for the afternoon session. Friday afternoon was reserved for the writing up of the report.

6. PRESENTATION OF PAPERS (Agenda item 6)

Twenty one papers were presented to the Workshop, and these dealt with a range of biological responses of different test species to marine contaminants (see Annex III). During the first half of the session devoted to paper presentations, Dr. Ken Renton, in his key-note paper, gave an overview of the molecular indicators of pollution, in which the utility and limitations of the present biomarkers were assessed and the need for the incorporation of biochemical indicators in pollution monitoring programmes was discussed. Lafaurie *et al.* presented results on the application of a range of biochemical markers in the assessment of environmental quality of sediments, water and biota in the Northern Mediterranean. This work illustrated the need for use of a range of biochemical markers for the evaluation of biological impact of complex mixtures of contaminants in the field.

Arinç and Şen presented results of a laboratory investigation on the induction of liver 7-ethoxyresorufin-O-deethylase (EROD) activity by exposure of fish to polycyclic hydrocarbons, while Pavičić *et al.* assessed the use of methallothionein-like proteins as metal pollution indicators in mussels. Kurelec proposed a multi-xenobiotic resistance mechanism in mussels and sponges, similar to the mechanism of multi-drug resistance found in tumor cells, supporting his proposals by a number of laboratory investigations. Krajnović-Ozretić and Ozretić investigated the use of liver toxicity biomarkers, plasma proteins and haematological parameters in assessing exposure of fish to contaminants, while Bolognesi *et al.* assessed the use of genotoxicity indicators in mussels exposed to contaminated sites from the Ligurian Sea.

Chassard-Bouchaud illustrated the use of micro-analytical techniques in the cellular and sub-cellular analysis of the localization and damage induced by uranium, and other radionuclides in three bivalves. Galdies and Axiak presented results of bio-kinetic studies of lead in another bivalve at the cellular and sub-cellular levels.

The second half of the session was devoted to whole-organismic responses to marine contamination. Dr. John Widdows, in his key-note paper, gave a review of the attributes of physiological energetic responses and their role in biological monitoring programmes. Investigations on the applicability of larval and developmental bioassays were presented by Quiniou and Toularastel on bivalves, and by Pagano *et al.* on echinoderms, respectively. Baldi and Pepi presented a review of chromium toxicity to a range of microorganisms, including tolerance phenomena.

Three papers on phytoplanktonic responses to contaminants as measured in the laboratory were presented. These included: effects of exposure to copper and mercury on growth rates in phytoflagellates (Gotsis-Skretas and Christaki); on diatoms by exposure to the herbicides, 2,4-D and trifluralin (Unsal); and on other unicellular algal species on exposure to lindane (Peñalva and Fernandez).

Verriopoulos presented results of a laboratory investigation of the sublethal effects of zinc, chromium and copper on the survival, fertility, feeding and respiration rates of two copepods, while El-Komi and Tayel provided lethal toxicity data on exposure to organophosphorus pesticides of adult and larval stages of barnacles. Abdel-Moati reported on laboratory and in situ investigations on the accumulation of mercury and lead by mussels as well as on the effects on protein and carbohydrate body contents and on the filtration rates of these test species.

Two papers reported on toxicological investigations carried out on cultured fish. Papoutsoglou and Tziha showed how low oxygen levels, low pH levels and high total ammonia levels which may result from organic pollution affected the blood chemistry and the body chemical composition of seabream and seabass, while Glamuzina investigated the effects of water soluble fractions of crude oil on the eggs, larvae and postlarvae of these same species. The papers appear in full in Annex IV of the report.

7. THE APPLICABILITY OF THE TECHNIQUES IN FIELD STUDIES ON A ROUTINE BASIS AND THEIR SIGNIFICANCE AND INTERPRETATION VIS-A-VIS MARINE POLLUTION RISK ASSESSMENT (Agenda item 7)

In opening the discussion section on the use of ecotoxicological tests for the regulation and control of marine pollution, Mr. Lloyd stressed the need to be clear about the definition of pollution. The GESAMP definition refers to the protection of marine resources from harm; this is an anthropocentric definition which focuses on the need for man's use of the marine resources to be protected. A second use of the term pollution is the presence of man-made chemicals, or man-made increases in natural chemicals, in the marine environment. This definition is based on the fact that all chemicals, at whatever concentration, will have some effect (even though imperceptible at very low concentrations) on marine organisms; it is therefore an ecocentric view. Whether water is polluted or not depends on the sensitivity of chemical and biological analysis to measure the presence of such chemicals.

Pollution control measures reflect to some extent the definition of pollution used. Protection of a resource can be achieved by setting water quality standards or selective controls applied to chemical products. Such procedures require good ecotoxicological concentration/response data for use in hazard and risk assessments. Removal of man-made chemicals from the environment can be achieved by using the best available technology (within economic feasibility) for waste treatment and by the control of chemical products. These procedures can be used independently of ecotoxicological hazard and risk assessments and they satisfy the need to reduce all chemical inputs as far as possible. It may not be a cost-effective approach.

Ecotoxicological hazard and risk assessments require data on concentration/effect relationships. These can be used to classify the hazardous properties of a chemical, to provide environmental quality standards for substances that can occur in the environment at potentially harmful concentrations, in the evaluation of biological monitoring procedures, and for bioassays.

Bioassays use measurements of biological effects to indicate the presence of chemicals in the water at significant concentrations. The main use of such techniques is to measure the concentrations of substances for which chemical analysis is insufficiently sensitive, and to indicate the presence of chemicals which are not included in chemical monitoring programmes. This requires that the tests should have a high degree of reproducibility and precision. Bioassays should not attempt to duplicate or replace chemical monitoring but should be integrated into such programmes. Results of such bioassays cannot be used alone to predict the risk of damage to living aquatic resources.

For those substances which are shown to be present or have the potential to be present in the marine environment, specific tests to derive concentrations/response relationship were required to make risk assessments and, where necessary, establish water quality standards to give adequate protection to marine communities. It is essential that these toxicological tests should incorporate a proper control of the chemical conditions of exposure; the concentrations should be kept as constant as possible, the chemical state of the substance should be identified, and the exposure conditions should be relevant to those encountered in the environment. Lack of such control destroys the usefulness of the data for hazard and risk assessments, and therefore for pollution control. There is a better need for harmful effects to be related to tissue concentrations, and the state of these levels should be identified, i.e. whether they are in stores of fat (lipophilic substances) or in inert granules (metals).

The effects that are measured should be capable of extrapolation to predict the risk of harm to aquatic communities. This cannot be done for biochemical effects but is possible for measurements of growth potential and embryonic development. The ability to obtain good concentration/response data diminishes with tests on higher levels of biological organisation i.e. on mesocosms containing complex communities.

Finally, ecotoxicological risk assessments should be incorporated into economic cost-benefit analyses in order to obtain a rational regulation of pollution that takes priorities into account. There is a need for a better education of the public in the scientific assessment of pollution risk in order to influence their risk perception and so contribute to rational political decisions. Disagreement among the scientific community as to what constitutes a risk has led to a general lack of confidence in ecotoxicological predictions.

After the presentation of Mr. Lloyd, the discussion concentrated on the suitability of biological effects techniques for marine pollution assessment purposes stressing their limitations. The main limitation identified was the fact that some of these techniques are undertaken under unrealistic experimental conditions and subsequent claims of the importance of the derived information may not be justifiable.

Many of these techniques are applicable only to certain species and to a small group of chemicals and therefore the physiological background of each species should be known before undertaking any toxicological experiments.

The idea was put forward that these techniques could be used to identify "hot-spot" areas where chemical monitoring should be initiated.

As the process of evaluation is still going on, intercalibration exercises are needed to ensure improved quality and comparability of results. Practical workshops taking place elsewhere could also be repeated in the Mediterranean region.

It was pointed out that the suggestions of committees entrusted with the recommendation of specific procedures to be used in monitoring programmes may be biased by the personal views of the individual committee members and such subjective judgement should be thoroughly discouraged.

Ecotoxicological risk assessment should also be incorporated into more general approaches such as control management and environmental policy analysis.

The view was expressed that more effort should be devoted to useful monitoring which could help in making proper decisions for pollution control purposes. The views of decision-makers are often influenced by public opinion pressure groups and, as a result, certain decisions taken are based on emotion and perception rather than on actual scientific facts.

8. FUTURE RESEARCH NEEDS (Agenda item 8)

The Workshop recognized an urgent need for marine research in environmental toxicology to be directed more specifically to the solution of perceived pollution problems. In particular, the role of ecotoxicology in the monitoring, regulation and control of pollution has potentially enormous benefits in optimising the allocation of scarce resources. Unless, however, agreement is reached on a number of issues, it is likely that national and international agencies will move as a result of public pressure towards pollution control strategies based wholly upon best technological means and/or control of pollution at source. While such strategies will result in some environmental improvement, they are not cost-effective, and therefore not an optimal solution for an economically deprived area such as the Mediterranean.

In order to make the most rapid progress towards routine applications, the choice of techniques (test species, experimental and monitoring procedure) should be based on a few selected approaches which are well-established and documented. A major research activity should therefore be to develop and apply these techniques within the Mediterranean, bearing in mind both the oceanographic and the biotic peculiarities of this area as well as laboratory organization and availability of resources.

8.1 Toxicity testing procedures

No single test procedure can be appropriate for all circumstances. It is therefore necessary to establish a repertoire of procedures which would meet the requirements of the various pollution situations which exist. In

particular, the Workshop concentrated on a range of biochemical and physiological approaches. Some of these are reasonably well established and are ready for universal application in the short-term. These may include:

- a) Molecular biomarkers, such as:
 - mixed-function oxidase (MFO) especially 7-ethoxy-resorufin-O-deethylase (EROD);
 - acetylcholinesterase (AChE);
 - metallothioneins (MTh);
 - DNA alterations.
- b) Toxicity testing on early life stages, including:
 - fertilization toxicity (both in vitro and in vivo), and
 - developmental toxicity (embryo/larval stages in bivalves, echinoids and fish).
- c) Bioenergetics (Scope For Growth) in mussels (or other species where appropriate).
- d) Cytogenetic testing, such as:
 - micronucleus induction, and
 - cytogenetic analysis (primarily in mussels and sea urchins).

Where these techniques are used as bioassays and in biomonitoring procedures, they should be fully integrated into chemical monitoring programmes, so that they can identify the occurrence of unknown chemicals present at biologically significant concentrations in the marine environment. The limitations, as well as the value, of these tests should be recognized; these should include evaluation of the reproducibility and precision of the results obtained.

Any additional set of procedures appearing to be of interest at the present time cannot be recommended for universal application. They may, however, be worth investigating in a few laboratories which have specific expertise, or may be susceptible of further development until their routine utilization may be recommended.

These approaches include, e.g.:

- a) the cellular/subcellular distribution of pollutants;
- b) the multi-drug resistance (MDR) system, as a possible indicator of adaptation to a polluted environment;
- c) the occurrence of neoplastic and preneoplastic lesions in fish populations (preferably non-migratory, benthic species);

- d) the use of remote sensing as a monitoring tool;
- e) toxicity testing techniques designed to investigate the effects of pollutants on primary producer organisms, particularly if they are sufficiently sensitive to be ecologically relevant.

The Workshop also recognized that studies carried out within the framework of aquaculture programmes may provide useful data within an ecotoxicological context.

8.2 Choice of testing conditions and substrates

It must be emphasized that environmentally relevant conditions (e.g. concentration, route of administration and duration of experiment) should be invariably adopted in toxicity testing. There remains scope for the use of "classical" toxicological techniques such as determinations of LC 50 or EC 50 using a variety of organisms such as phytoplankton, zooplankton, polychaetes, etc., provided that the purposes of the experiments are clearly defined and their limitations are clearly understood. In particular, the use of lethal toxicity tests or other tests involving the exposure of organisms or cells to high concentrations of toxicants for short periods of time may be viewed as a part of a more comprehensive risk assessment for regulatory purposes.

By utilizing environmentally realistic levels of toxicants, investigators should be aware, however, that in a few cases they may encounter a positive stimulation of the biological event being considered (e.g. growth, fertilization, etc.). This phenomenon is currently termed "hormesis" which has been reported extensively in the literature. If this phenomenon may be confirmed in the environment, hormesis might reflect potential alterations at the community level.

As for substrate choice in toxicity testing, in-field investigations may either focus on water or sediment toxicity, or both. Since it is recognized that marine contaminants of whatever class are rapidly deposited in sediments, it is essential that techniques be developed for studying the response of benthic organisms to contaminated sediments. On the other hand, the measurements of effects in the water column is made difficult by the low concentrations of chemicals present; this could be overcome if techniques to concentrate these substances (eg. absorption/elution) could be developed so that tests can be made on the concentrates. As for the utilization of sediment samples, it is currently controversial whether sediment toxicity should be tested on either solid phase or water extracts (elutriate), or both. Efforts should be made to determine the most appropriate techniques for studying sediment toxicity.

9. RECOMMENDATIONS (Agenda item 9)

The Workshop made the following recommendations:

- a) Ecotoxicological effect measurements in the Mediterranean should be directed more specifically to the solution or amelioration of existing pollution problems.

- b) Toxicity testing procedures should be developed and applied based on (i) existing knowledge and techniques directed primarily towards monitoring and regulation of pollution and (ii) the specific oceanographic and biotic conditions in the Mediterranean.
- c) Those techniques and approaches which are listed in section 8.1 should be promoted and applied as widely as possible. It is recommended that further aspects of ecotoxicological studies (such as responses at community level and multispecies toxicity tests) will be discussed and assessed in other further international meetings.
- d) The proper choice, control and description of the chemical conditions of exposure within toxicity tests and the integration of chemical and biological monitoring programmes need to be given a much greater emphasis.
- e) There remains a continuing need for training in specific techniques and in the development of relevant research strategies. It is recommended that a workshop is organized as early as possible to train Mediterranean scientists in a number of biological monitoring techniques.
- f) The Workshop recommends the establishment of a Working Group to formulate a pilot biomonitoring exercise for implementation by selected Mediterranean institutions in their respective areas. The purpose will be to evaluate the environmental impact of pollution sources and to correlate the results with chemical monitoring data. Moreover, this Group could recommend necessary training and intercalibration exercises.
- g) MED POL should increase its support to enhance communication and collaboration between laboratories especially between those in developed and developing countries.
- h) MED POL should seek ways to initiate informal and, at a later stage, formal co-operation with laboratories in the Black Sea countries.

10. ANY OTHER MATTER (Agenda item 10)

The publication of the proceedings was brought up under this agenda item and the Technical Secretary explained that these would be published in the MAP Technical Reports Series unless otherwise decided by the Workshop.

After an extensive discussion, it was decided that the best solution would be to publish the papers presented at the Workshop in the MAP Technical Reports Series. However, before publication, the papers should be reviewed by other participants of the Workshop. If the authors would not accept any of the comments of the referee, then this comment, together with the author's reply, would appear at the bottom of the paper. In the meantime, participants were also encouraged to publish their papers in international scientific journals mentioning that the paper was presented at the FAO/UNEP/IOC Workshop on the Biological Effects of Pollutants on Marine Organisms (Malta, 10-14 September 1991).

Authors wishing to submit a revised version of their paper for the review, should do so as soon as possible but not later than the end of October 1991.

11. ADOPTION OF THE REPORT (Agenda item 11)

The present report was adopted by the Workshop on Saturday, 14 September 1991.

12. CLOSURE OF THE MEETING (Agenda item 12)

The Hon. Minister of Education and Interior Dr. Ugo Misfud Bonnici, responsible for environment, stressed, in his speech, the importance of environmental protection in man's development and well-being and expressed his confidence that the deliberations of the Workshop would be useful in formulating control measures for the protection of the Mediterranean sea.

The Technical Secretary, in his closing remarks, expressed satisfaction for the results of the Workshop and thanked the participants for the constructive spirit in which it was conducted. He also thanked the Officers of the Workshop, the guest-speakers and everybody else who contributed directly or indirectly to its success. The work of the Director and staff of the Euro-Mediterranean Centre on Marine Contamination Hazards was gratefully acknowledged and appreciation was expressed for the warm hospitality.

After the usual exchange of courtesies the Chairperson closed the Workshop.

ANNEX I

LIST OF PARTICIPANTS

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ANNEX II

AGENDA OF THE MEETING

1. Opening of the meeting
2. Background and scope
3. Election of officers
4. Adoption of the agenda
5. Organization of the work
6. Presentation of papers
7. The applicability of the techniques in field studies on a routine basis and their significance and interpretation vis-à-vis marine pollution risk assessment
8. Future research needs
9. Recommendations
10. Any other matter
11. Adoption of the report
12. Closure of the meeting

ANNEX III

LIST OF PAPERS PRESENTED

Key-note papers

1. Biochemical techniques for the assessment of chemical pollutant exposure in the marine environment
by K.W. RENTON
2. Role of physiological energetics in ecotoxicology and environmental pollution monitoring
by J. WIDDOWS

Other papers

3. Biochemical and physiological responses of Mytilus edulis to Hg and Pb in the coastal waters of Alexandria region
by M.A.R. ABDEL-MOATI
4. Induction of liver 7-ethoxyresorufin O-deethylase in gilthead seabream by benzo(a)pyrene and its potential use in biochemical monitoring of environmental pollutants
by E. ARINÇ and A. ŞEN
5. Microbial response to chromium toxicity
by F. BALDI and M. PEPI
6. Carcinogenic and mutagenic pollutants: impact on marine organisms
by C. BOLOGNESI, M. PARRINI, P. ROGGIERI, C. ERCOLINI and C. PELLEGRINO
7. Biological effects of uranium and transuranium nuclides on marine bivalves, Mytilus edulis, Crassostrea gigas, and Cerastoderma edule: Microanalysis at the cellular and subcellular levels
by C. CHASSARD-BOUCHAUD
8. Influence of organophosphorus herbicides on barnacles (Crustacea:Cirripedia)
by M. EL-KOMI and F. TAYEL

9. The fate of lead in a benthic bivalve (Venus verrucosa)
by Ch. GALDIES and V. AXIAK
10. The early fish stages as an object of toxicological studies; case of seabass, Dicentrarchus labrax, and gilthead seabream, Sparus aurata
by B. GLAMUZINA
11. Physiological responses of two marine phytoplanktonic species to copper and mercury
by O. GOTSIS-SKRETAS and U. CHRISTAKI
12. Detection and evaluation of hepatic intoxication in fish
by M. KRAJNOVIĆ-OZRETIĆ and B. OZRETIĆ
13. The multi-xenobiotic resistance mechanism in aquatic organisms
by B. KURELEC
14. Biochemical markers in pollution assessment: field studies along the North coast of the Mediterranean sea
by M. LAFaurIE, A. MATHIEU, J.P. SALAUN, J.F. NARBONNE, F. GALGANI, M. ROMEO, J.L. MONOD and Ph. GARRIGUES
15. Sublethal toxicity testing in sea urchin fertilization and embryogenesis: a study of polluted water and sediment from two rivers in Campania, Italy
by G. PAGANO, A. ESPOSITO, M. GUIDA, G. MELLUSO and N.M. TRIEFF
16. Effects of water pollution, caused by organic material, on the physiology and body chemical composition of seabream (Sparus aurata) and seabass (Dicentrarchus labrax)
by S.E. PAPOUTSOGLOU and G. TZIHA
17. Metal binding proteins of Mytilus galloprovincialis, similar to metallothioneins, as a potential indicator of metal pollution
by J. PAVIČIĆ, B. RASPOR and M. BRANICA
18. Lindane effects on the growth, size and composition of two marine unicellular algae, Monochrysis lutheri (Droop) and Phaeodactylum tricornutum (Bohlin)
by S. PEÑALVA and F. FERNANDEZ

19. Biological effects of contaminated water tested by marine bivalve embryo-biassay
by F. QUINIOU and F. TOULARASTEL
20. Effects of herbicides on the growth of marine phytoplankton
by M. ÜNSAL
21. Effects of sublethal concentrations of zinc, chromium and copper on the marine copepods Tisbe holothuriae and Acartia clausi
by G. VERRIPOULOS

ANNEX IV

PAPERS IN FULL

BIOCHEMICAL TECHNIQUES FOR THE ASSESSMENT OF CHEMICAL POLLUTANT
EXPOSURE IN THE MARINE ENVIRONMENT

by

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A B S T R A C T

The use of cytochrome P-450 as a biochemical marker for the exposure of marine species to chemical pollution is reviewed. The most important methods for sample collection, preparation and analysis are discussed. Several case studies illustrate the use of catalytic, immunological and molecular biological approaches for the assessment of cytochrome P-450 in fish species. The measurement of cytochrome P-450 is recommended as an integral component in any chemical/biological monitoring program to the marine environment.

1. INTRODUCTION

In recent years increasing amounts of chemical pollutants have been added to the land and water based ecosystems around the world and have had a major impact on all living organisms. The marine aquatic environment is particularly vulnerable as it receives considerable amounts of chemicals as the end stage collector for the pollutants originating from the land, lakes, rivers and atmosphere (Fig. 1). Tidal estuaries are particularly vulnerable as they are usually closest to pollutant sources and the effects of dilution in these areas are minimal. The deposition of massive quantities of biologically active chemicals into the marine aquatic environment from anthropogenic sources involves a huge number of compounds used for industrial, urban and agricultural purposes. These original compounds are often degraded chemically or biologically as they pass through various systems and the result is exposure of the marine environment to literally thousands of different of chemical entities.

Because of the impact of chemicals in the marine environment it has become important to monitor the extent of pollution and its impact on marine species (Cairns *et al.*, 1984). Although a large number of chemical methods have been developed to measure chemical pollutants these procedures usually require prior knowledge of the structure and class of chemical involved and especially recently the methods have become so sensitive that they measure levels of pollutant that are of no biological significance. It is also possible to monitor the gross effects of chemicals on living systems, however, usually when such effects are easily observed, a large amount of irreversible damage to the environment has already taken place. Some of the most useful techniques for monitoring pollutant impact in the marine environment are methods which assess biological effects of environmental pollutants at a stage prior to permanent environmental damage and which do not require previous knowledge of the exact chemical structure of the pollutants.

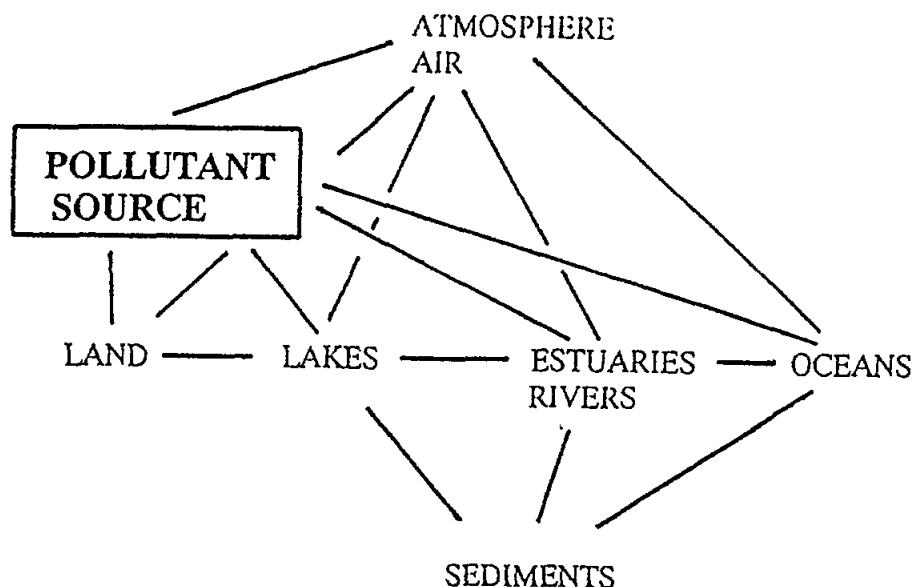


Fig. 1 Pollutant sources in the marine environment

Among several techniques that have been developed, changes to normal biochemical systems in marine organisms can often be used as an early signal that environmental damage is in progress. The advantage of a biochemical determination is that this is a measure of chemical load which is having a true biological impact and the exact chemical structure and make up of the pollutants do not have to be defined. Although these procedures can also be used for monitoring purposes on land or in fresh water this paper will be confined to biochemical techniques which have been useful in monitoring pollutants in the marine environment.

Although most biochemical systems in marine species are similar to those in other organisms, they must be carefully re-characterized in the species to be used for monitoring purposes. Ideally, the basic biochemical measurement from a clean area must be stable and have a low variation between individual organisms. The biochemical parameter must respond to the pollutants in question and at a magnitude which is easily distinguished from levels determined in a control situation. The parameter to be utilized can be selected from the known pharmacological, toxicological and biochemical effects of classes of chemical pollutants. Depending on the specificity required, it may be sufficient to examine a single biochemical pathway or, an entire suite of measurements may be required to cover the range of pollutants in question. In nearly all cases, samples obtained from a non-polluted reference site of a similar nature is required for comparison. For the purposes of this review, cytochrome P-450 has been selected as an example of a biochemical parameter that can be utilized to signal the exposure of marine life to pollutant chemicals.

2. SAMPLE ACQUISITION

When using any biochemical procedure for monitoring purposes the sample must be obtained, stored and processed under conditions that will protect the integrity of the biochemical parameter to be measured. This presents special difficulties in collecting samples from the field as many biochemical entities or activities are easily degraded and marine sample sites are often remote from laboratory and testing facilities. Although the tissue sample can be processed immediately on collection and measurements made immediately on fresh sample, many biochemical measurements require delicate and sophisticated equipment and this presents a real problem in the field. In some cases it is possible to transport the live organism to the laboratory and carry out the tissue preparation and measurements on fresh tissue. This obviously has to be carried out within a reasonable time of collection otherwise the biochemical parameter can change in vivo.

Another solution is to remove the tissue of interest in the field or field station and preserve the sample in ice, dry ice or liquid nitrogen for transportation back to the home laboratory. Intact tissues and subcellular fractions can be transported in this way. Most biochemical measurements can only survive short time periods in ice and more often dry ice or liquid nitrogen is employed to transport the sample prior to permanent storage in a deep freeze. In the case of cytochrome P-450 measurements we have collected liver samples from marine species in both warm and cold climate conditions and by utilizing liquid nitrogen transportation and subsequent storage at -90°C , values for cytochrome P-450 measurements could be obtained many months after collection. A major drawback to the use of dry ice or liquid nitrogen is its availability in some locations and the regulations for the transportation of such samples by air.

3. CYTOCHROME P-450

3.1 Background

The cytochromes P-450 are a group of hemoproteins which are products of a superfamily of genes which are distributed throughout living organisms (Nebert et al., 1989). Of particular interest to pollution monitoring is the P450IA gene family in vertebrates which can be induced by a number of chemical classes which are common environmental pollutants (eg. oil derived chemicals, polyaromatic hydrocarbons, polychlorinated biphenyls, dioxins). In fish, only a single member of this gene family is expressed (Cytochrome P-450IA1) and the induction of this hemoprotein can be used as a biochemical indicator for pollutant exposure. The exact chemical structure of the pollutants do not have to be known but this technique can obviously only signal the presence of chemicals which induce cytochrome P-450 (Stegeman, 1989). A major advantage in using cytochrome P-450 as a monitoring procedure is that it can be applied to any fish species provided control values are available for that species.

The use of cytochrome P-450IA1 induction as biochemical monitoring tool has become well established using measurements at three different levels of enzyme synthesis and function (Fig. 2). Since it was first proposed (Payne, 1976) numerous examples exist which indicate that induction of this enzyme system is a distinct and specific indication that a marine species has been exposed to one or more of the suite of chemicals known to cause this type of

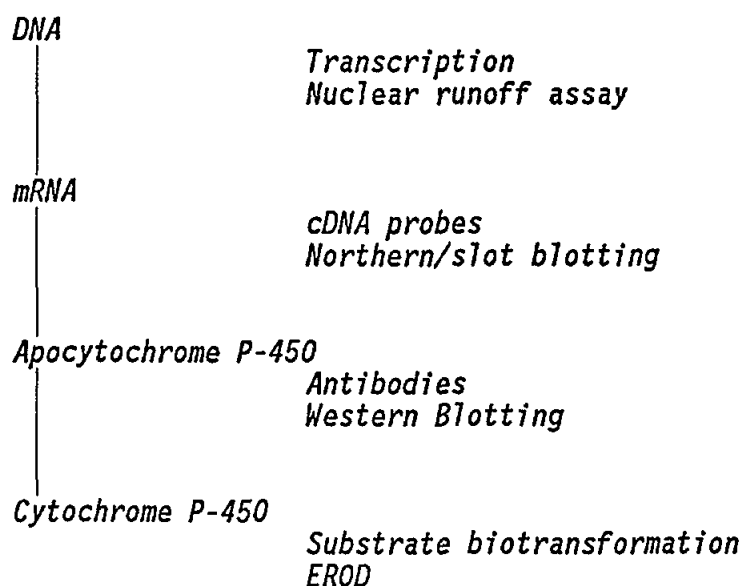


Fig. 2 The various steps in cytochrome P-450 synthesis that are utilized for the assessment of this enzyme system in environmental monitoring programs

response (Payne *et al.*, 1987; Haux and Forlin, 1988; Andersson, 1990). These various assays for cytochrome P-450 can provide a biomarker to detect the exposure of marine species to organic chemical pollutants in large scale monitoring programs. In marine mammals, a variety of cytochrome P-450s are expressed and the methods used to monitor pollutant exposure must be specific for cytochrome P-450IA1. Unfortunately, cytochrome P-450 is expressed at extremely low or non-detectable levels in invertebrates or other organisms and therefore its use in pollution monitoring in the ocean environment is largely confined to measurements in fish or marine mammals.

3.2 Sample preparation

The cytochrome P-450 system is located in the endoplasmic reticulum of cells and the highest concentrations are found in the liver of most species (Gibson and Skett, 1986). The hemoprotein degrades rapidly in intact tissue or subcellular fractions even when kept on ice and it is important to freeze the tissue immediately on collection or to prepare the subcellular fractions without delay. Subcellular fractions are prepared by ultra-centrifugation of homogenates from fresh or frozen liver. Cytochrome P-450 measurements and assessment of its activity can be made in the 10,000 x g supernatant, however, microsomes are the preferred subcellular fraction as the hemoprotein is concentrated in this fraction. Microsomes which normally require centrifugation at 100,000 x g can also be prepared by a calcium precipitation method which requires only low speed centrifugation (Gibson and Skett, 1986).

We have recently developed a procedure to prepare such microsomes in the field using a microfuge. Liver homogenates are centrifuged at full speed for 10 minutes in a Beckman microfuge (10,000 x g) and the supernatant which contains the microsomes and soluble fraction is treated with 8 mM CaCl₂ solution which causes the microsomes to aggregate and recentrifugation at 10,000 x g for 10 minutes produces a microsomal pellet. The pellet is then washed with 0.1 M TRIS (pH 7.4) solution. These small centrifuges can be operated in a cold room to maintain the sample at 4°C. The microsomal preparations can then be utilized for cytochrome P-450 assay or stored below -60°C until analysis. Recently, we have prepared microsomes in remote field locations or on board the ship using this technique and transported them back to the laboratory in powdered dry ice. Microsomes prepared in this way give identical values to those prepared conventionally in an ultra centrifuge.

3.3 Cytochrome P-450 measurement

The measurement of cytochrome P-450IA1 and its induction can be determined at a number of steps in protein synthesis as shown in Figure 2. The induction of this enzyme results from an increase in gene expression (Nebert *et al.*, 1989) and this can be detected by measuring the actual protein itself or its catalytic activity or by determining changes at the various stages of protein synthesis.

3.3.1 Catalytic activity

Traditionally, induction has been assessed by the measurement of the enzymatic activity in hepatic microsomes using specific substrates for P-450IA1 such as benzo(a)pyrene hydroxylase or ethoxyresorufin O-dealkylase. A large number of studies have indicated that induction in the activity of substrate metabolism can be detected in many different fish species following laboratory or field exposure to chemicals of environmental concern (Payne, 1976; Haux and Forlin, 1988; Stegeman, 1989; Andersson, 1990). A large number of different chemicals produce this response in most fish species tested to date and therefore this procedure has great value as an early sentinel for the biological response of pollutants in marine species. The procedures are however time-consuming and do require a fluorimeter to carry out the assay.

3.3.2 Cytochrome P-450 level

The actual levels of the hemoprotein can be assessed by measuring the magnitude of change in the carbon monoxide binding spectrum in hepatic microsomes. The levels of total cytochrome P-450 in fish is much lower than that found in mammals and only large inductive changes are apparent (Stegeman *et al.*, 1985). In recent years a number of immunological procedures have been developed using specific antibodies directed against Cytochrome P-450IA1 to detect increases in cytochrome P-450 protein levels following induction by chemical exposure (Stegeman, 1989). Low levels of cytochrome P-450 are easily detected by such methods and even minor levels of induction are readily apparent. Techniques of immunoblotting or ELISA (Goksoyr, 1991) can be developed into specific, rapid screening assays that can monitor a large number of samples with relatively simple equipment. The antibodies raised against cytochrome P-450IA1 from a specific fish species have a high degree of cross reactivity in other fish species likely allowing environmental studies to be carried out in any fish species present in the area to be

monitored (Goksoyr *et al.*, 1991). In marine mammals great care must be taken to ensure that the antibody recognises cytochrome P-450IA1 in the species being examined.

3.3.3 Cytochrome P-450 synthesis

In some recent studies, the levels of mRNA for cytochrome P-450IA1 have been utilized as a measure of chemical induction (Haasch *et al.*, 1989; Renton and Addison, in press). This procedure requires the isolation of RNA from liver and the construction of a specific cDNA probe with a nucleotide sequence complementary to cytochrome P-450IA1. Either full length probes or short oligonucleotides have been developed for this purpose. A major difficulty arises in that DNA has been sequenced in only a few species of fish to date (Heilman *et al.*, 1988) and the procedure requires a probe which will hybridize to the mRNA in the test species. To overcome this difficulty we have recently developed a synthetic 24 nucleotide cDNA probe which has a high homology in all mammalian species and is only three nucleotides different from the sequence determined in trout. The assumption is that this section of the sequence will have a high homology in all species and the probe will be universal in all species for the detection of cytochrome P-450IA1 mRNA.

In a recent study in the fish dab in the North Sea we have shown that induced levels of cytochrome P-450IA1 mRNA correlate with increases in substrate activity and the levels of cytochrome P-450IA1 protein determined by immuno-quantisation. This method provides a highly specific procedure that can handle a large sample volume in a pollution monitoring program. A disadvantage of this method is that samples must be removed from the fish immediately on death to prevent mRNA degradation by nucleotidases and a high level of skill is required to carry out the procedure. The transcription of DNA could potentially be used as a monitor of increased P-450IA1 gene expression in response to pollutants. This procedure is complex and to date has not been applied to monitor the biological effects of pollutants.

4. CYTOCHROME P-450 AS BIOMARKER IN FIELD STUDIES

4.1 Seasonal variation

Cytochrome P-450 is affected by a large number of exogenous factors which can influence basal levels of the hemoprotein (Gibson and Skett, 1986). Such changes are of major concern to a monitoring program as it is essential to identify pollutant based induction which is independent of any other change. Sex and seasonal breeding status are major determinants of basal levels and it is important to recognise this in any environmental study (Edwards *et al.*, 1988). In a population of winter flounder (*Pseudopleuronectes americanus*) we have shown that in the spring of two consecutive years during the spawning period the activity of EROD was elevated as much as six fold in both sexes of fish as shown in Figure 3. Male fish had approximately double the activity of females at all times of the year. This variability is generally below the induced activity which can be caused by realistic levels of some pollutants, however, the study points out that control values from clean areas must be obtained at the same time in the reproductive cycle for any comparison to be valid especially if low level contamination is being assessed.

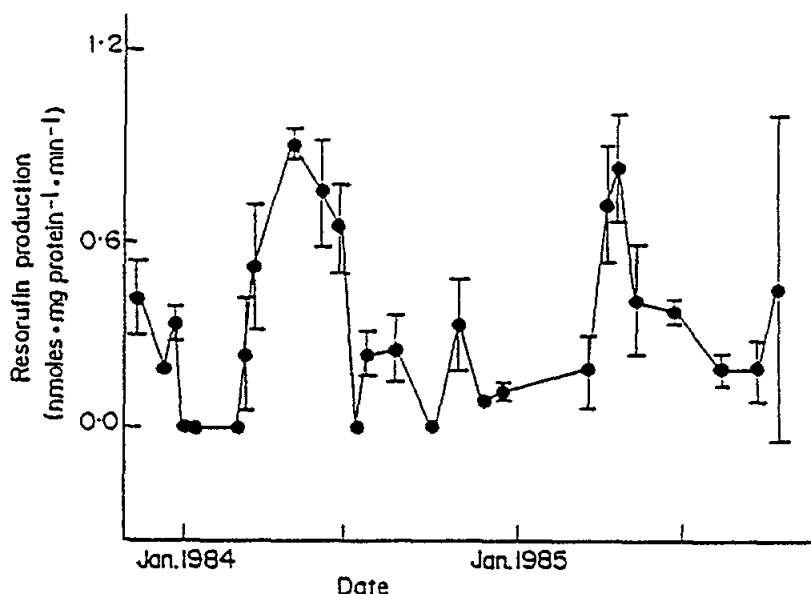


Fig. 3 Variation of female hepatic EROD in liver microsomes obtained from winter flounder over a two year period. Data is expressed as the mean \pm S.E.

4.2 Catalytic activity of cytochrome P-450 as a marker of pollutants in the Langesundfjord, Norway

Ethoxy resorufin O-dealkylase and total cytochrome P-450 were utilized to monitor a pollution gradient in a fjord (Addison and Edwards, 1988). The activities of EROD in *Platichthys flesus* were greatest closest to the site of pollution and diminished gradually at more distant sites (Fig. 4). Cytochrome P-450 levels demonstrated a similar trend, however, the differences were much less pronounced. The induction of cytochrome P-450 and its activity correlated closely with the pollutant load at the various sites indicating that these biochemical markers are a true reflection of the biological effects of effluent pollutants in a partially closed water system.

4.3 Laboratory and environmental induction of cytochrome P-450 in warm water fish species

Although most of the studies to date have concerned the use of cold water northern species of fish, cytochrome P-450 can also be used to monitor pollutant exposure in tropical, sub-tropical and reef fish (Stegeman *et al.*, 1990). In a laboratory experiment cytochrome P-450 was examined in blue striped grunt (*Haemulon sciurus*) exposed to a known cytochrome P-450IA1 inducer, β -naphthoflavone (BNF). These fish responded with a significant induction of cytochrome P-450 estimated by CO binding spectra, immuno-blotting and by the activity of ethoxy resorufin O-dealkylase as shown in Figure 5.

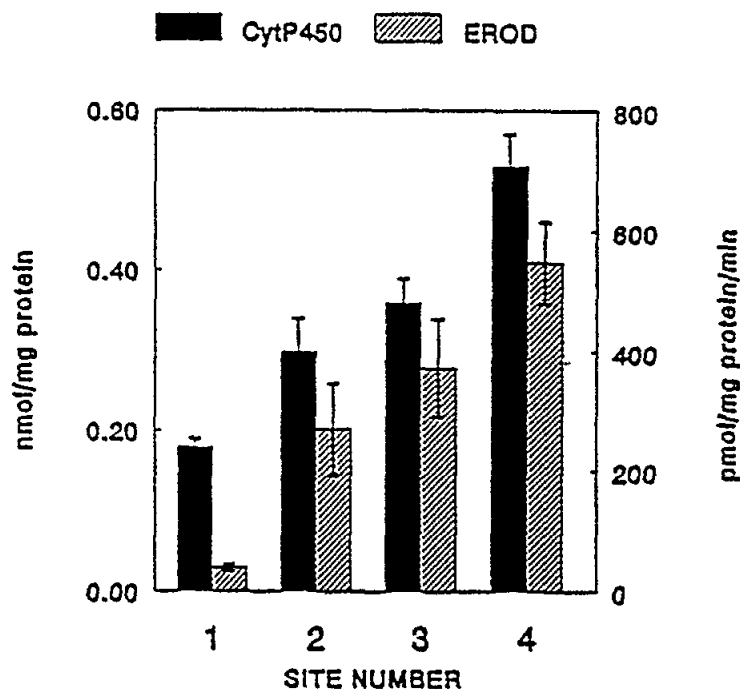


Fig. 4 The levels of cyt P450 and EROD in hepatic liver microsomes from Platichtys flesus along a pollution gradient in Langesundfjord, Norway. The highest levels of organic pollutants are at site 1 and diminished towards the reference site 1

These indicate that in tropical species cytochrome P-450 determinations can be indicative of pollutant exposure in a warm water environment. The only major difficulty encountered in studies in warmer climates is the difficulty in preserving the integrity of samples after collection.

Further studies were carried out in tropical fish species in Bermuda waters to assess the impact of potentially polluted areas on the cytochrome P-450 levels in fish liver (Stegeman *et al.*, 1990). At a site close to an automobile dump the levels of cytochrome P-450 and the activity of EROD were significantly elevated in seabream relative to an un-polluted site which received a high tidal water exchange (Table 1). On the other hand, these parameters were only marginally elevated in blue-striped grunt obtained in the inner part of Hamilton harbour relative to levels found in the liver of fish caught in the outer reaches of the harbour (Table 1). In the overall study in a number of different fish species in Bermuda the levels of EROD were significantly higher in sites characterized by having higher levels of sedimentary hydrocarbons and a strong correlation was found with the PCB content of Arca zebra collected at the same sample sites. These results indicate the value of cytochrome P-450 as an indicator of chemical contamination even in an area which can only be considered to be marginally polluted.

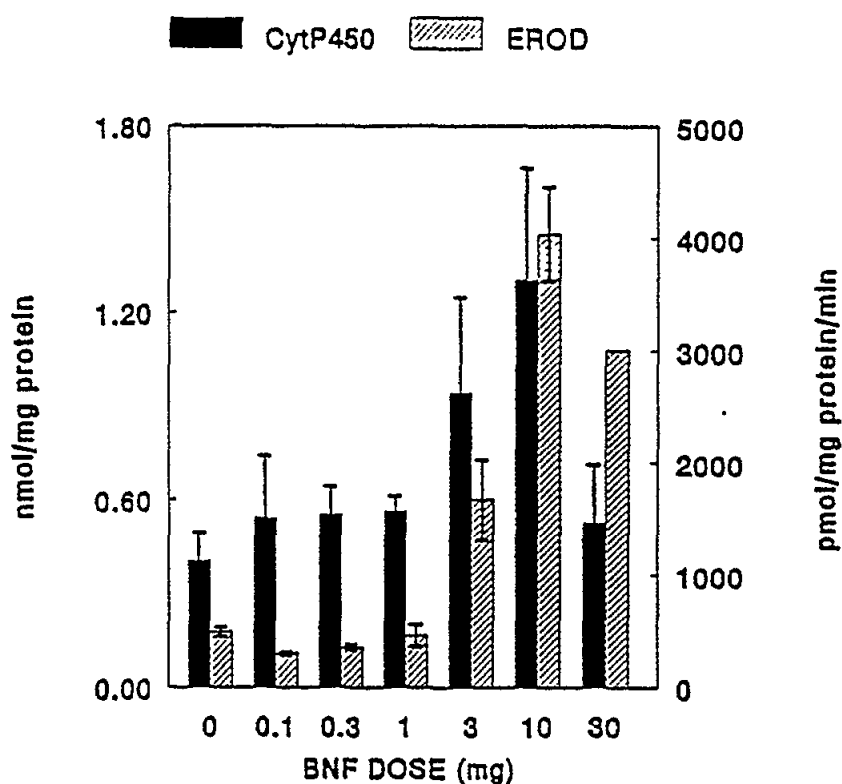


Fig. 5 Dose response for BNF induction of cytochrome P450 levels and EROD activity in blue-striped grunt

Table 1

Cytochrome P-450 levels and EROD activity in polluted sites in Bermuda.

Species/site	Cytochrome P-450 (nmoles/mg protein)	EROD pmoles/mg protein/min
Bream		
Control	0.17 ± 0.04	41.0 ± 5.4
Dump Site	0.46 ± 0.11	108.4 ± 4.9
Blue grunt		
Control	0.41 ± 0.09	408.0 ± 41.0
Inner Harbour	0.48 ± 0.03	551.8 ± 50.3

4.4 Cytochrome P-450 mRNA as a measure of pollutant exposure in the North Sea

We have used two specific substrates for cytochrome P-450IA, ethoxyresorufin and cyano-ethoxycoumarin (Burke and Mayer, 1974; White, 1988) and a cDNA oligonucleotide probe as a measure of cytochrome P-450IA mRNA to

assess pollution in the German Bight area of the North Sea which receives the effluents of the Rivers Weser and Elbe (Renton and Addison, in press). The cDNA probe had the sequence GGAAGTGGTAGGGGTGTCGTG corresponding to nucleotides 705-727 in rat cytochrome P-450d as listed in Genebank. This sequence differs by 3 nucleotides from the sequence published for Trout P450IA1 (Heilman *et al.*, 1988). *Limanda limanda* were collected along a transect of several hundred kilometres in the plume of the two rivers (54° 00'N, 8° 00'E to 55° 30'N, 4° 10'E) and at the site of an abandoned oil well (54° 06'N, 4° 45'E).

Total cytochrome P-450 levels in hepatic microsomes were significantly elevated in samples obtained from site 3 which was closest to shore compared to the values obtained at all other sites. The activities of EROD and CN-ECOD were elevated at site 3 and 5 and indicated a gradual decline along the transect as illustrated in Figure 6. Such results are consistent with the exposure of fish to chemical inducers in the plume of the two rivers. It is impossible to know if the activities found at the sites furthest from shore represent true "no effect" levels because of generalized pollution in the North Sea. The synthetic oligonucleotide hybridized to mRNA in *Limanda limanda*. The levels of mRNA in samples from site 3 were significantly higher than at sites 6 through 9 (Fig. 7) which is also indicative of chemical mediated induction of cytochrome P-450IA. The amount of total mRNA as measured using a poly T probe was identical in fish from all sample sites.

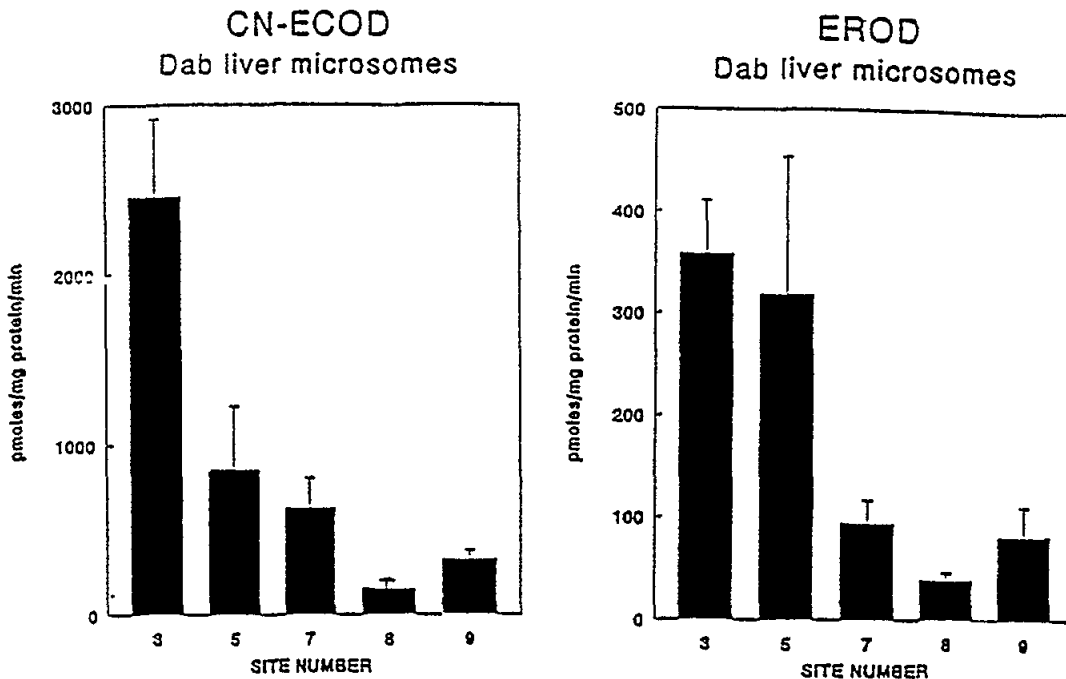


Fig. 6 The activities of EROD and CN-ECOD in hepatic microsomes obtained from *Limanda limanda* caught along a pollution gradient in the German Bight area of the North Sea

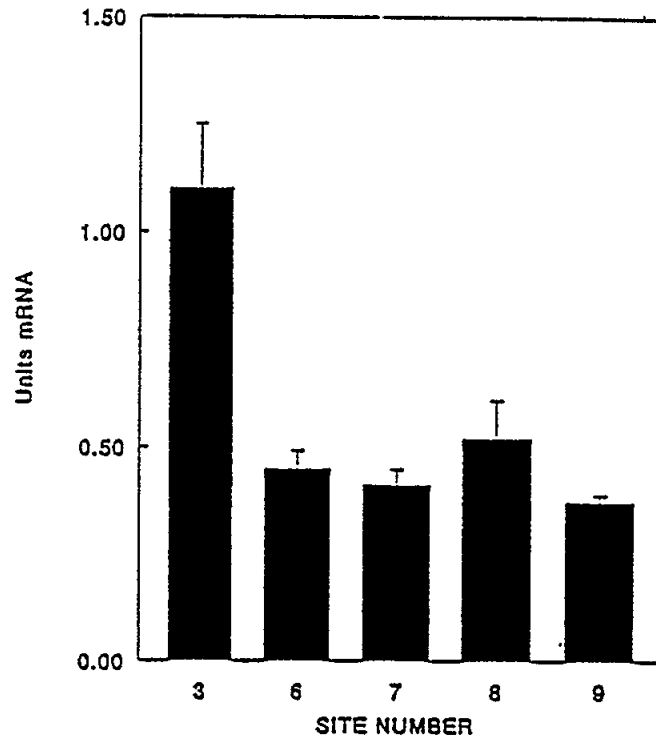


Fig. 7 The levels of cytochrome P450IA mRNA in the livers obtained from Limanda limanda caught along a pollution gradient in the German Bight area of the North Sea

The presence of measurable levels of mRNA in the samples taken from the less polluted sites might suggest that low levels of induction had taken place in fish obtained from these areas. In samples obtained from the area of an abandoned oil rig off the coast of the Netherlands, cytochrome P-450, CN-ECOD and cytochrome P-450IA mRNA values were identical at the site and at 5 and 10 km down stream. The values obtained were similar to those found at sites 8 and 9 of the transect samples from the German Bight indicating that a chemically mediated induction of cytochrome P-450 was absent.

These studies indicate that enzymatic biochemical markers for cytochrome P-450IA were able to identify a pollution gradient in the North Sea. Cytochrome P-450IA mRNA also correlated with the induction of substrate based indicators. The measurement of mRNA in marine species can therefore be used as a biochemical marker for the exposure of marine species to certain chemical pollutants.

5. CONCLUSIONS

The studies summarized here along with the large body of supporting literature indicate that cytochrome P-450 is an important indicator of chemical exposure in the marine environment. Induction of the cytochrome can be measured by a number of highly specific techniques which can process a

large volume of samples in a relatively short time period. Induction of the enzyme is a sensitive enough indicator to identify a biological effect resulting from exposure to small concentrations of chemical pollutants. A number of methods are now available which can be carried out in any endogenous species in a test area. Cytochrome P-450 therefore provides useful indication of biological damage at a stage prior to gross irreversible destruction and has become one of the most useful biochemical tools used for this purpose.

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ROLE OF PHYSIOLOGICAL ENERGETICS IN ECOTOXICOLOGY AND
ENVIRONMENTAL POLLUTION MONITORING

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A B S T R A C T

The role of physiological energetic measurements combined with chemical analyses of contaminants in body tissues of mussels in toxicological studies and pollution monitoring programs is outlined. Important features of this approach are reviewed, including bioaccumulation, sensitivity, quantitative concentration-response relationships, QSARs, mechanistic interpretation, integration, ecological relevance and its application in laboratory and field studies. Case studies are used to demonstrate, not only the quantification of pollution effects on growth of mussels, but also the relative importance of different toxicants in causing the reduction in growth.

1. INTRODUCTION

The primary objectives of Agencies concerned with aquatic environmental protection and pollution control are :-

- To ensure increasing quality of controlled waters (rivers, lakes, coastal waters and ground waters)
- To establish a full picture of any spatial and temporal changes in quality and the causes of such changes (stated objectives of the newly established U.K. National Rivers Authority [NRA], 1990).

To achieve these objectives it is essential that environmental managers and decision makers have the appropriate information on the quality of the aquatic environment. However, due its complexity there is no single/simple method of establishing environmental quality, it must ultimately be a combination of physical-chemical (cause) and biological (effect) measurements. Furthermore, these objectives cannot be met simply by selective chemical monitoring and community analysis (the two primary techniques currently deployed), for the following reasons :-

- All potentially toxic contaminants and their degradation products cannot be monitored solely by chemical measurements (due to the many thousands of compounds and their structural diversity), hence the need for an integrated biological measure of environmental quality.
- While the well-being of populations and communities are the ultimate concern, indices of community change are generally insensitive (i.e. reflecting lethal effects on certain species) with a slow response and slow recovery time, are largely

descriptive and retrospective (i.e. assessing damage following acute pollution incidents), and are labour intensive/time-consuming.

Consequently, chemical and community analyses need to be complemented by more sensitive, integrative and sublethal effects measurements that are both predictive (anticipatory) of likely population effects and allow toxicological interpretation of complex mixtures of contaminants and identification of causality through mechanistic understanding of readily established tissue concentration - response relationships and QSARs (Quantitative Structure- Activity Relationships).

Experience in marine/estuarine, and more recently in freshwater systems, has shown that physiological energetic measurement of "scope for growth", using indicator species such as mussels (*Mytilus edulis*), is able to provide a sensitive and graduated stress index from optimal to lethal conditions and is responsive to environmental levels of pollution (Widdows and Donkin, 1989; 1991). This paper reviews the attributes of physiological energetics and its role in ecotoxicology and pollution monitoring.

2. FEATURES OF COMBINED MEASUREMENT OF TISSUE RESIDUE CHEMISTRY AND PHYSIOLOGICAL ENERGETICS IN MUSSELS

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

- (i) establish relationships between the concentrations of chemical contaminants in the environment and in the tissues of biota (i.e. bioconcentration factors - BCF), and
- (ii) establish cause - effect relationships between [tissue] contaminant concentration and the resultant biological effects, based on an understanding of their mode of toxic action.

The combination of chemical analysis of contaminant levels in the body tissues of mussels and the measurement of biological effects in terms of physiological energetics is ideally suited to such a "cause - effect" framework (Fig. 1).

2.1 Physiological energetics (scope for growth)

Determination of the energy available for growth and reproduction, based on the physiological analysis of the energy budget rather than direct measurement of growth itself, has proved to be particularly useful in assessing the biological effects of pollution. It provides an instantaneous measurement of the energy status of the animal, as well as insight into the underlying components which effect changes in growth rate. Furthermore, there is good agreement between indirect estimates of growth (based on the energy budget) and direct measurement of tissue and shell growth (Riisgard and Randløv, 1981), and also determination of production based on detailed population size-class analysis (Gilfillan and Vandermeulen, 1978; Bayne and Worrall, 1980).

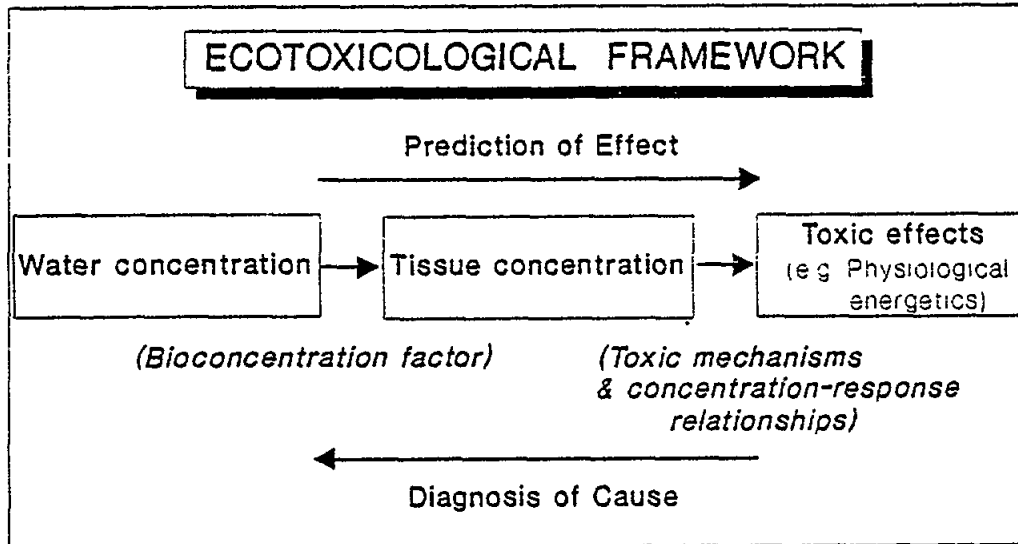


Fig. 1 Ecotoxicological framework enabling the prediction and diagnosis of toxic effects resulting from contaminant exposure

The energy budget of an animal represents an integration of the basic physiological responses such as feeding, food absorption, respiration, excretion and production. Each component is converted into energy equivalents ($J h^{-1}$) and alteration in the amount of energy incorporated into growth and production can be described by the balanced energy equation (Fig. 2).

When production is estimated from the difference between the energy gains (energy absorbed from the food) and the energy losses (energy expenditure via respiration and excretion), it is referred to as the "scope for growth" (Warren and Davis, 1967). Scope for growth (SFG) can range from maximum positive values under optimal conditions, but declining to negative values when the animal is severely stressed and utilizing its body reserves for maintenance. Details of the methods for measuring physiological energetic responses and SFG of bivalves are presented in Widdows (1985) and Widdows and Johnson (1988).

Physiological energetic measurements not only provide information on the key processes of energy acquisition, energy expenditure and thus energy available for growth and reproduction, but also reflect some of the major mechanisms of toxicity.

Important features of this toxicological approach involving the combined measurement of physiological energetics and chemical contaminants in mussels are:-

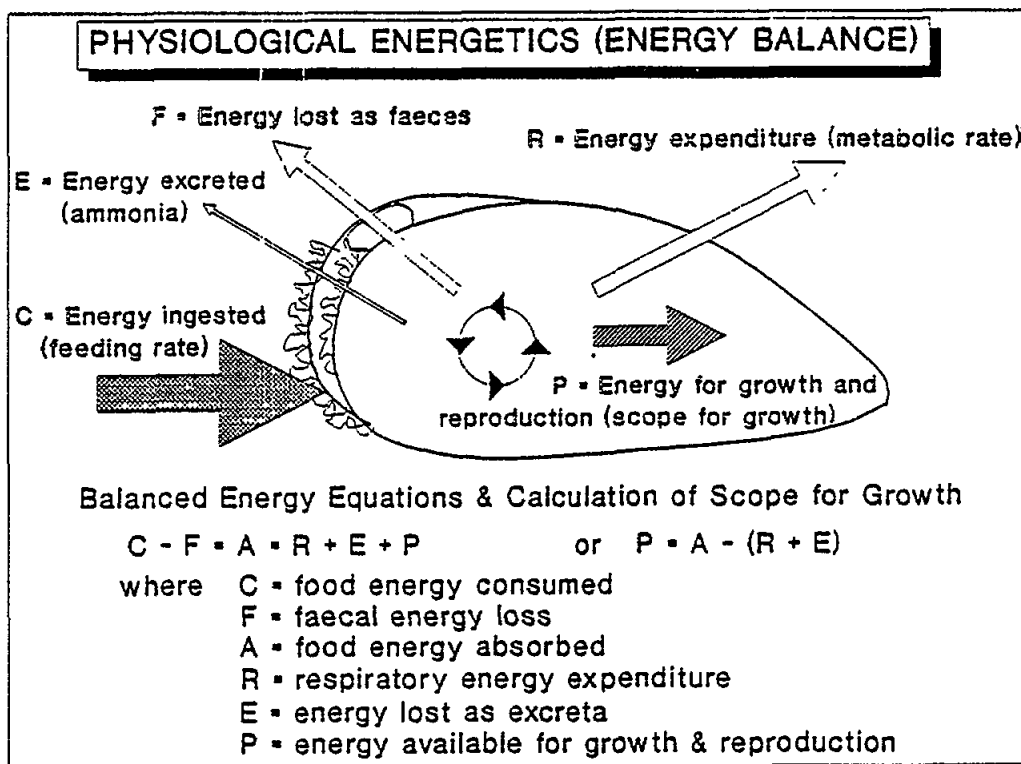


Fig. 2 Diagrammatic representation of the energy budget of a mussel

- bioaccumulation with minimal metabolic transformation of most organic contaminants;
- sensitivity to environmental levels of pollutants;
- mechanistic interpretation and ecological relevance;
- quantitative [tissue] concentration - response relationships;
- integration of effects of contaminant mixtures and multiple mechanisms of toxicity; and
- application to both laboratory and field studies.

2.2 Bioaccumulation

Mussels and other bivalves readily accumulate hydrophobic organic contaminants in their tissues with minimal metabolic transformation (Livingstone, 1991). Therefore, complex tissue residues largely reflect changes in the quality and quantity of ambient environmental levels of contaminants (Burns and Smith, 1981) and thus mussels are widely used in pollution monitoring programs (e.g. "Mussel Watch", Farrington *et al.*, 1983). This is in contrast to higher invertebrates and vertebrates which rapidly biotransform and excrete many xenobiotics, and under these circumstances body burdens do not simply reflect ambient levels of contaminants (Varanasi, 1989).

2.3 Sensitivity

Many biological responses are reported to be "sensitive" to toxicants without authors providing evidence in support of such statements. The relative sensitivities of SFG and other sublethal and lethal responses of mussels (Mytilus edulis), at different life stages, to environmentally important toxic contaminants such as Cu, TBT (tributyltin) and petroleum hydrocarbons are compared in Table 1. It shows that (a) the physiological components of the energy budget, which determine growth, are responsive to environmental levels of pollutants, (b) the physiological energetic and growth responses are considerably more sensitive than lethal responses, and (c) the larval stages are apparently less sensitive to some contaminants than adult mussels. In addition, mussels and their physiological responses appear to be of greater and equal sensitivity to environmental toxicants in comparison to other aquatic animals (hydrocarbons - Donkin et al., 1989; Cu - Davenport and Redpath, 1984; TBT - Widdows and Page, in press). One notable exception is the very sensitive and toxicant-specific "imposex" response of the gastropod, Nucella lapillus, to TBT. Imposex occurs at 0.005 µg TBT l⁻¹ or 0.5 µg TBT g⁻¹ d.w. (Gibbs et al., 1987) and is therefore an order of magnitude more sensitive to TBT than the growth response of mussels.

2.4 Mechanistic interpretation and ecological relevance

Physiological energetics and the disturbance of the mussel's energy balance can be interpreted both in terms of the underlying mechanisms of toxicity at the cellular level and in terms of the ecological consequences at the population level. Physiological energetics provides insight into, and integration of, some of the primary mechanisms of toxicity that are both biologically and environmentally important. Major mechanisms of toxicity reflected in physiological energetics include:-

- non-specific narcosis affecting the ciliary feeding activity of bivalves (e.g. hydrocarbons, Donkin et al., 1989);
- neurotoxic effects on the neural control of gill cilia (e.g. dinoflagellate toxins, Widdows et al., 1979; Cu, Howell et al., 1984; TBT, Snoeij et al., 1987);
- uncoupling of oxidative phosphorylation causing an increase in respiration rate (e.g. TBT, Snoeij et al., 1987; Phenols, Buikema et al., 1979);
- inhibition of oxidative metabolism thus reducing respiration rate (e.g. DBT (dibutyltin), Snoeij et al., 1987; Hypoxia, Widdows et al., 1989);
- toxic effects on membrane structure and function affecting processes of food digestion and absorption (e.g. hydrocarbons, Widdows et al., 1987a).

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

Responses measured at the organism level generally have the advantage of being readily interpreted as being either beneficial or deleterious. In addition, energetics offers a common currency [energy] enabling the consequences of primary toxic mechanisms at the cellular level to be translated into effects on growth, reproduction and survival at the individual

Table 1

Comparison of lethal and sublethal physiological responses of mussels (Mytilus edulis) to selected toxicants.

Toxicant	Biological response (50% of control)	Water ($\mu\text{g l}^{-1}$) concentration	Tissue ($\mu\text{g g dry wt}$) concentration	Life stage	Reference
Cu	Lethal (15d)	400 $\mu\text{g l}^{-1}$		Larvae	Beaumont <u>et al.</u> , 1987
Cu	Lethal (15d)	50 $\mu\text{g l}^{-1}$		Adults	Martin, 1979
Cu	Lethal (39d)	20 $\mu\text{g l}^{-1}$	59 $\mu\text{g g}^{-1}$	Adults	Martin, 1979
Cu	Shell growth	150 $\mu\text{g l}^{-1}$		Larvae	Beaumont <u>et al.</u> , 1987
Cu	Shell growth	5 $\mu\text{g l}^{-1}$		Adults	Redpath, 1985; Strømgren, 1986
Cu	Valve movement	10-20 $\mu\text{g l}^{-1}$		Adults	Davenport and Manley, 1978 Kramer <u>et al.</u> , 1989
Cu	Clearance rate, SFG	12 $\mu\text{g l}^{-1}$		Adults	Widdows (unpublished data)
TBT	Lethal and shell growth (15d)	0.4 $\mu\text{g l}^{-1}$		Larvae	Beaumont and Budd, 1984
TBT	Lethal (5d)		20 $\mu\text{g g}^{-1}$	Adults	Page and Widdows, 1991
TBT	Valve movement	10 $\mu\text{g l}^{-1}$		Adults	Kramer <u>et al.</u> , 1989
TBT	Shell growth	6 $\mu\text{g l}^{-1}$		Juveniles	Strømgren and Bongard, 1987
TBT	SFG	0.1 $\mu\text{g l}^{-1}$	4 $\mu\text{g g}^{-1}$	Adults	Page and Widdows, 1991

Table 1 (cont).

Toxicant	Biological response (50% of control)	Water ($\mu\text{g l}^{-1}$) concentration	Tissue ($\mu\text{g g dry wt}$) concentration	Life stage	Reference
Hydrocarbons (crude oil)	Lethal (6h)	$>10 \times 10^3 \mu\text{g l}^{-1}$		Larvae	Craddock, 1977
Hydrocarbons (crude oil)	Lethal (4d)	$1-10 \times 10^3 \mu\text{g l}^{-1}$		Adults	Craddock, 1977
Hydrocarbons (diesel oil)	Lethal (4m)	$125 \mu\text{g l}^{-1}$		Adults	Widdows <i>et al.</i> , 1987a
Hydrocarbons (crude oil)	Valve movement	$6 \times 10^3 \mu\text{g l}^{-1}$		Adults	Kramer <i>et al.</i> , 1989
Hydrocarbons (crude oil)	Shell growth	$1.5 \times 10^3 \mu\text{g l}^{-1*}$		Adults	Strømgren, 1986
Hydrocarbons (diesel oil)	Clearance rate, SFG	$30 \mu\text{g l}^{-1}$	$150 \mu\text{g g}^{-1}$	Adults	Widdows <i>et al.</i> , 1987a
Hydrocarbons (aliphatics & aromatics $\log K_{ow} < 5$)	Clearance rate		$95 \mu\text{g g}^{-1}$	Adults	Donkin <i>et al.</i> , 1989

* Total concentration in microcapsules and water.

Environmental ranges:

Cu:- In water: 1 to $30 \mu\text{g l}^{-1}$ (Davenport and Redpath, 1984)
 In tissue: 2.4 to $95 \mu\text{g g}^{-1}$ dw (Fowler and Oregioni, 1976)

TBT:- In water: 0.005 to $0.5 \mu\text{g l}^{-1}$ (Salazar and Salazar, in press)
 In tissue: 0.1 to $>10 \mu\text{g g}^{-1}$ dry wt (Page and Widdows, 1991)

Petroleum hydrocarbons:

In water: 1 to $74 \mu\text{g l}^{-1}$ (Law, 1981)
 19 to $560 \mu\text{g l}^{-1}$ (12-351d after oil spill; Blackman and Law, 1980)

In tissue: Aliphatic UCM 1 to $270 \mu\text{g g}^{-1}$ dw; Aromatic UCM <3 to $111 \mu\text{g g}^{-1}$ dw; Selected PAH <0.02 to $17.5 \mu\text{g g}^{-1}$ dw (Farrington *et al.*, 1982; Farrington *et al.*, 1983)

and population levels. The ultimate effects at the higher levels of biological organization are thus more readily interpreted and understood. Field and mesocosm studies have provided confirmation that the long-term consequences to growth and survival of individuals and the population can be predicted from measured effects on energy balance observed at the individual level (Gilfillan and Vandermeulen 1978; Widdows et al., 1987a).

2.5 Quantitative concentration - response relationships and QSARs

Tissue concentration - response relationships derived from controlled laboratory and mesocosm studies, not only facilitate the identification of chemical contaminants causing effects recorded in the environment, but also enable biological effects to be predicted from environmental levels of contaminants in water and body tissues. Such toxicological research therefore provides the information necessary to establish an appropriate database for the toxicological interpretation of tissue residue data derived from chemical monitoring programs (e.g. Mussel Watch).

While it may be feasible to determine tissue concentration - response relationships (Fig. 3) for individual contaminants with specific mechanisms of toxicity, for example Cu (Redpath, 1985), TBT (Widdows and Page, in press) and selected aromatic hydrocarbons (see Fig. 5; Widdows and Donkin, 1989), it is clearly unrealistic to examine the sublethal effects of the $>10^4$ individual organic contaminants which enter the environment. Consequently, the application of a Quantitative Structure-Activity Relationship (QSAR) approach (Hermens, 1986), which facilitates the prediction of toxicological properties of organic compounds from their chemical/structural properties, provides a means of overcoming this problem (Donkin et al., 1989; Donkin and Widdows, 1990). Furthermore, QSARs provide a unified approach to modelling and predicting the environmental behaviour, fate and effects of structurally diverse organic contaminants from their physicochemical properties.

A QSAR approach based on relationships between sensitive physiological responses and tissue toxicant concentrations is distinct from the approach usually adopted by aquatic toxicologists, based on relationships between LC_{50} or EC_{50} values and aqueous toxicant concentrations. Such laboratory derived relationships between sensitive responses and toxicant [tissue] concentrations are more relevant to environmental situations; where animals are typically exposed to sublethal and varying contaminant concentrations in the water, which are then accumulated and time-integrated by the body tissues, and which can subsequently induce the toxic effect.

A number of important features concerning QSARs and the sublethal toxic effects of a range of hydrophobic organic compounds are illustrated in Figure 4 (from Donkin et al., 1989):

- (a) There is an inverse linear relationship between the hydrophobicity of organic compounds (measured in terms of $\log K_{ow}$, the octanol-water partition coefficient) and the \log concentration in the water inducing a 50% reduction in the feeding (clearance) rate of Mytilus edulis.

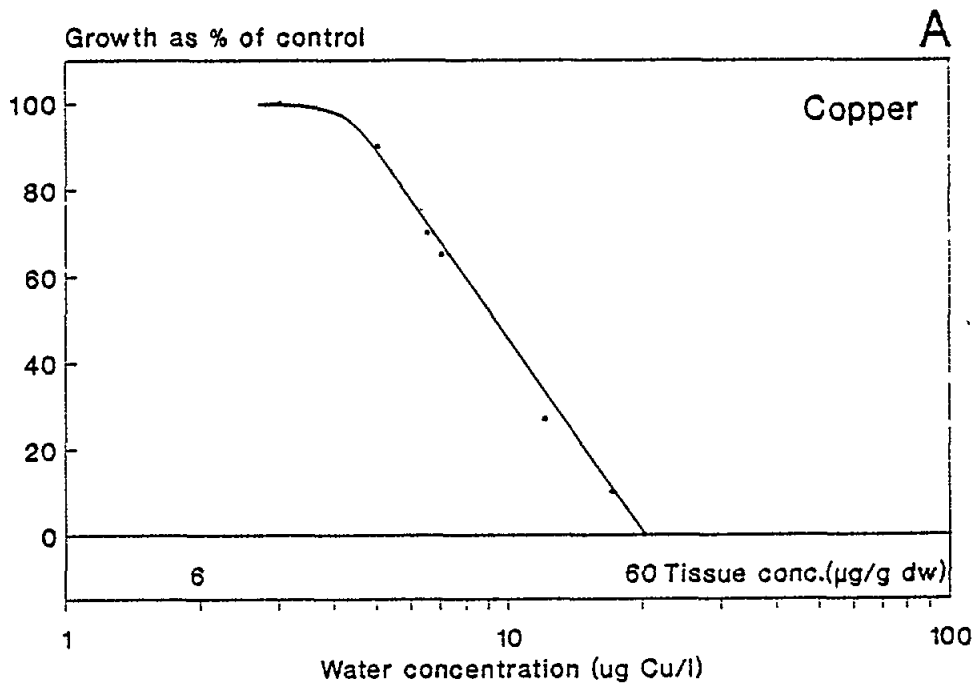


Fig. 3 (A). Effect of copper on growth of *Mytilus edulis* (from Redpath, 1985; Widdows and Johnson, 1988)

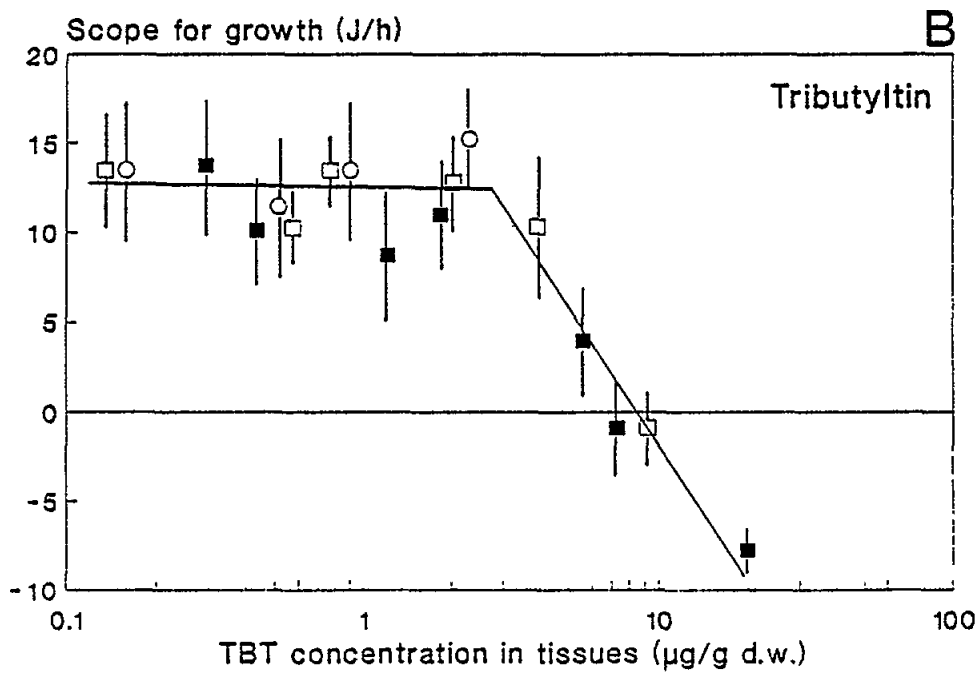


Fig.. 3 (B). Effect of tributyltin on growth of *Mytilus edulis*; mean + 95% CI (from Widdows and Page, in press)

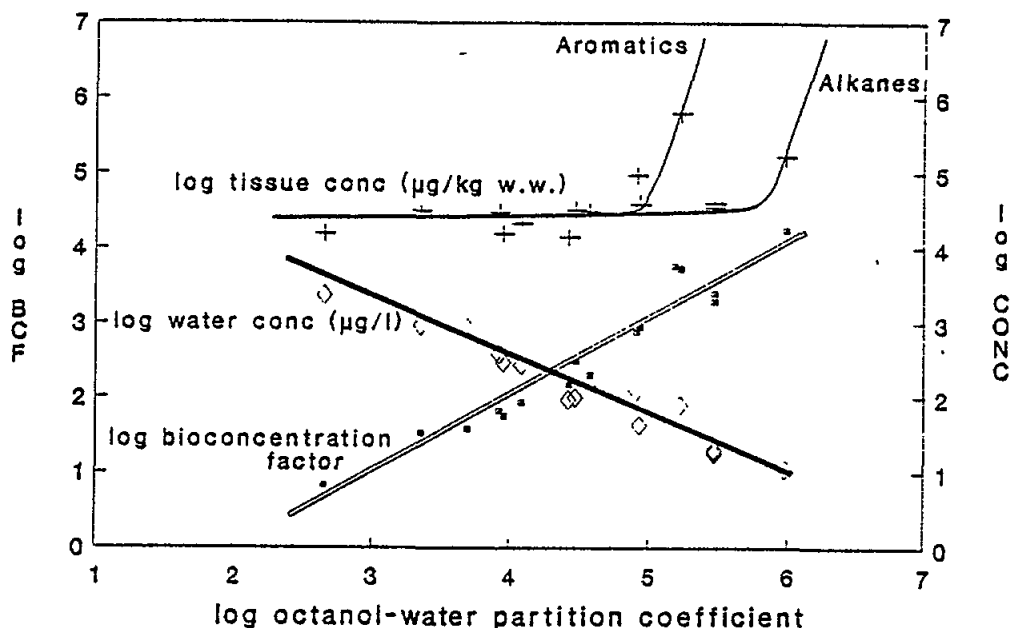


Fig. 4 Relationship between log Kow, BCF and toxicity of hydrocarbons to mussels. Toxicity in terms of log concentration reducing feeding rate by 50% (from Donkin *et al.*, 1989)

- (b) The log bioconcentration factor (BCF) increases linearly with log K_{ow} .
- (c) Changes in bioconcentration account for most of the differences in the water concentration based expression of toxicity. Consequently, hydrophobic organic compounds, such as aromatic and aliphatic hydrocarbons with log K_{ow} values <5 and 6 respectively, have equal toxicity when expressed on the basis of toxicant concentration in the tissues (i.e. horizontal line).
- (d) A single QSAR line implies a common mechanism of toxicity and a simple concentration additive effect when present as complex mixtures (Könemann, 1981; Widdows and Donkin, 1991). Conversely, QSARs can be used to identify outliers which then indicate different modes of toxic action or mechanisms of defence against xenobiotics.

- (e) There exists a molecular weight cut-off in the QSAR line occurring at a $\log K_{ow}$ of 5 for aromatic and 6 for aliphatic hydrocarbons; a well known phenomenon associated with compounds inducing narcosis. Compounds with a $\log K_{ow} > 5$ are accumulated in the tissues but induce little or no effect on feeding rate. This "cut-off" identifies the molecular/structural range over which compounds inhibit feeding rate and demonstrates that while many hydrocarbons may be detected in mussels sampled from contaminated environments, not all produce adverse effects on feeding rate.

Once established, a QSAR between a physicochemical descriptor (e.g. K_{ow}) and a biological response can then play an important role in: (a) predicting the toxicity of untested but structurally related compounds, (b) identifying potentially toxic environmental contaminants which need to be incorporated into chemical monitoring programs, (c) the systematic comparison of relative sensitivities of different organisms to classes of toxicants thus enabling extrapolation from a test organism (e.g. mussels) to other species, and (d) providing a sound basis for mathematical models of the fate and effects of organic chemicals in the aquatic environment. Current and future research is concerned with establishing QSARs for other major classes of organic compounds with different physico-chemical properties and for other biological responses reflecting different mechanisms of toxicity.

2.6 Integration of multiple mechanisms of toxicity and effects of contaminant mixtures

Many toxicants can induce effects via more than one mechanism of toxicity. For example, the biocides pentachlorophenol (PCP) and tributyltin (TBT) both act as uncouplers of oxidative phosphorylation, which results in enhanced oxygen consumption. In addition, PCP induces narcosis and TBT is neurotoxic at higher concentrations, thus reducing ciliary feeding activity. Physiological energetics can therefore provide an integration of these separate toxic effects as well as insight into the underlying mechanisms of toxicity (Widdows and Donkin, 1991; Widdows and Page, in press).

The toxic effects of structurally related organic compounds that form a single QSAR line, and therefore a common mechanism of toxicity, are predicted to be additive when present in a complex mixture (Könemann, 1981; Hermens, 1986). Oil is one of the best examples of a complex mixture of chemicals. QSAR studies have shown that aromatic hydrocarbons with $\log K_{ow}$ values < 5 and aliphatic hydrocarbons with $\log K_{ow} < 6$ have equal toxicity (inducing narcosis) and form a common QSAR line (Donkin *et al.*, 1989). Consequently, hydrocarbons above this "molecular weight cut-off" can be regarded as contaminants of significantly lower toxicity that are accumulated without exerting a direct toxic effect on the clearance rate (or metabolic rate) of mussels.

Recent experiments exposing mussels to mixtures of aromatic and aliphatic hydrocarbons have confirmed the additive nature of toxicity of related compounds forming a single QSAR line (Widdows and Donkin, 1991).

Comparison between results of laboratory based QSAR experiments and mesocosm/field studies involving complex mixtures have highlighted a discrepancy between the [tissue] hydrocarbon concentration required to reduce

clearance rate (ca. 23 μg hydrocarbons g^{-1} wet weight, Fig. 4) and the measured [tissue] concentration of "selected hydrocarbons" causing such an effect in the field (e.g. ca. 2 μg g^{-1} wet weight for 2 and 3 ring aromatic hydrocarbons - Fig. 5). It is apparent from the literature that the total petroleum derived body burden (hydrocarbons, substituted hydrocarbons and their polar oxygenated derivatives) is rarely measured in field studies and only a fraction of the "total toxic load" is routinely quantified in the chemical analysis of biota. Extraction and analytical procedures can lose a significant proportion of the more volatile toxic compounds (e.g. $\log K_{ow} < 3$; Farrington et al., 1988), and the unresolved complex mixture (UCM) component of chromatograms, which contains toxic compounds, is frequently not quantified. Furthermore, the precision and accuracy of some analytical procedures is inadequate for detailed toxicological comparisons (Farrington et al., 1988). Consequently it is often necessary to use selected groups of compounds (e.g. 2 and 3 ring aromatic hydrocarbons) which can be analyzed reliably, as "indicators" of the total petroleum derived toxic load.

3. PHYSIOLOGICAL ENERGETICS IN FIELD ASSESSMENT OF POLLUTION EFFECTS

The assessment of environmental pollution using physiological energetic measurements of bivalves in conjunction with analysis of contaminants in their tissues began with field studies by Gilfillan et al. (1977), Widdows et al. (1981) and Martin et al. (1984). These initial studies examined the biological effects of an oil spill in Maine, and pollution gradients in Narragansett Bay and San Francisco Bay in the USA. Since these early field studies, methodological development has continued and laboratory/mesocosm studies have enhanced toxicological understanding of [tissue] contaminant concentration - response relationships. Moreover, recent studies have shown that direct comparisons can be made between mussels from population/sites that are separated by considerable distances; by adopting standard conditions and procedures, using intertidal mussels and confining measurement to the summer period of growth. For instance, mussel transplantation experiments over >1000 km have demonstrated that physiological responses and growth rate reflect environmental rather than genetic differences (Kautsky et al., 1990; Widdows and Salkeld, unpublished data).

Recent applications of this approach and the environmental questions addressed are illustrated with reference to a field monitoring program in the vicinity of the North Sea oil terminal at Sullom Voe in the Shetlands (for details see Widdows et al. 1987b), and two practical Biological Effects Workshops which investigated pollution gradients in sub-tropical and northern temperate environments (Bermuda- Widdows et al., 1990; Oslo- Widdows and Johnson, 1988).

3.1 Effects of sullom voe oil terminal

Combined measurement of SFG and the concentration of two and three ring aromatic hydrocarbons (a major toxic component of oil) in the tissues of Mytilus edulis during the period from 1982 to 1989 has provided an assessment of spatial and temporal impact of oil pollution in the vicinity of the Sullom Voe oil terminal (1982-1985 reported in Widdows et al., 1987b). A unique feature of this case study has been the quantification of (1) environmental inputs of oil, (2) subsequent hydrocarbon accumulation in the mussel tissues, and (3) the resultant biological effects. Mussels living near the source of

oil inputs, the tanker loading areas, accumulate hydrocarbons to concentrations typically 10-fold higher than those at the "clean reference" site, and this results in a significant reduction in the SFG of mussels at these contaminated sites. The relationship between SFG and the log concentration of two and three ring aromatic hydrocarbons indicates that an order of magnitude increase in the tissue concentration can account for an approximately 50% reduction in the growth potential of *Mytilus edulis* (Fig. 5).

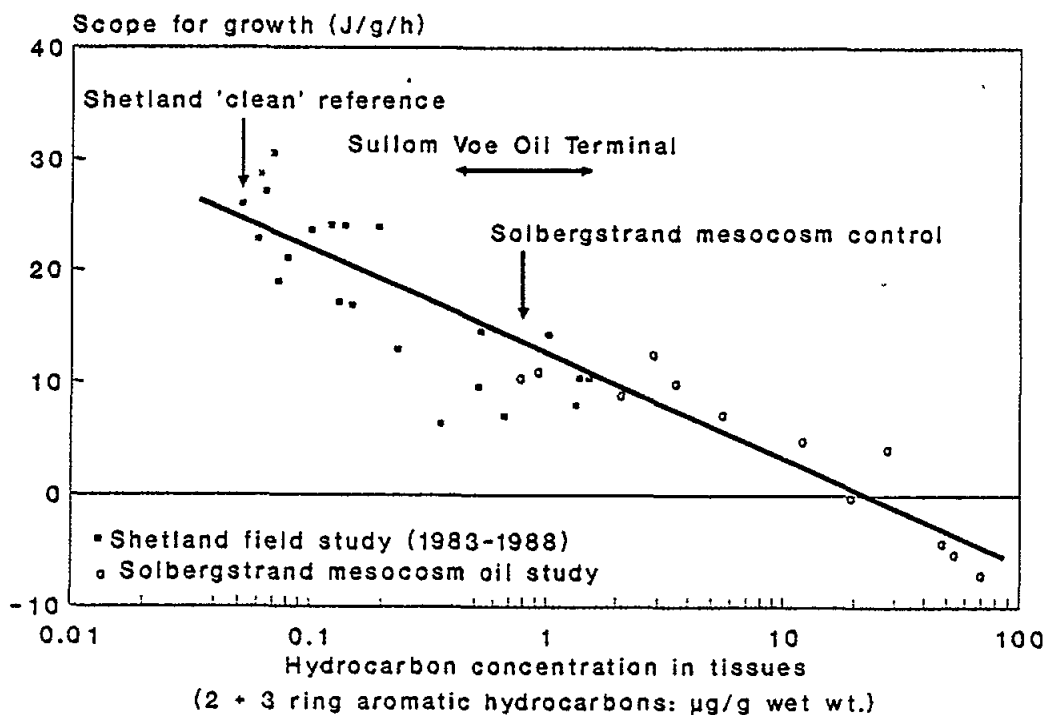


Fig. 5 Relationship between scope for growth and concentration of 2+3 ring aromatic hydrocarbons in mussels (Widdows *et al.*, 1987b)

Annual variations in SFG and hydrocarbon residues in the tissues of mussels sampled in the vicinity of the Sullom Voe oil terminal since it became operational, indicate that there has been neither a steady increase in the level of hydrocarbon contamination of the water column, nor a gradual deterioration in water quality (measured in terms of SFG). The year-to-year variation in hydrocarbon levels in the mussels is closely correlated ($r=0.89$; 1984-1989) with the total amount of oil spilt during the month preceding the annual field sampling (in July). These observations therefore suggest that the system can readily respond to and recover from any transient increases in oil inputs.

For purposes of environmental management, it is necessary not only to quantify the degree of contamination and the resultant deleterious biological effects, but also to address such questions as - "how bad or good are the conditions?" and "at what level of impact should managers start to be

concerned and take remedial action?". This requires the presentation and interpretation of data in a wider geographical and ecological context. Figure 5 shows a synthesis of data derived from the Sullom Voe study and from mesocosm oil experiments (Widdows *et al.*, 1987a). The semi-logarithmic relationship ($r=-0.87$) illustrates that:-

- (a) There is an inverse relationship between SFG and the log concentration of aromatic hydrocarbons in the tissues of Mytilus edulis over three orders of magnitude and without an apparent threshold effect; this suggests a simple mode of toxicity based on loading of body tissues and the absence of any significant physiological adaptation.
- (b) Mussels from the "reference" site near Sullom Voe have a very low level of hydrocarbon contamination compared to other sites studied (see below).
- (c) Mussels at the tanker loading jetties nearest the source of pollution are only moderately contaminated, but to no greater extent than many urban estuaries (e.g. River Tamar, S.W. England) and sites such as Solbergstrand on the Oslofjord (ca 35 km south of Oslo) in a region that is not in the vicinity of any point sources of industrial or urban pollution.
- (d) In comparison with other sites, mussels in Sullom Voe experience only moderate levels of pollution and appear to have sufficient capacity (i.e. SFG) to grow, reproduce and thus maintain a viable population.

3.2 Contaminant gradient in Bermuda

At the Bermuda Workshop, effects on the physiological energetics of transplanted mussels (Arca zebra) were quantified and related to contaminant gradients at a "dump site" and in Hamilton Harbour, a "non-industrialised" region receiving relatively low levels of contaminant inputs (Fig. 6; Widdows *et al.*, 1990). The results highlight several important features:-

- (a) In Hamilton Harbour, Arca accumulated petroleum hydrocarbons, their polar oxygenated derivatives, PCBs, TBT and Pb in their tissues and these were all significantly correlated with reductions in SFG. However, toxicological interpretation of the tissue residue data extended the analysis beyond statistical correlations and indicated that the lower molecular weight hydrocarbons [+ polar oxygenated derivatives] and TBT could account for the observed decline in SFG.
- (b) In addition, the overall reduction in SFG at the various sites could be "partitioned", such that at the most contaminated site TBT accounted for 21% and hydrocarbons for 79% of the observed effect.
- (c) The very conspicuous "dump site" caused only slight contamination of the marine environment and the recorded effects were not statistically significant.

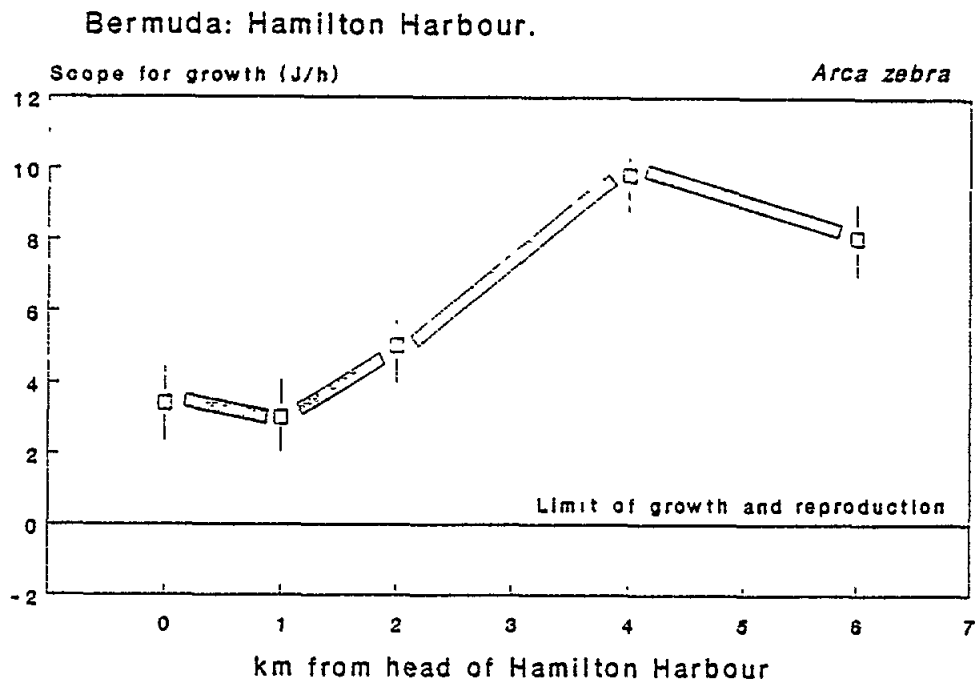


Fig. 6 Decline in scope for growth of mussels along a pollution gradient in Hamilton Harbour, Bermuda (from Widdows *et al.*, 1990)

- (d) Any reduction in SFG caused by relatively moderate levels of pollution is likely to shift the energy balance of a suspension feeding bivalve, living in a food limited subtropical environment, closer to the limit of growth and reproduction. Consequently, the effects of pollution are likely to be more marked in oligotrophic environments.

3.3 Contaminant gradient in Langesundfjord

In contrast, the Oslo Workshop examined a pollution gradient (in Langesundfjord) resulting from an "industrialised" environment (Widdows and Johnson, 1988; Widdows and Donkin, 1989). In this study the results (summarized in Figure 7) showed that:-

- (a) The "cleanest/outermost" site in Langesundfjord was significantly contaminated by hydrocarbons and TBT, and was therefore not an appropriate "reference" site.
- (b) Use of a well established "uncontaminated reference" site in the Shetlands enabled the contaminant levels and effects recorded at sites in Langesundfjord to be placed in a broader context.

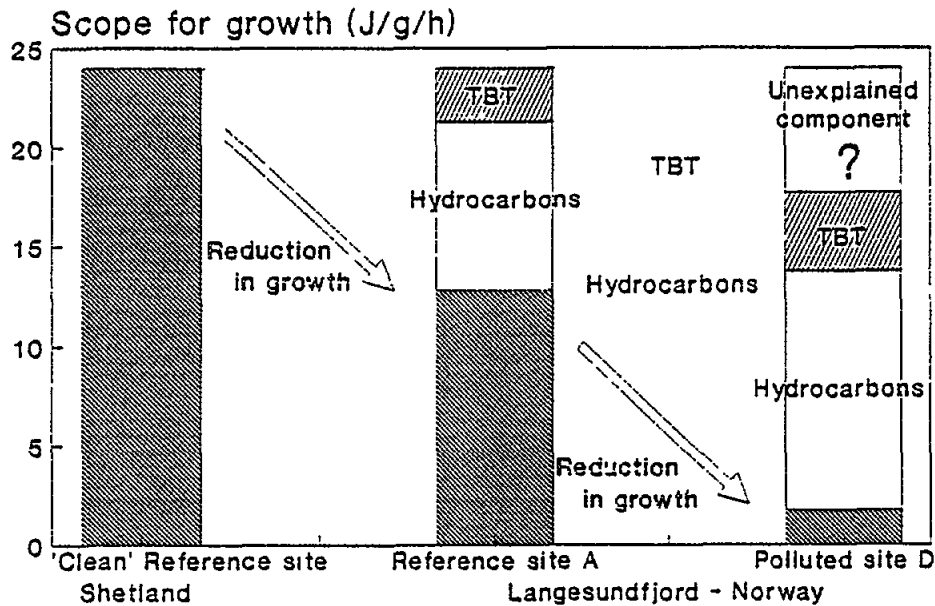


Fig. 7 Effect of pollution on scope for growth of *Mytilus edulis* from Langesundfjord (Norway) in comparison to a "clean reference site" in the Shetlands. Relative contribution of hydrocarbon and TBT towards observed decline in SFG (Widdows and Johnson, 1988)

- (c) There were marked reductions in SFG of mussels (*Mytilus edulis*) particularly at the inner northern end, nearest to the source of contaminants.
- (d) Observed effects at the most polluted site were far greater than could be explained by the tissue concentrations of contaminants analyzed (metals, hydrocarbons and TBT), thus indicating toxic effects resulting from both known (PCBs, dioxins and mercury) and as yet unidentified toxicants.

4. CONCLUSIONS

It is apparent that the degree and extent of pollution in the marine environment can only be assessed if combined chemical and biological effects monitoring programs are more widely adopted by Agencies concerned with environmental protection and management. While the toxicological approach outlined in this paper provides the possibility of extrapolation from laboratory to environmental conditions, with respect to bivalve sentinel organisms, knowledge at present does not allow extrapolation from the health of bivalves to the health of ecosystems. Further research is necessary to provide information on the relative sensitivities of different species which form components of the ecosystem, as well as the nature of the relationship

between responses of sentinel organisms and responses at the community/ecosystem level. Due to its widespread distribution and use as a sentinel organism, the growing toxicological database and the extent of understanding concerning its basic biology, the mussel provides an important reference species with which to compare not only different environments but also different species.

While significant progress has been made towards the long-term objective of "predicting effects and interpreting the causes of observed effects", further research is required to provide a more comprehensive toxicological interpretation of contaminant residues in body tissues. Future research within this toxicological framework needs to establish (a) QSARs for other important classes of contaminants, (b) relationships for other important biological responses (e.g. reproductive processes), (c) relationships enabling extrapolation to other key species, and (d) further understanding of the combined effects of complex contaminant mixtures.

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BIOCHEMICAL AND PHYSIOLOGICAL RESPONSES OF Mytilus edulis
TO Hg AND Pb IN THE COASTAL WATERS OF ALEXANDRIA REGION

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A B S T R A C T

Alexandria coastal waters are considered as one of the most polluted areas in the Mediterranean Sea as far as heavy metals are concerned. About 5 and 14 tons of Hg and Pb are discharged annually to the coastal waters. These metals are toxic causing several adverse effects on the mussel Mytilus edulis. There was a general tendency of decreasing dry body weight with increasing metal concentration. Mercury and lead were accumulated in the kidney, foot, muscles, hepatopancreas and gonads in regions affected by chlor-alkali, textile and dyes industries. Kidney showed the highest levels i.e. $11.31 \pm 8.5 \mu\text{g g}^{-1}$ Pb and $4.81 \pm 3.7 \mu\text{g g}^{-1}$ Hg. The natural biochemical response indicate a significant reduction in the soluble protein and carbohydrate contents near the effluent discharge points, especially opposite the chlor-alkali plant, relative to remote areas. Reduction in protein and carbohydrate contents with increasing Hg and Pb concentrations, reflected either a reduction in synthesis and/or an increase in their utilization under metal stressed conditions. The exposure of M. edulis to Hg and Pb (single or combined) drastically lowered the filtration rate to 48% for Pb, 75% for Hg and 85% for Hg+Pb maximum combination; thus clearly demonstrating additivity.

1. INTRODUCTION

The coastal waters of Alexandria, especially the semi-enclosed bays are subjected to the discharge of untreated urban and industrial wastes from major industrial centers as well as diffusive agricultural runoff. These types of discharges contained several trace metals which when introduced to the ecosystem are concentrated in marine organisms. Consequently, these organisms act as a store for these metals transferring them thereafter to human beings. In this case, economically important species could be health hazardous.

The edible filter feeding mussel Mytilus edulis is internationally used as a sentinel organism for heavy metal pollution monitoring (Goldberg et al., 1978). The appearance of high metal concentrations in healthy mussels render them unsuitable for human consumption. Due to the importance of the mussel as a food source, especially for inhabitants of coastal cities, studying the impact of industrial effluent on its nutritional value is of great importance.

The present work was undertaken to study the natural and experimental impact of industrial discharge (mainly Hg and Pb) on metal bioaccumulation, biochemical composition and physiological behaviour of M. edulis collected from the coastal waters of Alexandria. Examining the separate and interactive effects of both metals on the organisms were also objectives of this study.

2. MATERIAL AND METHODS

Specimens of Mytilus edulis were collected by hand (mean length 45-65 mm) from the intertidal zone of three different habitats covering the coastal waters of Alexandria (Fig. 1) varying in their extent of exposure to Hg and Pb. Regions 1 and 3 (Mex and Abu Qir bays) receive a mixture of agricultural and industrial discharge while Region 2 receives negligible amounts of sewage discharge. To Region 1, about 5 and 2 tons of Hg and Pb are discharged annually through a chlor-alkali plant and Alexandria Petroleum Company effluent. Region 3 receives about 12 tons of Pb annually, mainly through dyes, textile and weaving industries and an unspecified amount of Hg through agricultural runoff.

All specimens were scrubbed and allowed to purge gut contents for 24 hours in sea water (salinity 36). After depuration, weight and length measurements, specimens were opened and the soft part dissected to obtain gills, mantle/gonad, foot, adductor muscle, hepatopancreas, kidney and digestive gland. Samples were freeze dried after which Pb and Hg concentrations in different organs were determined using the 65% HNO₃ (Aristar) digestion, according to the method described in the FAO Fisheries Technical Paper No. 158 (Bernhard, 1976). Samples were placed in polyethylene prewashed screw top sample bottles and analyzed within 3 days using a Perkin Elmer 403 graphite furnace Atomic Absorption Spectrophotometer with deuterium background. For mercury determination, the cold vapour technique was used. Pb was analyzed in the presence of 0.5% (NH₄)₂HPO₄ as matrix modifier. The accuracy of the analysis was tested using the NBS (Oyster tissue) SRM- 1577. Deviation from certified values does not exceed 5%. Recoveries of Pb and Hg were examined using standard addition and were 100% and 98% with coefficients of variation 3.9% and 2.4%, respectively. Analyzing 10 replicates gave a coefficient of variation of 2.2% and 3.1% for Pb and Hg, respectively. Blanks were routinely run through the analysis to check for contamination.

Protein and carbohydrate contents were determined in samples collected from the previous mentioned locations. Soluble protein was determined according to Lowry et al. (1951) with albumin (BSA) standard. Carbohydrate was determined by the phenol-sulphuric acid method (Herbert et al., 1971) using glucose as standard reference. The method used comprises the determination of free as well as structurally bound sugars.

For the determination of single and joint effects of Hg and Pb on the protein and carbohydrate concentrations of M. edulis, live mussels were placed in 40 X 20 X 20 cm glass aquaria. Test dosing solutions used were analytical grade mercuric chloride (HgCl₂) and lead nitrate Pb(NO₃)₂. Animals were sacrificed after 5 days. Mean concentrations and standard deviations were calculated whilst paired sample t-test was used to determine significant differences between means. In each experiment, a control was run under similar conditions, except that metals were not added. Replication of experiments were tested for carbohydrates and soluble protein using high and low levels of Hg, Pb and combination effects on the adductor muscle. Results were remarkably consistent showing deviations from the mean of 3%, 2% and 6% for low concentrations and 8%, 3% and 12% for high concentrations of carbohydrates. Deviation from the mean for soluble protein were 5%, 5% and 8% for the lower concentration while 11%, 6% and 10% for the highest.

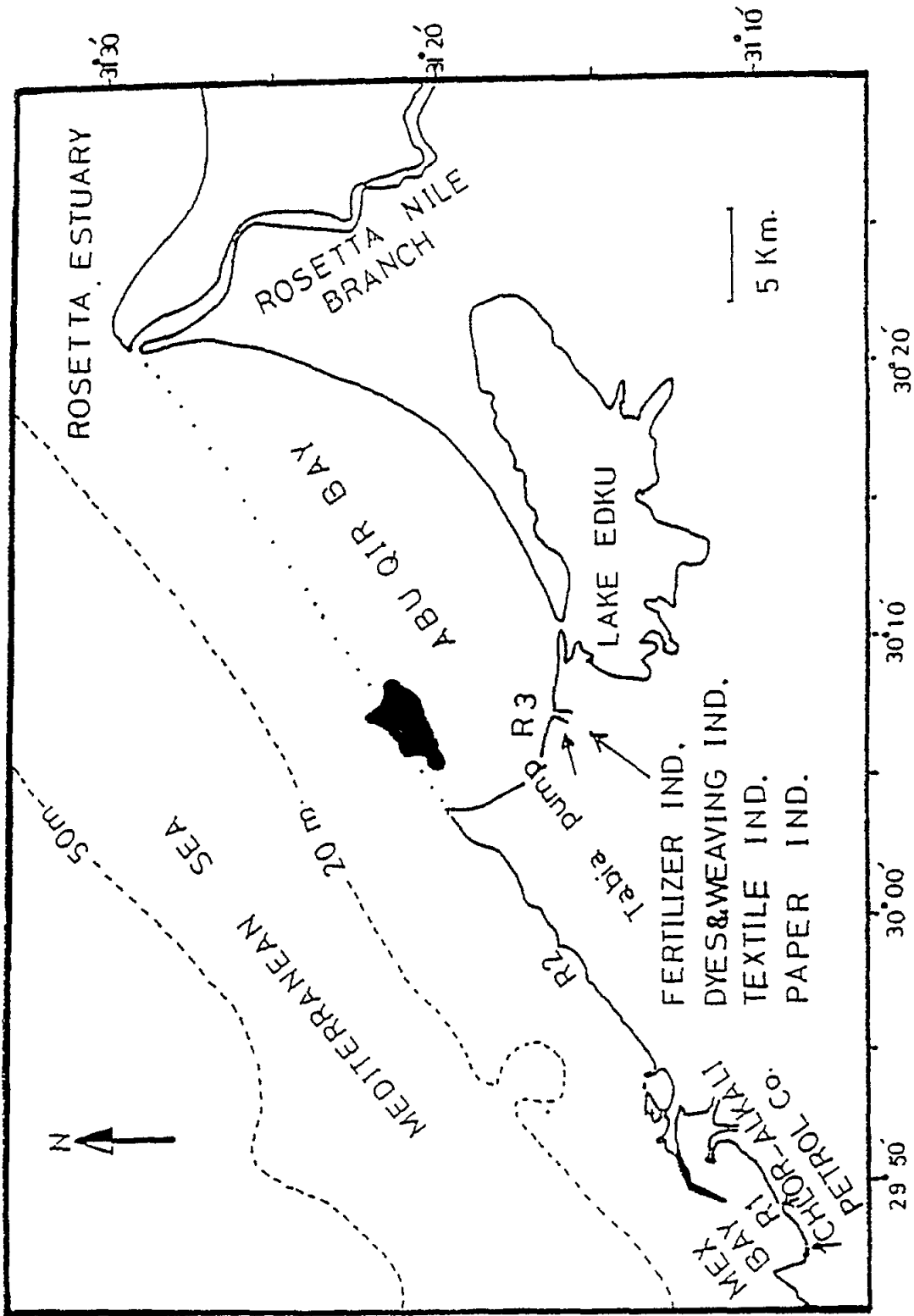


Fig. 1 Alexandria coastal area with sampling locations

For the filtration rate experiment, live specimens were collected and acclimatized for 1 week in aerated synthetic sea water media. Experiments were conducted in a constant temperature room at $18 \pm 2^\circ\text{C}$. The technique used was that described by Welsh et al. (1968) using glass experimental tanks with a ratio of 3 litres of water to 1 gram total wet weight tissue. A 4% colloidal graphite solution was used (Aquadag, Acheson Colloidal Corporation). Particles' removal was determined spectrophotometrically after 90 min. (Grace and Gainey, 1987). Filtration rates were determined from the equation:

$$FR = \frac{M \text{ Ln } (C_0/C_t)}{t}$$

where,

FR = filtration rate (ml min^{-1})
Co = initial concentration of particles
Ct = particle concentration at time t
M = solution volume (ml)
t = time in minutes

Changes in filtration rates for each metal concentration, as well as combined metal concentrations, were calculated using the mean control rate as 100%.

3. RESULTS AND DISCUSSION

3.1 Bioaccumulation of Hg and Pb

The growth parameters of M. edulis (length and dry weight) collected from Alexandria coastal waters showed maximum growth (ie. size) away from industrial outfalls coinciding with the lowest concentrations of Hg and Pb. The smaller sizes of mussels were collected from Region 1 followed by regions 3 and 2. The mean lengths of mussels at the three regions are 48 ± 9 mm, 55 ± 12 mm and 62 ± 4 mm for regions 1, 3 and 2 respectively. Higher concentrations of both metals observed near discharge points are inhibitory for growth parameters. There was a general tendency for metal concentrations to increase with the decrease in dry weight. For Hg the relation is: $\text{Log } C = -0.8473 \text{ Log } d \text{ wt} - 1.8415$ ($r=0.709$, $p < 0.001$) while for Pb: $\text{Log } C = -0.4301 \text{ Log } d \text{ wt} - 0.0552$ ($r=0.487$, $p > 0.1$). Both equations showed that metal concentrations are too high to be explained simply by changes in dry weight. The continuous variation in sea water concentration, spawning season as well as metal uptake rate and availability are important factors controlling the metal concentrations in the mussel. The increase in concentration with decreasing animal weight was suggested by Roméo and Gnassia-Barelli (1988) to be partially due to higher metabolic rate of small organisms.

Maximum Hg concentrations in different organs were observed for Region 1 near the chlor-alkali outfall (average Hg in kidney was $4.81 \pm 3.7 \mu\text{g g}^{-1}$ d wt). The concentration of Hg in water (average $1.16 \mu\text{g l}^{-1}$) and sediments ($38 \pm 12 \mu\text{g g}^{-1}$) at this location were also high (Table 1). A significant variation appeared between the concentration of Hg in mussels affected by the

Table 1

Concentrations of Hg and Pb ($\mu\text{g g}^{-1}$ d wt) for different body organs of M. edulis.

Metal	LOCATION ORGAN	Region 1		Region 2	Region 3	
		A	B	A	A	B
Hg	FOOT	3.15±2.97	1.68±1.16	0.59±0.33	1.01±0.06	0.85±0.63
	ADD. MUSCLE	1.87±1.27	1.12±1.42	0.29±0.24	0.63±0.47	0.44±0.39
	GONAD	2.86±2.67	1.65±0.93	0.17±0.05	0.59±0.38	0.38±0.31
	GILLS	1.98±1.23	0.74±0.55	0.36±0.14	0.55±0.33	0.22±0.10
	KIDNEY	4.81±3.70	2.16±1.16	0.76±0.32	1.95±0.89	1.22±0.96
	MANTLE EDGE	0.53±0.50	0.22±0.18	0.05±0.03	0.21±0.08	0.06±0.02
	H. PANCREAS	1.63±0.96	0.93±0.62	0.28±0.13	0.49±0.19	0.35±0.18
	DIG. GLAND	0.63±0.51	0.45±0.19	0.04±0.01	0.18±0.10	0.09±0.06
	S.W. ng kg ⁻¹	1160±694	587±210	143±75	215±88	91±18
	Sed. µg g ⁻¹	38±12	41±3	1.8±0.2	8±2	3±0.6
Pb	FOOT	2.92±1.34	1.82±0.49	0.95±0.21	5.42±1.00	2.31±1.54
	ADD. MUSCLE	2.11±1.92	0.93±0.66	0.52±0.09	4.31±2.60	1.96±1.12
	GONAD	1.71±0.95	1.01±0.30	0.46±0.11	6.29±4.15	2.85±1.03
	GILLS	1.06±0.83	0.63±0.24	0.39±0.08	3.95±2.40	1.20±1.16
	KIDNEY	3.63±2.17	1.90±1.82	0.48±0.11	11.31±8.50	4.65±2.86
	MANTLE EDGE	0.75±0.62	0.53±0.35	0.18±0.03	2.26±0.80	0.45±0.38
	H. PANCREAS	1.56±0.98	1.10±0.78	0.42±0.26	4.34±3.16	1.09±0.82
	DIG. GLAND	1.66±1.21	0.93±0.59	0.29±0.14	2.93±1.00	1.38±1.08
	S.W. ng kg ⁻¹	973±187	241±97	203±38	1785±493	618±180
	Sed. µg g ⁻¹	21±7	11±4	6±2	64±37	19±6

A = near outfall B = remote from outfall

chlor-alkali outfall and those collected from the control area, Region 2 ($F=11.13$, $p<0.004$) and Region 3 ($F=6.73$, $p=0.03$). In a similar pattern, an increase of Pb content in the mussel organs was observed at Region 3, in the vicinity of the discharge outfalls of 36 factories, the major ones being textile, fertilizer, dyes, weaving, pulp and paper industries. The average Pb concentrations near the outfall were $1.79 \pm 0.49 \mu\text{g l}^{-1}$ for water and $64 \pm 37 \mu\text{g g}^{-1}$ for sediments. The concentration of lead in mussels reached $11.31 \pm 8.5 \mu\text{g g}^{-1}$ and $6.29 \pm 4.15 \mu\text{g g}^{-1}$ in kidney and gonad, respectively. On the other hand, the effluents of an ethyl lead unit of the Alexandria Petroleum Company, located onshore in this area as well as the car traffic in the area, contribute to the elevated Pb concentration in Region 1.

A one way ANOVA test indicates significant differences in the metal levels of the different tissues analyzed. The order of Hg accumulation in the different organs of *M. edulis* in Region 1 was kidney > foot > gonad > adductor muscle = gills = hepatopancreas > digestive gland = mantle edge. In Region 3, Pb accumulates in kidney > gonad = foot > adductor muscle = hepatopancreas = gills > digestive gland = mantle edge. According to Eganhouse and Young (1976) the increase of Hg in gills, foot and adductor muscle indicate the extent of biological incorporation, while viscera and total flesh content exhibit information on relative environmental levels of the metal. As the organisms were purged of gut contents before analysis, Pb and Hg concentrations measured for the digestive gland were minimum.

Results from this work, combined with other observations, indicate that metal concentrations in mussels depend on multifactors related to environmental conditions, industrial wastes and organism physiological behaviour.

3.2 Effect of Hg and Pb on the biochemical composition of *M. edulis*

3.2.1 Natural effect

The average percentage composition of soluble protein and carbohydrate in *M. edulis* relative to its dry weight are presented in Table 2. The lowest protein averages were observed opposite the chlor-alkali plant. The most affected organs were adductor muscle (average $10.2 \pm 8.6\%$) and kidney (average $11.9 \pm 7.1\%$). Variations in the protein content of the different organs at the area near the outfall of Region 3 were not significant ($F=3.11$, $p<0.1$). However, maximum concentrations were observed for Region 2 located away from industrial outfalls. The same trend was observed for the carbohydrate content of different organs except that no significant differences ($F=1.01$, $p<0.5$) were obtained between the concentrations of adductor muscle, mantle, kidney and digestive gland in Region 1 ($F=1.63$, $p<0.5$). The reduction in the carbohydrate content in Region 3 was of lesser magnitude. The lowest average was recorded for the hepatopancreas (average $4.6 \pm 1.1\%$). In the control Region 2, the carbohydrate content of the same organ was $12.4 \pm 4.8\%$.

These data indicate that there is a reduction in synthesis and/or an enhanced utilization of protein and carbohydrate in *M. edulis* near the effluent discharge points.

Table 2
 Percentage composition of protein and carbohydrate in M. edulis in relation
 to its dry weight.

Metal	LOCATION ORGAN	Region 1		Region 2		Region 3	
		A	B	A	B	A	B
PROTEIN	FOOT	16.6±5.5	22.6±8.3	26.5±14.9	19.9±7.2	23.2±9.8	
	ADD. MUSCLE	10.2±8.6	25.8±7.9	28.6±12.3	16.5±9.5	24.4±20.6	
	GONAD	18.0±8.2	23.5±11.4	26.7±6.9	20.3±8.6	23.2±12.3	
	GILLS	18.3±4.8	23.9±6.6	28.2±11.1	21.7±7.1	25.2±10.6	
	KIDNEY	11.9±7.1	20.3±8.2	22.9±18.2	16.7±4.2	20.8±9.7	
	MANTLE EDGE	13.4±6.9	21.6±5.1	26.2±8.5	17.4±5.4	24.8±11.6	
	H. PANCREAS	12.8±10.2	19.3±2.5	23.8±7.9	16.8±9.3	21.4±5.3	
	DIG. GLAND	16.4±7.8	21.3±6.9	25.3±6.4	19.8±11.1	22.6±12.8	
CARBO- HYDRATE	FOOT	6.4±1.8	9.1±1.3	10.5±2.7	7.3±2.6	9.8±5.2	
	ADD. MUSCLE	1.8±2.0	8.0±2.3	9.4±3.6	5.9±1.3	6.5±3.9	
	GONAD	5.3±2.9	8.9±1.4	10.1±2.4	7.7±3.4	9.2±2.1	
	GILLS	5.6±3.8	7.3±2.6	8.5±1.9	6.2±1.9	8.1±3.7	
	KIDNEY	4.8±2.2	7.9±1.7	9.9±2.8	5.8±3.7	7.5±2.2	
	MANTLE EDGE	4.5±1.4	9.5±3.3	11.3±3.6	5.9±1.8	10.2±1.9	
	H. PANCREAS	3.2±1.6	10.4±4.2	12.4±4.8	4.6±1.1	10.9±3.6	
	DIG. GLAND	4.9±1.8	8.7±3.1	10.5±2.9	6.6±8.5	9.2±2.5	

A = near outfall

B = away from outfall

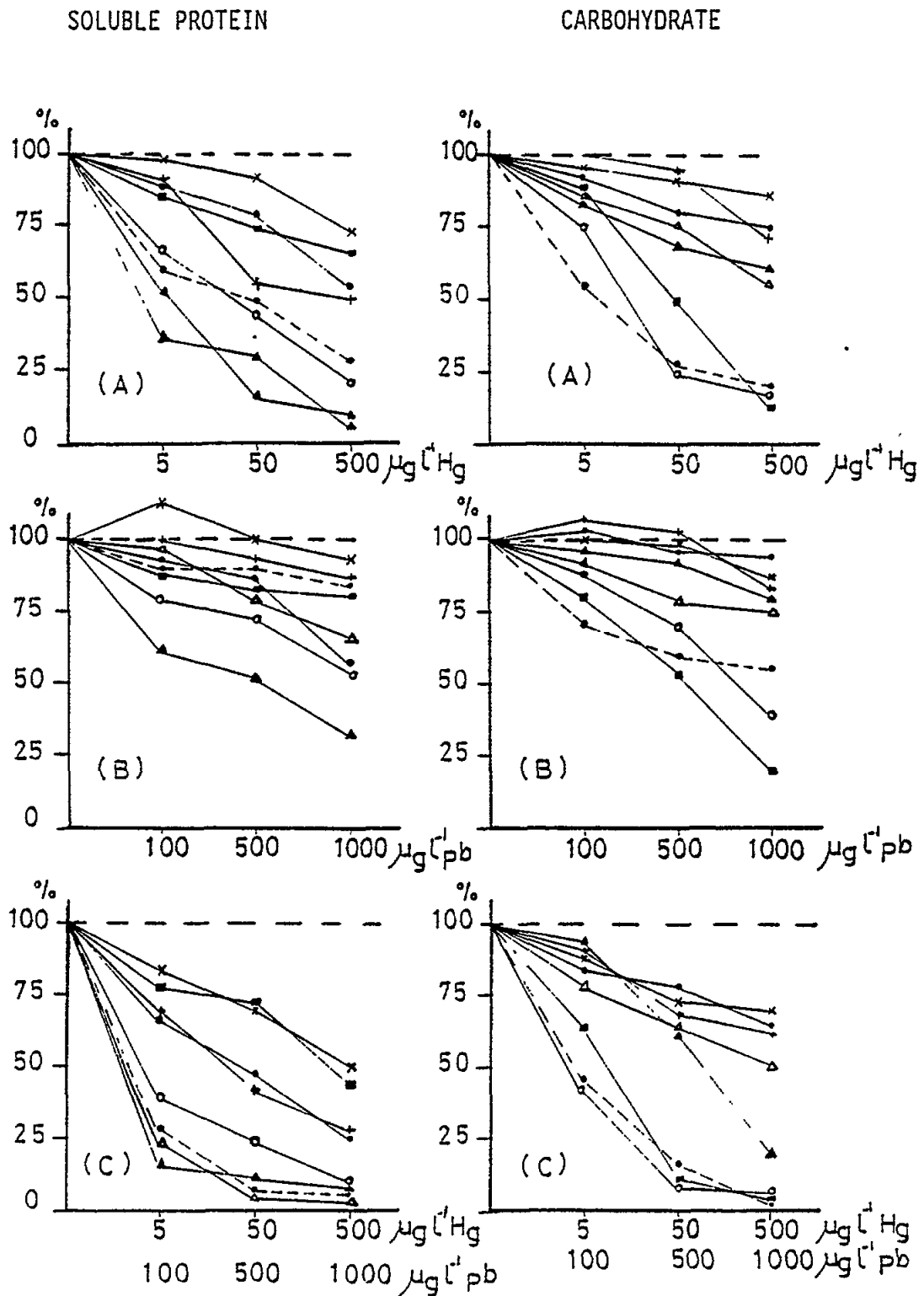


Fig. 2 Changes in protein and carbohydrate content of different organs of *M. edulis* (as percent of control) in relation to the different concentrations of (a) Hg, (b) Pb and (c) Hg + Pb. Adductor muscle Δ , Kidney \blacktriangle , Gills ---, Hepatopancreas -.- Foot x, Mantle +, Gonad ·-·-·, Digestive gland \blacksquare

3.2.2 Experimental effect

Figure 2 presents the experimental changes of soluble protein and carbohydrate contents of different organs of *M. edulis* (as percent of control values) in response to individual and combined effects of different Hg and Pb concentrations. Each metal seemed to affect the same organ differently; Hg appeared to be more effective than lead in reducing the protein as well as the carbohydrate contents of the mussel. Significant variations appeared between the effect of Hg and Pb especially on reducing muscle protein ($F=9.37$, $p=0.005$) and the carbohydrate content of hepatopancreas ($F=8.607$, $p=0.0036$). Lead was characteristically effective in reducing the protein content of the kidney and the carbohydrate contents of the digestive gland, gonads and hepatopancreas. About 35% reduction in the protein content of the kidney was exerted by the lowest Pb concentration. However, mercury especially in high concentrations i.e. 50 and 500 $\mu\text{g l}^{-1}$, reduced the protein of the adductor muscle (about 50% at the lowest concentration) and kidney (about 65% at the lowest concentration), and the carbohydrate contents of the hepatopancreas, gills and digestive gland (about 80% at the highest concentration). The interaction of Hg and Pb when combined is significantly more effective in reducing the protein and carbohydrate contents of different organs compared to single metal treatments (Fig. 2c). More than 95% reduction in the total protein content of the adductor muscle and gonads was exerted by the joint effect of the middle (50 $\mu\text{g l}^{-1}$ Hg/ 500 $\mu\text{g l}^{-1}$ Pb) and higher (500 $\mu\text{g l}^{-1}$ Hg/ 1000 $\mu\text{g l}^{-1}$ Pb) concentration mixtures. On the other hand, the reduction of the carbohydrate content of the digestive gland, hepatopancreas and gonads was much pronounced at the same concentration levels.

The effect of metal pollutant on protein is due to its affinity to functional groups including enzymes which are blocked by the metals, resulting in the disruption of fundamental physiological and biochemical mechanisms in the organism. Moreover, Sigel (1974) added that the formation of protein complex with Hg may change the conformation and solubility of protein.

It can be concluded, from the results presented here, that combinations of toxic concentrations of the heavy metal pollutants Hg and Pb promote the reduction in the soluble protein content of *M. edulis* by enhancing protein utilization and/or impairing protein synthesis (Jana and Choudhuri, 1984; De et al., 1985). The effect was highest for adductor muscle, gonad and kidney and lowest for digestive gland and foot. Comparing these results with the effects of individual heavy metal pollutants, it appears that the degree of protein reduction by combinations of toxic concentrations of heavy metal pollutants is much higher due to interactive additive effects rather than that by individual metal pollution.

3.3 Effect of Hg and Pb on filtration rate of *Mytilus edulis*

The average filtration rate of unexposed *M. edulis* controls was $8 \pm 1.6 \text{ ml min}^{-1} \text{ animal}^{-1}$. Other reported values were 5-10 $\text{ml min}^{-1} \text{ animal}^{-1}$ (Abel, 1976) and 6 $\text{ml min}^{-1} \text{ animal}^{-1}$ (Howell et al., 1984).

Figure 3 shows a significant difference between the filtration rates of control and treated animals, even those exposed to low concentrations of Pb ($F=6.255$, $p < 0.1$) and Hg ($F=9.384$, $p < 0.001$). The lowest concentrations of Pb and Hg lowered the filtration rate of *M. edulis* by 25 and 46% respectively,

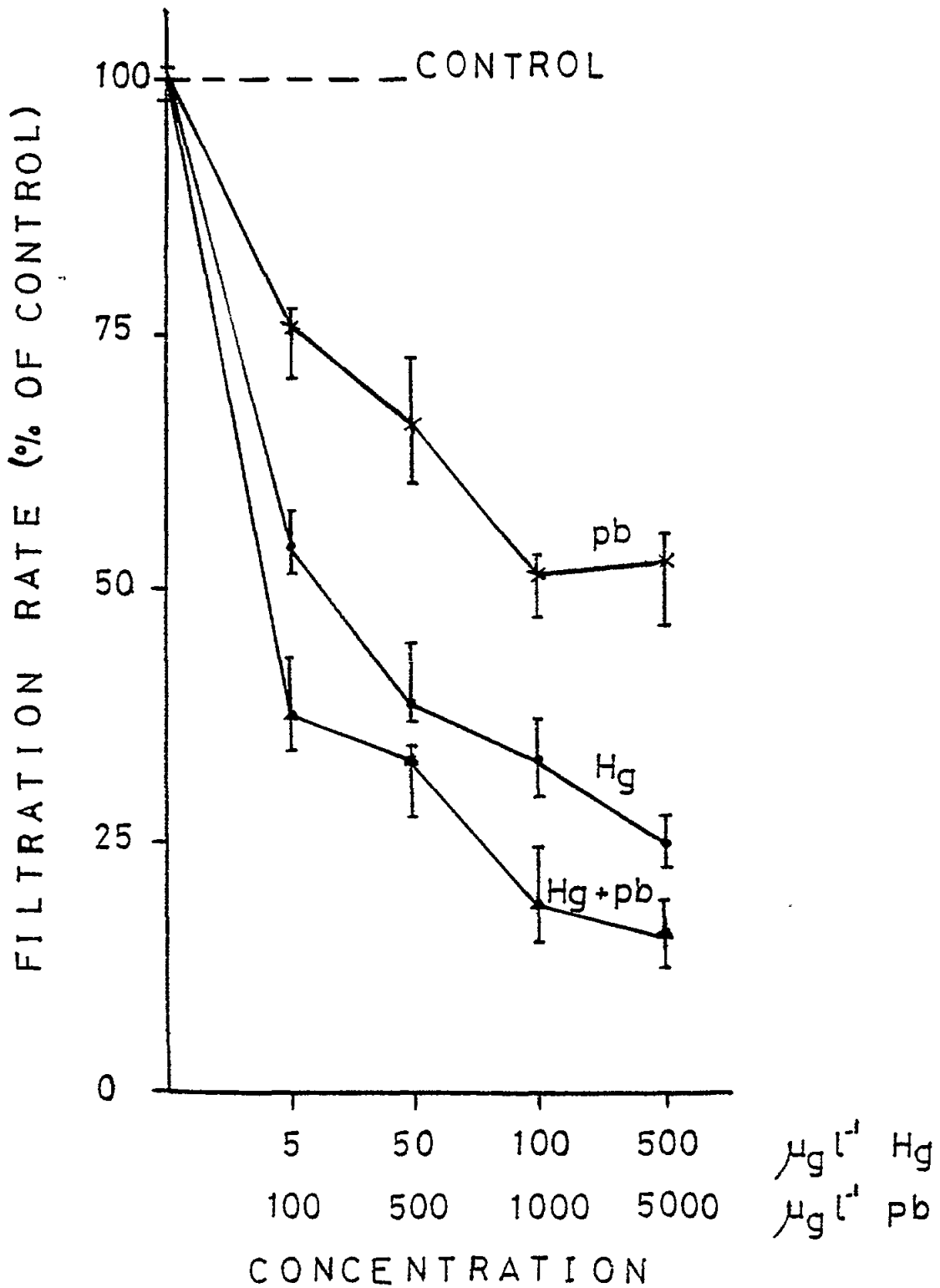


Fig. 3 Effect of Hg and Pb (single and combined) concentrations (as percentage of control) on the filtration rate of *Mytilus edulis*

while their lowest combined concentration lowered it by 63%. The maximum percent inhibition of mussels' filtration rate was 48% for Pb, 75% for Hg and 85% for Pb+Hg. Such results indicate that Hg, compared to Pb is significantly more effective in lowering the filtration rate of Mytilus ($F=11.203$, $p < 0.0007$). Grace and Gainey (1987) observed an inhibition in the filtration rate of 74% and 40% for M. edulis exposed to 0.15 ppm and 0.0002 ppm Cu, respectively. Experiments on bivalves showed similar decrease in the filtration rates when applying individual metals (Grace and Gainey, 1987; Papathanassiou, 1990). The joint effect of Hg and Pb indicate that their interaction on the filtration rate is additive.

The mechanism of filtration rate reduction was explained by Grace and Gainey (1987) to be a result of a combination of depressed lateral ciliary activity, caused by neural inhibition and separation of gill filaments.

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INDUCTION OF LIVER 7-ETHOXYRESORUFIN O-DEETHYLASE IN
GILTHEAD SEABREAM BY BENZO(A)PYRENE AND ITS POTENTIAL USE
IN BIOCHEMICAL MONITORING OF ENVIRONMENTAL POLLUTANTS

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A B S T R A C T

Polycyclic hydrocarbons found in waste materials of industry, in oil spills of ships, in natural seapages from under-water oil deposits are known to be toxic to the living organisms. These compounds are usually transformed by the liver mixed-function oxidase (MFO) system of fish. In this study, MFO system of gilthead seabream was characterized and the effects of benzo(a) pyrene [B(a)P] on fish liver microsomal MFO enzymes were investigated. B(a)P was administered, 25 mg per kg body weight per day for five consecutive days, i.p. in corn oil. The fish was killed 18 hours after the last treatment. Liver microsomes were prepared by differential centrifugation. While the rates of aniline 4-hydroxylase and cytochrome P450 reductase activities of liver microsomes were found to be unaltered by B(a)P treatments, that of 7-ethoxyresorufin O-deethylase activity increased approximately three folds.

The results of this study suggest, among other things, that the degree of elevated levels of liver microsomal 7-ethoxyresorufin deethylase activity of gilthead seabream can be used as an indicator of the presence of polycyclic hydrocarbon pollution.

1. INTRODUCTION

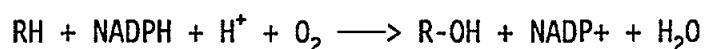
The sea is a repository for a number of foreign compounds (xenobiotics) which occur as environmental pollutants. Among these pollutants, polycyclic hydrocarbons hold an important place because of the significant health risks. Polycyclic hydrocarbons found in waste materials of industry, in oil spills of ships, and in natural seapages from under-water oil deposits are known to be carcinogenic to animals and man. The ability of fish and other marine species to accumulate, biotransform, and excrete these xenobiotics as well as the effect of these pollutants on the marine species are of considerable interest. These xenobiotics are metabolized in the liver of vertebrates and invertebrates by four types of enzymatic reactions: oxidations, reductions, hydrolyses, and conjugations. The oxidative metabolism of many xenobiotics is largely catalyzed by mixed-function oxidase enzymes present in the endoplasmic reticulum of liver tissue.

2. MIXED-FUNCTION OXIDASES

Although an early report (Brodie and Maickel, 1962) suggested that fish lacked the ability to oxidatively metabolize foreign compounds,

subsequent studies (Chan *et al.*, 1967; Dewaide and Henderson, 1968; Buhler and Rasmusson, 1968) have demonstrated that both freshwater and marine fish have cytochrome P450 dependent mixed-function oxidase activity.

Mixed-function oxidases, a type of oxygen consuming enzymes carrying out special oxidative reactions are present in several tissues and are complex in their action. As shown in the following equation, they catalyze reactions in which one atom of the molecular oxygen is incorporated into substrate while the other is being reduced to water. Thus, they are also called monooxygenases.



MFO can catalyze a wide range of different types of reactions. These include oxidative hydroxylation of aromatic and aliphatic hydrocarbons, O-, N-, and S-dealkylation reactions, N-oxidation, sulfoxidation and deamination reactions. MFO is a general term. These enzymes are also named on the basis of the type of the reaction they catalyze and the identity of the substrates involved. For example, an enzyme catalyzing 4-hydroxylation of aniline is named aniline 4-hydroxylase and the one catalyzing O-deethylation of 7-ethoxyresorufin is called 7-ethoxyresorufin O-deethylase. The enzyme system which oxidatively hydroxylates polycyclic hydrocarbons is given the name arylhydrocarbon hydroxylase (AHH).

Substrates for MFO are of two categories: Endogenous and exogenous. Endogenous substrates include steroids, steroid hormones, fatty acids, vitamin D, and bile acids. Exogenous substrates include almost all drugs, organic chemistry laboratory reagents including benzene, ethanol, and acetone, insecticides, ingredients in soaps and deodorants, certain fungal toxins and antibiotics, many of the chemotherapeutic agents used to treat human cancer, strong mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine and nitrosamines, aminoazo dyes and diazo compounds, numerous aromatic amines such as those found in hair dyes, nitro aromatics, and heterocyclics, N-acetylacrylamines and nitrofurans, wood terpenes, epoxides, antioxidants, other food additives, environmental pollutants including polycyclic aromatic hydrocarbons such as biphenyl and benzo(a)pyrene, halogenated hydrocarbons such as polychlorinated and polybrominated biphenyls (Conney and Burns, 1972; Lu and Levin, 1974; Pelkonen and Nebert, 1982).

The rate at which these various compounds are metabolized by this enzyme system varies widely and depends on the species, strain, age, tissue, nutritional status, and pretreatment of the animals (Conney, 1967; Conney and Burns, 1972; Lu and Levin, 1974).

The main function of MFO is to convert relatively insoluble organic compounds into water-soluble metabolites which may be further conjugated and excreted in urine or bile. Thus, they play a critical role in detoxification of foreign compounds. On the other hand, it is now recognized that many toxic and carcinogenic compounds exert their toxicity after being converted to chemically reactive species by MFO enzymes. Examples include selected polycyclic hydrocarbons (Heidelberger, 1973; Wiebel *et al.*, 1975), aflatoxins (Swenson, 1981), 4-ipomeanol (Minchin and Boyd, 1983).

Many researchers have shown that metabolically activated epoxides can be formed as intermediate metabolites from benzo(a)pyrene (Wiebel *et al.*,

1975) and aflatoxin B1 (Swenson, 1981). Binding of several epoxides to DNA has been implicated in the process of chemical carcinogenesis and mutagenesis.

2.1 Components of MFO

It has been now established that MFO system functions as a multicomponent electron transport system and has been resolved into three components: Cytochrome P450, NADPH-cytochrome P450 reductase, and lipid.

2.1.1 Cytochrome P450

Cytochrome P450, terminal oxidase of NADPH dependent MFO system contains Fe^{3+} and protoporphyrin IX and is a b type cytochrome. Recently, it has been shown that it exists as a family of isozymes. Up to now, at least 154 genes for cytochrome P450 have been identified in different species (Nebert *et al.*, 1991). Their monomer molecular weights range from 48000 to 60000 (Philpot and Arinç, 1976; Lu and West, 1980; Arinç and Adali, 1983; Black and Coon, 1986). Some of these forms are inducible, i.e., the levels are elevated by challenge of the animals with xenobiotics. Other forms have not been inducible yet. However, such forms have been shown to be responsive to developmental changes *in vivo*, sexual development, and pathophysiological conditions (Schenkman, 1991). From biochemical and toxicological viewpoint, *in vivo* studies carried out with chemical carcinogens have demonstrated that different isozymes of P450 are responsible for the activation and toxicity of the most of the chemical agents (Nebert and Felton, 1976; Wolf *et al.*, 1979; Nebert and Gonzalez, 1987; Arinç *et al.*, 1991).

2.1.2 Cytochrome P450 reductase

NADPH dependent cytochrome P450 reductase enzyme functions in the transfer of electrons from NADPH to cytochrome P450. It is a membrane-bound amphipatic protein containing both hydrophobic peptide and hydrophilic peptide. Monomer molecular weight of P450 reductase was determined to be 78000 (Gum and Strobel, 1981; Black and Coon, 1982; Işcan and Arinç, 1986 and 1988). Hydrophilic peptide having Mr of 71000 contains 1 mol each of FAD and FMN. Hydrophobic peptide is responsible for proper interaction of reductase with cytochrome P450) and anchoring the reductase to endoplasmic reticulum.

2.2 Mechanisms of mixed function oxidases

The mechanisms postulated for the hydroxylation of organic substances by microsomal cytochrome P450 dependent MFO is illustrated in Figure 1 (taken from Lehninger, 1975). The reducing equivalents from NADPH are transferred through NADPH-cytochrome P450 reductase to cytochrome P450 during hydroxylation of various compounds. The substrate RH first combines with Fe^{3+} form. The latter is then oxygenated and a second electron from NADPH converts bound oxygen to O_2^- radical. An internal oxidoreduction takes place by the formation of the hydroxylated substrate and H_2O , which contained the oxygen atoms introduced as O_2 . Free cytochrome P450 is regenerated in its Fe^{3+} form (Fig. 1A).

It has been proposed that lipid facilitate transfer of electrons from NADPH-cytochrome P450 reductase to cytochrome P450 (Lu and Levin, 1974). This is shown in Figure 1B.

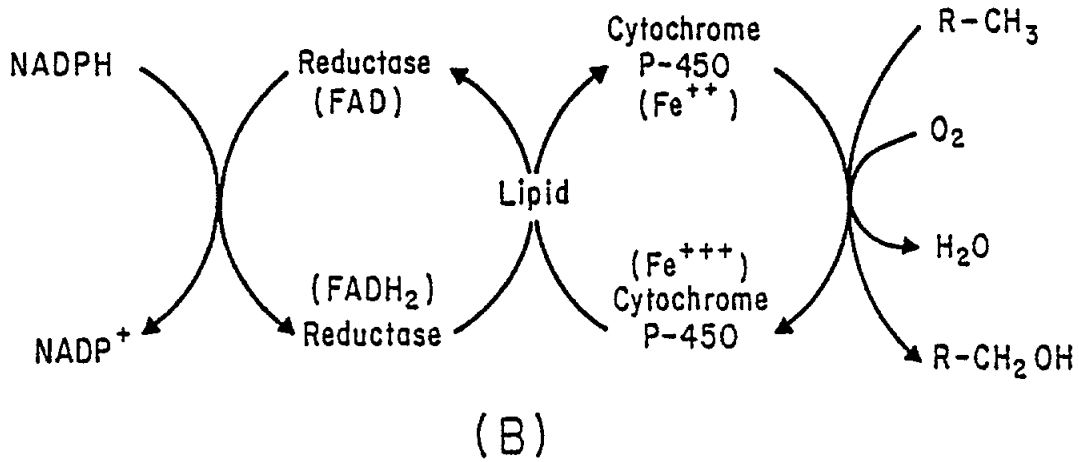
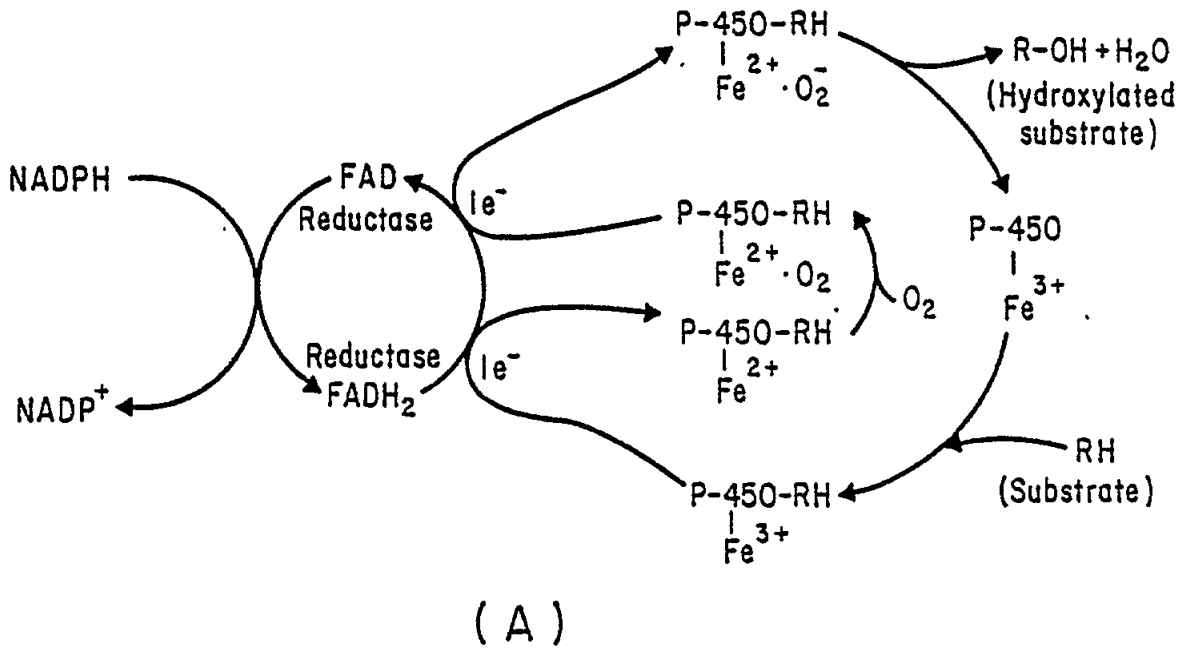


Fig. 1 Mechanism postulated for the hydroxylation of organic substrates by the liver microsomal cytochrome P450 dependent MFO system.
A. Oxygenated intermediates
B. The role of the lipid fraction

All of the three components, cytochrome P450, NADPH-cytochrome P450 reductase, and lipid (phosphatidyl-choline) are required to reconstitute the full hydroxylation activity. (Lu and Coon, 1968; Lu and Levin, 1974; Arinç and Philpot, 1976; Black and Coon, 1986; Adali and Arinç, 1990).

2.3 Resolution of fish liver MFO system

It is now clear that a fish liver MFO system, with the capability of metabolizing a variety of chemicals, exists in both freshwater and marine fish. Resolution of fish liver microsomal MFO system was first described by Arinç et al. in 1976. Little skate liver microsomal MFO system was resolved into three components: cytochrome P450, NADPH cytochrome P450 reductase, and lipid (Arinç et al., 1976; Bend et al., 1977; Arinç et al., 1978). Liver microsomes obtained from untreated scup (Klotz et al., 1983) and β -naphthoflavone (BNF) treated scup (Klotz et al., 1984) and Atlantic cod (Goksoyr, 1985) also resolved into these three components.

2.4 Toxicological significance of MFO induction in fish

It has been clearly established that although MFO catalyzes the detoxification reactions, in some cases the reactive intermediates formed are more mutagenic, more carcinogenic and ultimately more toxic than the parent compound. More than 200 chemicals have been shown to induce MFO activity. Induction of MFO is commonly associated with an apparent increase in the level of cytochrome P450 content (Lu and Levin, 1974; Stegeman and Kloepper-Sams, 1987). However this is not always the case. In some cases, changes in the level of P450 isozymes cannot be determined spectrophotometrically and can only be assessed by immunological methods which result in no apparent alteration in the total level of cytochrome P450 (Payne et al., 1987; Arinç et al., 1991).

Induction of MFO in aquatic species by polycyclic hydrocarbons such as 3-MC and B(a)P, polychlorinated biphenyls (PCB), 2,3,7,8-tetra-chlorodibenzo p-dioxin (TCDD) and BNF has been shown. In all cases, AHH activity measured by hydroxylation of B(a)P was induced several folds (Bend et al., 1977; Arinç et al., 1978; Elcombe and Lech, 1979; Klotz et al., 1983; Goksoyr, 1985).

In general, induction of AHH activity was associated with induction of a specific cytochrome P450 isozyme, P4501A1. This P450 is called P450E in scup (Klotz et al., 1983) and P450c in cod (Goksoyr, 1985). Spectral, biocatalytic, and immunological properties of scup P450E and cod P450c showed that these fish enzymes have properties similar to those of rat cytochrome P450c (Stegeman and Kloepper-Sams, 1987). It is suggested that induction of P450E, P450c, or their counterparts may be responsible for initiation of carcinogenesis by some polyaromatic hydrocarbons in fish (Stegeman and Kloepper-Sams, 1987). Field studies carried out with some fish species collected from oil-contaminated areas also showed high AHH activity (Payne, 1976; Kurelec et al., 1977; Rijavec et al., 1981; Lindström-Seppä et al., 1985). Recently, the B(a)P hydroxylase assay has been replaced by a more simple and accurate 7-ethoxyresorufin O-deethylase assay (Klotz et al., 1983 and 1984; Goksoyr, 1985).

2.5 The aim of the present study

It is well known that differences among species are notable for the liver microsomal MFO systems. Up to now, no study is available in the context of xenobiotic metabolism and toxicology of gilthead seabream which is a valuable fish of the Aegean Sea. In this study, MFO system of gilthead seabream (Sparus auratus) liver microsomes were partially characterized and response of gilthead seabream to B(a)P was investigated.

3. MATERIALS AND METHODS

3.1 Chemicals

Resorufin was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, USA. p-Aminophenol (pAP) was obtained from Fluka A.G., Buchs S.G., Switzerland. Ethylene diamine tetra acetic acid disodium salt (EDTA), dimethylsulfoxide (DMSO), and sodium dithionite were purchased from E. Merck, Darmstadt, Germany.

Benzo(a)pyrene [B(a)P], bovine serum albumin (BSA), 7-ethoxyresorufin (7-ER), N-2-hydroxyethylpiperazine-N'-2, ethane sulfonic acid (HEPES), 3-methylcholanthrene (3-MC), nicotinamide adenine dinucleotide, reduced form (NADH), nicotinamide adenine dinucleotide phosphate (NADP+) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA. All other chemicals were of analytical grade and were obtained from other commercial sources at the highest grade of purity available.

3.2 Fish

The fish used in this study, gilthead seabream (Sparus aurata), were obtained from the fish farm of Pinar Sea Products Ltd., Ildir, Çesme, Izmir, Turkiye. The fish were grown in special hatcheries until they reached the required weight and then were taken into adaptation tanks to be inured to sea conditions. Finally, they were transferred to the cages in the Aegean Sea. The fish used in this study weighed 250 ± 25 g and were about 24 months old.

3.2.1 Benzo(a)pyrene treatment

Fish were transferred from their cages in the sea to two-ton tanks equipped with circulating filtered seawater at $21 \pm 0.5^\circ\text{C}$ three days before the start of the experiment and feeding was stopped 12 hours before the first injection. The treatment solution was prepared first by dissolving 62.5 mg B(a)P in 15 ml of DMSO by immersing in a water bath at 100°C . Then, the solution was completed to the final volume of 50 ml with corn oil. The ratio of DMSO was 3:7 and the final concentration of B(a)P was 12.5 mg ml^{-1} . During the treatment, the fish were held gently with fish strainer and 0.5 ml of this solution was administered intraperitoneally to each fish for five consecutive days. Some of the control fish were treated with DMSO and corn oil. No other treatment was done to anesthetize the fish for handling.

3.2.2 Preparation of liver microsomes

Gilthead seabream liver microsomes were prepared as described by Arinç and Adali (1983) with slight modifications. The fish were killed by decapitation. The livers, each weighing 2.5 to 4.5 g, were removed immediately. The gall bladders were removed carefully to avoid spillage of their content which is known to be inhibitory to MFO activities. The livers were placed in liquid nitrogen and transported from Izmir to the University laboratories in Ankara, about 450 miles away.

Fish liver microsomes were prepared by differential centrifugation. About three to ten fish livers were used for preparation of microsomes.

Fish homogenate was centrifuged at 13,300 g for 40 minutes to remove cell debris, nuclei, and mitochondria. The microsomes were sedimented from the supernatant solution at 133,000 g for 70 minutes and washed. The washed microsomal pellet was resuspended in 10% glycerol containing 1 mM EDTA. For each gram of liver, 0.5 ml of suspension solution was used. The enzymatic reaction rates and cytochrome P450 concentrations in microsomes were determined with these freshly made preparations. Additional microsomal suspensions were gassed with nitrogen and immediately immersed into a liquid nitrogen tank for storage.

3.3 Analytical procedures

3.3.1 Protein determinations

The protein concentrations of the microsomes were determined by the method of Lowry et al., 1951. Crystalline bovine serum albumin was used as a standard.

3.3.2 Determination of cytochrome P450

Cytochrome P450 concentrations were determined by carbon monoxide difference spectra of dithionite-reduced samples on Hitachi 220A double beam recording spectrophotometer using an extinction coefficient of $91 \text{ mM}^{-1}\text{cm}^{-1}$ for the difference in the absorption between 450 and 490 nm (Omura and Sato, 1964).

3.3.3 Determination of cytochrome b5

Cytochrome b5 concentrations of microsomes were determined according to the method of Nishibayashi and Sato (1968). The concentration of cytochrome b5 was estimated from the initial dithionite-reduced minus oxidized difference spectrum using an extinction coefficient of $185 \text{ mM}^{-1}\text{cm}^{-1}$ for the difference in the absorption between 424 and 410 nm.

3.3.4 Determination of NADPH cytochrome P450 reductase activity

NADPH-dependent cytochrome P450 reductase activity was measured spectrophotometrically according to the procedure of Masters et al. (1967) except that the reaction was carried out in 0.3 M potassium phosphate buffer, pH 7.7, at room temperature. The extinction coefficient of $19.6 \text{ mM}^{-1}\text{cm}^{-1}$ was used (Yonetani, 1965).

One unit of enzyme activity is defined as the amount of enzyme causing a reduction of 1 nmol cytochrome c per minute under the described conditions.

3.3.5 Determination of aniline 4-hydroxylase activity

Aniline 4-hydroxylase activity of gilthead seabream liver microsomes was determined by measuring the quantity of p-aminophenol formed as described by Imai et al. (1966) with some modifications (Arinç and Işcan, 1983).

3.3.6 Determination of 7-ethoxyresorufin O-deethylase activity

The gilthead seabream liver microsomal 7-ethoxyresorufin O-deethylase (7-EROD) activity was measured by modifying the methods described by Pohl and Fouts (1980) and Klotz et al. (1984). This activity was determined by measuring the intensity of the pink color at 572 nm, which was produced by the conversion of 7-ethoxyresorufin into resorufin.

Assay conditions such as incubation time and protein and substrate concentrations were optimized for the gilthead seabream liver microsomes.

The assay mixture contained 100 mM potassium phosphate buffer, pH 7.8, 100 mM NaCl, 1.2 mg BSA, 1.5 micromolar 7-ethoxyresorufin, 0.2 mg microsomal protein, and 0.5 mM NADPH generating system described above in a final volume of 1 ml. The enzymatic reaction was carried out at 25°C for 10 minutes.

4. RESULTS AND DISCUSSION

4.1 Cytochrome P450 of gilthead seabream liver microsomes

Cytochrome P450 content of three different liver microsomal preparations obtained from 4 to 10 control fish were found to be 0.084, 0.074 and 0.064 nmol of P450 per mg of microsomal protein.

The studies were carried out for accurate measurement of cytochrome P450 content of gilthead seabream. Figure 2 shows the influence of time on apparent P450 content of microsomes during the spectrophotometric measurements. During this procedure, P450 was first reduced by dithionite and then gased with carbon monoxide. Reduction of P450 (Fe³⁺) to P450 (Fe²⁺) is time dependent. As seen in Figure 2, the maximum P450 content was calculated at 8th and 9th minutes after the dithionite reduction. At 4th and 6th minutes, only 75% and 85% of the P450 was reduced, respectively. Apparent low P450 contents calculated at 10, 12, and 15 minutes were due to the formation of cytochrome P420, denatured form of P450 (Omura and Sato, 1964; Arinç and Philpot, 1977).

4.2 Mixed-function oxidase activities

The quantitative data on liver microsomal MFO enzymes and on components of MFO of gilthead seabream, rainbow trout, and rabbit are given in Table 1. The values obtained from two different fish species for aniline 4-hydroxylase, NADPH-cytochrome c reductase, cytochrome P450, and cytochrome b5 are compatible. However, these values are much lower than the respective values determined in rabbit liver microsomes.

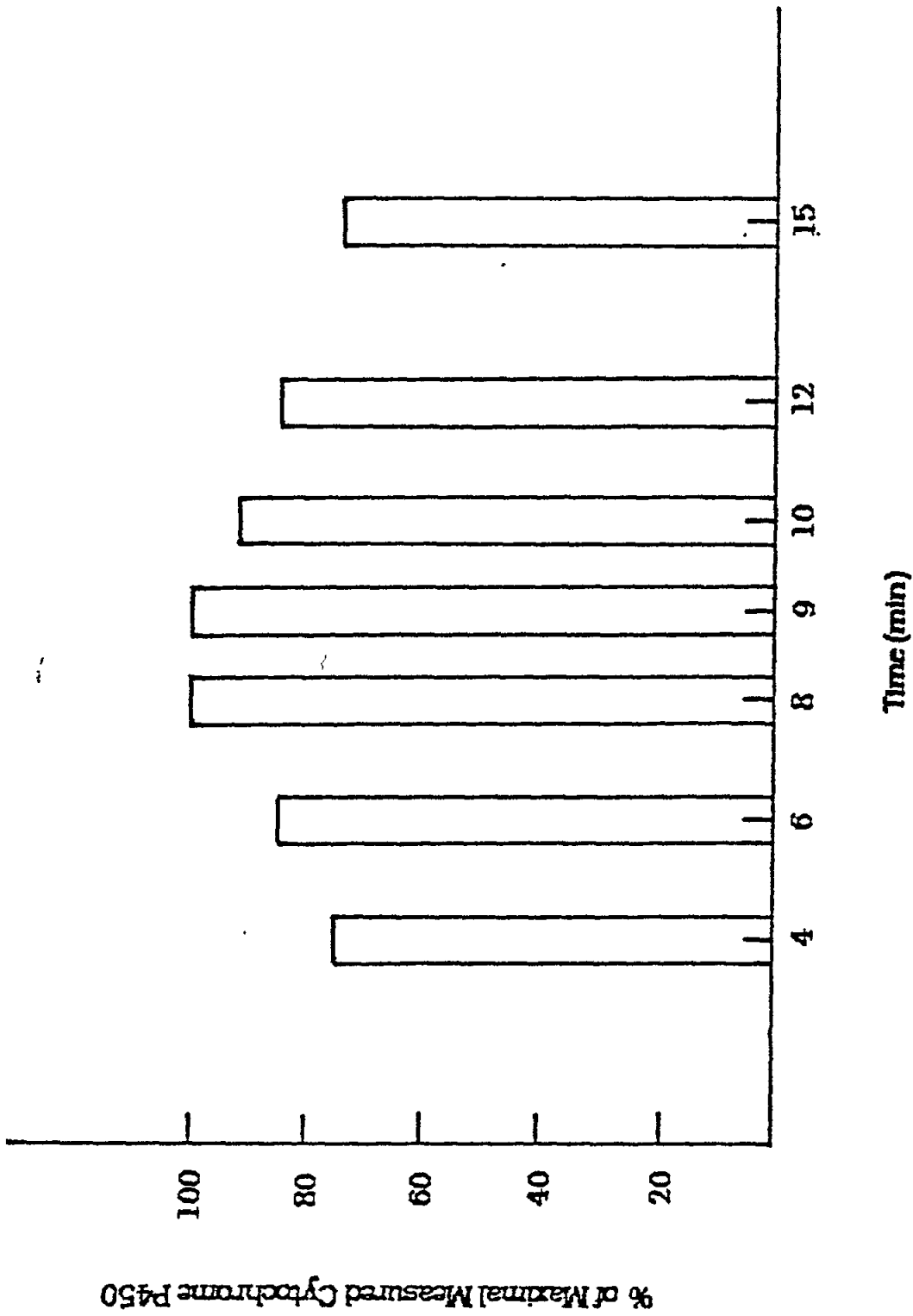


Fig. 2 Influence of time on apparent cytochrome P450 content of gilthead seabream liver microsomes. The values are averages of 3 sets of data

In gilthead seabream liver microsomes, the specific activity of aniline 4-hydroxylase associated with P450IIE1 was found to be low. In contrast, The activity of 7-ethoxyresorufin O-deethylase associated with P450IA1 was higher.

Table 1

Aniline 4-hydroxylase, 7-ethoxyresorufin O-deethylase P450 reductase activities and cytochrome P450 and b5 content of fish and rabbit liver microsomes.

MFO	Gilthead ^a Seabream	Rainbow ^b Trout	Rabbit ^b
Aniline 4-hydroxylase (nmol pAP min ⁻¹ mg ⁻¹)	0.019±0.003	0.040	0.65
7-Ethoxyresorufin O-deethylase (nmol resorufin min ⁻¹ mg ⁻¹)	0.407±0.046	N.D.	N.D.
Cytochrome P450 Reductase (U mg ⁻¹)	23.3±2.34	38.0	100
Cytochrome P450 (nmol mg ⁻¹)	0.074±0.01	0.160	0.76
Cytochrome b5 (nmol mg ⁻¹)	0.30±0.038	0.26	0.77

a : Determined in our laboratory

b : Taken from Arinç and Adalı, 1983. Untreated control fish, Salmo gairdneri

N.D. : Not determined

4.2.1 Characterization of assay conditions for 7-ethoxyresorufin O-deethylase

Because of the potential use of deethylase in assessment of organic pollution, this enzyme were characterized in gilthead seabream microsomes. The microsomal protein concentrations ranging from 0.05 to 0.6 mg per ml were tried in the presence and absence of BSA, and 0.2 mg microsomal protein and 1.2 mg BSA per ml of incubation mixture were chosen for routine analysis. O-deethylation of 7-ethoxyresorufin was measured at different temperatures such as 15, 20, 25, and 30°C. No significant difference was observed among the enzyme activities at these temperatures.

The effect of varying incubation period on enzyme activity was studied. The time course of 7-ethoxyresorufin O-deethylation of fish liver microsomes was found to be linear for 12 minutes under the described conditions. During the enzymatic assays, a 10-minute incubation period was routinely used.

4.2.2 Stimulation of 7-ethoxyresorufin O-deethylation activity by B(a)P

The effects of benzo(a)pyrene treatment on cytochrome P450 content and mixed-function oxidase activities were investigated. The rates of aniline 4-hydroxylation, NADPH-dependent cytochrome P450 reductase activities of fish liver microsomes were found to be unaltered by this treatment. In contrast, the cytochrome P450 content and 7-ethoxyresorufin O-deethylase activities of microsomes prepared from treated fish were found to be 160% and 300% higher than those of the control (Fig. 3).

These results clearly showed that gilthead seabream responded to benzo(a)pyrene treatment and 7-ethoxyresorufin O-deethylase activity was stimulated three fold. The induction of MFO in aquatic species by polycyclic hydrocarbons and related compounds such as 3-MC, B(a)P, PCB, TCDD, and BNF has been shown (Bend et al., 1977; Arinç et al., 1978; Elcombe and Lech, 1979; Klotz et al., 1983; Goksoyr, 1985). In the field studies, some fish species collected from oil-contaminated areas also showed high AHH activity. Payne (1976) observed that canners (Tautoglabrus adspersus) collected at the site of a large oil refinery in Newfoundland had elevated levels of liver AHH activity (approximately three folds). Kurelec et al. (1977) observed that blennies (Blennius pavo) collected in an area of No.2 fuel-oil spill in Northern Adriatic Sea showed a marked level of liver AHH induction and AHH levels remained elevated for a 2-3 week period. Rijavec et al. (1981) observed that blennies taken from the area of a refinery outfall in the Adriatic Sea had highly elevated AHH activity. Lindström-Seppä et al. (1985) observed that perch (Perca fluviatilis) collected at the site of an oil spill in the Vassa Archipelago in Finland had elevated levels of AHH enzyme in comparison to the fish taken from control sites. In this case, the enzyme activity remained elevated for four months and returned to normal in 9 months. A review article by Payne et al. (1987) has further examples. In the same review, it is indicated that such studies demonstrate the value of MFO system as a sensitive monitoring tool for petroleum hydrocarbons.

In all the above field trials, the AHH activity was measured by hydroxylation of substrate benzo(a)pyrene. Recently, B(a)P assay has been replaced by 7-ethoxyresorufin O-deethylase assay for the following reasons: First of all, substrate B(a)P is a carcinogenic chemical. Secondly, during the incubation with liver microsomes, B(a)P produces a complicated mixture of about 10 metabolites that are best studied by time-consuming high-pressure liquid chromatography. In contrast, 7-ethoxyresorufin O-deethylase assay is a simple assay. Its activity can be determined by using a visible spectrophotometer allowing measurement of large number of samples within a short period of time. In addition, the quantitation is both highly reliable and reproducible. 7-ethoxyresorufin O-deethylase assay was developed by Prough et al. (1978), Pohl and Fouts (1980) and Klotz et al. (1984). Similar to B(a)P hydroxylase activity, ethoxyresorufin O-deethylase activity is also most commonly associated with forms of P450 which are induced by polycyclic hydrocarbons and other similar inducers.

Recently, scup (Klotz et al., 1983 and 1984), cod (Goksoyr, 1985), perch (Förlin et al., 1985), and winter flounder (Payne et al., 1987) liver microsomal 7-ethoxyresorufin O-deethylase activities were determined and employed as a probe for polycyclic hydrocarbon or related chemical induction of fish MFO system.

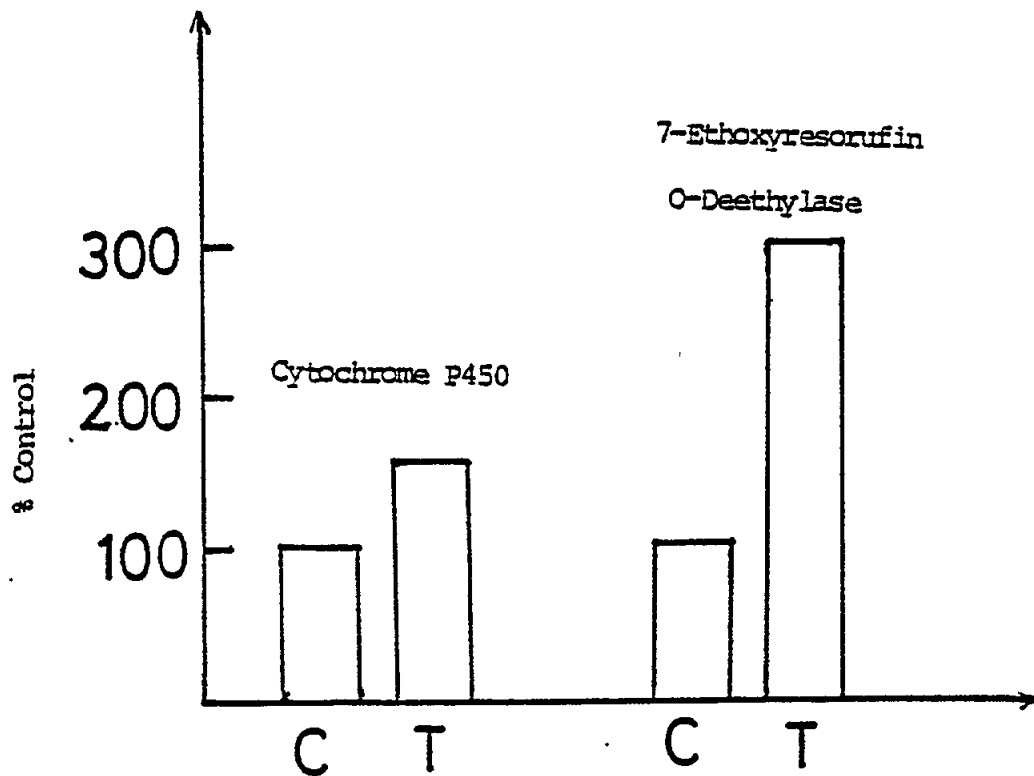


Fig. 3 Effect of benzo(a)pyrene administration on cytochrome P450 content and 7-ethoxyresorufin O-deethylase activity of gilthead seabream liver microsomes. Control values are given as 100%. T and C correspond to treated and control fish. O-deethylase activity of the treated fish was significantly different from control, $P < 0.05$

Although the concentration of benzo(a)pyrene and of other polycyclic hydrocarbons may be lower in a true field situation, for induction studies of 7-ethoxyresorufin O-deethylase 6.25 mg B(a)P was administered i.p. to each fish for five consecutive days in order to get a fast response. The concentration of B(a)P in gilthead seabream liver was not measured. Short-term injection experiments have shown to be a rapid means of screening PAHs for MFO inductibility (Gerhart and Carlson, 1978). Aromatic hydrocarbons enter the marine environment from a number of sources. A large portion of these compounds eventually settles in sediments. Fish can accumulate aromatic hydrocarbons via the food chain and via interstitial water. A study carried out in 1978 in U.S.A. showed that when rainbow trout was exposed to B(a)P concentration of $0.4 \mu\text{g l}^{-1}$ in water the amount of B(a)P in fish liver reached the value of $368 \mu\text{g kg}^{-1}$ (Gerhart and Carlson, 1978). B(a)P concentration was determined to be $1-2 \mu\text{g l}^{-1}$ and $0.05-3.5 \mu\text{g l}^{-1}$ in waters of Metropolitan areas in U.S.A. and in rivers having connections with petroleum industries in Russia, respectively (Gerhart and Carlson, 1978).

In this study, 7-ethoxyresorufin O-deethylase assay is successfully used and assay conditions are characterized for gilthead seabream liver microsomes. As stated above, gilthead seabream responded to polycyclic hydrocarbon treatment and 7-ethoxyresorufin O-deethylase activity was stimulated three folds. As noted by Payne *et al.* (1987), the induction of MFO enzymes in response to polycyclic hydrocarbon and mixed organic contamination has been validated in a large number of field studies and induction of MFO enzymes is the most sensitive biological response for assessing a variety of organic pollution conditions. The results of this study suggest, among other things, that the degree of elevated levels of liver microsomal 7-ethoxyresorufin O-deethylase activity of gilthead seabream can be used as an indicator of the presence of polycyclic hydrocarbon pollution.

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REFEREE'S COMMENT

The manuscript contains a large section of very basic information that can be obtained in text book form. I would suggest that this be eliminated as follows: (a) p.74 lines 4 all the way to p.77 line 5 and (b) p.77 lines 16 to 26.

AUTHOR'S RESPONSE TO REFEREE'S COMMENT

I do not agree with this suggestion. People working in the fields of toxicology, pharmacology, biochemistry, biophysics, molecular biology, endocrinology, immunology, environmental sciences, genetics and physiology are all interested in mixed-function oxidases (MFOs). However only some pharmacology books cover this subject somewhat in detail. For example, a recent biochemistry textbook written by C.K. Mathews and K.E. Van Holde, Benjamin/Cummings Publishing Co.Inc, 1990 gives half-a-page of information on cytochrome P450 (p. 533) and does not mention the term MFO. Another biochemistry book written by J.D. Rawn, Neil Patterson Publishers, 1989, covers mitochondrial cytochrome P450 in steroid hormone biosynthesis (p.564), but again it does not mention the term MFO.

MICROBIAL RESPONSE TO CHROMIUM TOXICITY

by

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A B S T R A C T

The present review on chromium toxicity in microorganisms, outlines the various known mechanisms of chromium genotoxicity and tolerance to its trivalent and hexavalent forms. Cr-sensitive microorganisms are inhibited by low concentrations of hexavalent Cr, which is very toxic. Cr(III) is less harmful because it is relatively insoluble and remains outside the cell. Cr(VI) enters the cytoplasm via the sulphate transport system, where it is reduced to Cr(III). This chemical form interacts with different intracellular targets, especially nucleic acid, causing genetic damage or changes, such as petite mutations in yeasts.

Microorganisms have developed several strategies to thrive in the presence of high concentrations of Cr(VI) and Cr(III). Genetic Cr(VI)-resistance has been reported for several bacteria and it is often harboured in plasmids (e.g. in Pseudomonas aeruginosa and Streptococcus lactis) or chromosomes (Neurospora crassa mutants). Tolerance to Cr(VI) is also developed by marine microorganisms in coastal sediments, e.g. sulphate-reducing bacteria, which reduce Cr(VI) to Cr(III) with production of H₂S. Under anaerobic conditions, Cr(VI) can be utilized by the Enterobacter cloacae strain HO1 as an electron acceptor, consuming glycerol and acetate and reducing Cr(VI) to Cr(III) more rapidly.

In marine environments, mucoid coryneform bacteria have also been demonstrated to induce exopolysaccharide production, and hence to complex Cr(III) extracellularly.

The mechanism of Cr-resistance in microorganisms has been studied mostly for the more toxic form, Cr(VI). Bacteria, cyanobacteria and yeasts can tolerate high concentrations of Cr(VI) because of the reduced uptake of this form. The inefficient transport of sulphate in microorganisms is always related to high Cr(VI) resistance and in some strains can be induced by the presence of thiols or S-aminoacids like cysteine, which inhibits the sulphate transport system. Under aerobic conditions, a Pseudomonas putida strain can reduce Cr(VI) to Cr(III) extracellularly. This mechanism was suggested as a form of Cr(VI)-tolerance, but it was later demonstrated that the high Cr(VI)-resistance was mostly due to reduced uptake of Cr(VI). Other tolerance mechanisms probably exist and should be investigated further.

1. INTRODUCTION

Chromium is one of the most widely used metals in industry and its abundance in the environment is due to urbanization and industrialization

(Kimbel and Panulas, 1984; Hayes, 1988). Discharges from tanneries, steelworks, chromeplating and paint factories are sources of Cr which accumulates in sediments and water (Duedall et al., 1983; Moore and Ramamoorthy, 1984). The high volume of sewage from cities also contributes significant amounts of Cr to the environment (Jan and Young, 1978). Natural sources of Cr include the weathering of chromite (CrFe_2O_4) deposits (Papp, 1985), volcanic debris (Moore and Ramamoorthy, 1984) and deep-sea vents (Jeandel and Minster, 1984).

The element Cr exists in nature in a variety of compounds, although only the trivalent and hexavalent forms are biologically important (Mertz, 1969). Considerable attention has been paid to the form of Cr occurring in seawater: in the open ocean (Fukai, 1967; Cranston and Murray, 1978; Nakayama et al., 1981) and in freshwater (Luli et al., 1983) Cr(VI) is dominant whereas in the littoral environment, the Cr(III) form is mostly found (Elderfield, 1970; Jenkins, 1982).

In sediments, Cr does not form sulphides but may form oxides and hydroxides (Curl et al., 1965; Schroeder and Lee, 1975). This feature supports the contention that, once deposited, Cr is unavailable to biota. In marine sediments of Sawyers Bay, Otago Harbour (New Zealand), which received tannery wastes for 100 years, most Cr occurred as Cr(III) (Loutit et al., 1988) and was associated with insoluble floc. The rest (4%) was in the form of oxides and hydroxides. Cr(VI) is regarded as toxic for eukaryotic and prokaryotic cells (Venitt and Levy, 1974; Bianchi and Levis, 1988), whereas Cr(III) is less toxic and is regarded in some cases as micronutrient, especially for mammals (Mertz, 1969) and probably also for plants (Babich et al., 1982).

Studies of the interactions between microorganisms and Cr in the environment and laboratory can help to understand the mechanisms of Cr toxicity in higher organisms and microbial survival strategies can tell us much about Cr effects. The toxicity of Cr compounds is also of interest for sewage biotreatment. Cr compounds inhibit microbial cell growth and respiration in activated sludge (Kaneko and Nambu, 1973).

2. CHROMIUM GENOTOXICITY

The mutagenicity and carcinogenicity of Cr compounds has been studied from the medical point of view (IARC, 1987). Much experimental evidence has been gathered on the mechanism of genotoxicity in cultured mammalian cells (Sugiyama et al., 1986) and laboratory animals (Tsapakos et al., 1983). Genotoxic effects of Cr have been demonstrated in bacteria (Venitt and Levy, 1974; Ogawa et al., 1989; De Flora et al., 1990; Brandi et al., 1990). Bianchi and Levis (1985) reported that Cr(VI) is actively transported through cell membranes and can thus reach genetic targets. This form of the metal is an active genotoxic agent in whole cells, whereas Cr(III) is inactive. This reduced form of Cr gives a positive mutagenic response only when tested in vitro on purified DNA. On the other hand, Cr(VI) gives contradictory results in vivo. Several hypotheses have been formulated for the mechanism of genotoxic action of this metal. Schoental (1975) proposed that Cr(VI) produces reactive aldehydes, because of its oxidizing power. This mechanism may be operative only in some in vivo mutagenic assays. The alternative hypothesis is that Cr(VI) enters the cell and that cytoplasmic products reduce Cr(VI) to

Cr(III). This reduced form becomes genetically active only after it has reached the chromosome. At this point, mutations can arise through direct oxidation of DNA-protein complexes (Petrilli and De Flora, 1978a), through the interaction of intra-reduced Cr with DNA or more indirectly, through the interaction of Cr(III) with precursor nucleotides or DNA polymerases that diminish the fidelity of DNA replication (Ogawa *et al.*, 1989). These mechanisms are not mutually exclusive. Cr(III) is confirmed as the final genotoxic agent by the fact that the targets identified through exposure of purified DNA to Cr(III) are the same as those affected by treatment of whole cells with Cr(VI) (Levis and Bianchi, 1982). The affected DNA regions are rich in guanidine and cytosine and contain nucleophilic sites, indicating that the metal produces DNA single-strand breaks, DNA-protein crosslinks and DNA-DNA crosslinks. Crosslinking is observed when isolated cell nuclei are treated with Cr(III) or Cr(VI) in the presence of reducing systems such as microsomes (Bianchi and Levis, 1985). The requirement of a reducing system demonstrates that the reduction of Cr(VI) to Cr(III) is crucial for the induction of genotoxic effects (Fornace *et al.*, 1981).

Genotoxic effects of Cr compounds are extensively studied in bacteria for predicting their carcinogenicity. In particular, the Salmonella typhimurium his⁻ reversion test is universally recognized as a versatile and flexible model for the assessment of mutagenic properties of both pure chemicals and complexed mixtures. The mutagenic potency of Cr(VI) compounds in different tester strains can yield some indication of the mutagenicity mechanisms (Table 1). De Flora and Wetterhahn (1989) reported extensive findings on the mutagenic potency of sodium dichromate on eleven strains of S. typhimurium his⁻. Bacterial targets have been exploited for assessing the reactions of Cr with various substances affecting its oxidation state and solubility. For example nitrilotriacetic acid (NTA), a substitute for

Table 1

A list of genetic targets investigated in vitro and in vivo for assessing the genotoxicity of the transforming ability of Cr compounds (from De Flora and Wetterhahn, 1989).

Targets	Parameters monitored
Purified nucleic acids (DNA, RNA)	-Physico-chemical changes -Infidelity of DNA replication
Bacteria (<u>Salmonella typhimurium</u> , <u>Escherichia coli</u> , <u>Bacillus subtilis</u>)	-Reverse point mutations -Forward point mutations -Selective lethality in DNA repair-deficient strains -Induction of SOS DNA repair -Induction of L-prophages
Yeasts (<u>Schizosaccharomyces cerevisiae</u> , <u>Schizosaccharomyces pombe</u>)	-Reverse point mutations -Forward point mutations -Mitotic gene conversion -Mitotic recombination -Meiotic disomic and diploid gametes

polyphosphate household detergents, solubilized water-insoluble Cr compounds at neutral pH in sufficient amounts to exert mutagenic effects in Salmonella and other in vivo and in vitro systems (Loprieno et al., 1985). Conversely, the chelating agent EDTA produced little loss of mutagenicity of Cr(VI). Oxidation of Cr(III) to toxic and mutagenic Cr(VI) was not obtained in the presence of a weak oxidizing metabolite, i.e. oxidized glutathione (GSSG), but only of a strong oxidizing agent like potassium permanganate (Petrilli and De Flora, 1978b). On the other hand, the mutagenicity of Cr(VI) in S. typhimurium was decreased and eliminated, with dose-related effects, by reducing chemicals such as sodium sulphite, ascorbic acid, cysteine, and N-acetylcysteine (Fig. 1) (Petrilli and De Flora, 1978b). The same effect was produced by physiological concentrations of reduced glutathione (GSH) and by non-physiological concentrations of reduced pyridine nucleotides (NADH and NADPH) (De Flora and Wetterhahn, 1989).

Another toxicity effect of Cr(VI) was well investigated in Saccharomyces cerevisiae mitochondria (Egilsson et al., 1979; Bruce et al., 1987) and recently also by Henderson (1989). The Cr(VI) caused anti-mitochondrial effects and induced petite mutations (small colonies). The yeast was grown in two different media, YED and YEG. The first contained D-glucose, a fermentable substrate, and the second, glycerol, a non-fermentable compound. In the presence of glycerol, S. cerevisiae was forced to oxidize the substrate by the respiration pathway, which is carried out by the mitochondrial system. The effect of Cr(VI) toxicity was 10 times higher with glycerol than with D-glucose, which can also be degraded by the fermentation pathway. In addition, Cr(VI) causes induction of mitochondrial petite mutations, which result from extensive deletions in the mitochondrial genome, so that the yeast is unable to respire and can only grow glycolytically (Bernardi, 1979).

3. CHROMIUM TOLERANCE

When toxicity of Cr compounds is tested by studying growth inhibition of microbial cells, one indirectly studies the result of the active transport of Cr through the cell envelope and the related damage to the cell metabolism and chromosome interaction. Phenotypic features of microbial cells show different strategies towards Cr-toxicity.

3.1 Genetic resistance to chromium

Cellular protection against the chemical form of metals has been widely studied from the genetic and physiological points of view. The genetic Cr resistance was described for the first time by Shimada (1976) and then more extensively by Efstathiou and McKay (1977) in Streptococcus lactis. Cr(VI) resistance was found to be harboured in the same plasmid as glucose fermentation genes, but in a different locus. Summers and Jacoby (1977) investigated Cr(VI) tolerance in a strain of Pseudomonas aeruginosa isolated from the sputum of a patient transferred to the Massachusetts General Hospital from Athens (GR). The Cr-resistance was harboured in plasmid pMG6, which also conferred resistance to mercury and tellurium compounds. Streptococcus and Pseudomonas both were ten times more tolerant to Cr(VI) than the plasmidless strains.

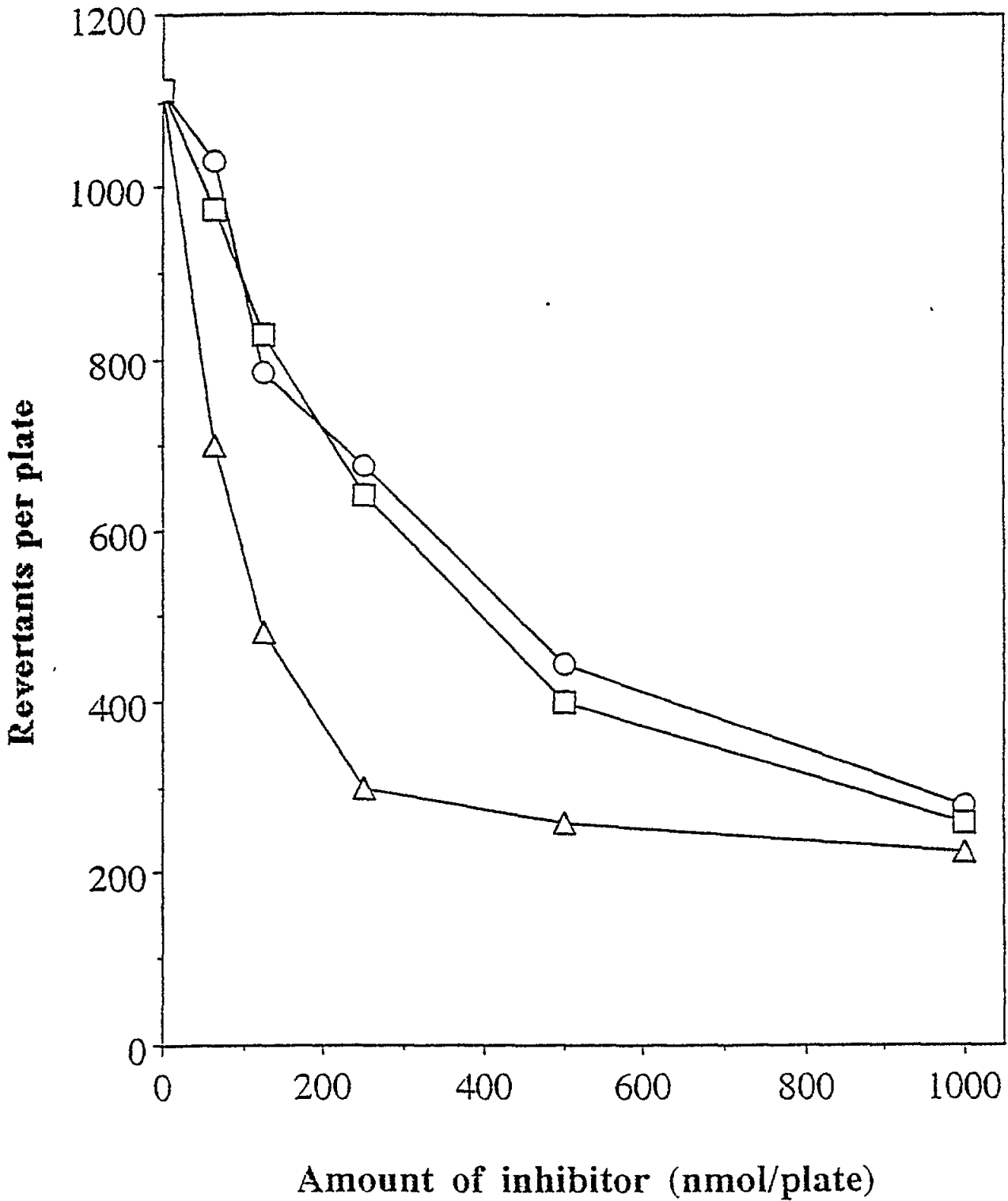


Fig. 1 Effect of ascorbic acid (Δ), reduced glutathione (O) N-acetylcysteine (\square) on the mutagenicity of sodium dichromate (100 nmol/plate) in strain TA102 of *S. typhimurium* (from De Flora and Wetterhahn, 1989)

Horitzu *et al.* (1978) isolated a *Pseudomonas ambigua* strain G-1, from activated sludge. The bacterium tolerated up to 2,000 $\mu\text{g ml}^{-1}$ of Cr(VI) and high concentrations of copper and cadmium in agar plates. In liquid media the strain could tolerate up to 750 $\mu\text{g ml}^{-1}$, but above 250 $\mu\text{g ml}^{-1}$, growth slowed down. The uptake of Cr by the *P. ambigua* cells was 16.5% of the soluble metal in the medium. 86.5% of the Cr accumulated by the cells was determined in the intracellular soluble fraction, whereas 13.5% was bound to the lipid fraction (67%) and the polyphosphate polysaccharide (21%). The soluble fraction was spun down at 105,000 g for 120 min. to collect the microsomal fraction and divided into two components: nucleic acids and proteins. Cr was bound to both components.

Environmental studies on the distribution of Cr-resistant bacteria were carried out by Luli *et al.* (1983). They isolated 89 strains from Cr-polluted sediments in the Ottawa River in north-eastern Ohio (USA). Of the isolates tested, 47% were resistant to 100 $\mu\text{g ml}^{-1}$ of Cr(VI), and 88% were resistant to 100 $\mu\text{g ml}^{-1}$ of Cr(III). 64% were *Pseudomonaceae*, 17% were gram-positive and were resistant only to 50 $\mu\text{g ml}^{-1}$ of Cr(VI) (*Streptomyces* and *Corynebacteria*), and three *Bacillus* were resistant only to 50 $\mu\text{g ml}^{-1}$ of Cr(VI). During the experiment of Cr(VI) toxicity, the oxyanion was not reduced or lost through volatilization after 48 hours.

The main mechanisms of Cr tolerance so far investigated are: a) exopolysaccharide production; b) inefficiency or lack of sulphate transport system; c) enzymatic and non-enzymatic reduction of Cr(VI) to Cr (III) in aerobic and anaerobic environments.

3.2 Chromium tolerance by exopolysaccharide production

Production of exopolysaccharides is considered to be universal feature of microbes in the natural environment (Costerton *et al.*, 1981). Microorganisms are known to bind metals and to protect themselves from metal toxicity. Toxic elements such as copper and cadmium influence the production of polysaccharides by *Klebsiella aerogenes* (Bitton and Friehofer, 1978).

Aislabie and Loutit (1986) reported very high sequestration of Cr(III) by polysaccharides of coryneform bacterium strain 3-31 and *Enterobacteriaceae* strain 4/6 (Fig. 2). The importance of polysaccharide in Cr tolerance was well demonstrated in an experiment with a coryneform strain L11+ and its non-mucoid variant L11. This mutant was totally inhibited by 3 mM of Cr(III), whereas the wild-type grew well. The accumulation of Cr(III) by the L11+ strain was significantly higher ($p > 0.001$) than its mutant L11 (Fig. 3).

The chemical composition and the amount of polysaccharides produced by microorganisms are reported to be dependent on many environmental factors (Geesey, 1982; Sutherland, 1985). Polysaccharides seemed to be induced in a coryneform strain 3-31 (Fig. 4), which was grown on increasing concentrations of Cr(III) (Aislabie and Loutit, 1986). In another strain, belonging to the *Enterobacteriaceae* group, this polysaccharide-Cr(III) correlation was not found.

Exopolysaccharides are not always negatively charged, but can still sequester metals. For example, *Alcaligenes faecalis* produces a capsule of a neutral-charged polyglucose polymer, curdelin, which accumulates uranyl and lead ions (Beveridge, 1989).

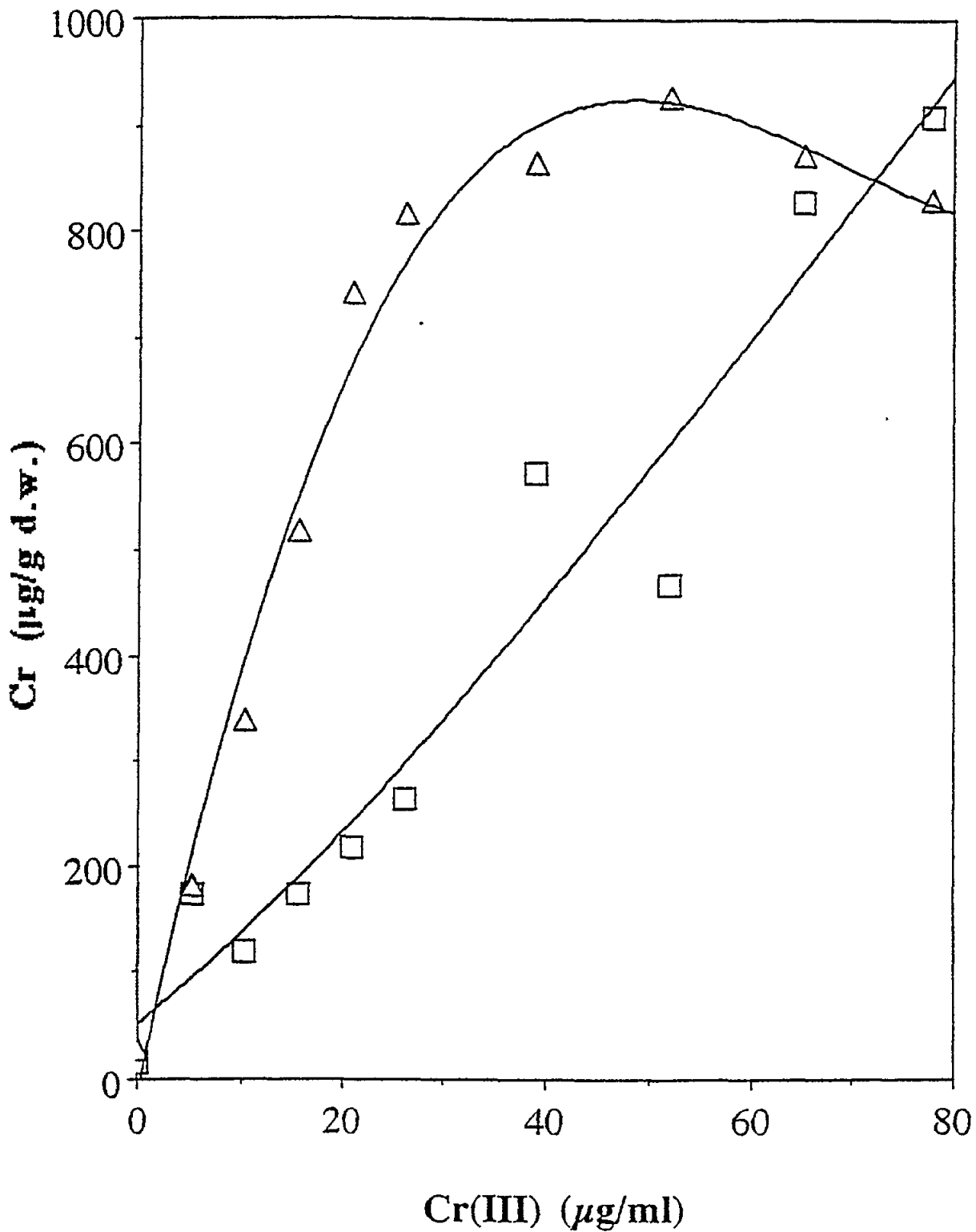


Fig. 2 Cr concentration ($\mu\text{g g}^{-1}$ dry weight) in polysaccharide extracted from coryneform strain 3-31 (□) and Enterobacteriaceae strain 4/6 (Δ) when grown on plate count agar amended with increasing concentration of Cr(III) (modified from Aislabie and Loutit, 1986)

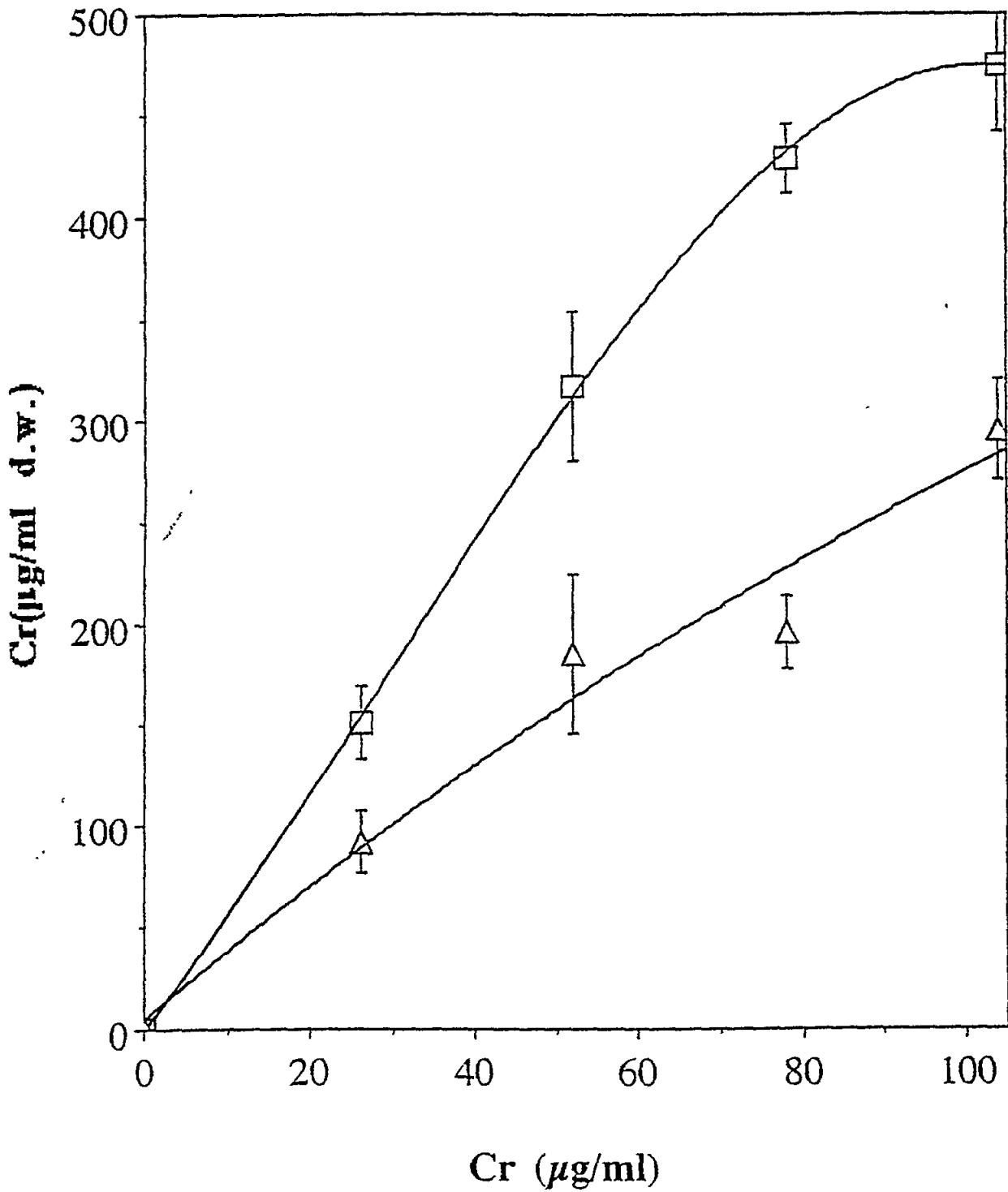


Fig. 3 Cr concentration ($\mu\text{g g}^{-1}$ dry weight) accumulated by coryneform strain L11⁺ (\square) and its non-mucoid variant L11 (Δ) at 15°C and increasing concentrations of Cr(III) (modified from Aislabie and Loutit, 1986)

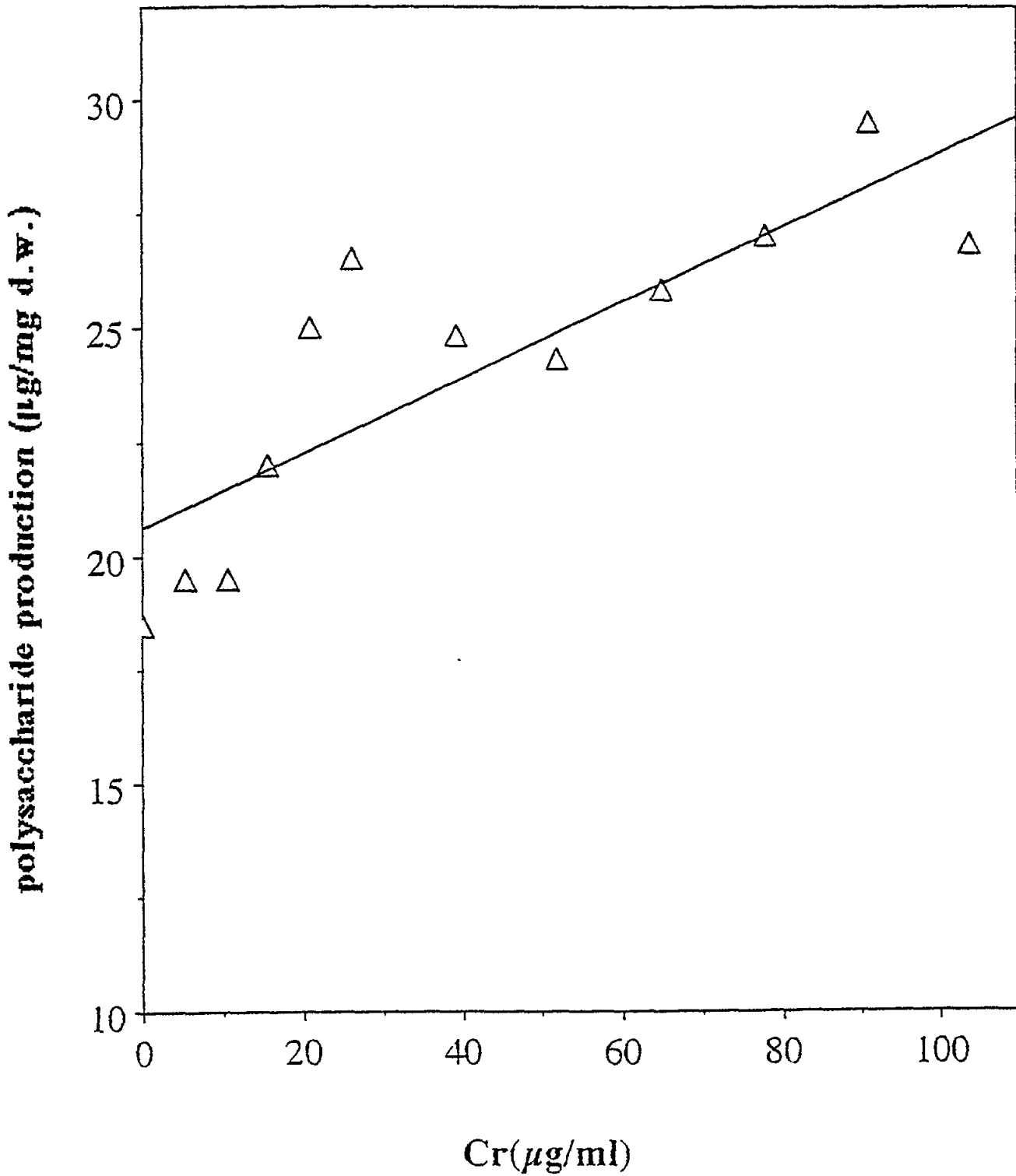


Fig. 4 Effect of increasing concentration of Cr(III) on the polysaccharide production (µg of glucose equivalent units per mg of dried cells) of coryneform strain 3-31 (from Aislabie and Loutit, 1986)

Coleman and Paran (1983) isolated 362 strains from Cr-polluted soil and selected them for Cr(VI) tolerance. The range of tolerance was found to be from $50 \mu\text{g ml}^{-1}$ to $450 \mu\text{g ml}^{-1}$. All strains resistant to $>100 \mu\text{g ml}^{-1}$ of Cr(VI) formed large mucoid colonies, especially isolates Nos. 96 and 227, which were identified respectively as an Arthrobacter sp. and an Agrobacter sp. Strain No. 96 was chosen because it was resistant up to $450 \mu\text{g ml}^{-1}$ of Cr(VI) and strain No. 227 because it took up greater quantities of chromate. Both strains accumulate large amounts of Cr and for the Arthrobacter the uptake was related to Cr(VI) concentrations in the medium, whereas for the Agrobacter the uptake was comparable with that of Arthrobacter only at low concentrations of Cr(VI). At higher concentrations of the oxyanion, the accumulation rate decreased. No further investigations were made to detect polysaccharide production, even though the authors noticed that both species formed mucoid colonies.

3.3 Chromium tolerance related to sulphate transport

Marzluf (1970) selected mutants of Neurospora crassa, an ascomycetes, by UV irradiation of its spores. All the mutants were Cr(VI) resistant and all of them had partially defective sulphate transport, especially during the conidial stage. It turned out that this microorganism had two sulphate transport systems. Sulphate permease I° was produced mainly in the conidial stage and the genes were located in chromosome 1. Sulphate permease II° was produced mainly in the mycelian phase. Both permeases were repressed by methionine. This amino acid acts as corepressor for severe repression of related enzymes, including aryl-sulphatase, choline sulphatase, choline-O-sulphate permease, and sulphate permease (Metzenberg and Parson, 1966) and conferred resistance to Cr(VI).

A similar mechanism was further investigated in a wild-type Pseudomonas fluorescens, strain LB300, isolated from Hudson River sediments (Bopp et al., 1983). Reduced uptake of chromate was detected and this feature was related to the sulphate transport system (Ohtake et al., 1987). The strain LB300 with plasmid pLHB1 and its plasmidless Cr(VI) sensitive variant LB303 were grown with different concentration of cysteine and djenkolic acid. The radioisotope $^{51}\text{Cr(VI)}$ was transported by active sulphate transport; thus, cells grown with 0.15 mM cysteine, a repressor of sulphate transport, were much more resistant to chromate than those grown with 0.15 mM djenkolic acid, a derepressant of the sulphate uptake system. Kinetics studies of Cr(VI) uptake by P. fluorescens with and without the plasmid showed that the V_{max} for $^{51}\text{Cr(VI)}$ uptake with the resistant strain was 2.2 times less than V_{max} for the sensitive strain (Ohtake et al., 1987).

In yeasts Cr(VI) toxicity was also related to the sulphate transport system. Baldi and Pepi (1991) investigated the mechanism of Cr(VI) resistance of two yeasts: Candida sp. DBVPG 6502 and Rhodospiridium sp. DBVPG 6662. These species were isolated from a tannery (Baldi et al., 1990) and from metallurgical wastes respectively. Neither accumulated Cr(VI) after 24 h of exposure from 0.5 to $10 \mu\text{g ml}^{-1}$ of Cr(VI), whereas the control Cr(VI) sensitive yeasts with similar physiological and morphological characteristics to Candida 6502 accumulated 5 times more. A Cr(VI)-sensitive strain of Saccharomyces cerevisiae from the pharmaceutical industry took up 10 times more Cr than the Cr(VI)-resistant yeasts (Fig. 5). In strains Candida 6502 and Rhodospiridium 6662, Cr(VI)-resistance was constitutive and was related to the sulphate

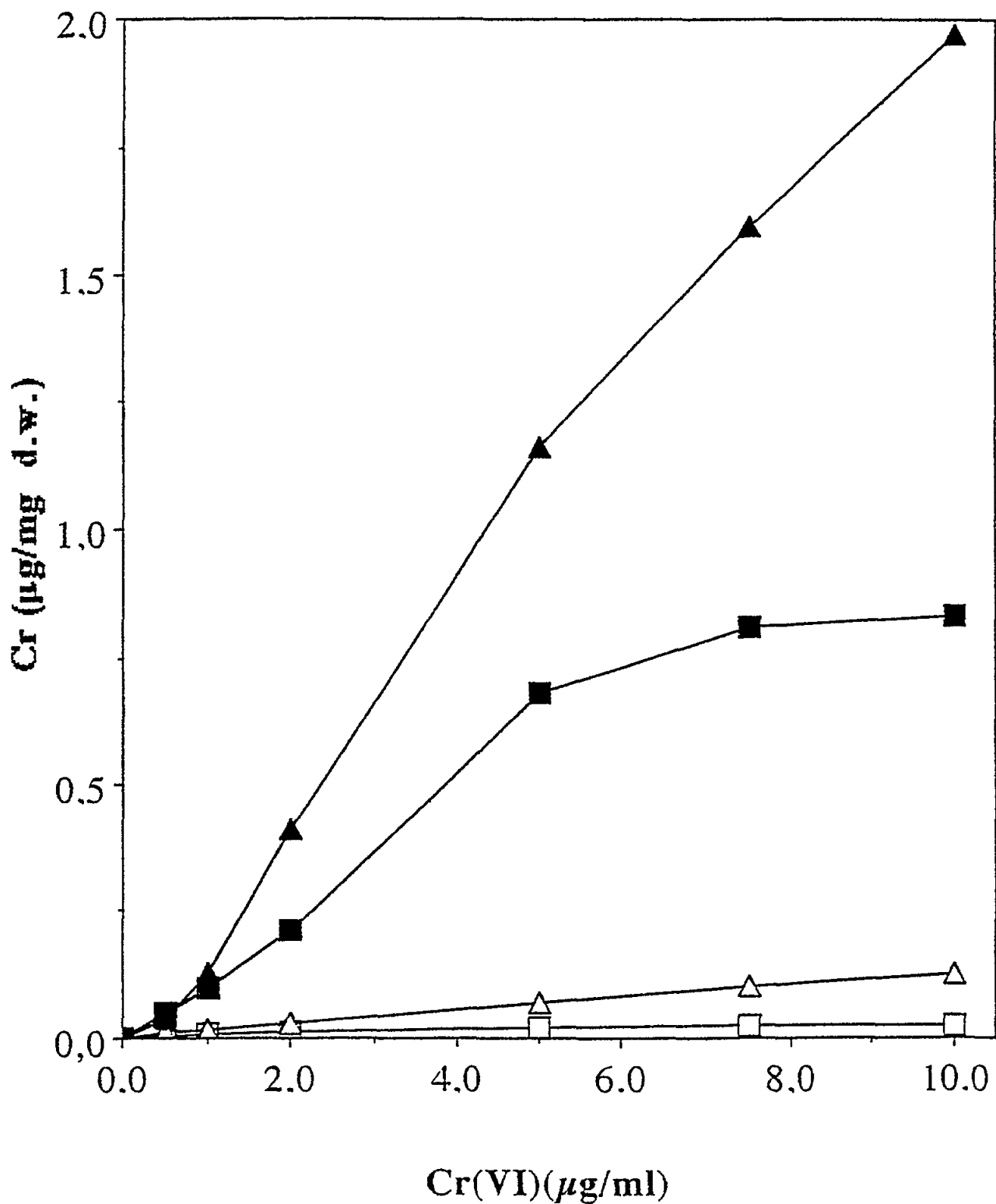


Fig. 5 Uptake of chromium by Cr-sensitive (*Saccharomyces cerevisiae* (▲) *Candida famata* 6016 (■)) and by Cr-resistant strains (*Candida sp.* 6502 (□) and *Rhodosporidium sp.* 6662 (△)) with increasing concentrations of Cr(VI) in media after 24 h of exposure (from Baldi and Pepi, 1991)

uptake system. Rhodospiridium 6662 did not grow in minimal medium (Yeast-Nitrogen Base) with glucose as carbon source and with sodium sulphate as sulphur source, but grew well with cysteine, methionine and even with sodium thiosulphate. Candida 6502 grew well in minimal medium with sulphate, but in the presence of Cr(VI) ($10 \mu\text{g ml}^{-1}$) became Cr-sensitive and with djenkolic acid, a stimulant of sulphate transport, hypersensitive. If cysteine was added to the medium, Candida 6502 again became Cr(VI) resistant (Fig. 6). Addition of djenkolic acid to the Rhodospiridium culture with Cr(VI) was ineffective. Cr(VI)-resistance in Candida 6502 and Rhodospiridium 6662 also seemed related to be related to good utilization of sulphurized aminoacids, which did not occur in Cr(VI)-sensitive strain. For example, in Candida famata DBVPG 6016 cysteine was toxic at a concentration of 0.05 mM. Both Candida 6502 and Rhodospiridium 6662 were also very resistant to selenate (125 mM) (a sulphate analogue), whereas the Cr(VI) sensitive yeasts showed a similar minimum inhibitory concentration of 0.1 mM of Se(VI).

Anabena doliolum, a N_2 -fixing cyanobacteria, was exposed to different concentrations of Cr(III) as chromium dioxide (Dubey and Ray, 1989). At $40 \mu\text{g ml}^{-1}$ of Cr(III) CO_2 uptake was improved by the addition of sulphur containing aminoacids: cysteine, methionine and cystine. Attenuation of Cr(III) toxicity was also produced by the addition of thiols such as mercaptoethanol and dithiothreitol. Dithiols were more protective than monothiols and cysteine, while methionine was better than cystine. Nitrogenase activity and heterocyst (a specialized N_2 fixing cell) frequency was also restored by adding sulphur containing aminoacids and thiols. The Cr tolerance investigated in this experiments is probably related to the inhibition of sulphate transport in A. doliolum, although it is not specifically investigated, it is well known that organic sulphur compounds such as methionine and cysteine inhibit the sulphate transport system (Metzenberg and Parson, 1966).

3.4 Cr(VI) reduction

Elderfield (1970) reported that from according to a thermodynamic reasoning hexavalent chromium should predominate in seawater, but in a study of the coastal waters of Wales, he found that Cr was present predominantly as Cr(III). He suggested that microorganisms may be responsible for chromate reduction. Smillie et al. (1981) studied Cr(VI) reduction in marine environments by bacteria-produced H_2S . In Sawyer Bay, Otago harbour (New Zealand) most of the Cr and organic matter from a tannery was deposited within 100 m of the outlet. In an experiment 1 mg l^{-1} of Cr(VI) was added to a flask containing 1 l of seawater collected from a coastal area with high concentrations of microbial H_2S . The same amount of Cr was added to another flask from the same site but was aerated to remove as much H_2S as possible, and also to a control flask with seawater but no H_2S . The Cr(VI) was significantly reduced to Cr(III) after 24 h only in the flask with the original seawater sample (Table 2). Certainly the conversion of Cr(VI) to Cr(III) by H_2S is thermodynamically favoured (Elderfield, 1970), but in separate studies it has been demonstrated that biological reduction of Cr(VI) to Cr(III) is a detoxification process. There is evidence of both aerobic and anaerobic reduction systems with different microbes.

Pseudomonas and Aeromonas strains have been reported to anaerobically reduced Cr(VI) to Cr(III). Horitzu et al. (1983) showed that a Cr(VI)-sensitive derivative (S-1) of a Cr(VI)-resistant strain of Pseudomonas ambigua

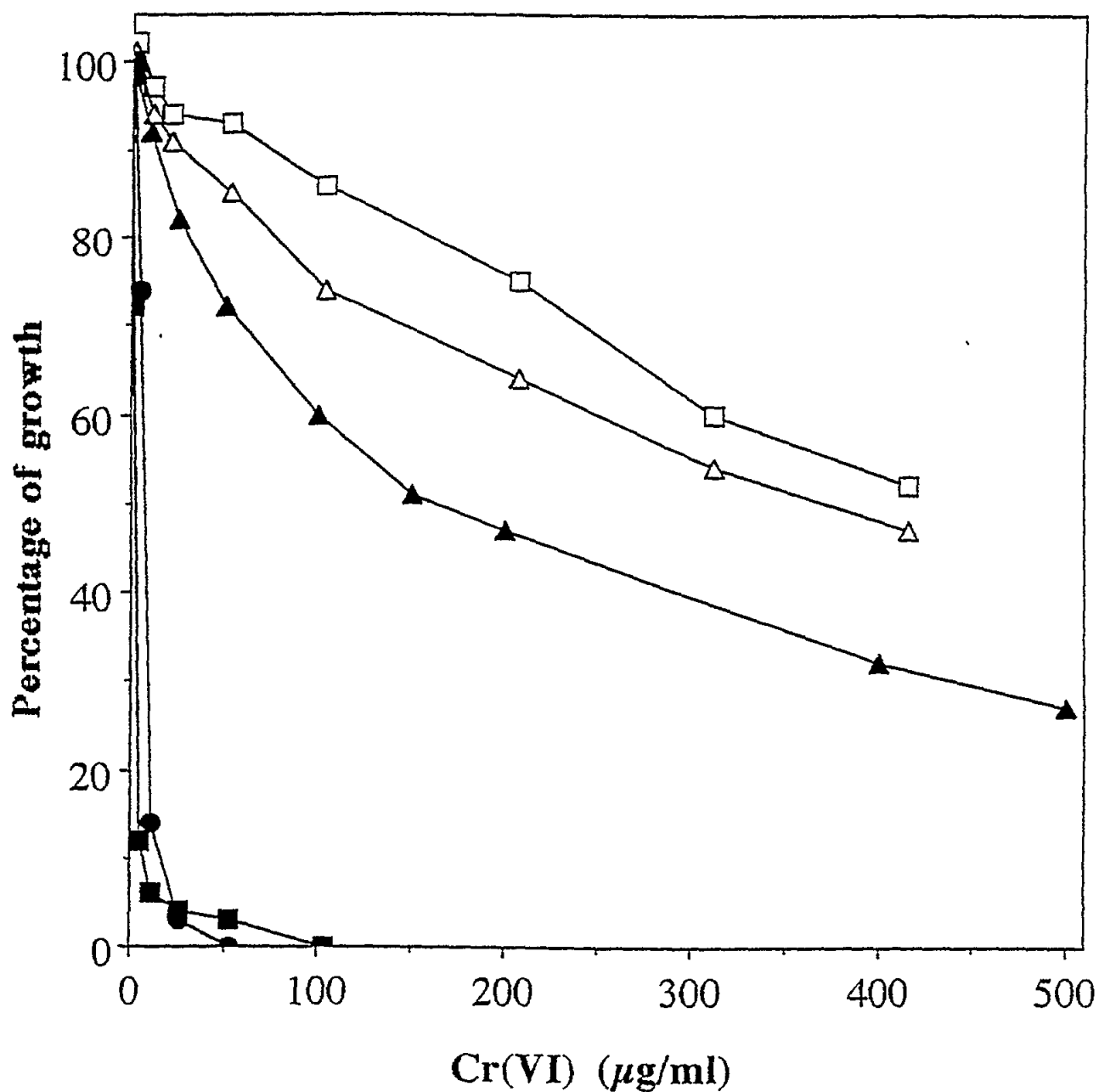


Fig. 6 Minimum inhibitory concentrations (MICs) obtained by additions increasing concentrations of Cr(VI) in different media: Yeast Nitrogen Base (YNB), 2% glucose, and 0.16 M of sulphates, plus 0.1 mM of cysteine (Δ) and plus 0.2 mM of djenkolic acid (\square) and both inoculated with *Rhodosporidium* sp. 6662. Besides, YNB, 2% glucose, and 0.16 M of sulphates (\bullet) or plus 0.2 mM of djenkolic acid (\blacksquare), or plus 0.1 mM of cysteine (\blacktriangle), were inoculated with *Candida* sp. 6502 (modified from Baldi and Pepi, 1991)

(G-1) accumulated six times more Cr than the resistant parental strain. The mutant strain showed thinning of the cell envelope, suggesting that the membrane barrier for the permeation of Cr(VI) might be injured. Later, a Cr(VI)-reducing enzyme was detected in the cell-free extract of the parent G-1. In the Cr(VI)-sensitive mutant S-1 the specific activity of this enzyme had dropped to about one-fourth.

Table 2

Reduction of Cr(VI) to Cr(III) as potassium dichromate (mg l^{-1}) by hydrogen sulphide produced by bacteria in natural seawater (from Smillie et al., 1981).

	No H ₂ S	H ₂ S oxidized by aeration	H ₂ S present
Concentration S-2 mmol l^{-1}	0.0	0.09	2.8
At zero time			
total Cr	1.99	1.89	1.86
Cr(III)	ND	ND	ND
After 24 h			
total Cr	1.92	1.88	1.77*
Cr(III)	0.03	0.26	1.02

(*) Some loss of total Cr was due to deposition with colloidal sulphur onto the side of the flask. (ND) - not detectable at $10 \mu\text{g l}^{-1}$

Bopp and Ehrlich (1988) showed that the P. fluorescens strain LB300, which has lowered Cr uptake (Bopp et al., 1983), is also capable of Cr(VI) reduction in aerobic and anaerobic environments with glucose as electron donor. When growing in a liquid glucose-mineral salt, P. fluorescens converts Cr(VI) to Cr(III). Cell-free extract reduces chromate only when acetate is available as energy source. Furthermore, although P. fluorescens LB300 will reduce Cr(VI) aerobically at an initial concentration up to at least $100 \mu\text{g ml}^{-1}$, it will reduce chromate anaerobically only at concentrations below $50 \mu\text{g ml}^{-1}$. By contrast P. dechromatica and P. chromophila will only reduce Cr(VI) anaerobically with organic electron donors but not glucose (Ehrlich, 1990).

Kvasnikov et al. (1985) reported a bacterium belonging to the Aeromonas genus, isolated from Cr-polluted industrial wastes. Because the strain has a mixed type of flagellation and reduces Cr(VI) at a high rate, it was classified as Aeromonas dechromatica.

Wang et al. (1989) isolated an Enterobacter cloacae strain H01 from activated sludge. It was resistant to Cr(VI) under aerobic and anaerobic conditions, and could grow at chromate concentrations exceeding 10 mM chromate. Only the anaerobic culture of E. cloacae showed a rate of Cr(VI) reduction dependent on cell density. With $5 \times 10^8 \text{ cells ml}^{-1}$, 0.5 mM Cr(VI) was completely reduced within 5 h. The rate of Cr(VI) reduction was dependent

also on Cr(VI) concentration; complete Cr(VI) reduction at 3 mM did not occur. This E. cloacae probably utilized Cr(VI) anaerobically, as an electron acceptor because: a) the anaerobic growth of OH1 cells accompanied the decrease of Cr(VI) in the culture medium; b) Cr(VI) reducing activity was rapidly inhibited by O_2 and c) the reduction occurred more rapidly in glycerol or acetate-grown cells than in glucose-grown cells. Ohtake *et al.* (1987) reported that Cr(VI) resistance in P. fluorescens LB300(pLHB1) was related to the decreased uptake of Cr(VI) under aerobic condition. Decreased Cr(VI) uptake is probably also the mechanism of Cr(VI) resistance in E. cloacae HO1 under aerobic conditions.

The Cr(VI) reductase activity is membrane-associated in P. fluorescens (Bopp and Ehrlich, 1988) and in E. cloacae (Wang *et al.*, 1990). Ishibashi *et al.* (1990) found that Cr(VI) reductase activity in Pseudomonas putida was associated with soluble protein (Fig. 7). The crude enzyme activity was heat labile and showed a K_m of 40 μM of Cr(VI).

3.5 Other tolerances to chromium toxicity

Clover seedlings of Trifolium incarnatum inoculated with Rhizobium leguminosarum biovar trifolii and treated with increasing concentrations of Cr(III) up to 100 $\mu g\ ml^{-1}$ affects the size of root nodules, although their number was higher than in untreated roots. In addition a Cr(III) concentration exceeding 10 $\mu g\ ml^{-1}$ decreased specific nitrogenase activity (Casella *et al.*, 1988). Plant-partner symbiosis is increased by chromium and the free-living rhizobia tend to enter the root, probably to protect themselves from Cr toxicity.

4. CONCLUSIONS

The environmental input of chromium is mostly anthropogenic. The metal is transported to the sea from tannery, metallurgical and paint factory outlets. The role of microorganisms in chromium interactions is very important in natural and especially marine environments, where high concentrations of sulphate (28 mM) occur. Chromate transport into microorganism cytoplasm has so far always been related to sulphur demand.

In coastal areas, Cr(VI) is reduced in sediments and the water column by sulphate reducing bacteria, which utilize sulphate as electron, producing H_2S . No chromium sulphide is ever produced by this reaction; there is only chemical reduction of Cr(VI) to Cr(III) followed by its precipitation to the bottom where it is buried in the sediment. Studies on Cr removal from sediment are scarce and little data is available. It has been demonstrated in laboratory experiments that Cr can be leached from chromite ($FeCr_2O_4$) by the chemolithotrophic acidophilic Thiobacillus thiooxidans (Ehrlich, 1983). Loutit and Pillidge (1986), observed that Cr(III) is removed from sediment by the biological activity of heterotrophic bacteria from Sawyer Bay sediment. Bartlett and James (1979) showed that Mn(IV) can interact with Cr(III) in soil to oxidize this form to the more soluble Cr(VI). This interaction should be investigated further, especially in estuarine sediments, where common Mn(II)-oxidizing bacteria precipitate Mn(IV) as oxide. This Mn species might then oxidize back from Cr(III) to the toxic Cr(VI).

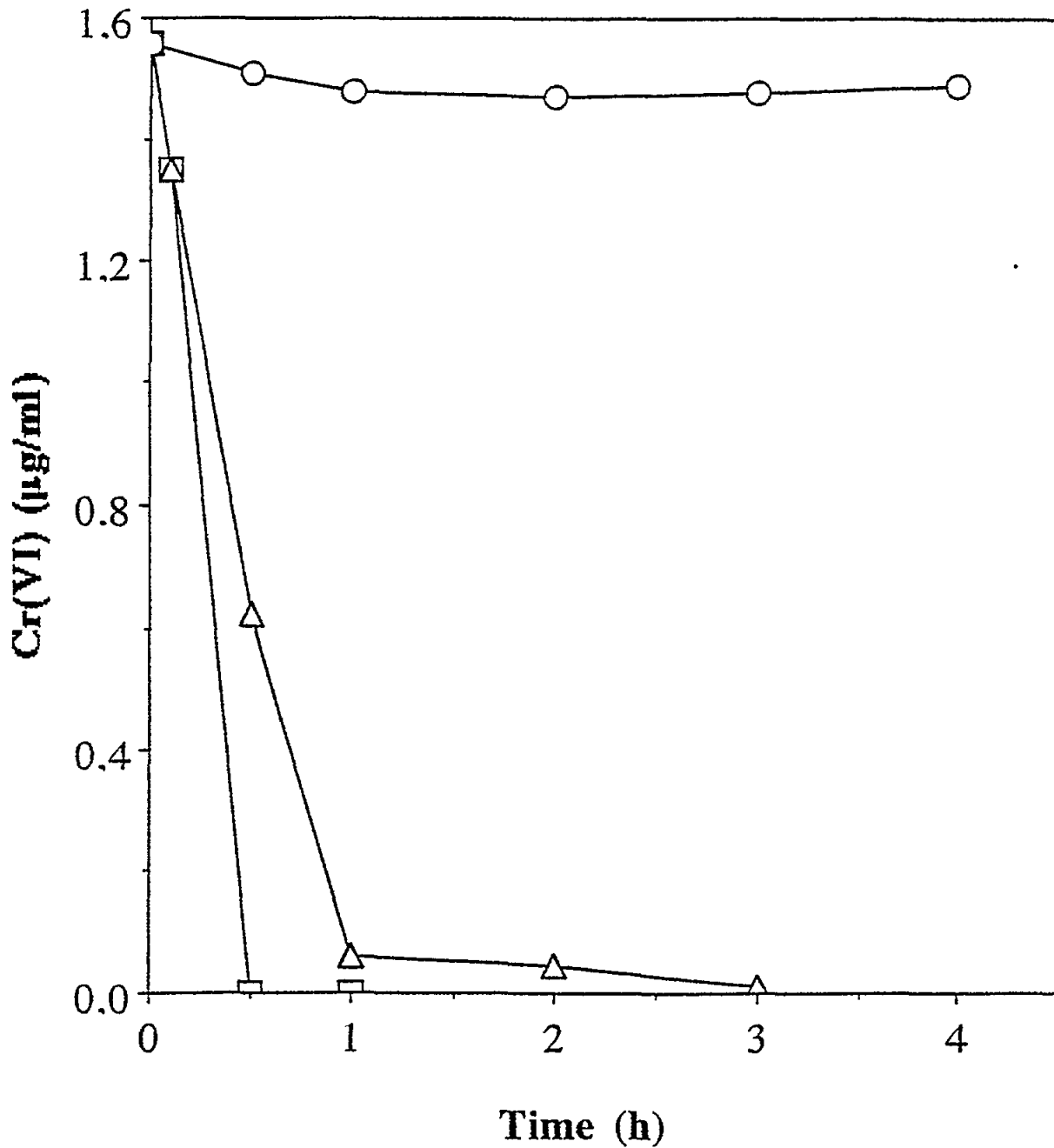


Fig. 7 Cell-free chromate reduction was measured in cell fractions from strain *Pseudomonas putida* PRS2000. Cells were disrupted by French Press, and after various centrifugation and ultracentrifugation passages, equivalent volume of cell extract (\square) supernatant fluid (Δ) and resuspended membranes (O) were tested spectrophotometrically for Cr(VI) reduction by adding 200 μM NADH and 30 μM of K_2CrO_4 (from Ishibashi *et al.*, 1990)

Further research is needed, especially in the marine environment, where bacteria are induced to produce exopolysaccharides from interactions with Cr(III). Today in the Mediterranean Sea, especially around the Italian coast, polysaccharides are produced in large quantities in summertime. Little is known about this phenomenon. The manner in which Cr(III), the most common species in the marine environment, interacts with the polysaccharide metabolism of marine microorganisms, would be an interesting field of study.

The response of the microbial community to high concentrations of different forms of chromium is also an interesting topic, which could be developed using Cr-resistant bacteria as bioindicators of Cr pollution in aquatic and terrestrial environments, in the same way as for mercury (Baldi *et al.*, 1991). There has recently been a new development in the area of pollution monitoring: whole-cell bacteria can be used in new biosensors (Rawson *et al.*, 1989), in which the biological signal is transduced into optical or electronical signals. A further advance in heavy metal biosensors can be achieved by improving the biosensing agent: for example a Cr-resistant strain can be engineered for "lux fusion", namely, the gene cluster of chromate resistance can be fused with the gene for luciferase synthesis. When Cr(VI) is present in the environment, the immobilized biosensing microorganism will induce Cr(VI)-resistance and photons by the luciferase reaction at the same time. These photons are related to Cr(VI) content and can be detected and amplified by a variety of detectors.

Studies in this area are promising and will certainly lead to new applications in environmental and basic research.

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CARCINOGENIC AND MUTAGENIC POLLUTANTS:
IMPACT ON MARINE ORGANISMS

by

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A B S T R A C T

Short-term biomarkers could be applied to predict the impact of pollution on marine organisms. These include the early biological events of the carcinogenic process, such as DNA damage, gene and chromosome mutations. Our studies attempt to evaluate the evidence of genotoxic effects in mussels. The abundance, wide distribution and the sedentary nature of mussels suggest that this species may have a general usefulness as a biomonitor. The genotoxicity has been evaluated in terms of DNA damage and frequency of micronuclei in gill cells of mussels sampled by polluted and unpolluted sites. We have analyzed specimens of Mytilus galloprovincialis collected from different locations in the Ligurian sea, and have observed a significant increment of the two parameters in polluted compared with the unpolluted sites. Evidence of DNA damage could reflect the occurrence of recent pollution. Conversely, an increased micronucleus frequency persists for a very long time after an exposure to pollution. The evaluation of both parameters could provide information of great significance about the pollution status of waters.

1. INTRODUCTION

Over 9 million chemicals are listed on the Chemical Abstract Service's Registry of Chemicals, although only an estimated 76,000 are in common use (Cairns and Mount, 1990). The marine environment is the recipient of many of these chemicals, generally at very low concentrations. Many potentially hazardous substances are quickly transformed to less hazardous intermediates or are eliminated from the aquatic ecosystem. However, many chemicals are persistent and water and sediments in heavily polluted areas may contain a variety of carcinogenic and mutagenic compounds.

Marine organisms are able to bioconcentrate lipophilic organic compounds to levels thousands of times greater than the concentrations in the water in which they live. As a possible result of this contamination, several pathological conditions associated with pollutant stresses may occur in aquatic animals.

Neoplasms have been observed in many marine species and they affect virtually all organs.

Epizootic neoplasms are known to occur in localized populations of several bottom-feeding and bottom-dwelling fish exposed to sediments containing toxic chemicals, from US East and West coast and Japan (Bolognesi,

1990). Recent studies on the etiology of hepatic neoplasms in English sole suggest that there is a direct link between the exposure to sediment-associated contaminants, mainly aromatic compounds, and the development of hepatic neoplasms (Myers et al., 1990).

Neoplasms analogous to a cancerous growth in vertebrates were shown to occur in bivalve molluscs (Table 1).

A high incidence of neoplastic diseases can occur in bivalves living in highly polluted waters. These studies generally lacked quantitative information of water quality, so the correlations between prevalences of tumors and environmental concentrations of toxic chemicals could not be evaluated.

Short-term biomarkers have been applied to predict the impact of carcinogens on marine organisms. Their endpoints are different effects at the molecular and cellular level, such as mutations and induction of DNA damage and repair. In order to select an aquatic organism, as an indicator of water pollution by carcinogenic agents, we have focussed on the mussel. These wide and abundant species are likely to be found in all aquatic ecosystems. Moreover, the sedentary nature of these organisms results in continual exposure within a specific microhabitat, compared to fish which tend to migrate over a much greater distance. Therefore, our studies have attempted to evaluate the genotoxic effects induced by marine pollutants in mussels.

These effects are measured in terms of DNA damage and frequency of micronuclei in gill cells from mussels at polluted and unpolluted sites.

2. MATERIALS AND METHODS

2.1 Mussel collection

Our sampling stations (1-4) were located in the La Spezia Gulf, Ligurian sea (Fig. 1). These sites are highly polluted by sewage and industrial plant discharges. Mussel farms at the inner sites of the La Spezia Gulf breakwater are considered to be a reference area (RA).

Only adult specimens of Mytilus galloprovincialis (major axis about 5-6 cm) collected from natural substrates were used. The samples were transported on ice and immediately processed for the evaluation of the genotoxic effects. Gills were excised and cells were isolated by enzymatic digestion in a solution of dispase-HBSS for 20 minutes at 37°C. The cellular suspension obtained was centrifuged at 1,000 rpm for 5 minutes.

2.2 Alkaline elution of DNA

The alkaline elution procedure was carried out as previously described (Bolognesi et al., 1985). The elution was performed under reduced light using Durapore filters (25 mm diameter, 0.65 μ m pore size) placed on filter-holders (Millipore Corp., USA). The elution chambers were connected by silicone tubes to a 4-channel peristaltic pump having a flow-rate of 0.15 ml min⁻¹. The nuclear suspension was first washed with the saline solution. The nuclei were

Table 1

A summary of surveys on neoplasms in Mytilus edulis.

GEOGRAPHIC LOCATION	NEO-PLASMS	NUMBER EXAMINED (% AFFECTED)	POSTULATED CAUSE	REFERENCE
UNITED STATES				
Pacific Northwest				
Oregon	Hemic	100 (10.0%)	None	Farley, 1969
Yaquina Bay	Hemic	1.861(9.8%)	None	Mix, 1982; 1983
Yaquina Bay	Hemic	1.073(0.4%)	None	Mix, 1982; 1983
Yaquina Bay	-	600(0.0%)	-	Mix, 1982; 1983
Washington	-	372	(0.0%)	Pauley, 1969
Sequim Bay				
CANADA				
British Columbia	Hemic	180(28.9%)	None	Emmett, 1984
Nanaimo				
Bamfield	Hemic	159(17.6%)	None	Emmett, 1984
Cowichan Bay	Hemic	66(19.7%)	Virus	Cosson-Mannery et al., 1984
McLoughlin Pt.	Hemic	50(8.0%)	Virus	Cosson-Mannery et al., 1984
Hatch Pt.	Hemic	50(8.0%)	Virus	Cosson-Mannery et al., 1984
ENGLAND				
Plymouth	Hemic	994(1.6%)	None	Lowe and Moore, 1978
Twenty-one coastal sites	Hemic	4470(0.6%)	None	Green and Alderman, 1983

then lysed at room temperature, using 5 ml of a solution containing 2.0 M NaCl, 0.02 M Na₃EDTA and 0.2% sarkosyl, pH 10.2. The pellet was washed with 2.5 ml of 0.02 M Na₃EDTA, pH 10.2. Single-stranded DNA fractions were then eluted through membrane micropores with 20 ml of a solution containing 0.04 M EDTA (acid form), tetraethylammonium hydroxyde (TEA, 10% in water) to provide a pH of 12.3.

Fractions were collected at 6-min intervals for 1 h at a flow rate of 0.15 ml min⁻¹. At the end of the elution step, the membranes were removed and the DNA retained was solubilized with 4 ml of the elution buffer. Aliquots of 1 ml from each fraction and 1 ml from the solution prepared after recovery of the DNA left on the filter were used for the fluorometric microdetermination of the DNA content. Samples were neutralized with 0.2 M KH₂PO₄, pH 4.8, to a pH value in the range 7-7.5.

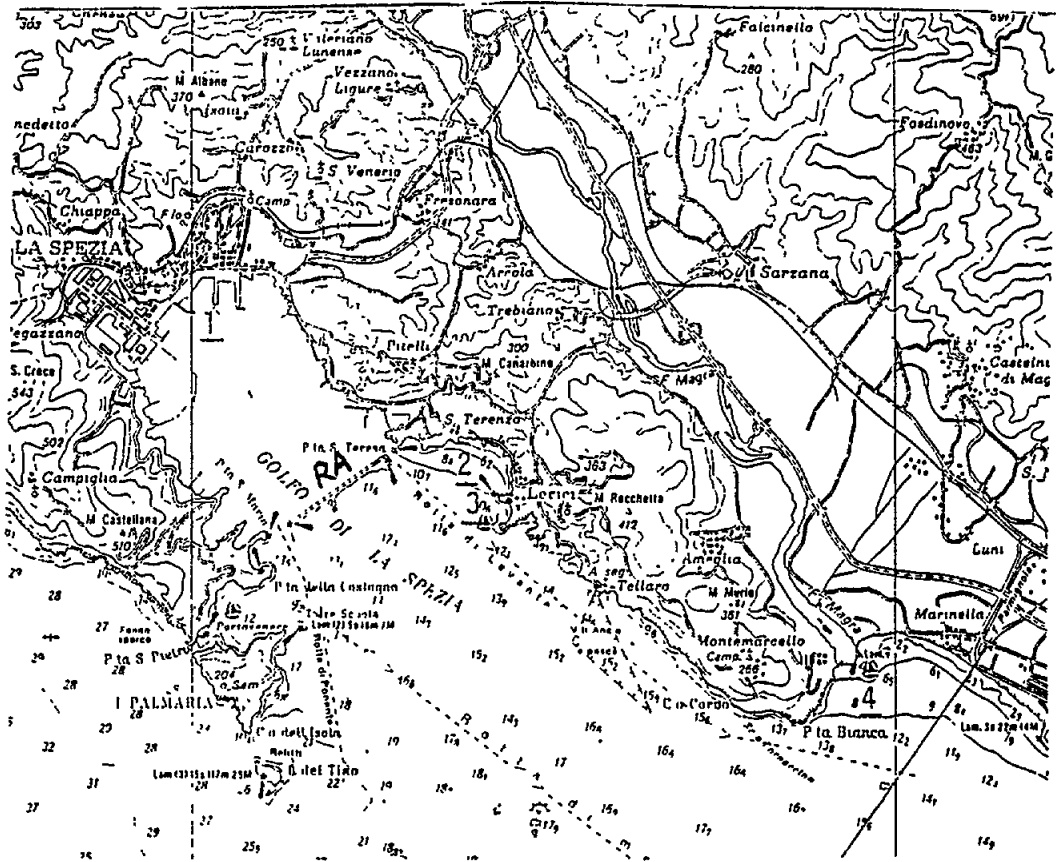


Fig. 1 Map showing the sampling stations in the Gulf of La Spezia

The fluorometric microdetermination of DNA was performed with 33258 Hoechst, using the method developed in our laboratory and described elsewhere (Cesarone *et al.*, 1979). 1 ml of reagent (33258 Hoechst 3.0×10^{-6} M in saline citrate buffer SSC) was added to the neutralized sample solution diluted to 2 ml. The tubes were stirred and the increased fluorescence, obtained after fluorochrome binding to DNA, was determined 10 min. later in an Hitachi-Perkin-Elmer spectrophotofluorometer mod 202 A with the excitation set at 360 nm and the emission recorded at 450 nm. The elution profiles were plotted as DNA left on the filter versus the eluted volume, and the results were expressed as elution rate constant (K):

$$K(\text{ml}^{-1}) = \frac{-\ln \text{fraction of DNA retained on the filter}}{\text{eluted volume}}$$

This parameter was computed on the first fractions to avoid artifacts due to prolonged exposure of DNA to alkali, which may induce additional breaks in the molecule. The standard deviation and the non-parametric test of Mann-Whitney (Siegel, 1956) were used for the statistical analysis.

2.3 Micronuclei

Aliquots of the cellular pellets obtained from mussel gills were fixed in methanol:acetic acid (3:1) for 20 min then centrifuged at 1,000 rpm for 10 min. The resuspended cells were spread on slides, air dried and stained with 3% Giemsa. Two thousand cells with preserved cytoplasm per mussel were scored to determine the frequency of micronuclei.

3. RESULTS

Table 2 shows the preliminary results for the frequencies of micronuclei and DNA damage, expressed as elution constants in marine mussels from different sites of the La Spezia Gulf.

We observed a significant increment of the two parameters in polluted in comparison with the unpolluted sites, except for station 1.

The low extent of genotoxic effects observed in mussels from La Spezia harbor is clearly in contrast with the high degree of pollution present. These results might be due to the presence of lethal concentrations of genotoxic pollutants. The specimens collected might represent the most resistant to the toxic agents. Similar results are reported in a study performed on marine mussels collected from stations in the Venetian Lagoon (Brunetti *et al.*, 1988). Relatively high frequencies of micronuclei are scored in stations 2, 3 and 4. These values are similar to those found in mussels exposed for 48 hrs to benzo(a)pyrene in aquarium (Scarpato *et al.*, 1990).

Table 2

Frequency of micronuclei and DNA single-strand breaks evaluated in gill cells of marine mussels from different sites of the La Spezia Gulf.

Station	No. of micronucleated ^a cells/1000	Elution constant ^b K(ml ⁻¹)X10 ³
1.(La Spezia Harbour)	4.0 ± 1.41*	42 ± 9*
2.(S.Terenzio)	21.5 ± 0.7	94 ± 17
3.(Lerici)	17.0 ± 8.5	62 ± 9
4.(Magra River Estuary)	30.5 ± 12.02	73 ± 8
Reference area	3.5 ± 2.16	27 ± 10

a. mean ± SD of 3 samples (2000 cells scored per sample)

b. mean ± SD of 4 samples

The values were significantly different from the reference site (P<0.005) by the Mann-Whitney test (Siegel, 1956): * not significant.

Moreover, a significant amount of DNA damage was observed in gills of mussels from stations 2, 3 and 4. The extent of DNA breakage was less relevant with respect to clastogenic damage, as revealed by micronucleus test. The greatest value of K was about 3-fold higher with respect to the figures obtained in the same tissue of mussels from reference areas.

4. DISCUSSION

The mussels have been widely used as bioindicators of marine pollution. It is well known that these species concentrate many genotoxic compounds (PAH, heavy metals, chlorinated compounds) that are expected to be present in polluted waters.

It has been shown that xenobiotics' uptake can occur in gills. Bioaccumulation of toxic compounds was observed in this tissue (Nolan *et al.*, 1984). The extent of genotoxic effects, evaluated in gills of mussels could reflect the quality of their respective environment. A relationship between accumulation of carcinogenic agents, such as polycyclic aromatic hydrocarbons in mussel tissues and occurrence of genotoxic events, in terms of DNA damage and frequency of micronuclei, has been observed (Bolognesi *et al.*, 1990).

It has been demonstrated that mussels have some potential for metabolizing promutagens into genetically active metabolites (Stegeman, 1985; Kurelec *et al.*, 1986; Livingstone, 1987).

Chemical carcinogens are transformed to DNA reactive forms leading to lesions in target-cells DNA. DNA-damage can be converted into mutations and related biological effects, cell killing, chromosomal aberrations and sister chromatid exchanges.

Alkaline elution technique, first performed by Kohn *et al.* (1976), measures DNA damage, as breaks or weak points in alkali in single-stranded DNA. DNA breaks can be repaired by different mechanisms. The time taken for DNA repair differs with the damaging agents. It has been demonstrated that mussels exposed to alkylating agents, such as methylnitrosourea, or ethylnitrosourea, showed a significant level of DNA alterations. Incubation periods of up to 72 hrs resulted in a reduction of the DNA damage, providing evidence for the repair capacity of mussels (Turner and Parry, 1989). Therefore, evidence of DNA damage could reflect a recent pollution status of the marine environment. The micronucleus assay has been utilized to detect chromosomal damage due to both clastogenic effects and interference with the normal segregational mechanism of the cells. Animals exposed to mutagenic compounds may exhibit increased micronucleus frequency long after the exposure has ceased. Studies on experimental animals indicate that elevated levels of micronucleated cells in liver and bone marrow persist for up to 60 days from the exposure to the clastogenic agents (Tates *et al.*, 1983; Rithidech *et al.*, 1988). A recent study (Majone *et al.*, 1990) demonstrated that the micronucleus frequency induced by genotoxic agents in gill cells of mussels decreased after treatment but it remains at a level significantly higher than the control value for up to 28 days. These considerations suggest the suitability of this test to monitor a genotoxic effect of marine pollutants which was caused long before the time of sampling.

These short-term assays, measuring different genetic endpoints, could be applied to monitor marine environment for the presence of genotoxic agents. The use of these tests is clearly restricted to the areas in which the environmental conditions do not induce high mortality rate.

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REFEREE'S COMMENTS

There is no correlation made between the results of the bioassays and the chemical constituents of the sediments and the water. Only a qualitative distinction is made of the pollution status of the sampling sites. Even so, the most "polluted" site provided a very low bioassay result. The explanation given, that of adaptation of the stock of mussels, may or may not be correct. Whatever the truth is, it does detract from the use of these bioassays as a predictor of the presence of potentially harmful chemicals.

AUTHOR'S REPLY TO REFEREE'S COMMENTS

The explanation that in the highly polluted areas the toxic effects could induce a drastic selection in the natural population is only an hypothesis.

This hypothesis could give the reason of the low results obtained in mussels from a highly polluted site, such as La Spezia harbor. A relationship between the concentration of key-pollutants and the biological effect observed in a marine species have to be established in the validation of a short-term test for the application in pollution monitoring.

Our previous studies had revealed a correlation between the extent of genotoxic effects and PAH concentration in samples collected in the polluted and in the reference areas.

Further research is necessary to define the role of these tests in the prediction of marine pollution.

BIOLOGICAL EFFECTS OF URANIUM AND TRANSURANIUM NUCLIDES
ON MARINE BIVALVES Mytilus edulis, Crassostrea gigas
AND Cerastoderma edule: MICROANALYSIS AT THE
CELLULAR AND SUBCELLULAR LEVELS

by

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A B S T R A C T

Investigations on the biological effects of uranium 238, plutonium 239 and americium 241 were performed on several marine bivalves: the marine mussel Mytilus edulis, the oyster Crassostrea gigas and the edible cockle Cerastoderma edule. Two microanalytical methods were employed: secondary ion mass spectrometry, using the ion microscope and the ion microprobe, and X-ray spectrometry using the electron microprobe associated with a transmission electron microscope. A post-acquisition image processing system was used in association with the ion microscope and the ion microprobe.

Mechanisms involved in the uptake, storage and elimination were studied with a special emphasis on cellular and subcellular aspects of the biological effects. Target organs were identified. The target organelles appeared to be the lysosomes where radionuclides were associated with phosphorus in an insoluble form. Moreover radionuclides were also observed free in the cytoplasm where they were likely to persist and to provoke injuries. Damages were observed within nuclei, together with a decrease of the mitochondria population. Data are discussed in connection with the transfer of radionuclides through the different levels of food chains leading to man, the final consumer.

1. INTRODUCTION

Humans are exposed to radioactive materials that enter the general environment. The mechanisms are often complex and an understanding of them for the purpose of risk assessment involves information that comes from many scientific disciplines, including biology, chemistry and oceanography. Humans can be exposed to environmental radioactivity in a number of ways and the most complex mechanisms are those that involve contamination of food chains.

The understanding of the behaviour of radionuclides within food chains is important and necessary on several accounts: they have long half-lives, are radiotoxic α emitters, and persist within the environment.

Natural radioactivity originates from extraterrestrial sources as well as from radioactive elements in the earth's crust. The naturally occurring

uranium isotopes ^{238}U , ^{235}U and ^{234}U are dissolved during chemical weathering and form the stable uranyl carbonate species in oxidizing aqueous environments. The flux of uranium to oceans can be determined from estimates of the concentration of ^{238}U (major isotope) in river water, assuming conservative behaviour in the estuarine regime. Although the uranium concentration in rivers varies considerably, the average value is approximately $0.3 \mu\text{g l}^{-1}$. It has been reported that the higher uranium values might be due to contamination from phosphate fertilizer processing plants and from phosphogypsum produced as by-product as well as from uranium mining activities.

Artificial radionuclides are introduced in the marine environment by way of nuclear-weapons testing, nuclear fuel cycle operations and accidental releases.

Among transuranium radionuclides, americium (^{241}Am) and plutonium (^{239}Pu) appear to be of a major interest. Both of them have been investigated in laboratory experiments to localize these elements in tissues and cells by histo autoradiography. Experiments were performed for americium on the european lobster Homarus vulgaris and the edible crab Cancer pagurus (Miramand et al., 1989), while other experiments concern the localization of americium and plutonium in the edible cockle Cerastoderma edule (Miramand and Germain, 1985), microanalytical techniques allow faster progress as they are more sensitive, not time consuming and bring complementary informations (Chassard-Bouchaud, 1991).

We focused our investigations on several marine edible bivalves such as the marine mussel Mytilus edulis, the oyster Crassostrea gigas and the edible cockle Cerastoderma edule.

2. MATERIAL AND METHODS

The following species were investigated:

Mytilus edulis: samples were collected from Carentan, not far from the reprocessing plant of La Hague.

Crassostrea gigas were uranium exposed in laboratory experiments, with ^{238}U concentrations likely to be found in sea water.

Cerastoderma edule were ^{241}Am and ^{239}Pu exposed in laboratory experiments, with concentrations likely to be found in sea water (Miramand and Germain, 1985).

The samples were dissected to separate the different tissues, which then underwent several treatments depending on the microanalytical technique to be used.

For Secondary Ion Mass Spectrometry (SIMS Ion Microscopy) tissues were fixed in formaldehyde, embedded in paraffin, cut to sections ($5\mu\text{m}$) and deposited on a highly pure gold specimen holder, with removal of paraffin. For X-Ray Spectrometry (Camebax Electron Microprobe) and for Electron Microscopy, samples were fixed in 2.5% glutaraldehyde in 0.2 M sodium dimethylarsenate buffer at pH 7.0. After washing with the buffer, the samples were dehydrated and embedded in Epon. Ultrathin sections were then deposited on copper grids and coated with carbon. Grids to be observed in electron microscopy were

stained (uranyl acetate and lead citrate) while grids to be examined with the electron microprobe were unstained.

A post-acquisition image processing system was recently developed (Cavellier et al., 1989), and it was used in association with the ion microscope and the ion microprobe. Additional information concerning the principles of the microanalytical methods and instrumentation can be obtained from a recent review (Chassard-Bouchaud, 1991).

3. RESULTS

3.1 Mytilus edulis

Samples were investigated, using ion microprobe, ion microscope, electron microscope and electron microprobe.

Figure 1 presents two ion microprobe micrographs which show the topography of the section with the calcium image. The plutonium image demonstrates that the digestive gland diverticula are the target cells of radionuclide concentration. A high plutonium emission (white points) is obtained from the diverticula, while a very faint emission is obtained from the digestive tractus and from ovocytes.

Figure 2 presents results obtained from the digestive gland, which is the target organ of uranium concentration. A and B are ion microscope images showing in A the calcium repartition which gives the topography of the section and in B the localization of uranium which appears as white points in the apical region of the cells. The highly emissive uranium points correspond to lysosomes. In C, the electron micrograph of a digestive cell shows two lysosomes containing numerous micro needles. Using electron microprobe, we were able to analyze these microstructures: they appear to be made of uranium and phosphorus according to the X-ray emission spectra obtained from these lysosomes. The target organelle of uranium concentration is the lysosome where the radionuclide is associated with phosphorus in an insoluble form due to an acidic phosphatase enzymatic reaction.

Figure 3 is an electron micrograph of a digestive gland cell where lysosomes are to be seen. Moreover, we can observe a pycnotic aspect of the nucleus.

Electron micrographs of kidney are presented in Figure 4. In A, the low magnification allows us to observe a vacuolated cytoplasm, several nuclei with pycnosis figures and lysosomes with electron dense contents. In B, a higher magnification allows us to observe a large secondary lysosome which corresponds to an auto or heterophagous vacuole, in which cellular wastes and contaminants are concentrated before being extruded out of the cell. Using this process the cell can detoxicate.

3.2 Cerastoderma edule

Samples were investigated using ion microprobe. Figure 5 shows four ion images obtained from the same section of the digestive gland, by scanning. The diverticula are shown to concentrate, uranium (Fig. B), plutonium (Fig. C) and americium (Fig. D): the radionuclide emissions appear as white points.

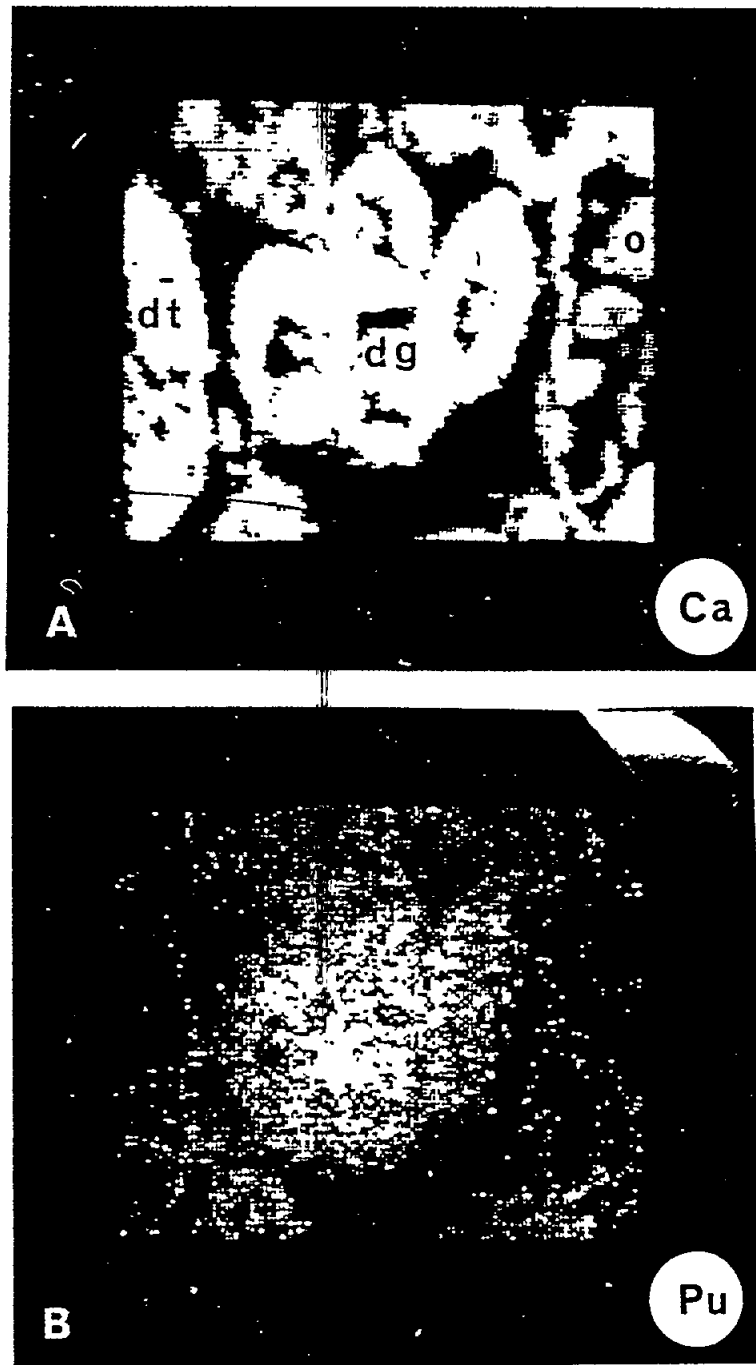


Fig. 1 Mytilus edulis (Mussel), collected from Carentan, near La Hague. Ion microprobe micrographs obtained by scanning (RIBER MIQ 156 ion microprobe) of an histological section showing digestive gland diverticula (dg), ovocytes (o) and digestive tractus (dt). x 500. A. $^{40}\text{Ca}^+$ image showing the topography of the section. B. $^{248}\text{Pu}^+$ image, from the same area as A, showing a high plutonium emission from the digestive gland diverticula, while a very faint emission is observed from the other tissues.

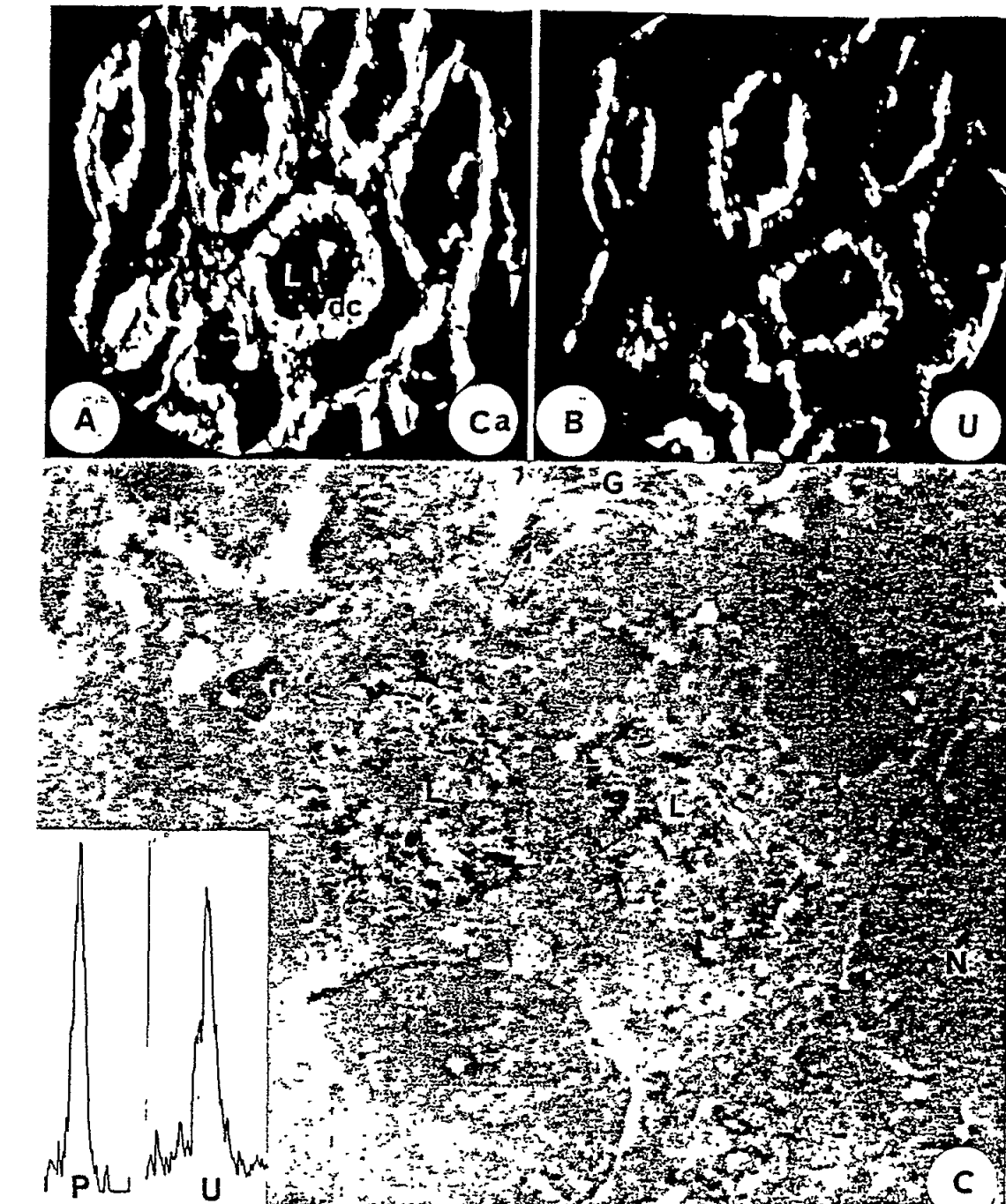


Fig. 2 Mytilus edulis (Mussel). Uranium exposed digestive gland. A & B. Ion microscope micrographs (CAMECA IMS 300) of an histological section. X 800.
A. $^{40}\text{Ca}^+$ image showing the topography of the section: digestive cells (dc) and lumen (L).
B. $^{238}\text{U}^+$ image obtained from the same area as A showing a high uranium emission from the digestive cells.
C. Electron micrograph of a digestive cell (non osmicated and unstained material) showing two lysosomes (L) containing microneedles of uranium associated with phosphorus. G: Golgi apparatus; N: nucleus. Inset: X-ray emission spectra of uranium and phosphorus obtained from these lysosomes. X 40000



Fig. 3 Mytilus edulis (Mussel). Uranium exposed digestive gland. Electron micrograph showing an epithelial cell with nucleus (N), lysosomes (L) and rough endoplasmic reticulum (RER). X 40000

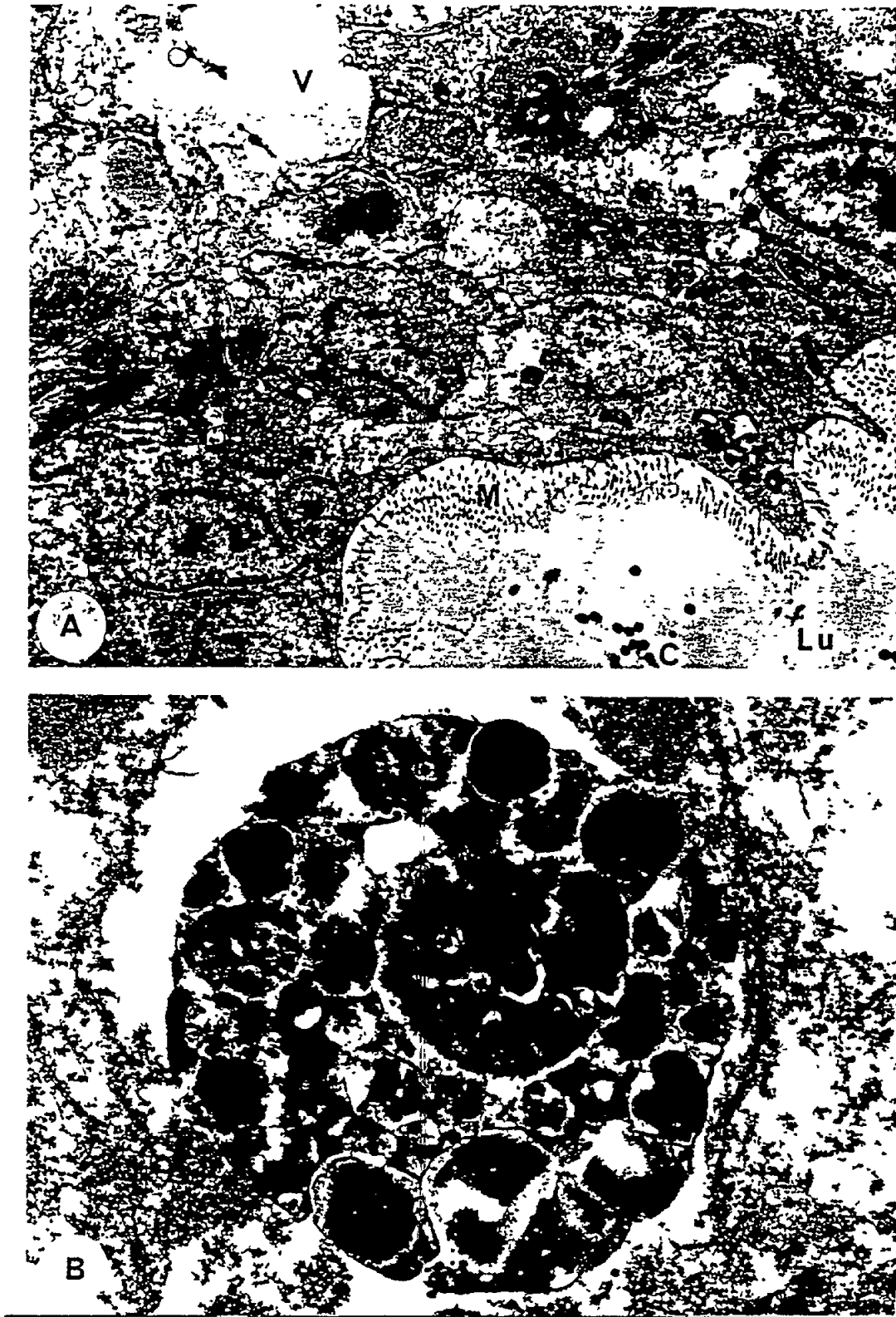


Fig. 4 Mytilus edulis (Mussel). Uranium exposed kidney.
A. Electron micrograph showing nuclei (N), lysosomes (L), vacuoles (V) and in the lumen (Lu): microvilli (M) and cilia (C). X 9200
B. Electron micrograph showing a large secondary lysosome (auto or heterophagous) overloaded with dense material. X 32000.

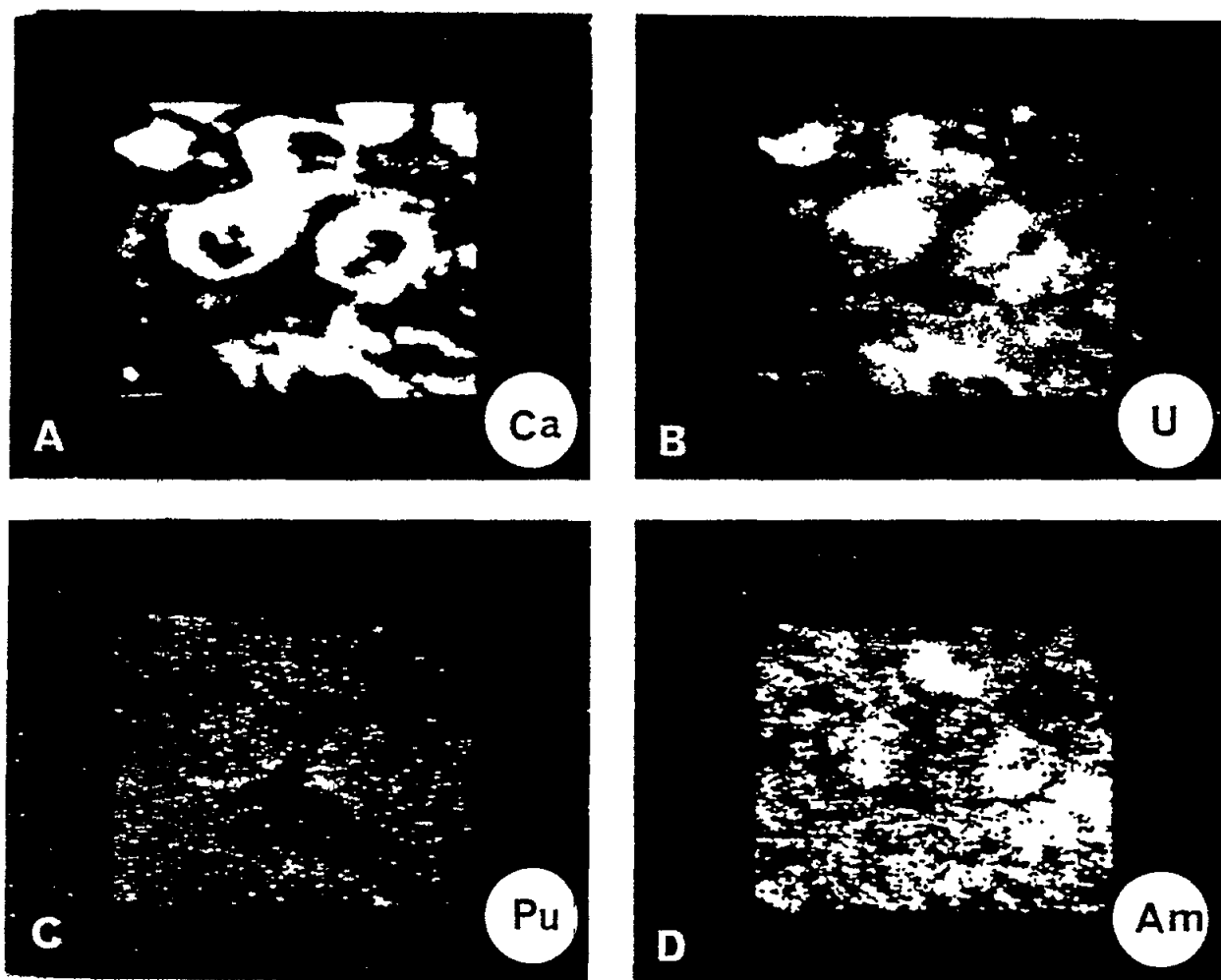


Fig. 5 Cerastoderma edule (edible cockle). Uranium and transuranium elements exposed digestive gland. Ion microprobe micrographs (RIBER MIQ 156 ion microprobe) obtained by scanning of an histological section. X 500.

A. $^{40}\text{Ca}^+$ image showing the topography of the section with the digestive gland diverticula.

B. $^{238}\text{U}^+$ image from the same area as A, showing a high uranium emission from the diverticula.

C. $^{239}\text{Pu}^+$ image from the same area as A showing a faint emission from the diverticula.

D. $^{241}\text{Am}^+$ image from the same area as A, showing a high americium emission from the diverticula

3.3 Crassostrea gigas

Samples were investigated using electron microscope and electron microprobe. Figure 6 shows two electron micrographs obtained from uranium exposed organs.

Fig. 6A: a kidney cell with many lysosomes containing microgranules. Using electron microprobe, we analyzed these microstructures which appeared to be made of uranium and phosphorus. The target organelle of uranium concentration is the lysosome where the radionuclide is associated with phosphorus in an insoluble form due to acidic phosphatase enzymatic reaction. These results have to be compared with the ones obtained from the mussel as they are in complete agreement.

Fig. 6B: a labial palp cell with microneedles of uranium which are not located in membrane-limited organelles.

4. DISCUSSION AND CONCLUSIONS

Considering tissue and cell structure, complementary results were obtained by using several different microanalytical methods, secondary ion mass spectrometry associated with electron microscopy. At the ultrastructural level, the electron microprobe was very useful as it allowed determination of the precise location of elements.

At the structural level, we were able to determine organs involved in uranium and transuranium nuclide bioaccumulation. Uptake happens via the labial palp and gill as we were able to demonstrate it in a previous paper (Chassard-Bouchaud and Escaig, 1984). The target organ of concentration is the digestive gland and excretion happens mainly via the kidney.

At the ultrastructural level, the target organelle is the lysosome where nuclides are associated with phosphorus in an insoluble form. The electron microscopy investigations demonstrate the occurrence of two types of bioconcentration:

- (a) When toxic elements are trapped into special organelles such as membrane-limited lysosomes, then they cannot induce any cell damage and they are generally extruded out of the tissues as secondary lysosomes.
- (b) When toxic elements are scattered in the cytoplasm and not located in membrane-limited organelles, such as uranium in the oyster labial palp, they are likely to persist within the cell and to provoke injuries.

Our present data contribute to the understanding of nuclide cytotoxicity. Damages appear within nuclei; it is well known that deoxyribonucleic acid damage are produced in the marine environment by pollution and chemicals (Zahn, 1991). Concerning cytoplasmic organelles, a decrease of mitochondria and an increasing activity of Golgi apparatus producing lysosomes are observed as well as a vacuolization of the tissues.



Fig. 6 *Crassostrea gigas* (Oyster). Uranium exposed organs.
A. Electron micrograph of a kidney cell with lysosomes (L) and nucleus (N) (non osmicated and unstained material). Lysosomes which are membrane limited, contain microgranules of uranium associated with phosphorus. X 32000.
B. Electron micrograph of a labial palp cell with microneedles of uranium. (non osmicated and unstained material). These uranium microdeposits are non membrane limited. X 84000

The problem of release of uranium and transuranium nuclides into the aquatic environment and their accumulation into marine organisms are a cause for widespread concern. Plutonium contents of several species of molluscs sampled from several sites along the French coast were measured in order to establish plutonium levels (Guary and Fraizier, 1977). The influence of the La Hague reprocessing plant was apparent in the immediate proximity of the waste-disposal outfall.

Another point which is of high interest is the transfer of environmental radionuclides through the different steps of food chains including, in particular, shellfish ingested by man. Transfer of environmental plutonium and americium across the human gut has been demonstrated (Hunt *et al.*, 1986) by analyzing urine from people near the nuclear installation at Sellafield in the United Kingdom, who eat a lot of local seafood and from volunteers living far from this area, after a single meal of shellfish obtained near Sellafield. The results showed that radionuclides passed through the intestinal barrier and an increasing excretion of plutonium and americium following contaminated food consumption was observed.

Complementary investigations are under way to get a better understanding of the mechanisms of transfer of transuranium elements through the different steps of the food chains.

5. ACKNOWLEDGEMENTS

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THE FATE OF LEAD IN A BENTHIC BIVALVE (Venus verrucosa)

by

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A B S T R A C T

The bio-kinetics of lead in a benthic clam, Venus verrucosa were investigated. Lead contents in various body organs of bivalves freshly collected from contaminated coastal sites, and then after 1 month and 2 months depuration, were investigated by anode stripping voltammetry. Histochemical and secondary-ion emission spectroscopy techniques were employed to localize lead at the cellular and subcellular levels.

Most of the lead body burden was found to be sequestered in the kidneys, presumably after entry through ingestion of lead contaminated particles and passage through the digestive gland. Some loss of lead to the gills was evident during the 2 month depuration period. Renal spherocrystals were found to be the most important subcellular depositary sites for this contaminant. A likely transport mechanism of lead from one body organ to another is suggested.

1. INTRODUCTION

A rapid increase in the number of operating automobiles over the past decades has greatly accelerated the deposition of lead in the marine environment (Zarogian et al., 1979). This is more so in a small and densely populated island like Malta which has one of the highest densities of car traffic in Europe. Semi-enclosed inshore coastal sites near densely urbanized areas may be expected to receive a significant amount of lead contamination. These include, the local harbours which are also exposed to industrial activities.

The benthic bivalve, Venus verrucosa is an important component of benthic communities in such harbour areas, because of its relative abundance. Moreover, it is often consumed by man and has a significant commercial value.

Bivalves have often been used as bioindicator species to monitor levels of contamination in the marine environment. However, while much is known about the fate, bioaccumulation, and depuration of contaminants in Mytilus spp., very little information is available with respect to Venus spp. Various biological responses of this species to a range of contaminants, have been investigated in our laboratory (Axiak and George, 1987a,b; Axiak and Galea, 1988; Axiak et al., 1988). In the present study, the fate and mobilization of accumulated lead within the various body compartments of this clam were investigated.

2. MATERIALS AND METHODS

Specimens of Venus verrucosa (of size range: 45-55mm) were collected by SCUBA divers from Marsamxett and Grand Harbour between July 1990 and February 1991. They were immediately transported to the laboratory in clean polythene plastic bags, within a maximum of 1 hour of being collected.

2.1 Investigations on freshly collected specimens

A subsample (9 individuals) of freshly collected specimens were dissected and their main organs (namely: digestive gland, kidney, mantle folds, muscular foot, gills and siphons) were fixed in 70% alcohol saturated with hydrogen sulphide for 41 hours. They were then dehydrated, cleared in chloroform and embedded in paraffin wax. Sections were cut at 6 μm with a rotary microtome, deparaffinized and brought down to water. Histochemical staining for lead was carried out according to Sumi *et al.* (1983). 2-3 mg of the chelate Bromopyridylazo-diethylaminophenol (Br-PADAP) were dissolved in 1 ml dimethylsulphoxide and a drop of 0.5MNaOH. To the solvent 15 ml of 0.1M thiourea and 15 ml of 0.1M potassium cyanide (masking agents for iron, copper and zinc) were added. The different sections were stained in this solution for twenty minutes and afterwards washed in deionized water. They were allowed to dry completely in air at room temperature and mounted in DPX.

Another subsample of three bivalves were similarly dissected and fixed in 70% gluteraldehyde for 30 minutes. Tissues were washed in three changes of 3.5% cacodylate buffer and stored in same buffer until further analysis using secondary-ion emission spectroscopy. Such analysis were carried out by Prof. C. Chassard-Bouchaud at the Laboratoire de Biophysique (Université Paris, France). A Cameca SMI-300 fitted with an electrostatic deflector, with ionic oxygen as primary ions, was employed. The images of the distribution of the secondary ions (lead) were obtained directly with appropriate optics (Chassard-Bouchaud, 1991).

A further subsample of 12 individuals was used for lead analysis using anode stripping voltametry. These specimens were stored at 8°C overnight. Shells were then washed with distilled water to remove any source of metal contamination. Bivalves were then dissected after the internal soft parts were rinsed with deionized water and allowed to drain on clean tissue paper. Organs were pooled according to type, frozen at -20°C overnight and then homogenized. Digestion was carried out in concentrated nitric acid for 15 hours at room temperature and then for two 6-hour digestion cycles at 115°C. The clear digests were diluted to a known volume with deionized water and then analyzed for lead content using a Metrohm 646 VA anode stripping voltameter.

2.2 Investigations during depuration phase

Freshly collected bivalves were introduced to clean sea water in a recirculating seawater system, within a maximum of 1 hour of being collected from the field. Animals were placed in glass aquaria measuring 60 by 39 by 40 cm and each holding 55 l of water. Seawater was circulated through these aquaria at a rate of 1.5 to 2 l min⁻¹. The bottoms of these aquaria were covered with clean sediments to a depth of 5 cm, and in which the clams could easily burrow. Water temperatures were kept at 19 to 21°C and salinities at 36 to 38 ppt throughout the depuration investigation period which lasted for two months. During this period, bivalves were fed on 'Liquifry Marine', supplied by Liquifry Co. Ltd., Dorking, U.K. (Batch Code: 0308).

After 1 month and 2 months of depuration in clean seawater, samples were collected from the aquaria and analyzed histochemically (9 individuals), and for tissue lead content (12 individuals) using anode stripping voltammetry as indicated above.

2.3 Sediment analysis

Sediment samples were collected from Marsamxett at the same site from which animals were collected. Superficial sediments (top 5 - 8 cm) were collected by SCUBA divers from a depth of 7.2 m, stored in prewashed plastic containers and immediately transported to the laboratory. Here they were dried at 100°C, sieved to a fine fraction (<2mm in particle diameter) and digested in concentrated nitric acid at room temperature for 15 hours, and then for a further period of 6 hours at 115°C. The digest was filtered and then diluted to 20 ml with deionized water before being analyzed by Anode stripping voltammetry. Sediments contained in depuration aquaria were similarly treated for subsequent analysis of lead content.

3. RESULTS

The superficial sediments from Marsamxett Harbour were found to carry a lead content of 118 $\mu\text{g g}^{-1}$ dry weight, while the sediments in the depuration aquaria had 12 $\mu\text{g g}^{-1}$ dry weight of lead.

The lead concentrations in the various body parts of freshly collected bivalves and then after 1 month and 2 months depuration are presented in Table 1. Also shown are the percentage distribution of lead within the various body compartments and the percentage of fresh weight of the given body part to the whole soft body weight of the animal. Freshly collected bivalves had a total lead body burden of 1.45 $\mu\text{g g}^{-1}$ of fresh weight. Over 83% of this contaminant was located in the kidney, though this organ constituted only 7.4% of the total soft body weight of the animal. No lead was detected in gills, siphons and muscular foot of this group of bivalves, while the remaining body burden of lead was localized in the digestive gland (16%) and the mantle and mantle folds (0.5%).

After one month depuration in clean seawater, 86% of the body burden of lead was still localized within the kidneys, with the remaining being found in the muscular foot. No lead was found in the gills, siphons, mantle or digestive glands.

The overall lead body burden did not decrease even after two months of depuration in clean seawater. In fact during this period, the relative distribution of lead within the kidneys was actually higher than in freshly collected bivalves. Therefore, by this time over 93% of the lead body burden was located within the kidneys, with almost 6% being located in the digestive glands and only 0.9% being found in the gills.

The above findings were confirmed by the histochemical localization of lead at the cellular level. Positive control tissue sections which, were previously immersed in a saturated solution of lead nitrate for 24 hours and then treated histochemically as described above, exhibited a magenta to purple depositions of Br-PADAP within various parts of the tissues. This was considered as a positive reaction towards lead.

Table 1

Lead concentrations and % distribution of lead load in the various body parts of Venus verrucosa.

Body Part		Freshly Collected	1 month Depuration	2 months Depuration
Gills	Pb Conc. ($\mu\text{g g}^{-1}$)	-	-	0.173
	% Distrib. of Pb	0	0	0.86
	% of Body Weight	9.5 + 1.3	9.8 + 2.0	8.9 + 2.6
Siphons	Pb Conc. ($\mu\text{g g}^{-1}$)	-	-	-
	% Distrib. of Pb	0	0	0
	% of Body Weight	6.6 + 3.2	6.1 + 0.7	5.4 + 1.7
Kidney	Pb Conc. ($\mu\text{g g}^{-1}$)	15.89	9.22	26.8
	% Distrib. of Pb	83.5	86.1	93.2
	% of Body Weight	7.4 + 2.1	7.3 + 1.6	6.2 + 2.7
Mantle Margin	Pb Conc. ($\mu\text{g g}^{-1}$)	0.03	-	-
	% Distrib. of Pb	0.5	0	0
	% of Body Weight	26.6 + 3.9	26.1 + 3.7	27.2 + 3.1
Muscular Foot	Pb Conc. ($\mu\text{g g}^{-1}$)	-	0.41	-
	% Distrib. of Pb	0	13.9	0
	% of Body Weight	34.6 + 4.2	35.4 + 5.2	32.9 + 5.2
Digestive Gland	Pb Conc. ($\mu\text{g g}^{-1}$)	1.96	-	0.551
	% Distrib. of Pb	16.0	0	5.963
	% of Body Weight	14.8 + 5.2	14.8 + 5.0	19.3 + 3.9

N.B. Lead concentrations in the various body parts are shown in $\mu\text{g g}^{-1}$ of fresh soft body weight.
 % Distribution of Lead in the various body parts is calculated as the percentage ratio between the lead burden of the organ to the total lead body burden.
 % of Body Weight shows the percentage fraction of the weight of the organ to the weight of the total fresh soft body.

Sections taken from freshly collected bivalves, exhibited a highly marked light red precipitate in the renal concretions. These concretions or spherocrystals, were amorphous and had pleiomorphic radial structures (especially evident in sections stained with heamatoxylin and eosin). They were located within the renal glandular cells forming the walls of the kidney tubules.

A weak Br-PADAP precipitate was detected in the ciliated epithelium of the mantle folds; the connective tissues between the pallial muscles and outer epithelia, and the digestive cells of the tubules of the digestive glands of freshly collected specimens.

Tissue sections which were analyzed with secondary-ion emission spectroscopy, exhibited lead in the renal spherocrystals. The digestive gland also showed distinct localization of lead within the digestive cells and the interstitial regions.

After one month and two months depuration in clean seawater, only the renal spherocrystals continued to exhibit a positive reaction to Br-PADAP.

4. DISCUSSION AND CONCLUSIONS

The level of lead in the sediments ($118 \mu\text{g g}^{-1}$) collected from the same site as that of the bivalves, was found to be comparable or higher than those of other Mediterranean inshore sediments exposed to urban and industrial pollution (eg. Thermaikos Gulf; $71.2 \mu\text{g g}^{-1}$ dry weight; Voutsinou-Taliadouri, 1981; Gulf of Venice: $45 \mu\text{g g}^{-1}$ dry weight, Angela *et al.*, 1981). Marsamxett Harbour is in fact a semi-enclosed body of water which is surrounded on all its sides by heavily urbanized areas. Though a number of yacht marinas as well as a small ship repairing yard are located here, it may not be considered as a heavily industrialized area. Therefore it may be suggested that the main source of lead contamination in this area is car traffic.

The total lead body burden of freshly collected specimens from this area was found to be $1.45 \mu\text{g g}^{-1}$ fresh weight. *Mytilus galloprovincialis* collected from other similarly urbanized areas such as the Gulf of Genoa and Rijeka Bay (UNEP/FAO, 1986) showed maximum lead body burdens of approximately $2 \mu\text{g g}^{-1}$ fresh weight.

Uptake of lead by bivalves can either occur through adsorption of ions at membrane-water interphases, by absorption or passive diffusion across semi-permeable membranes into body fluids or by ingestion of the metal with food. No significant levels of lead were located within the external body parts (eg. siphons, gills, and muscular foot) of freshly collected *Venus verrucosa*. However, 16% of the total body burden was located within the digestive glands. This suggests that the major uptake mechanism is through ingestion of lead contaminated particles by this filter feeder. Bryan (1973) observed that on exposure to lead, *Mytilus edulis* showed a lower concentration of lead in the gills as compared to the mantle margin, with much higher levels in the kidneys and digestive gland.

The present study has shown that once inside the body, lead is almost completely sequestered by the kidney of *Venus verrucosa*. Histochemically, this lead was found to be mostly associated with renal spherocrystals. These renal refractory concretions are only found in invertebrates and are concerned with synthesis of an inorganic matrix in which inorganic matter may be precipitated as phosphates, carbonates or oxalates (Martoja and Truchet, 1983). Accumulation of soluble metal-binding proteins within the lysosomal system of the bivalve kidney has also been postulated (Simkiss and Mason, 1984). Both the renal lysosomes as well as the renal spherocrystals may act as the major depositary sites of this contaminant in *Venus verrucosa*, thus limiting the injury to the other body parts.

The present study have shown that during depuration, most of the lead originally found in the digestive gland of this species was transported to the kidney. Moreover, at the end of 2 months depuration, traces of lead were found

to increase in the gills. This suggests that these sites may also be sequestering lead from other body organs. The transport of lead from one organ to another may be occurring via some metallo-protein plasma ligand. No amoebocytes loaded with lead were ever detected histochemically throughout the depuration period.

The total lead body burden of the present species did not decrease over a two-month depuration period. Similarly, Mauri and Orlando (1982) have reported that renal spherocrystals of the clam Mercenaria mercenaria are only eliminated after prolonged depuration in clean seawater. This long residence time of lead within the body of Venus verrucosa suggests the possibility of biomagnification to higher trophic levels. Moreover, it may constitute a health hazard through its consumption by man.

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THE EARLY FISH STAGES AS AN OBJECT OF TOXICOLOGICAL STUDIES;
CASE OF SEABASS, Dicentrarchus labrax, AND
GILTHEAD SEABREAM, Sparus aurata

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A B S T R A C T

Effects of the water soluble fraction of Iraq crude oil on the early life stages, (eggs, larvae and post-larvae) of seabass Dicentrarchus labrax and gilthead seabream Sparus aurata are presented. Hydrocarbons from this oil preparation, greatly affect eggs of both species, including high mortality of eggs and hatched larvae, different kinds of deformities along the entire body, and narcotic effects manifested as slowing down of heartbeat rate in developing embryos. Long-term exposure of seabream larvae leads to total mortality, while short-term exposure of seabass larvae exhibit significant mortality in highest water soluble fraction concentration. Post-larvae are most resistant of all studied stages.

Problems and critical aspects of these toxicological experiments are discussed and our recommendations for future investigations are given.

1. INTRODUCTION

Oil and oil derivatives are the most frequent pollutants of marine waters, particularly in inshore areas. The oil input to the world sea has been assessed to range from 3.2 million tons (Terrell, 1987) to 10 million tons (Blumer, 1973). This oil and oil derivatives input has already caused an increase of hydrocarbon levels in the sea (Jeffrey, 1971; Samra et al., 1986).

From observations made of the possible adverse effects of hydrocarbons on fish, taking into account all fish developmental stages (from immobile eggs, poorly mobile larvae to juvenile and adults) it might be concluded that the effects will be most strongly felt by the immobile or poorly mobile stages. Pelagic stages will be most at risk because they tend to float at the sea surface where the concentrations of hydrocarbons are highest. This applies to the eggs and larvae of most commercially important fish species.

Toxicological studies of the effects of oil, oil derivatives and oil hydrocarbons on early fish developmental stages started at the end of the sixties (Mironov, 1967) and were very popular during the seventies (Kühnold, 1974, 1977; Anderson et al., 1974; Linden, 1975; Anderson, 1977; Lonning, 1977, and many others). Even though limited to several fish species such as cod, herring, plaice, these experiments showed exceptionally harmful effects of oil on early developmental stages of fish; different effects on the survival, deformities and metabolism were described. During the eighties, the studies were extended to include the effects at the level of cell and

chromosomes. This paper presents the results of the experiment of the effects of the water soluble fraction of Iraq crude oil on early developmental stages (eggs, larvae and post-larvae) of seabass Dicentrarchus labrax and gilthead seabream Sparus aurata, describes the problems encountered during the experiments and gives recommendations for future standardization of toxicological studies in this area of research.

2. MATERIALS AND METHODS

2.1 Studied fish species

Seabass Dicentrarchus labrax and gilthead seabream Sparus aurata are typical Mediterranean species. Both inhabit inshore waters, especially estuaries with lower salinities. Spawning grounds in the Adriatic are unknown. Post-larvae and juveniles were caught along the coast, in the bays and on sand beaches with macroalgae beds.

Eggs of both species are pelagic, float near the sea surface, so they are very likely to be exposed to oil hydrocarbons originating from marine traffic or from oil refinery plants that discharge along the coast. Ripening fish were caught from the polluted estuaries during autumn, so the gonads may be contaminated by hydrocarbons at this stage.

These species have a very high market value, and there is a high fishing effort to exploit the natural populations. A decrease of populations in Adriatic of both species has therefore been observed, especially that of fingerlings. This decrease may be partly due to pollution effects. However, no such investigations have yet been performed.

Market interest for these fish has led to their artificial rearing in hatcheries and cages all along the Mediterranean coast, and this is now a big industry. Therefore, it is easy to obtain eggs and other early stages for different studies, including toxicology, all year round from commercial production. This is one of the major reasons why we have chosen these fish species for our past and future toxicological studies.

2.2 Preparation of the experimental oil solution

Crude Iraq oil (Kirkuk) was obtained from the INA oil company. The water soluble fraction was prepared according to the method described by Anderson et al. (1974). One part of oil and nine parts of ambient sea water (18°C for seabream; 11.2°C for seabass and salinity 38) were stirred in glass jars for 20 hours. The mixture was then allowed to separate for one hour, after which the water phase was decanted and used as a 100% water soluble fraction. Dilutions of WSF (10, 20, 30 and 50%) used for the experiments were prepared by mixing this stock with ambient sea water. Experimental temperatures were maintained by flow of ambient sea water in a water bath, and jars were gently aerated from the bottom.

The qualitative analysis of WSF showed this oil preparation to be rich in aromatic hydrocarbons, especially benzene, toluene and xylene. The naphthalene concentration in 100% WSF stock solution was 0.189 mg l⁻¹.

3. RESULTS

3.1 Effects on eggs

Eggs of gilthead seabream exposed to the WSF of crude Iraq oil showed a steady mortality during the 72 hours test from fertilization to hatching. Dead eggs were in the blastula and gastrula stage, as well as in different stages of embryogenesis. Embryos in higher WSF concentrations died in the earlier stages of embryogenesis compared to the lower concentrations. About 40-45% of larvae were hatched from the eggs exposed to 10, 20 and 30% WSF, and only 8% in 50% WSF concentration. Between 67% (in 10% WSF) and 100% (in 30 and 50% WSF) of hatched larvae had spinal deformities, lowered heartbeat rate, deformed head, and cytolysis of the whole body epithelium. These deformities lead to total immobility or abnormal swimming modes of the hatched larvae.

On the other hand, seabass eggs exposed to the WSF at the gastrula stage showed high resistance during the first 72 hours exposure, but dramatic increase of mortality was found later. The embryos in all WSF treatments (except 4.1% in 10% WSF) died before hatching, while in control groups hatching was about 75%. Most of the dead eggs were in different stages of late embryogenesis. Except for only sporadic and arrhythmic heartbeats in some embryos in 10% WSF, there was no heartbeats in other WSF concentrations. All hatched larvae in 10% WSF had spinal deformities, arrhythmic heartbeat and were immobile on the jar bottom.

Both experiments confirm the present knowledge of hydrocarbon effects on fish eggs, including reduced hatchability and high mortality during whole embryogenesis. Most of the experiments on other species also reported a reduction in hatching (Kühnhold, 1974; Lonning, 1977) and this is a general consequence of different pollutant impacts on fish eggs (Rosenthal and Alderdice, 1976). The egg membrane is most permeable immediately after fertilization, so hydrocarbons can enter eggs and then produce different effects in tissues, leading to a steady mortality during embryogenesis (as in our seabream experiments). Crosby Longwell (1977) described chromosomal damages and mutagenic effects, and this is a reason for a high incidence of deformities in embryos and hatched larvae. Abnormal mitoses per embryo and survival were correlated in cod eggs exposed to naphthalene (Stene and Lonning, 1985).

However, the sensitivity of eggs is reduced as development progresses, which is due to the reduction in permeability of egg membrane, and to the development of mechanisms for detoxification of hydrocarbons (Binder and Stegeman, 1980). This is maybe a reason for the low mortality in the first 72 hours of exposure of seabass eggs. After that, due to long exposure, low metabolism and high WSF concentrations, the embryos died.

So, eggs and embryos of marine fish are very sensitive to the levels of oil hydrocarbons used in our tests, exhibiting high mortality, deformities and narcotic effects, which under experimental conditions lead to an almost total loss of tested eggs.

3.2 Effects on yolk-sac larvae

Effects on larvae were examined in short-term tests on the seabass larvae and long-term tests with seabream larvae. This long exposure (96 hours)

killed all seabream larvae in all WSF concentrations. In contrast, short-term (24 hour) tests with seabass larvae shows a slight increase in mortality at higher WSF concentrations. However, the significant effect was a slowing down of yolk-sac resorption in the two highest concentrations. These larvae also moved more slowly, which is an indication of the narcotic effects of oil hydrocarbons. In the test with seabream we did not find a significant difference in the yolk-sac resorption and growth between larvae in all treatments during the first 48 hours. Later on, we found a difference in growth rate between controls and 10 and 20% WSF concentrations, but no difference in the yolk-sac resorption. It is possible that this energy was consumed for detoxification of hydrocarbons and other stress reactions.

The larval behaviour was typical of that described earlier by Kühnhold (1977), with increasing activity at the beginning of exposure, then narcosis and reduction of swimming followed by complete immobility and dying at the bottom.

3.3 Effects on post-larvae

Although we found significant mortality in the treatments and in the controls for both species, we can conclude that post-larvae is the most resistant stage. Resistance was high especially in seabass post-larvae, which were 30 days older than seabreams. In the initial stages of exposure in all WSF concentrations, post-larvae gather on the bottom but a few hours later they leave it, moving freely around. Only in the highest WSF in test with seabream did they remain on the bottom, moving from time to time, and died by the end of the test.

4. DISCUSSION

During the experiments a number of problems were encountered, which frequently resulted in unpredictable losses and death of early fish stages. These difficulties were due to the following factors.

- i) A high mortality of early life stages was caused by their susceptibility to the environmental conditions and to handling, both in controls and in replicates of individual treatments. This frequently resulted in dramatic differences in mortality. Therefore, replicate experiments are indispensable. Mortality was particularly higher due to the counting of individuals and putting them in the experimental jars.
- ii) Problems arose from the different compositions of different oils and due to the evaporation of hydrocarbons from the media. This leads to serious difficulties in possible comparisons of the effects of different oils or attributing of toxic effects to individual components.
- iii) The replacement of the medium to overcome the problem of hydrocarbon volatilisation presents a particular problem. It may lead to damage and even the death in particular of larvae which are poorly mobile and distributed throughout the jar. Eggs become grouped at the surface under conditions where there is no aeration, whereas post-larvae are mobile and might easily evade the current caused by the replacement of the medium.

All these problems, made worse by the susceptibility of individual stages and individual phases of the same stage, present serious difficulties in accounting for the specific effects of oil. They also impede the description of specific effects of oil on the stages which could then be used for the analysis of oil effects under natural conditions. However, these experiments provided qualitative results which may serve in the optimization of experiments with the early life stages as well in the standardization of experiments in order to carry out other toxicological studies.

On this basis, we would like to recommend that toxicological tests should be carried out on the following phases or stages of development.

- For short-term toxicological tests: eggs of seabass during early embryogenesis.
- For tests of long-term effects on egg development: gilthead seabream eggs since complete embryonic development takes 70 hours compared with 120 hours in seabass (at ambient temperature).
- Seabass larvae can be used because of their higher resistance to handling, but only 24 hours after hatching, since newly hatched larvae are most susceptible to handling; larvae at the end of yolk-sac resorption are not suitable since the effects on yolk-sac resorption or larval growth are not easily identifiable.
- Post-larvae of both species are suitable for testing, but not earlier than a month after hatching, since younger fish are very susceptible to handling.

5. CONCLUSIONS

Even though these experiments have given qualitative information on the effects of oil on early developmental stages of seabass and gilthead seabream, they do no more than confirm the results of a series of earlier studies on other fish species. They confirm the expected susceptibility of these stages which may indicate that the pollution of inshore waters is one of the significant factors affecting the reduction of natural populations of these fish, primarily by affecting their eggs and larvae.

To establish the effects of these pollutants on the early life stages of these fish in the natural environment, studies on mutagen and teratogen effects are required. These experiments could be conducted so as to describe these effects under experimental conditions, and to obtain data on chromosome changes (which are more or less specific for respective toxicants) which would be used as a reference in the same analyses of eggs and larvae from the natural environment. Thus, it would be possible to obtain information on the effects of the toxicants present as well as on the effects of toxicants accumulated in fish gonads during their residence in inshore polluted waters.

Development of such methods would much add to the understanding and explanation of the effects of pollutants on fish populations under natural conditions.

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REFEREE'S COMMENTS

- a) The main problem is that only qualitative data were obtained and/or presented. The concentrations of hydrocarbons to which the test organisms were exposed is not known; aeration of the test solutions suggests that the concentrations of harmful components would diminish rapidly during the exposure period. Data on the mortalities or abnormalities in the test and control vessels are not given; only qualitative descriptions of observations are given. Therefore, the data cannot be used to forecast the effects of oil pollution on these life-stages in the marine environment.
- b) Also, the concentrations of hydrocarbons in the local marine waters are not given, and it is stated that abnormal embryos are not found here. Therefore, there are no possible grounds for suggesting that the existing hydrocarbon concentrations in local coastal waters are causing damage to existing stocks of these species. It may be true that such effects are being caused in practice, but the data presented in this paper do not support such a causal relationship.
- c) Future research may be better expended on trying to overcome the practical difficulties encountered in these tests and to obtain proper concentration effects data, rather than branching out into other techniques (e.g. mutagenic and teratogenic measurements) of dubious value.

AUTHOR'S RESPONSE TO REFEREE'S COMMENTS

- a) Answers to many of the referee's questions can be found in detail in previously published papers on this subject, especially in:

B. Glamuzina, M. Tudor and J. Katavić. The effects of the water soluble fraction of Iraq Crude oil on eggs, larvae and postlarvae of gilthead sea bream, Sparus aurata Linnaeus, 1758. Oil and Chemical Pollution, 7(1990)283-298

and this was clearly indicated during the workshop.

The aim of the paper is not to present the detailed effects of oil on early fish stages, but to present the experimental problems facing scientists and make suggestions for future investigations with these stages in toxicology.

- b) It is possible to give concentration values of some hydrocarbons in the coastal waters of the Eastern Adriatic, but as there is no good statistical data on effects of oil on early fish stages in natural waters, the referee's statement about this matter holds good.
- c) I believe that both types of investigation have a place in future studies.

PHYSIOLOGICAL RESPONSES OF TWO MARINE PHYTOPLANKTONIC
SPECIES TO COPPER AND MERCURY

by

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A B S T R A C T

Some physiological responses of the marine phytoflagellates Pavlova lutheri and Dunaliella minuta to cupric chloride and mercuric chloride were studied. Both species were grown in batch cultures in an enriched sea water, over a maximum period of 18 days. Both copper and mercury at concentrations higher than 10 ppb, increased the lag phase of P. lutheri, which, in the majority of the cases, was followed by a period of recovery, during which the growth rate was not significantly reduced; D. minuta reacted to copper and mercury with a significant reduction in growth rate and final cell yield. At sublethal concentrations, the examined metals caused a significant increase of cell size, probably due to an inhibition of cell division process, and a loss of mobility of cells, especially in the early stages. Cell numbers and chlorophyll-a concentration per volume basis were inhibited to almost the same extent, while, the chlorophyll-a content per cell remained almost constant and independent of the cell size. Of the two species examined, P. lutheri appeared to be more sensitive to both metals than D. minuta.

1. INTRODUCTION

Copper is an essential micronutrient for algal growth (Provasoli and Pintner, 1953), being required for both photosynthesis and respiration (Sandman, 1985). At concentrations above those required for optimal growth, copper may have inhibitory effects and eventually become lethal (Saifullah, 1978; Florence and Stauber, 1986; Bozeman et al., 1989). Mercury is considered as one of the metals most toxic to marine life. It has been recorded that various organic and inorganic forms of mercury depress primary production and growth in freshwater and marine phytoplankton (Davies, 1976; Overnell, 1976; Rai et al., 1981; Aliotta et al., 1983).

The effects of copper and mercury on the growth and metabolism of various phytoplanktonic organisms have been investigated in laboratory cultures (Davies, 1978). However the results about metal toxicity to aquatic organisms are quite variable depending on the studied metal, the organism tested and the experimental procedure used by each investigator (Goudey, 1987; Wang, 1987; Hall et al., 1989). The physiological responses of phytoplanktonic organisms to a particular metal also vary. For some species of algae, there have been reports of an increase in the length of lag phase (Steenmann Nielsen and Wium-Andersen, 1970; Morel et al., 1978; Fuse, 1987), of a growth rate reduction (Morel et al., 1978; Fisher et al., 1981; Nakamura et al., 1986), of a photosynthetic rate inhibition (Overnell, 1976; Davies, 1978; Aliotta et al., 1983; Rao and Sivasubramanian, 1985), and of an increase in the rate of respiration (Rao and Sivasubramanian, 1985) as symptoms of

copper or mercury toxicity. Although, the effects of copper and mercury on some species of Dunaliella (e.g. tertiolecta, primiolecta and bioculata, tertiolecta and euchlora, respectively) have been studied to some extent (Davies, 1978), there is no available information on the effects of the above metals on Dunaliella minuta, as far as the authors know.

The present work examines the effects of copper and mercury on the growth of the phytoplanktonic organisms Pavlova lutheri and Dunaliella minuta in batch cultures. The purpose of this work was to study the tolerances of the above species to copper and mercury. The chosen parameters of growth were exponential growth rate, length of the lag phase, final cell yield and cell size. In addition, the effect of mercury on the chlorophyll content, either per volume of culture or per cell basis, of D. minuta was studied.

2. MATERIALS AND METHODS

The haptophyte Pavlova lutheri (Droop) Green = (Monochrysis lutheri Droop) and the chlorophyte Dunaliella minuta Lerche were studied. The cultures were grown in 125 ml Ehrlenmeyer flasks containing 50 ml of enriched seawater medium. Offshore seawater was filtered through a glass fibre filter (Whatman GF/C) in order to remove the large amount of particulate material present, and then through a Sartorius membrane filter of 0.45 μm average pore diameter. The filtrate was stored in darkness at laboratory temperature in plastic containers for at least a month. Before use, the water was passed through a column of a chelating ion-exchange resin (supplied in sodium form) to remove any copper and mercury naturally present in the water and to prepare a medium of as nearly constant physico-chemical composition as possible, by reducing the concentrations of other trace metals to a consistently low level (Davey et al., 1970).

The "f/5" medium of Guillard and Ryther (1962), without copper as a trace element was used for the growth of the examined species. After enrichment, the pH was adjusted to 7.8 and the medium was dispersed into the experimental flasks and autoclaved at 15 lb/in for 15 minutes. The media were allowed to stand at room temperature for approximately 24 hours for the dissolved gases (CO_2 , O_2 etc.) to equilibrate with those in air. Then the metal was added from freshly prepared working solutions in a proportion 1:100 with the medium. As a control, cultures without the addition of any metal were used.

The cultures were grown at a temperature of $22 \pm 1^\circ\text{C}$ and continuous light intensity 7000 ± 300 lux. At suitable time intervals, two 1 ml aliquots were taken from each of the duplicate flasks, fixed with Lugol's iodine solution (ca 0.5%) and the cells subsequently counted with a Fuchs-Rosenthal haemocytometer. In diluted cultures, at least 400 organisms were counted, thus achieving an accuracy of 10% of the count (Lund et al., 1958). On several occasions, the dimensions of each species were measured with an eye-piece micrometer and its cell volume was determined from appropriate geometric formulae (Vollenweider, 1974). Growth rates (μ), expressed as divisions per day, were calculated according to the expression $\mu = (3.5) (\ln n_t - \ln n_{t_0}) / (t - t_0)$ where, $t - t_0$ represents elapsed time in hours and n_t and n_{t_0} are the population densities at times t and t_0 , respectively (Eppley and Strickland, 1968).

For the determination of chlorophyll-a two 50 ml suspensions from the duplicate control and the cultures exposed to different mercury concentrations were filtered through a Whatman GF/C filter and the chlorophyll was immediately extracted into 90% aqueous acetone. After centrifugation, the fluorescence of the extracts was measured with an Aminco fluoromicrophotometer and the values were converted to chlorophyll-a concentrations by using the procedure of Strickland and Parsons (1972).

3. RESULTS

3.1 Effects on Pavlova lutheri

The cell population of P. lutheri (as % of the control) and its growth curves over a period of 18 days, in cultures containing different copper or mercury concentrations, are presented in Table 1 and Figures 1A & 1B, respectively. Comparison of the toxicity of the studied metals to P. lutheri shows that mercury is more toxic than copper, especially in the early stages of growth. For example, 2.5 days after inoculation, 20 ppb of mercury reduced growth by 90%, whereas the same concentration of copper reduced it by only 50% (Table 1).

Table 1

Effects of copper and mercury on the cell population (% of control) of P. lutheri, 1.5 and 2.5 days after inoculation.

Time (days)	Copper (ppb)	%	Mercury (ppb)	%
1.5	0	100	0	100
	10	77	10	58
	15	70	15	35
	20	65	20	22
	25	60	25	17
	35	45	35	13
	50	36	50	12
	75	25	75	10
2.5	0	100	0	100
	10	88	10	37
	15	74	15	23
	20	50	20	10
	25	30	25	5
	35	20	35	3
	50	15	50	2
	75	9	75	2

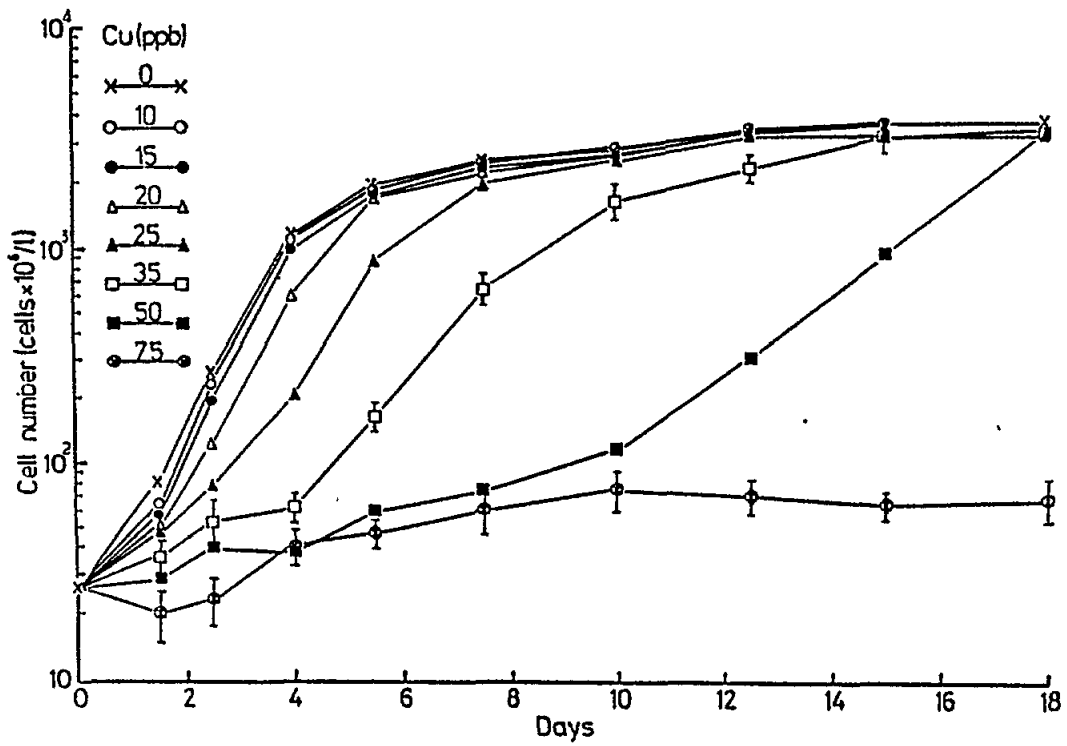


Fig. 1A Effects of copper on the cell population of Pavlova lutheri. Each point is the mean $\pm 2SE$ of 4 replicates

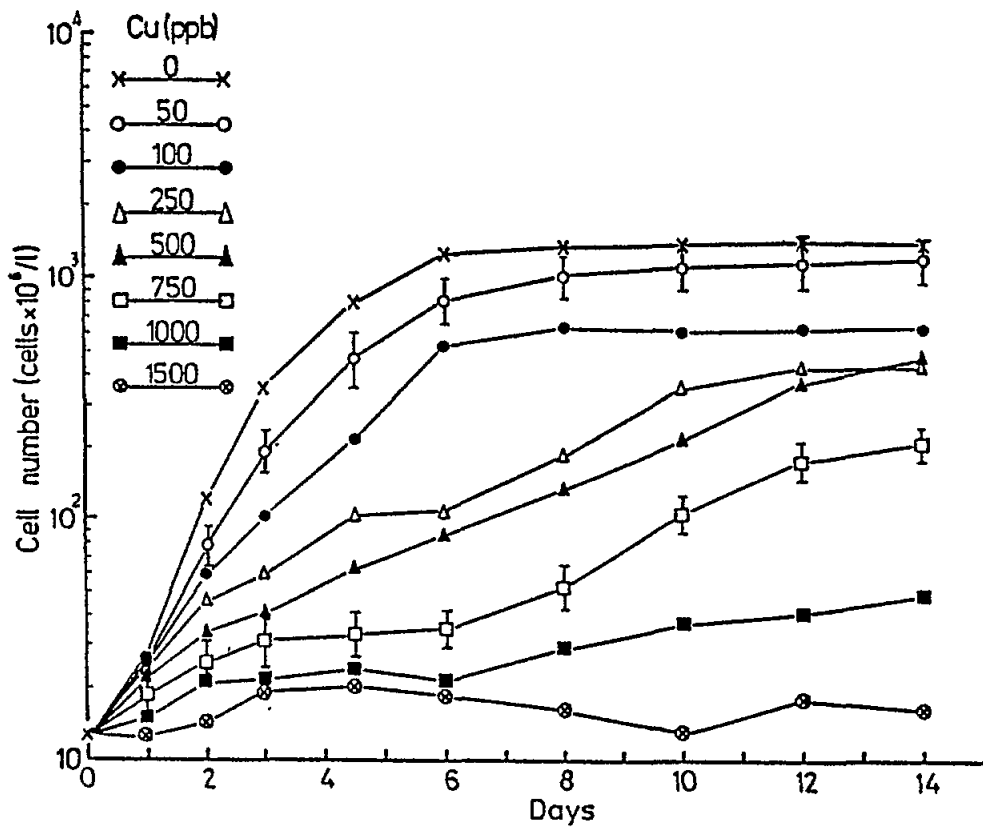


Fig. 1B Effects of mercury on the cell population of P. lutheri. Each point is the mean $\pm 2SE$ of 4 replicates

The effect of copper and mercury on the duration of the lag phase, growth rate in the logarithmic phase, generation time and final cell yield are presented in Table 2. As defined, the lag phase is the time necessary for the cell population to return to and exceed the level provided by the original inoculum, and is likely to reflect the degree of adaptation required for multiplication under given culture conditions.

Table 2

Effects of copper and mercury on the duration of lag phase, growth rate, generation time and final cell yield of P. lutheri.

Metal tested	Metal conc. (ppb)	Duration of the lag phase (days)	Growth rate (μ) (div./day)	Generation time (hrs/div.)	Final cell yield ($\times 10^6$ /ml)
copper	0	0.0	1.18	20.3	3.88
	10	0.0	1.18	20.3	3.82
	15	0.0	1.14	20.4	3.76
	20	0.0	1.09	21.9	3.32
	25	0.0	0.84	28.3	3.75
	35	4.0	0.94	25.4	3.46
	50	10.0			3.07
	75	18.0			0.07
mercury	0	0.0	1.13	21.1	4.05
	10	0.0	1.09	22.0	3.75
	15	1.5	0.99	24.0	3.86
	20	2.5	1.16	20.6	3.96
	25	3.0	1.01	23.6	4.24
	35	4.0	0.93	25.8	3.84
	50	7.5	1.07	22.5	2.90
	75	7.5	0.88	27.3	1.38

Comparison of the growth curves (Fig 1A & 1B) and examination of Table 2 indicates that Pavlova lutheri reacted to the two metals in a similar manner, by a progressive increase in the lag phase with increasing metal concentration. For example, in cultures exposed to 35 ppb of copper, the lag phase was 4 days, while in those exposed to 75 ppb it was 18 days. Then, after a time which depends on the metal concentration, recovery of the cultures occurred, so that ultimate cell levels were not considerably different from those of the control. This recovery was still more clearly demonstrated in the case of mercury, where the cell population actually declined with increasing mercury concentrations in the early stages of growth (Fig. 1B). For example, 2.5 days after inoculation, the cell population in the cultures exposed to 35, 50 and 75 ppb of mercury was 7.3×10^6 , 4.4×10^6 and 4.0×10^6 cells/l, respectively, which values correspond to 28%, 17% and 15% of the initial cell population (2.6×10^7 cells/l). After that marked decline, the extent of which depended upon the initial mercury concentrations, a progressive recovery occurred. Finally, the culture reached cell levels not significantly different from that of the control.

Microscopic examination of the different populations of P. lutheri, showed that populations growing in media with copper or mercury consisted of cells larger than those from the controls, particularly during the lag phase. At 4 days after inoculation, the diameters of 50 cells each from the control and the cultures containing 50 ppb of either copper or mercury, were measured. In these cultures, copper and mercury increased the mean cell volume approximately three times to 318% and 271% of the control, respectively.

From the above experiments the following conclusions may be drawn:

- a) Concentrations of either copper or mercury higher than 10 ppb significantly inhibited the growth of P. lutheri.
- b) There was an increase in the lag phase in cultures exposed to any of the two metals, proportional to the initial metal concentration. This lag phase was followed by a period of recovery during which the exponential growth rate did not differ considerably from that of the control. Finally, the cultures reached cell concentrations similar to those of the control.
- c) Both copper and mercury increased the cell size considerably, especially during the lag phase.

3.2 Effects on Dunaliella minuta

The effects of copper and mercury on the cell population (% of the control) of D. minuta is presented in Table 3, while corresponding growth curves are shown in Figures 2A & 2B. Two days after inoculation, 50 ppb of either copper or mercury considerably reduced growth by 37% and 31% of the control values respectively (Table 3). Growth inhibition was higher at higher concentrations of these metals. Comparison of the growth curves and examination of Table 2 shows that D. minuta reacted to copper and mercury in a similar manner, by progressively decreasing the growth rate and final cell yield with increasing metal concentration (Fig. 2A-B; Table 4). Both copper and mercury considerably increased the mean cell volume. At 6 days after inoculation, the mean cell volume in cultures containing 250 ppb of either copper or mercury was 202% and 181% of the control, respectively.

Because of the observed increase of cell size of both species exposed to copper and mercury, the chlorophyll-a content per cell was examined in order to find whether it remained constant or increased proportionally. The results (Table 5) revealed that the chlorophyll-a content per cell remained almost constant and independent of cell size. For example, on day 2, the average chlorophyll-a content of cells in the control culture was 0.46 pg l⁻¹ and values for the cultures exposed to mercury were generally somewhat higher, ranging from 0.52 to 0.63 pg l⁻¹. On day 4, there was even less variation and similar results were obtained on day 7, too. Comparison of the percentage reduction in cell numbers and chlorophyll-a concentration shows that both indices of growth were inhibited by mercury to almost the same extent.

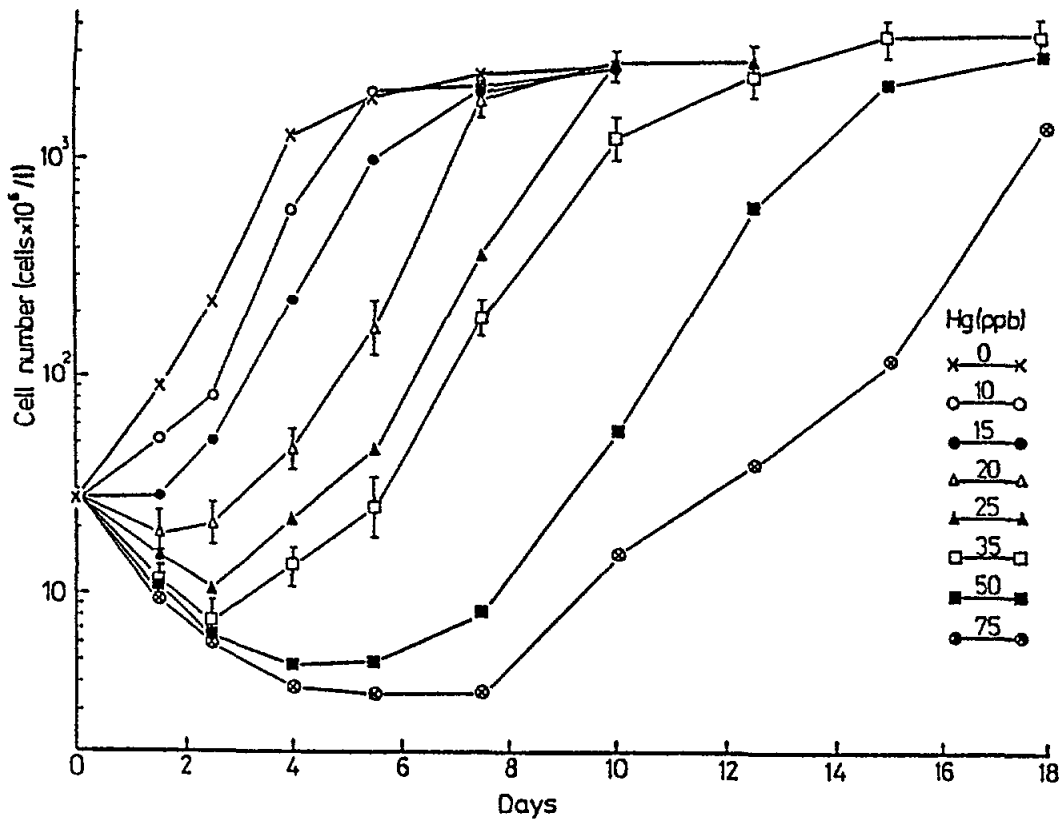


Fig. 2A Effects of copper on the cell population of *D. minuta*. Each point is the mean $\pm 2SE$ of 4 replicates

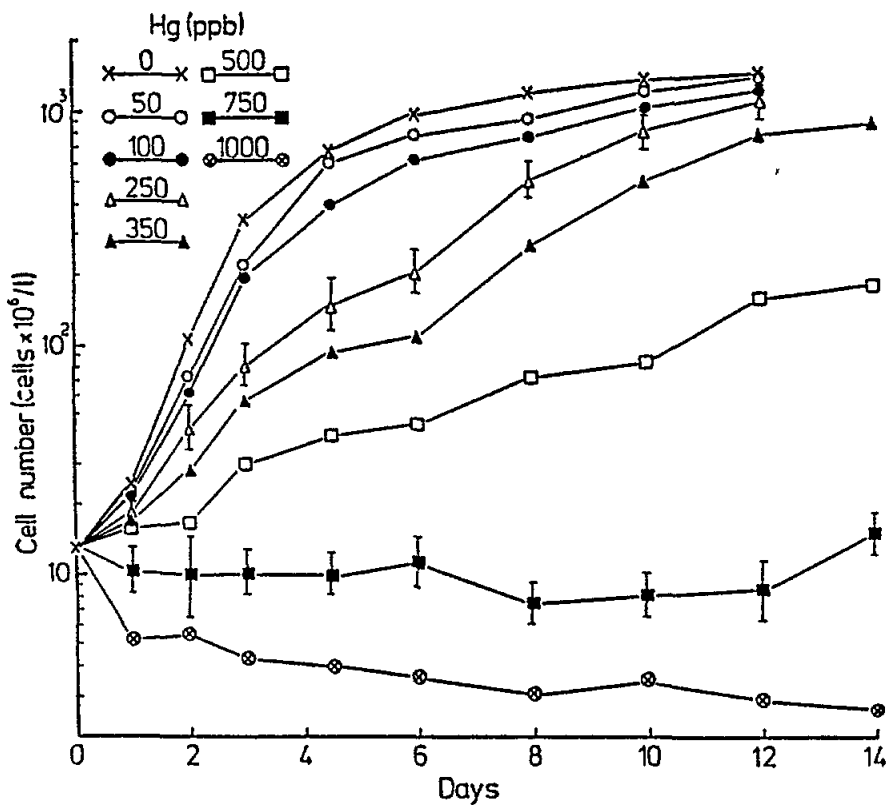


Fig. 2B Effects of mercury on the cell population of *D. minuta*. Each point is the mean $\pm 2SE$ of 4 replicates

Table 3

Effects of copper and mercury on the cell population (% of control) of D. minuta, 1 and 2 days after inoculation.

Time (days)	Copper (ppb)	%	Mercury (ppb)	%
1	0	100	0	100
	50	98	50	96
	100	93	100	92
	250	94	250	78
	500	83	500	71
	750	70	750	67
	1000	60	1000	43
	1500	47	1500	22
2	0	100	0	100
	50	63	50	69
	100	49	100	59
	250	38	250	41
	500	28	500	27
	750	21	750	16
	1000	18	1000	9
	1500	12	1500	5

Table 4

Effects of copper and mercury on the growth rate, generation time and final cell yield of D. minuta.

Metal tested	Metal concentr. (ppb)	Growth rate (μ) (div./day)	Generation time (hrs/div.)	Final yield (cells $\times 10^6$ /ml)
copper	0	1.41	17.0	1.32
	50	19.8	1.20	1.20
	100	0.98	24.4	0.62
	250	0.56	42.2	0.42
	500	0.44	54.0	0.49
mercury	0	1.37	17.5	1.53
	50	1.29	18.5	1.62
	100	1.04	23.0	1.35
	250	0.61	38.9	1.12
	350	0.54	44.3	0.90
	500	0.33	72.6	0.18

Table 5

Effect of mercury on the chlorophyll-a content of D. minuta.

Time (days)	Mercury (ppb)	Chl-a ($\mu\text{g l}^{-1}$) Mean \pm 2SE	Chl-a (%)	Chl-a (pg/cell) Mean
2	0	25.3 \pm 0.8	100	0.46
	25	22.1 \pm 0.4	87	0.60
	50	20.7 \pm 0.8	82	0.46
	100	20.5 \pm 0.6	82	0.62
	150	18.3 \pm 0.4	72	0.63
	250	15.6 \pm 0.6	62	0.52
	400	7.8 \pm 0.6	31	0.59
4	0	252 \pm 5.2	100	0.51
	25	168 \pm 8.4	67	0.52
	50	48 \pm 7.0	19	0.52
	100	39 \pm 2.0	16	0.50
	150	32 \pm 2.8	13	0.54
	250	20 \pm 1.2	7.9	0.53
	400	9.3 \pm 0.4	3.7	0.48
7	0	634 \pm 18.4	100	0.55
	25	625 \pm 16.4	98	0.58
	50	470 \pm 13.0	74	0.65
	100	100 \pm 7.2	16	0.55
	150	40 \pm 1.6	6.3	0.48
	250	17 \pm 1.2	2.7	0.50
	400	5.9 \pm 0.8	0.9	0.55

The following conclusions may be drawn:

- a) D. minuta was more resistant than P. lutheri to both copper and mercury; concentrations which caused slight inhibition of growth were about five times greater than those for P. lutheri.
- b) D. minuta reacted to copper and mercury by decreasing the growth rate and final cell yield, with increasing metal concentration.
- c) Both copper and mercury increased the cell volume, during the whole growth period.
- d) Cell numbers and chlorophyll-a concentration per volume basis were inhibited to almost the same extent, while the chlorophyll-a per cell remained almost constant and independent of the cell size.

4. DISCUSSION

This study of the effects of copper and mercury on the growth of the phytoplanktonic organisms Pavlova lutheri and Dunaliella minuta not only illustrates the differing toxicities of the metals, but does also reveal marked differences between the two species in respect of their sensitivity and physiological response to each metal. Of the two algae, P. lutheri was the more sensitive to metals used. It exhibited a prolongation of the lag phase, while D. minuta reacted to both metals by decreasing its growth rate. A great resistance to mercury of Dunaliella tertiolecta has been reported by Overnell (1976) and Davies (1976). Mandelli (1969), also, reported that D. tertiolecta continued to grow in a medium saturated with copper (ca 600 ppb).

From the two metals mercury was the most toxic to both species, especially in the early stages of growth. The concentrations of copper and mercury which were found to depress growth in P. lutheri and D. minuta are in general agreement with previous findings for several species of Haptophyceae and Chlorophyceae (Davies, 1978; Rai et al., 1981; Hall et al., 1989). However, it is difficult to make direct comparisons between the different studies because of differences in the organisms tested and the experimental conditions and indices of growth used by each investigator.

The degree of inhibition of growth in both experimental organisms brought about by each of the metals tested varied as a function of concentration and time of exposure. As expected, toxic effects increased with increasing metal concentration. They also increased with time of exposure up to a point where various physical, chemical and biological changes in the medium, acting alone or in combination, caused the cultures to recover. This was more pronounced in the cultures containing mercury, where a severe reduction of the cell population occurred at relatively high concentrations, especially in the early stages of growth (Fig. 1B). This decline in the cell population may be due to a disruption of the cell membrane by mercury, leading eventually to lysis (Rothstein, 1959).

Morel et al. (1978) suggested "conditioning" of the medium and/or adaptation of the cells to the high metal concentrations as a possible mechanism for the recovery of growth. Several studies have demonstrated the excretion of organic exudates by microalgae (e.g. McKnight and Morel, 1979; Fisher and Fabris, 1982). These substances will alter the copper speciation in the medium by binding the metal and may render it less bioavailable. One can, therefore, suggest that the medium becomes "conditioned" by the cells themselves and that the conditioning occurs during the extended lag phase. Another possibility is that the surviving cells may become "adapted" to the high metal(s) concentration during the extended lag phase. This adaptation may involve an intracellular physiological response, for instance, the activation of detoxification processes.

One of the reasons for the recovery of growth is probably the reduction of metal concentration in the growth medium because of: (a) taking up by the test organism (Chipman et al., 1958), (b) adsorption on particulate matter (Tomlinson and Renfro, 1972) or glass surfaces (Robertson, 1968) and possibly to precipitation (Lewin and Chen, 1971). Especially in the case of mercury, it has been reported that a significant fraction is lost from solutions containing microorganisms, due to the volatilization of the elemental metal, produced by reduction of the ionic forms (Toribara et al., 1970).

During the lag phase, especially in cultures containing high copper or mercury concentrations, the majority of cells had lost their ability for movement and appeared large, with relatively thick cell walls, as if they had been transformed into resting forms to reduce their metabolic needs to a minimum. A rapid loss of mobility of the dinoflagellate Gonyaulax tamarensis (Anderson and Morel, 1978) and of the chlorophyceae Dunaliella salina (Stom et al., 1984), both growing in media containing metals, has been reported as a criterion of the organism's short term sensitivity to metals. Kellner (1955) has reported that certain species can be adapted to relatively high concentration of toxicants, and, in some cases, resistant types may develop.

Another effect characteristic of both copper and mercury, was the increase in the cell size in both experimental species. Similar cases have been reported by Nuzzi (1972), who described morphological abnormalities in Phaeodactylum tricornutum and Chlorella sp., and by Gupta and Arora (1978) who reported increased cell size with increasing Cu^{+2} activity. The precise mechanism by which mercury and copper cause the production of large cells is not clear, but this may be because of disruption of the link between growth and cell division (Fisher et al., 1981), possibly mediated via a change in membrane function (Huntsman and Sunda, 1980) or affecting the metabolism of the organisms after the metals penetrate the membrane (McBrien and Hassal, 1967; Steemann Nielsen et al., 1969).

The results obtained, also, indicate that both cell numbers and chlorophyll-a concentration in the cultures of D. minuta exposed to mercury were reduced to approximately the same extent at the concentrations of the metal tested. Similar results have been obtained by Saifullah (1978), who reported that chlorophyll concentrations of Scrippsiella faeroence, as measured in batch cultures, were influenced by copper in a manner similar to the cell population. The almost identical reductions in population and chlorophyll concentration suggest that the chlorophyll content per cell of this species also remained approximately constant and independent of metal concentrations. An approximately constant chlorophyll-a content per cell, independent of cell size, was reported by Jorgensen (1964) for Cyclotella meneghiniana. The finding that the chlorophyll-a content per cell is almost constant, suggests that the number and size of the chromatophores do not change considerable when the cell volume varies, and that probably the volume of the cytoplasm also remains approximately constant. Thus, the change in the cell volume may be attributed to a change in the volume of the vacuole inside the cytoplasmic layer.

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DETECTION AND EVALUATION OF HEPATIC INTOXICATION IN FISH

by

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A B S T R A C T

Besides glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and lactic dehydrogenase (LDH) we have studied the possibility to use sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GLDH) and alkaline phosphatase (AP) to examine liver poisoning in grey mullet. Fish were treated with CCl₄, phenol and cyanide. The increased activity of GOT, GPT, SDH and GLDH and the change in concentration of triglycerides in plasma can be used as positive indicators of liver damage by poisoning. The change in activity of AP, and the decreased concentration of plasma proteins, haemoglobin and haematocrit are discussed.

1. INTRODUCTION

Hepatotoxicity and hepatic damage in fish are the most common responses to various toxic substances (Couch, 1975; Gingerich, 1982) but because of the lack of suitable tests, an accurate evaluation of fish liver toxicity has been hindered. Since a large part of serum enzymes are derived from liver, the measurement of their activity changes in serum or in blood plasma can provide an estimation of liver damage or dysfunction. However, owing to specific anatomical, physiological and biochemical differences between homeothermic mammals and poikilotherm fish, for the application of these methods it appears pertinent to evaluate their significance in fish physiology and biochemistry before they can be considered of diagnostic value in assessing fish health.

As it was ascertained for rainbow trout (Raccicot *et al.*, 1975; Pfeifer *et al.*, 1977) and English sole (Casillas *et al.*, 1983), we have previously confirmed that alteration of aminotransferase activity in plasma (GOT and GPT) could be used as useful labels of liver toxicity in grey mullet (Krajnović-Ozretić and Ozretić, 1987). The present study examines further the various enzymes and metabolites in the blood of grey mullets to assess additional aspects of liver toxicity following exposure to different toxicants. CCl₄ was used as specific hepatotoxic agent, phenol was chosen as general protoplasmatic poison and cyanide was adopted as extremely poisonous but non hepatotoxic substance. Their effects were studied measuring the activity of SDH, GLDH and AP in mullet plasma. The route of intoxication and the high doses of chemicals used were rather aggressive and do not represent those expected in case of environmental contamination. They were used to provide a massive, model toxic insult to identify large enzyme and metabolite changes in fish plasma as potential markers of fish liver intoxication. The activity of the same enzymes was also measured in selected tissues to examine their distribution and to identify potential sources of enzyme activity in

plasma. These enzymes were used as indicators of liver dysfunction in mammals (Tietz, 1976). In fish, the use of sorbitol dehydrogenase was evaluated only in rainbow trout (Dixon *et al.*, 1987), while Raccicot *et al.* (1975) and Casillas and Ames (1986) analyzed the potential use of GLDH and AP as possible indicators of liver dysfunction in rainbow trout and English sole respectively. We have also examined the concentration of lipids and triglycerides in plasma as possible indicators of altered fat metabolism in liver tissue. Total proteins, albumin and some metabolites such as ammonia and lactate were also measured. All parameters were determined 24 h after the injection, the time when the maximum changes in blood enzyme activities were observed (Krajnović-Ozretić and Ozretić, 1987). The actual liver damage was confirmed by comparison with the changed aminotransferase activity and by histological inspection of the liver tissue.

2. MATERIALS AND METHODS

2.1 Experimental animals

Grey mullet (Mugil auratus Risso) average weight 205 ± 25 g were used as test animals. Fish were acclimatized at least 2 weeks in 250 l aerated basins with a continuous flow of sea water (salinity 37.2 ± 0.4 , temperature $18 \pm 0.5^\circ\text{C}$). Fish were fed daily to satiation and the remaining food was removed.

2.2 Toxicant administration

Ten grey mullets per group were injected intraperitoneally with 2 ml CCl_4 and 200 mg of phenol per kg body weight. In a separate basin, ten mullets were exposed for 24 h to a constant concentration of $300 \mu\text{g}$ (NaCN) kg^{-1} that was maintained with a continuous flow doser for cyanide. Phenol and sodium cyanide were first dissolved in distilled water, while CCl_4 was injected without diluent.

2.3 Experimental techniques

Blood samples were taken after 24 hrs, by cardiac puncture. Blood sampling and i.p. injections were performed without the use of anaesthetics. Heparin was used as anticoagulant. Blood was kept iced and the plasma was immediately separated with a refrigerated centrifuge (10 min at 2000 g). The activity of LDH (EC 1.1.1.27), GOT (EC 2.6.1.1.) and GPT (EC 2.6.1.2.) was measured by NADH-linked spectrophotometric method (Bergmeyer and Bernt, 1974; 1974a; 1974b) with modifications to optimize the assay conditions for grey mullet (Krajnović-Ozretić and Ozretić, 1987). GLDH (EC 1.4.1.2) and SDH (EC 1.1.1.14.) activity was determined with the kinetic UV method (Schmidt, 1974; Gerlach and Hiby, 1974) and AP (EC 3.1.3.1.) according to the method of Klaus and Schutt (1974). The activity of the same enzymes was also measured in the extracts of liver, heart, kidneys, gills, white and red muscle. Tissue samples were homogenized with a Polytron grinder in 10 parts of a cold 0.2 M Na-phosphate buffer (7.4) in 20% glycerol with 5 mM mercaptoethanol. Homogenates were centrifuged at 17000 g for 30 min. and the supernatant was immediately used for enzyme assay. For plasma, the enzyme activity was calculated in relation to the unit volume (l) or to the unit weight (g) for body tissues and

it was expressed as International Units ($UI^{-1} = \mu\text{Moles substrate metabolised min}^{-1} l^{-1}$). Biuret reaction was used to determine total protein in plasma (Weichselbaum, 1946). Plasma albumin was determined spectrophotometrically using a commercial diagnostic test produced by Sigma. Boehringer biochemical test kits were used to estimate total plasma lipids (colorimetric method of Zollner and Kirsch, 1962) and triglycerides (enzymatic colorimetric GPO-PAP test, Wahlefeld, 1974). Samples of liver tissue were carefully isolated and fixed with Bouin's fluid. After routine histological processing, the tissue was embedded in paraffin and sections of 5-6 μm thickness were stained with hematoxylin and eosin, and examined by light microscopy.

3. RESULTS

The activity of LDH, GOT, GPT, GLDH, SDH and AP in several body tissues of a control group of grey mullet are displayed in Table 1. These values give an estimation of the potential sources of the enzymes from where, in particular circumstances, they could be transferred into fish plasma. The highest activity and the largest absolute content of LDH was found in white muscle tissue, the lowest in liver. As in other vertebrates, mullet liver was distinguished by the highest content of GPT, SDH and GLDH. The activity of GOT was highest in red muscle and heart but it was only slightly higher than in liver. AP activity was highest in kidney, nearly 3 times higher than AP in liver. The activity of all six enzymes was lowest in plasma: for GPT and SDH very near to the detection limits.

Some aspects of liver response to the toxic effects of CCl_4 , phenol and cyanide are illustrated in Table 2. As we have previously observed, both CCl_4 and phenol induced a significant increase of GOT, GPT and LDH activity in plasma ($p < 0.05$). The activity of LDH in plasma increased about 45 times higher than that of the control group, while GOT raised nearly 125 and 184 times respectively. The activity change of GPT in mullet plasma was particularly affected by CCl_4 and, an average increase of 465 times was detected, while phenol increased GPT only 21 times above the average activity in the control group but, still significant at the $p < 0.05$ level. The activity of SDH, GLDH and AP in mullet plasma also increased. SDH in fish treated with CCl_4 and phenol, compared with the control group increased 16 and 8.8 times, GLDH 5.5 and 27, whereas the activity of AP increased about 7.5 and 4.9 times respectively. Plasma lactate concentration was not notably changed while the concentration of ammonia was significantly increased. The concentration of triglycerides increased significantly only in fish injected with CCl_4 . Total lipids, proteins and haemoglobin decreased significantly in fish treated with phenol. Exposure to cyanide did not cause any change neither in enzyme activity nor in other plasma parameters.

Within 24 h after the injection of CCl_4 and phenol the structure of liver was injured (Fig. 1). The most evident structural changes were characterised by vacuolation of cells around the central veins, accompanied by fatty infiltrations. Lobular organization and the typical arrangement in multiplex cells thick muralia nearly disappeared due to the intensive cellular vacuolation, which compressed the liver sinusoidal structures.

Table 1

Grey mullet (*Mugil auratus* Risso). Distribution of LDH, GOT, GPT, GLDH, SDH and AP in mullet tissues (Ug^{-1} wet wt) and in cardiac-sampled plasma (U^{-1}). Each value represents the mean \pm s.d. (N=30).

	White muscle	Red muscle	Liver	Kidney	Heart	Gill filaments	Plasma
LDH	926 \pm 84	417 \pm 76	8 \pm 6	69 \pm 23	482 \pm 21	81 \pm 12	110 \pm 23
GOT	13.9 \pm 4.8	61.3 \pm 22.2	39.8 \pm 18.1	18.2 \pm 5.66	41.8 \pm 14.4	10.9 \pm 4.5	19.6 \pm 11.6
GPT	0.45 \pm 0.17	4.4 \pm 2.7	17.7 \pm 4.4	4.3 \pm 1.9	0.50 \pm 0.25	0.27 \pm 0.12	1.95 \pm 0.23
GLDH	0.22 \pm 0.13	4.1 \pm 2.4	66.0 \pm 35.5	9.9 \pm 5.3	19.5 \pm 7.3	3.9 \pm 1.0	18.3 \pm 9.4
SDH	0.05 \pm 0.01	0.19 \pm 0.02	4.2 \pm 0.39	1.7 \pm 0.26	0.39 \pm 0.02	0.13 \pm 0.01	1.16 \pm 0.20
AP	0.21 \pm 0.02	0.23 \pm 0.05	4.5 \pm 1.48	13.7 \pm 3.0	0.40 \pm 0.01	2.4 \pm 0.27	20.0 \pm 5.98

Table 2

Enzyme activity, hematological and biochemical parameters (\pm s.d.) in mullet (*Mugil auratus* Risso) injected with CCl_4 , phenol and cyanides compared to a control group.

	Control	Phenol	CCl_4	Cyanide
Ammonia ($\mu\text{g } 100 \text{ ml}^{-1}$)	194.0 (83.7)	371.8* (83.8)	322.4* (37.0)	-
Lactate ($\text{mg } 100 \text{ ml}^{-1}$)	13.9 (3.1)	14.9 (3.6)	17.6 (4.4)	15.1 (3.5)
LDH (U l^{-1})	218.8 (105.5)	9925.0* (6236.1)	9700.0* (5970.0)	244.0 (95.9)
GLDH (U l^{-1})	18.3 (9.4)	495.6* (324.0)	102.4* (71.3)	16.1 (6.2)
GOT (U l^{-1})	19.6 (11.6)	2451.3* (1361.9)	3600.0* (1067.0)	24.2 (7.8)
GPT (U l^{-1})	1.95 (0.23)	41.8* (5.9)	907.0* (394.0)	2.6 (0.4)
SDH (U l^{-1})	1.16 (0.20)	10.2* (1.7)	19.1* (2.7)	1.09 (0.2)
AP (U l^{-1})	20.0 (5.98)	75.9* (38.8)	150.9* (51.7)	21.8 (5.9)
Triglycerides ($\text{mg } 100 \text{ ml}^{-1}$)	91.1 (50.1)	98.3 (55.4)	196.6* (59.2)	90.8 (48.3)
Total lipids ($\text{mg } 100 \text{ ml}^{-1}$)	1404.0 (297.6)	913.5* (186.8)	1296.0 (268.5)	1358.0 (263.0)
Total Proteins ($\text{g } 100 \text{ ml}^{-1}$)	4.1 (0.6)	2.9* (0.7)	3.5 (0.9)	4.4 (0.4)
Albumin ($\text{g } 100 \text{ ml}^{-1}$)	1.2 (0.1)	1.4 (0.2)	1.1 (0.5)	1.4 (0.2)
alb./prot.	0.29	0.48*	0.31	0.34
Hemoglobin ($\text{g } 100 \text{ ml}^{-1}$)	9.1 (1.0)	6.4* (1.3)	10.6 (1.2)	9.8 (1.0)
Hematocrit (%)	31.0 (3.5)	22.0* (5.0)	35.3 (8.7)	34.5 (4.3)
MCHC (%)	30.8 (2.9)	29.0 (2.9)	30.8 (4.7)	28.4 (3.1)

* Values significantly different from controls ($P < 0.05$)

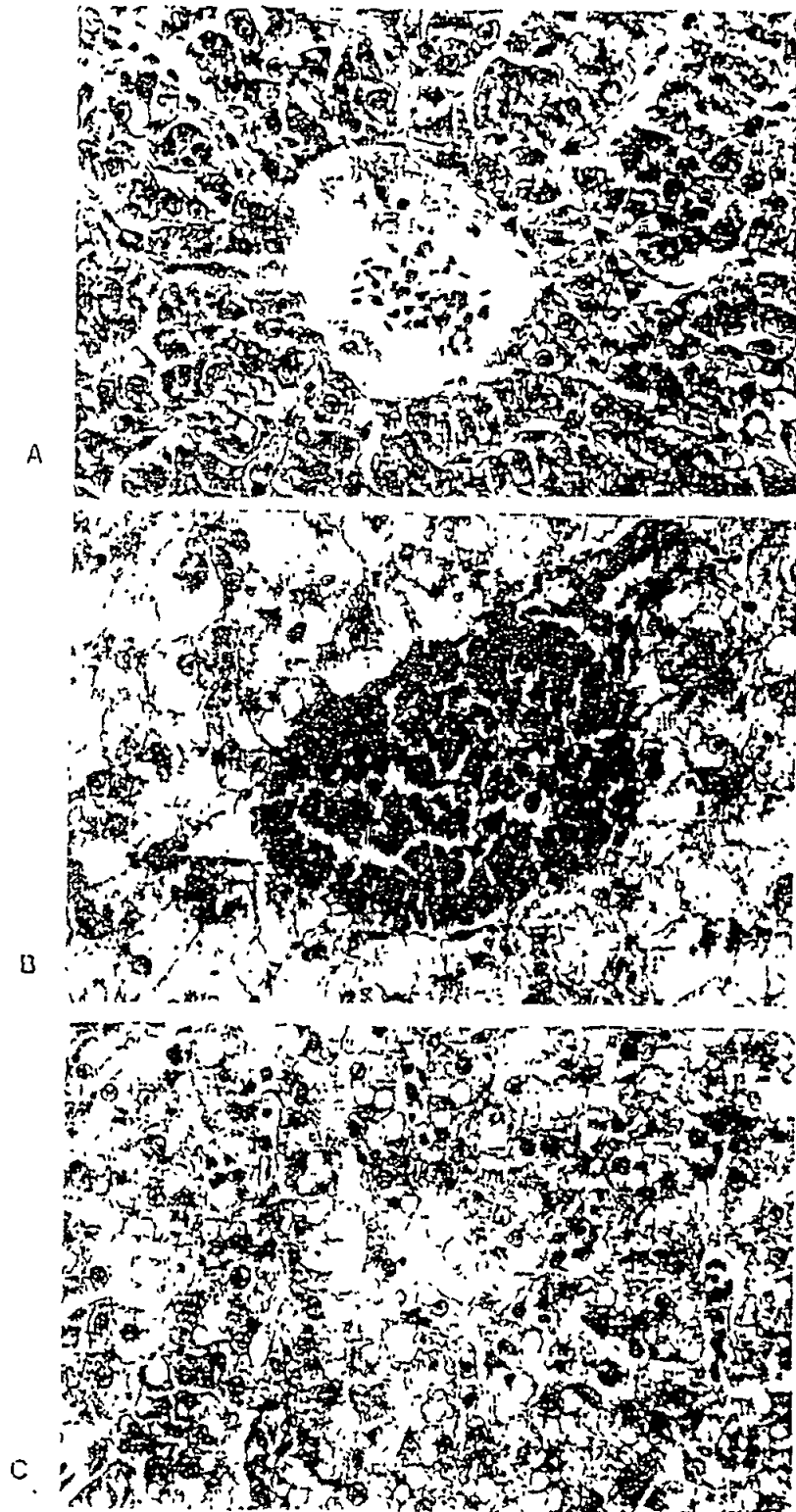


Fig. 1 Grey mullet (Mugil auratus Risso). Liver tissue sections stained with hematoxylin-eosin (x300) showing various degrees of hepatocyte vacuolization. A: control, B: CCl₄ and C: phenol injected fish

4. DISCUSSION

Liver injury induced by chemicals has been very early recognized as one of the main toxicological problems (Zimmerman, 1978). The most useful tools in hepatotoxic studies involves the determination of the activity of hepatic enzymes released into the blood due to the impaired liver organization on the cellular and molecular level (Kaplan and Szabo, 1983). The most frequently used enzymes by fish biologists to diagnose sublethal harm to liver are GOT and GPT (Bell, 1968; Raccicot *et al.*, 1975; Casillas *et al.*, 1983). Correlation between the increased activity of GOT and GPT in serum and histological damage of fish liver have been already demonstrated (Raccicot *et al.*, 1975; Statham *et al.*, 1978; Casillas *et al.*, 1983). Although they are from different origin, according to Zimmerman (1978) categorization, both enzymes are very symptomatic to hepatic cytotoxic injury. Actually GOT and LDH are tissue non specific enzymes (Table 1) and, in general their presence in plasma can reflect injury of extrahepatic tissues. On the contrary GPT is mainly located in liver (Table 1) and it is therefore expected that any change of GPT activity in plasma exhibits the functional state of liver. We have previously reported a detailed analysis of GOT, GPT and LDH activity in grey mullet plasma following intoxication with CCl_4 and phenol (Krajnović-Ozretić and Ozretić 1987, 1987a). According to the isozyme pattern determination and heat denaturation tests in plasma and in body tissues extracts, the increased LDH activity in plasma of grey mullet treated with CCl_4 and phenol can be considered as a leakage from the white muscle tissue and thus cannot be considered as indicator of hepatotoxic disfunctions. In addition, the electrophoretic patterns of GOT from different tissues, and the tissue distribution of both GOT and GPT suggested the liver to be their origin. As in mammals, GPT appeared to be a specific "liver-guiding enzyme" that can be used as a sensitive indicator of hepatotoxic effects.

Among other useful diagnostic enzymes, SDH belongs to the group of enzymes that are almost exclusively located in liver and their increased activity in plasma is a reliable reflection of hepatic injury. Actually SDH is properly used to evidence liver damage in mammals (Wolf *et al.*, 1973; Alemu *et al.*, 1977), and the utility of SDH to diagnose chemically induced liver damage in fish was also confirmed by Dixon *et al.* (1987). GLDH is also a liver specific enzyme in man and mammals. Owing to his intramitochondrial location, GLDH emerges as a highly positive marker of enzyme release from the mitochondrial matrix of hepatocytes. Increased activity of GLDH was observed in plasma of fish intoxicated with CCl_4 (Raccicot *et al.*, 1975; Casillas and Ames, 1986). In mammals, among the enzymes which evidence specific types of hepatic disfunction resulting from acute cholestasis, AP had attracted considerable attention.

We found that SDH activity markedly increased in mullet plasma 24 hr after exposure to CCl_4 and phenol. According to the highest activity of SDH in liver and to its substantial body burden, we presumed that impaired liver could release comparably more SDH into fish plasma than any other body tissue. That was supported by the high positive correlation with the increased activity of GOT and GPT as exclusive liver enzymes (+0.818 and +0.945 respectively). The activity of SDH in liver was nearly one order of magnitude higher than in any of the six other tissues. Thus it was assumed that damaged liver would release relatively more SDH into plasma than other tissues. In plasma of control grey mullet, SDH activity is very low, so that very small changes in the activity of this enzyme can be easily detected. The activity

of AP in plasma was also increased, but there are contradictory statements about the interpretation of the usefulness of this enzyme system in fish toxicology studies. While Raccicot *et al.* (1975) evidenced the inconsistency between liver intoxication with CCl_4 and AP activity in rainbow trout plasma, Casillas and Ames (1986) proposed it as useful to assess the hepatotoxic effects of CCl_4 in English sole. We found that kidneys contained the highest activity of AP but, since we were not able to prove its liver origin we can not speculate about the use of this enzyme as indicator of intrahepatic cholestasis.

The concentration of ammonia in plasma increased simultaneously with transaminases and GLDH activity. GLDH, in relation to GOT and GPT, plays an important function in ammonia detoxification in fish (Hochachka and Somero, 1973) and may determine the direction of amino acid metabolism (catabolism versus anabolism). Actually, the increased activity of GLDH jointly with the increased concentration of ammonia in blood, suggested that protein catabolic process prevailed as a result of toxicological stress.

In clinical medicine, serum triglycerides are used to evaluate lipid metabolism (Tietz, 1976). Since homeostasis of lipids is one of the principal liver functions, any change in serum triglyceride concentration is used as indicator of liver dysfunction. As in mammals, also in fish triglycerides are central metabolites in lipid metabolism (Love, 1980). In grey mullet's plasma, after exposure to CCl_4 , we found increased levels of triglycerides and histological analysis revealed cytological changes and fatty accumulation. These findings associated with the increased plasma triglycerides may be indicative of altered or impaired synthesis and transport of lipids in liver. In mullets treated with phenol, the increased enzyme activity was accompanied by decreased plasma proteins and lipids. Haemoglobin and haematocrit also decreased confirming our previous findings (Krajnović-Ozretić and Ozretić, 1988). Theoretically we can suppose that they can result from haemodilution, loss of proteins with urine following kidney damage, or increased protein utilization without replenishment. Since the concentration of proteins in mullet plasma decreased at the same time as total lipids, haemoglobin, and haematocrit, the dilution of plasma with water could perhaps be assumed.

Similarly, in plasma of laboratory animals treated with CCl_4 , Berryman and Bollman (1943) and in rainbow trout, Gingerich and Weber (1979), Pfeifer and Weber (1979) and Dixon *et al.* (1987) observed that total plasma protein also decreased. On the contrary, in mullet we did not find any significant decrease of plasma proteins, neither was the albumin/protein ratio altered.

Histological changes of mullet liver exposed to CCl_4 and phenol were characterized by extensive lipophilic vacuolization of parenchymal cells, which was more pronounced in fish injected with CCl_4 . This type of liver lesion is indicative of intermediate and nonspecific pathological response. In this occurrence, significant increase of GOT, GPT, SDH, GLDH and AP activity in plasma was observed. The absence of more severe histological lesions (focal or zonal necrosis) indicates that biochemical lesions (release of enzymes into plasma) precedes histological damage. The response of enzymes to CCl_4 , which is primarily considered as hepatotoxin is more pronounced than the response to phenol. This was particularly evident by the higher response of GPT, which is a specific "liver guiding enzyme".

The absence of altered enzyme activity and induction of hepatic lesions by cyanide is consistent with the mode of action of HCN as metabolic inhibitor rather than as an overall hepatotoxicant.

Searching for confident methods to assess the impaired metabolism and biochemistry in fish exposed to toxic substances we have previously confirmed the validity to check the activity of aminotransferases in mullet plasma as indicators of liver intoxication (Krajnović-Ozretić and Ozretić, 1987). We have currently extended the number of possible indicators of hepatotoxicity in grey mullet by demonstrating that changes of SDH and GLDH activity in plasma are correlated with toxicity of CCl₄ and phenol, while the appearance of triglycerides in plasma is correlated only with CCl₄.

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THE MULTI-XENOBIOTIC RESISTANCE MECHANISM
IN AQUATIC ORGANISMS

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A B S T R A C T

The fact that many aquatic organisms survive and reproduce in polluted waters indicates that they are equipped well with defense system(s) against several toxic xenobiotics simultaneously, since water pollution is typically caused by a mixture of a number of pollutants. We have found that the biochemical mechanism underlying such "multi-xenobiotic" resistance in freshwater and marine mussel as well as in several marine sponges is similar to the mechanism of multi-drug resistance (MDR) found in tumor cells that became refractory to treatment with a variety of chemotherapeutic agents. All these organisms possess a verapamil-sensitive potential to bind 2-acetylaminofluorene (AAF) and vincristine (VCR) onto membrane vesicles. In in vivo experiments, the accumulation of xenobiotics AAF and VCR is enhanced in all investigated organisms in the presence of verapamil, the inhibitor of the P-170 extrusion pump. It is obvious that knowledge that the presence of one xenobiotic may block the pumping out, and hence accelerate accumulation of other(s), may help us to understand and interpret our present and past data on different environmental parameters obtained using indicator organisms.

1. INTRODUCTION

For those of us interested in pathobiological effects of xenobiotics, i.e. in the environmental xenobiotic-risk assessment, the standard methodology in field studies includes the choice of the sites where a so called "scale of pollution" exists. In a typical case we have a site A, which is a point source of pollution, and then sites B, C, and D, which represent downstream sites where the pollutant is more and more diluted, and finally site H, which represent an unpolluted, control site. In our papers, in the Section Materials, Subsection Animals, we often use the phrase, for example: "6 specimens were collected at each site on a biweekly basis over the period of three years". This and similar statements, discover one fact: there are organisms that live and reproduce at polluted sites, including even a heavily polluted site A. These organisms are obviously resistant to pollution, and, since water pollution is caused by a complex mixture of different chemicals, it is equally so obvious that these organisms are simultaneously resistant to a number of different pollutants. Moreover, the concentration of pollutants in these organisms is often lower than is the concentration of these pollutants in a surrounding environment, for example in surrounding recent sediments (Sieben et al., 1983; Malins et al., 1984). Simultaneous resistance to a variety of pollutants and a decreased accumulation of pollutants are characteristics that are apparently similar to characteristics observed in

"multi-drug resistant" tumor cells. There, a multiple resistance to a variety of drugs is accompanied by a decrease in drug accumulation (Juliano and Ling, 1976). The multidrug resistant cells take up drugs just as well as sensitive cells but they fail to accumulate these drugs (Juliano and Ling, 1976).

The phenomenon of multidrug resistance (MDR) is associated with the increased production of a membrane glycoprotein of approximately 170,000 daltons, termed "P-glycoprotein" (Kartner *et al.*, 1983; Endicott and Ling, 1989), or P170 (P standing for permeability). In the MDR cell lines P170 binds a drug and facilitates its efflux by an energy-dependent process (Cornwell *et al.*, 1986). *mdr-1*, the gene coding for P170, has been cloned (Ueda *et al.*, 1986), and its overexpression was found to be proportional to the degree of resistance in resistant cell lines (Shen *et al.*, 1986). Expression of the *mdr* gene, both as mRNA and protein, has been detected in a number of normal tissues, such as liver, adrenal, pancreas, kidney, jejunum, colon (Thiebaut *et al.*, 1987), placenta (Sugawara *et al.*, 1988), and in specialized endothelial cells in the brain and testis (Gordon-Cardo *et al.*, 1989). The localization of P170 in specialized cells in tissues indicates that it might be involved in transepithelial secretion of toxic substances, steroid hormones, or unknown cell metabolites into bile, urine, or the lumen of gastrointestinal tract (Kane *et al.*, 1990). Many compounds inhibit drug binding to P170 (Cornwell *et al.*, 1986) and reverse MDR by acting as competitive inhibitors of the P170-pump. Such reversing agents, like verapamil, quinidine and reserpine, became a useful scientific tool in studies on the nature of MDR. P170-pump is activated by protein kinase C and inhibited by protein kinase C inhibitors (Chambers *et al.*, 1990). The molecular mechanism involved in MDR includes also alterations in drug metabolism: the increased expression of anionic glutathione transferase (Batist *et al.*, 1986), encoded by GST-P gene (Moscow *et al.*, 1989), with a simultaneous alteration in the glutathione redox cycle (Kramer *et al.*, 1988).

Although recent findings suggest that the mechanism of MDR may be more diverse and complex, the enhanced efflux appears to be the major determinant of reduced drug accumulation and the dominant feature in a model of classical multidrug resistance (Morrow and Cowan, 1988). The apparent similarity between the basic characteristics of MDR and the resistant aquatic organisms suggested that the mechanism of MDR might be operative also in resistant aquatic organisms. In order to check this possibility, we measured several parameters that offer direct or indirect evidence of the presence and function of MDR-mechanism components in several species of mussel and sponge. These included the measurement of potential of membrane vesicles to bind radiolabelled 2-acetylaminofluorene and vincristine in the presence or absence of verapamil and ATP, as well as measurements of a verapamil-sensitive bioaccumulation of radiolabelled xenobiotics in tissues of exposed organisms. This review will focus on recent data indicating the presence of biochemical elements of MDR mechanism in tissues of investigated aquatic invertebrates *in vitro* as well as their function *in vivo*.

2. MATERIALS AND METHODS

2.1 Chemicals

The sources of chemicals used in this work were as follows: 2-acetyl-amino-(9-¹⁴C)fluorene (¹⁴C-AAF; 55.3 mCi/mmol) and (G-³H)vincristine

sulphate (^3H -VCR; 2.4 Ci/mmol) from Amersham, England; 2-acetylaminofluorene (AAF), trypsin, soybean trypsin inhibitor, and verapamil from Sigma, FRG; phosphate buffered saline (PBS) from Boehringer, FRG.

2.2 Animals

Specimens of freshwater mussel *Anodonta cygnea*, 14-18 cm, 150-210 g, were from a brook supplying waters for carp-farm Draganici, near Zagreb. Specimens of adult mussel *Mytilus galloprovincialis*, were collected from the low water neap tide level in the protected area of Limski Kanal, Northern Adriatic. Sponges *Geodia cydonium*, *Verongia aerophoba*, and *Tethya aurantium* were collected by diving 3 n. m. offshore from the town of Rovinj, Northern Adriatic, from a depth of 30 m. Within 1 h after collection, the organisms were used for the preparation of membrane vesicles, or in laboratory exposure experiments.

2.3 Membrane vesicles preparation and binding assay

Membrane vesicles were prepared from homogenates (500 mg wet weight tissue in 2 ml PBS) following differential centrifugation steps according to the method of Riordan and Ling (1979). Total and nonspecific binding of radiolabelled substrates was measured according to the method of Cornwell *et al.* (1986) as modified by Kurelec and Pivcevic (1989).

2.4 Bioaccumulation experiments

A group of 4 mussel *Mytilus galloprovincialis* specimens was exposed to 200 ml of seawater polluted with 1 μCi ^{14}C -AAF in the presence or absence of verapamil (5 $\mu\text{g ml}^{-1}$) for 4 h-period. The procedure of Jorgensen *et al.* (1988) was used to ensure physically the space (2 mm) for the separation of the valves and the passive function of mussel pump during the exposure. After the exposure period, mussels were held for 10 min in a flow of seawater, the organs were separately removed and extracted. One g of sponge cubes (5 mm) were exposed to 20 ml of seawater polluted with 1 μCi ^3H -VCR for 4 h periods, in the presence or absence of verapamil (5 $\mu\text{g ml}^{-1}$). After the exposure period, organs of the mussel or sponge cubes were extracted in 1.5 volumes (w/v) of ethylacetate-acetone (2:1) by homogenization in a Polytron homogenizer and the radioactivity was measured in aliquots of the organic phase after centrifugation by liquid scintillation counting.

3. RESULTS

Incubation of membrane vesicles isolated from gills of both the freshwater and the marine mussel with ^{14}C -2-acetylaminofluorene (AAF) led to binding of the radiolabel to the membranes (Table 1). Similarly, ^3H -vincristine (VCR) binds to the membrane vesicles isolated from three marine sponges or from human kidney cortex (Table 1). In the presence of verapamil (10 $\mu\text{g ml}^{-1}$), a drug which is effective in overcoming resistance in MDR cells by inhibiting the outward transport of antitumor agents, the amount of radiolabel bound to the membranes was inhibited between 58 and 87% (Table 1). In the absence of ATP, the amount of radiolabel bound to the vesicles was reduced for more than 65% in all samples (not shown), demonstrating the energy dependence of the binding process. Pretreatment of vesicles with trypsin reduced binding in all samples for more than 60% (not shown), indicating that

the binding process is protein-dependent. Additions of 100-fold molar excess of unlabelled AAF or VCR to incubation media reduced the binding of radiolabel to vesicles in all samples by 82-94% (not shown), suggesting that binding involves a saturable process. This capacity for verapamil- and trypsin-sensitive binding of xenobiotics like AAF and VCR in a saturable manner strongly suggest that binding in membrane vesicles isolated from mussels and sponges involves a molecular mechanism similar to that found in membrane vesicles from MDR cells and identified as glycoprotein P170 (Kartner *et al.*, 1983).

Table 1

The effect of verapamil on ^{14}C -2-acetyl-aminofluorene (AAF) and ^3H -vincristine (VCR) binding to membrane vesicles isolated from mussels, sponges and kidney tissues.

Species	Substrate ¹	Binding ²		% inhibition
		-verap.	+verap.	
<u>Anodonta cygnea</u>	AAF	24.2	8.0	67
<u>M. galloprovincialis</u>	AAF	78.0	9.6	87
<u>V. aerophoba</u>	VCR	2.00	0.85	58
<u>G. cydonium</u>	VCR	2.45	0.95	61
<u>T. aurantium</u>	VCR	4.50	1.80	60
Kidney	VCR	4.50	1.80	60

The binding studies were performed in the absence or presence of verapamil ($10 \mu\text{g ml}^{-1}$) as described in Materials and Methods.

¹Hundred μg of proteins isolated from the digestive glands of mussels, whole tissues of sponges, or cortex region of human kidney were used.

²Binding is expressed in pmoles of substrate bound to a mg of protein within a 20 min incubation period. Numbers in columns represent means of binding determinations with 4 parallel experiments. The SD was less than 12% and thus the differences in binding were statistically significant.

If this *in vitro*-demonstrated mechanism is accompanied with an *in vivo* decrease in xenobiotic accumulation, similarly as in drug-resistant cells, then it is likely that such mechanism for the elimination of xenobiotics in aquatic organisms may explain at least a part of their resistance to pollution. In order to check whether this mechanism functions in the investigated organisms also *in vivo*, we exposed mussel Mytilus galloprovincialis and cubes prepared from tissue of two sponge species to seawater polluted with radiolabelled AAF and VCR, respectively. After the 4 hour exposure period, the presence of verapamil ($5 \mu\text{g ml}^{-1}$) increased the amount of the radiolabel recovered from gills (62%), mantle (33%) and digestive gland (5%) by ethylacetate-acetone extraction from 12.5% to 25% of the added radioactivity. Similarly, verapamil ($5 \mu\text{g ml}^{-1}$) increased the accumulation of the radiolabel in sponge cubes from Geodia and Verongia exposed for 4 hours to seawater polluted with radioactive VCR for 43 and 69%, respectively. The time-dependent increase of radioactivity in extracts from

gills tissue of mussel exposed to ^{14}C -AAF in the presence or in the absence of verapamil indicates that most of the radioactivity was accumulated within the first hour of exposure, irrespective of the presence or absence of verapamil (Fig. 1A). Similar dynamic of accumulation was observed in extracts from *Verongia*-cubes exposed to ^3H -VCR (Fig. 1B). The enhancement of accumulation in the presence of verapamil demonstrates that MDR-like binding mechanism found in cell-free vesicle preparation from mussels and sponges tissues in vitro, functions also in vivo.

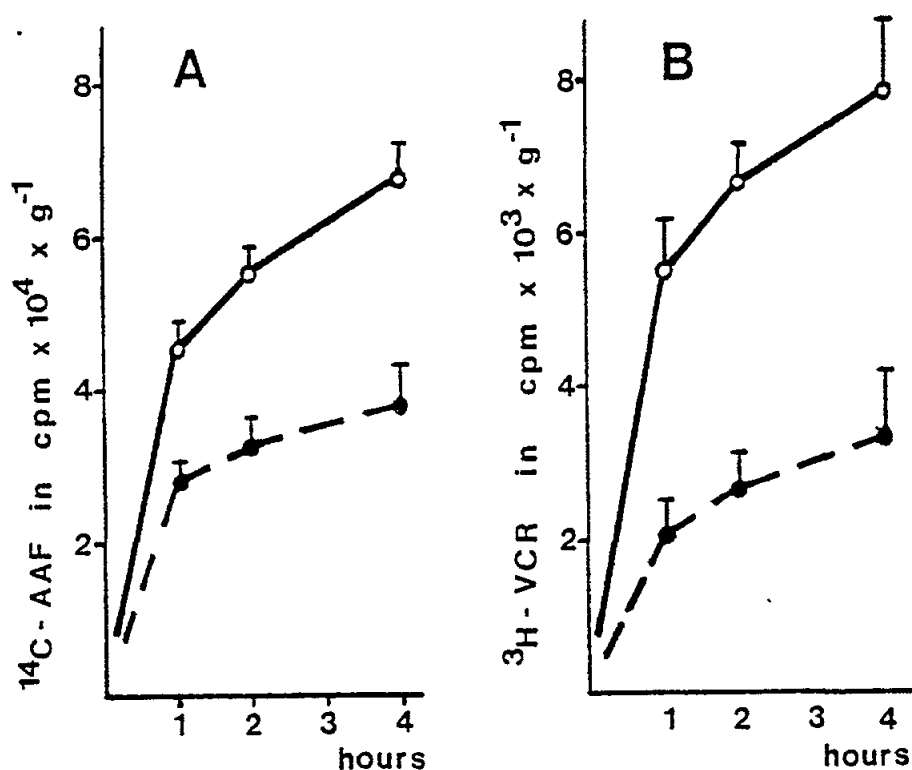


Fig. 1 Time-dependent accumulation of ^{14}C -AAF in gills of *M. galloprovincialis* (A) and of ^3H -VCR in cubes of *V. aerophoba* (B)

The time dependent studies were performed as described in Materials and Methods in the absence (●----●) or presence (○----○) of verapamil ($5 \mu\text{g ml}^{-1}$). Means and SD from four parallel experiments are given.

4. DISCUSSION

The fact that verapamil causes in mussels and sponges both the inhibition of binding in vitro and the enhancement of accumulation in vivo of AAF and VCR, argues strongly that the MDR-like mechanism is present in these organisms. In drug-sensitive cells, verapamil had little if any effect either on drug binding or on drug accumulation (Cornwell et al., 1986). However, in

contrast to the MDR phenomenon in tumor cell lines, where elements of the glycoprotein P170-mechanism involved in the resistance were acquired after a treatment of cells by a single drug, the presence of the verapamil-sensitive binding and accumulation in mussels and sponges investigated in this work was found in specimens collected from a pristine area, i.e. in specimens that have not experienced exposure to pollutants. This argues strongly that the MDR-like mechanism is inherent in these species. Several recent studies indicate the widespread de novo expression of *mdr1* gene also in normal, healthy kidney, liver, intestine, adrenal, pancreas, placenta, pregnant uterus and blood-brain barrier sites (Thiebaut et al., 1987; Arceci et al., 1988; Gordon-Cardo et al., 1989). In these tissues P170 is characteristically confined to the membranes of the luminal surfaces reflecting their possible physiological transport function, perhaps to excrete toxic natural products present in the diet, or unknown endogenous metabolites (Gottesman and Pastan, 1989). Recently it has been demonstrated that P170 may play a role in the excretion of progesterone and other steroids (Qian and Beck, 1990). To these functions, one should add the function of pumping out toxic chemicals originating from the polluted environment (Kurelec and Pivcevic, 1989). However, nontoxic lipophilic agents may also be recognized and processed by this molecular mechanism, and at high concentrations they might, consequently, saturate the system and thereby reverse multidrug resistance (Hofsli and Nissen-Meyer, 1990). Among a wide variety of organic compounds that are transported by P170 are also dyes like rhodamine 123, rhodamine 6G, acridine red, ethidium bromide, safranin O, pararosanilin, and methylene blue. They have been used in studies on the mechanism of MDR in human cell lines (Neyfakh, 1988; Ichikawa et al., 1991). Recently, fluorescent dyes have been used also to demonstrate the presence of MDR in eggs of several marine invertebrates (Barbara Toomey, Hopkins Marine Station, Stanford University, personal communication) and in the fresh water mussel (Pivcevic and Kurelec, unpublished).

The recognition of the presence of such a multixenobiotic resistance mechanism in aquatic organisms may have important implications in our present understanding of risks posed by various pollutants. It implies that the presence of one xenobiotic that is good substrate for the MDR extrusion pump, may inhibit or block the pumping out of other xenobiotic(s). The result is absence of accumulation of the first, and an unusually high accumulation of the second, or other xenobiotics. This fact may help us to interpret our present and past data on bioaccumulation, bioavailability, metabolism, toxicity, dose-effect relationships and exposure experiments, and, probably may improve our present criteria for "bioindicator" organisms, our criteria in designing biomonitoring programs, and consequently, the foundations of our present regulatory policy.

5. ACKNOWLEDGEMENTS

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REFEREE'S COMMENTS

Although the hypothesis is interesting the author has shown that verapamil can prevent binding as it does in MDR cells. He should expand on the final paragraph of the discussion to present any evidence that exists that pollutants are indeed pumped out of the cell. Many pollutants are so lipid soluble (e.g. PAHs) that they probably cross membranes by simple diffusion. The author should either present some convincing evidence that this mechanism can play a role in pollutant resistance or state that it is only an hypothesis at the present time.

AUTHOR'S RESPONSE TO REFEREE'S COMMENTS

Yes, there is no evidence that pollutants are indeed pumped out of the cells (organisms) in our paper. However, there are elegant experiments done with a pair of sensitive/resistant cells, demonstrating fast pumping out of fluorescent dye Rhodamine G6 accumulated in resistant cells in the presence of verapamil after their transfer to a pure medium. In comparison, under the same conditions, there was a lack of pumping out of dye accumulated in sensitive cells (Yoshimura *et al.*, 1990 Cancer Letters 50, pp.45-51). Unfortunately, we can not use the same approach with our organisms, since we do not have a corresponding "sensitive" partner of the needed pair. Therefore we had to rely, at present, on indirect evidences, like binding and accumulation, and these are the same in resistant cells as in aquatic organisms tested so far. Actually, recent evidence, accumulated in the meantime, and certainly after the presentation of my paper in Malta, strongly suggests that such a "drug-efflux" fulfills the expectation of a primordial non-specific (general) defense system: "this truly primordial system has been around for eons to protect healthy cells from cytotoxic substances in the environment and represents an essential factor for survival" (Kellen, 1991 Anticancer Research, 11:917-920).

Yes, lipid soluble pollutants (e.g. PAHs) cross membranes by simple diffusion. The whole MDR-concept is based on this fact: it explains how these pollutants, after they entered the cell by diffusion, are actively extruded out of the cell -- this defense mechanism, however, similar to any other detoxication mechanism, spends energy (ATP).

Thus, it is not an hypothesis: fish exposed to 2-aminoanthracene induce significantly more DNA adducts, as measured by ³²P-postlabelling analysis, and significantly more single strand breaks in ³²P their DNA, as measured by alkaline elution method, in the presence rather than in the absence of verapamil (Kurelec, Rev. Aquatic Toxicol., in press), be caused by pollutants that may inhibit, or block ("revert") the multixenobiotic mechanism.

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

by

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A B S T R A C T

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

1. INTRODUCTION

Utilization of biochemical factors to evaluate biologic responses to pollutants has increased considerably over the past 10 years. The basic concept appeared some 20 years ago, following the discovery that marine organisms possess enzymatic systems specialized in the biotransformation of liposoluble xenobiotics (oxidation by cytochrome P-450-dependent monooxygenases and conjugation by phase II enzymes such as glutathione-transferases) (Bend and James, 1978; James et al., 1979). In addition, the activity of these enzymes can be induced in a very short period of time by exposure to the substrate or compounds with a similar conformation (Payne, 1976; Stegeman, 1981). Various studies, most concerning mammals, have shown that biotransformation enzymes include a number of isoenzymes with different catalytic activities that respond to specific inducers (Parkinson et al., 1983). This research demonstrated the role of the molecular conformation (planar or globular) in the specificity of induction, in particular for

polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). These findings prompted searches for biochemical markers of the presence of different types of pollutants. For example, the induction of metallothioneins in marine animals exposed to metal contaminants has been the subject of numerous studies (Viarengo et al., 1980; Thomas et al., 1985; Overnell et al., 1987).

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

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

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

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

2. MATERIAL AND METHODS

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

2.1 Water and sediment samples

Sediment samples were collected by skin divers in zones around mussel beds at depths of 5 to 10 meters, in proximity to areas of Posidonia. After any organic matter had been pushed aside, the superficial layer of sediment (approximately 2 cm) was collected over an area of about 0.1 m². In each case, 2 samples were taken and stored at - 20°C. Sea water samples (8 to 12 liters) were taken in glass bottles opened by the divers at a depth of 5 meters, then kept refrigerated in the dark after addition of hexane.

2.2 Biological samples

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

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

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

2.3 Physicochemical analyses

2.3.1 Treatment of sediment

After removal of organic matter by floatation, sediment samples were screened to separate the fine fractions from coarse fractions, then dried. Treatment depended on the parameter to be measured: for PAH assays, Soxhlet extraction using dichloromethane was followed by purification on a column of Florisil (Garrigues et al., 1987); for PCBs, Soxhlet extraction with hexane was followed by purification through a mixed column of Al₂O₃-SiO₂.

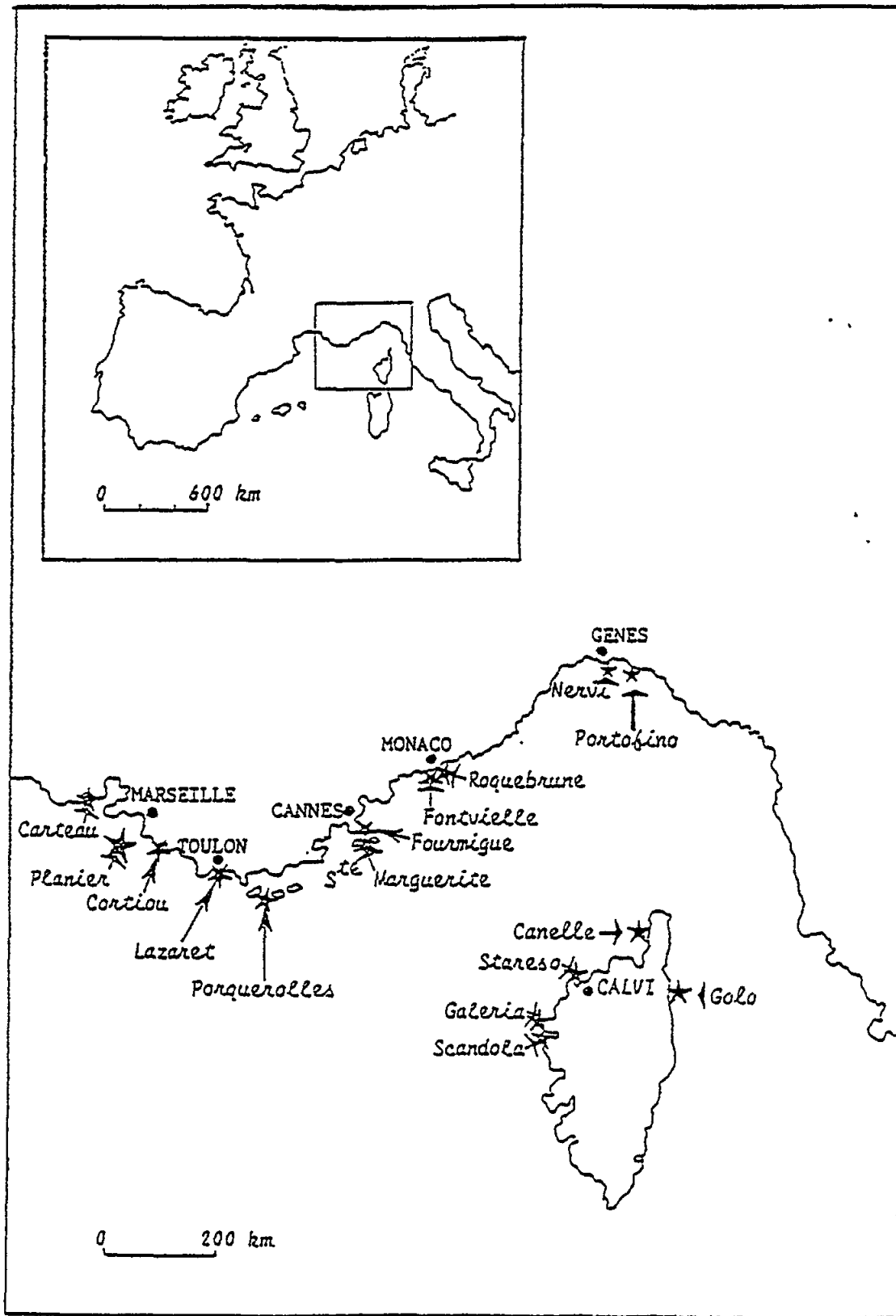


Fig. 1 Sampling sites in the north west coast of the Mediterranean sea

2.3.2 Treatment of sea water

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

2.3.3 Treatment of biologic samples

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

2.3.4 Quantification of parameters

The 12 PAHs recognized as major pollutants (phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, perylene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-C,D)pyrene) were separated and assayed by high performance liquid chromatography (Vydac 201 TP 54 column, 25 cm x 4.6 mm id) using a linear gradient of solvents CH₃CN/H₂O with programmed fluorimetric detection (Perkin-Elmer LS5) (Soclo et al., 1986). PCBs were analyzed by gas phase chromatography (Varian 3400, capillary column DB5 60m and DB210 30m) using a Ni 63 electron detector (Monod and Arnoux, 1979). The 7 congeners used for computations (CB 28, 52, 101, 118, 153, 138 and 180, according to the classification of Ballschmitter and Zell, 1980) are the most representative of the industrial mixtures used in France (DP5 and DP6). This methodology is recommended by the EEC Community Bureau of Reference (BCR). Metals were analyzed by fluorescence X-ray spectrometry according to the method of Lapaquellerie. Particulate organic carbon in sediment was evaluated as described by Etcheber (1983).

2.4 Biochemical analyses

2.4.1 Biotransformation activities

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

Samples of mussels were homogenized in a buffer of 50 mM K₂HPO₄, pH 7.5, supplemented with 1 mM EDTA and 15% glycerol. The homogenate was centrifuged at 9,000 x g for 30 min. The supernatant was then centrifuged at 100,000 g for 60 min, and the microsomal pellet was resuspended in the buffer. Benzo(a)pyrene monooxygenase (BaPMO) activity was evaluated by the radiometric

technique of Van Cantfort et al. (1977). Epoxide hydrolase (EH) activity was measured radiometrically according to Oesch et al. (1971) using styrene oxide as the substrate. Proteins were assayed according to Lowry et al. (1951); cytochrome P-450 was assayed as described by Estabrook and Werringloer (1978).

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

2.4.2 Lipid peroxidation activities

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

2.4.3 Intracellular calcium

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

2.4.4 Acetylcholinesterase activity (AChE)

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

2.5 Data analysis

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

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

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

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

3. RESULTS

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

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

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

Table 1

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

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

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

3.2 Correlations between biochemical markers and pollutant concentrations measured in the environment and in organisms (Table 2)

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

3.2.1 Biochemical measurements in the mussel

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

3.2.2 Biochemical measurements in fish

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

Table 2
Rank correlations between measures of chemical parameters and concentrations of a range of contaminants in water sediments and some organisms.

n	Sediments				Water				Orga- nisms				Sediments				Water				Orga- nisms				Sediments											
	total PAH		heavy PAH		total PAH		heavy PAH		total PCB		diortho PCB		total PCB		heavy PAH		total PCB		total PCB		total PCB		Total Metals		Copper		Nickel		Lead		Zinc		%POC		% fine	
	16	16	10	8	14	14	14	11	16	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	16	16			
	Mytilus galloprovincialis																																			
16	.870	.876	.252	.830	.697	.677	.606	.326	.631	.528	.408	.714	.422	.547	.654																					
15	-.167	-.169	.207	.359	.386	.309	.177	-.168	-.011	-.002	-.348	-.131	0.69	.353	.179																					
13	-.081	-.089	.034	-.404	-.062	-.058	-.272	-.088	-.290	.161	-.270	-.351	0.22	.124	.027																					
13	.146	.175	.276	.532	.676	.674	.627	-.234	.569	.473	-.288	.687	.524	.144	.008																					
7	-.588	-.596	ND	ND	-.280	-.158	.307	.031	.865	-.330	-.458	-.450	-.483	-.799	-.853																					
10	.444	.456	.184	.770	.255	.604	.765	.348	.092	-.176	.033	.373	-.264	.272	.299																					
	Sertanus scriba																																			
9	.307	.316	.259	ND	.486	.549	.866	.151	.472	.154	.626	.691	.100	.189	.176																					
6	.643	.659	-.339	ND	.417	.371	.699	-.458	-.180	-.720	-.043	-.036	-.487	.571	.791																					
11	.424	.446	-.010	ND	.274	.274	.608	-.278	-.069	-.398	.177	.162	-.369	.347	.560																					
	Sertanus Cabrilla																																			
11	.647	.677	.640	ND	.413	.437	.352	.259	.500	.524	.218	.544	.415	.422	.507																					
6	.693	.678	.434	ND	.647	.708	.475	.936	.711	.578	.805	.771	.579	-.138	.094																					
13	.539	.539	.534	ND	.636	.623	.689	.939	.671	.588	.601	.674	.486	.692	.587																					
8	-.367	-.333	-.666	ND	.653	.706	.006	-.164	-.614	-.034	-.971	-.358	-.090	.007	-.064																					
9	-.114	-.098	.221	ND	.099	.231	.105	.244	.563	.819	.313	.522	.723	-.396	-.268																					
	Posidonia oceanica																																			
5	.884	.895	-.326	ND	-.217	-.074	.551	ND	-.361	-.281	-.495	-.232	-.347	-.409	-.536																					
5	-.416	-.491	-.345	ND	-.631	-.576	.234	ND	-.223	-.446	-.416	-.216	-.412	-.320	-.656																					
5	.707	.712	-.497	ND	-.268	-.243	.238	ND	-.357	-.243	-.478	-.244	-.221	-.423	-.416																					
5	-.152	-.212	-.728	ND	-.934	-.914	.430	ND	-.763	-.869	-.877	-.714	-.789	-.492	-.315																					

3.2.3 Biochemical measurements in Posidonia

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

4. DISCUSSION

4.1 Physicochemical parameters

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

4.2 Responses of biochemical markers

4.2.1 Cytochrome P-450 and MFO

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

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

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

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

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

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

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

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

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

4.2.2 Conjugation activities

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

At the Oslo Workshop, Van Veld and Lee (1988) described their investigations on the response of GST activity in the intestine of P. flesus using CNDB as the substrate. The maximum amplitude of response was 2.2, with a weak correlation with the pollution gradient. A low correlation was observed in S. scriba between GST activity and the degree of environmental pollution

by PAHs or PCBs. By contrast, in S. cabrilla, significant correlations were observed with the PCB levels in water, sediment, and especially tissues. Our findings are similar to those reported by Lee (1988), who cited a coefficient of correlation of 0.795 between tissue PCB levels and GST activity in the hepatopancreas of Carcinus maenas. The response of GST to inducers thus differs as a function of the species.

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

4.2.3 AChE activity

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

4.2.4 Lipid peroxidation and intracellular calcium

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

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

In the mussel, a good amplitude of response was observed for MDA, and especially for calcium. The discriminator factor in this case was over 5. The results presented at Oslo by Viarengo et al. (1988a) corresponded to an amplitude of 1.8 and a discriminator factor over 4 for calcium. In the two fish species studied, response and discriminator factors were below 2. According to the previously described mechanism of action, these parameters should have been significantly correlated with P-450 inducers (PAH and PCB) and metals such as copper and zinc. Significant correlations were in fact observed in the mussel between MDA and Ca⁺⁺ and between tissue PAH and contamination of water and sediment by PCBs. In the Oslo mesocosm experiment, metals (Cu⁺⁺ in particular) had a significant effect only at high doses (Viarengo et al., 1988a). By contrast, our analyses of fish gills revealed excellent correlations between cellular calcium and environmental pollution by metals (especially copper and zinc). Recent research (Gnassia-Barelli et al., 1990) has also established a relation with contamination by cadmium. However, no correlation has been found with the gradient of pollution by PAHs or PCBs. Responses thus differ not only between species but also between tissue types. For example, the gills are less sensitive to P-450 induction than the liver, but they are more exposed than the internal tissues to metal pollution because they serve as a barrier in direct contact with the outside environment.

5. CONCLUSION

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

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

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

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

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

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

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

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SUBLETHAL TOXICITY TESTING IN SEA URCHIN FERTILIZATION
AND EMBRYOGENESIS: A STUDY OF POLLUTED WATER AND SEDIMENT
FROM TWO RIVERS IN CAMPANIA, ITALY

by

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A B S T R A C T

Echinoid gametes and embryos may be used in evaluating the toxicity of individual chemicals or of complex mixtures, or in mapping sediment toxicity in defined water bodies (Pagano *et al.*, 1986; 1989). This may be accomplished by exposing sea urchin embryos or sperm to the test chemical or mixture, and scoring a number of different outcomes according to quantitative criteria. The effects on developing embryos are evaluated by scoring developmental defects as observed at the pluteus larval stage. Sperm exposure to the agent may turn into effects on fertilization and/or on offspring quality. The above methods have been applied to evaluating the topographic distribution of embryo- and spermiotoxic activity in water and sediment from two rivers, the Sarno River and the Volturno River, and the Regi Lagni drainage system, in the Campania region, Italy. There were ten sampling sites from the rivers and four from coastal locations, close to the two river estuaries and the mouth of the Regi Lagni. Water sampling was carried out biweekly for a year, and sperm or embryo bioassays were run alternatively. Sediment was collected close to the river banks or at approximately 200 m from the coast close to the estuaries. The most polluted sites (at the Sarno River and at the mouth of the Regi Lagni drainage system), displayed highest activity in inducing larval malformations and mortality, as well as a decrease in fertilization success. As for coastal sediment samples, their toxicity was dramatically decreased compared to the estuary sediment samples.

1. INTRODUCTION

The use of sea urchin embryos and gametes in testing developmental, reproductive and cytogenetic effects of chemicals and complex mixtures has led to the establishment of a few bioassay methods applied by a number of laboratories worldwide (reviewed by Kobayashi, 1984; Pagano *et al.*, 1986; Dinnef *et al.*, 1988). Sea urchin embryo and sperm bioassays display a number of advantages, including: a) quantitative response; b) multi-parameter information; c) metazoan organism; d) year-round availability of test animals; e) large numbers of embryos in each treatment schedule; f) sublethal toxicity testing. The information obtained from sea urchin bioassays encompasses both the testing of some action mechanisms of defined chemicals (e.g. pollutants, pharmaceutical drugs etc.) and evaluating the environmental toxicity of

complex mixtures (Pagano *et al.*, 1986; 1989). The following endpoints may be detected in this test system: a) developmental defects (in embryos exposed during early development, or in the offspring of pretreated sperm); b) cytogenetic abnormalities, and c) changes in fertilization success.

The present study aimed to apply our sea urchin bioassay to a study of two water bodies in Campania region, Italy, the Sarno River and Volturno River (Fig. 1). The Sarno River is a relatively small water body, which has raised substantial concern locally for its polluted state; it is affected by a number of industrial, domestic and agricultural effluents. Unlike the Sarno River, the Volturno River is characterized by a much larger flow (approx. 100 m³/sec), and the vast majority of the wastewater from the Volturno River catchment is discharged into the Regi Lagni; thus, the Volturno River is considered to be relatively less polluted than the Regi Lagni. We planned to verify the above assumption, which has been the subject of a few previous investigations that focussed mainly on some parameters related to chemical and domestic pollution (Amodio-Cocchiari and Arnese, 1988), without providing any evidence for health effects. Therefore, we envisaged a multi-disciplinary study including: a) the analysis of some major indicators of industrial, domestic and agricultural pollution (Melluso *et al.*, 1991), and b) a bioassay model based on sea urchin embryos and sperm (Pagano *et al.*, 1986; 1989). Analytical and microbiological data will be reported elsewhere (Melluso *et al.*, 1991; unpublished data).

2. MATERIALS AND METHODS

2.1 Sampling sites and procedures

The sampling sites for water and sediment were located along the Sarno River and its tributary, the Solofrana Stream, and the Volturno River and its drainage system, the Regi Lagni, as depicted in Figure 1. Water samples were refrigerated and transported to the laboratory where they were then processed by diluting each sample (100 to 10,000 times) in filtered natural seawater and tested for the effects on sea urchin early development and egg fertilization.

Sediment samples were collected at: a) three sites in the Sarno River; b) three sites in the Volturno River and Regi Lagni, and c) four marine sampling sites. The samples were refrigerated and transported to the laboratory; thereafter, the samples were kept frozen up to the time of the experiments. A thawed sediment aliquot was used (0.3 to 5 mg ml⁻¹ dry weight) for each test. The sediment aliquots were laid on a wet disc of filter paper, and allowed to settle gently in 50 ml filtered natural seawater.

2.2 Sea urchins

Sea urchins from the Mediterranean species Paracentrotus lividus and Sphaerechinus granularis were used and the two species were utilized alternatively, in order to obtain spawning animals year-round. Gametes were obtained as reported previously (Pagano *et al.*, 1986). Ten minutes after fertilization, a 1-ml aliquot of zygote suspension (approx. 500 embryos ml⁻¹), was added gently to: a) river water-contaminated seawater (rwcs), or b) sediment-contaminated seawater (scs), and reared to the larval stage of pluteus at 19° ± 1°C (P. lividus) or 17°C ± 1°C (S. granularis). Similar



Fig. 1 Location of sampling sites (arrows) in Campania, Southern Italy

treatment was performed on sperm suspensions (50 μ l sperm pellet/50ml seawater) added to rwcs or scs; after pretreatment (60 min. for *P. lividus* or 15 min. for *S. granularis*), sperm was used to inseminate untreated eggs (200 μ l sperm/50 ml eggs). The endpoints were: a) sperm fertilization success, and b) offspring quality. Embryological analysis was performed as reported by Pagano *et al.*, 1983; 1986.

3. RESULTS

3.1 River water samples

Few, if any effects were observed by rearing *P. lividus* or *S. granularis* embryos to river water samples (data not shown). No effect on fertilization success could be detected when sperm of either species were

suspended in rwcs from any of the sampling sites. A similar lack of any effects was observed in the offspring of S. granularis sperm exposed to rwcs 15 min. before insemination (data not shown). Unlike S. granularis, P. lividus sperm pretreatment (60 min.) appeared to result in a slight increase in offspring malformations following exposure to rwcs from the sampling site S.3 (S. Marzano), although this result was slightly above the level of statistical significance ($p < 0.06$).

3.2 Sediment samples

3.2.1 Embryotoxicity experiments

A triplicate experiment was performed on sediment from three sites at the Sarno River, namely: Mercato S. Severino (S.1), which includes leather tannery effluents, S. Marzano (S.3), an agricultural site, and Castellammare di Stabia (S.4), the river mouth. A dramatic arrest of P. lividus embryogenesis was exerted by the sediment at S.1, invariably resulting either in the lack of larval differentiation or in early embryonic death. Less dramatic, yet significant was the damage exerted by sediment samples from the sites S.3 and S.4, which resulted in larval defects or mortality. A second experiment was performed on the sediment samples collected from the above Sarno River sites and from three sites at the Volturno River and the Regi Lagni (V.3, V.5, and V.6). As shown in Figure 2, the highest developmental toxicity was exerted on P. lividus embryos by the samples from the Regi Lagni mouth (V.6), and Mercato S. Severino (S.1). A third experiment was performed on sediment samples collected at the mouths of the two rivers and of the Regi Lagni, and on four marine sediment samples collected nearby. Again the Sarno River and Regi Lagni sediment samples showed a higher embryotoxicity, compared to the Volturno River. A dramatic decrease in sediment toxicity appeared at the marine sites.

Sediment samples affected the fertilization success of P. lividus sperm; the sites apparently displaying spermiotoxic effects were V.5 and S.1 (data not shown). Along with the effects observed on fertilization success, the embryogenesis and viability of the offspring of exposed sperm were most affected by the sediment samples from the sites S.1 and S.3; the Volturno River sediment (sites V.3 and V.5) appeared to be ineffective on the offspring of pretreated sperm.

4. DISCUSSION

The effects of river water samples (collected year-round, in both sea urchin species and for the different parameters tested) were significant only in a few cases. Whether water samples may be toxic with longer exposure times (than utilized in our bioassays), or via bioaccumulation, is a question that could be investigated by utilizing either long-term test systems or some devices for pollutant concentration. Dilutions of 100 to 10,000 fold were used throughout this study, both in order not to affect salinity and, above all, in order to maintain river water dilutions within an environmentally realistic range. These dilutions cannot be regarded, however, as too great to produce an effect, since we observed the embryo- and/or spermiotoxic action of effluents at the above dilutions in other water bodies (Pagano et al., 1989; unpublished data).

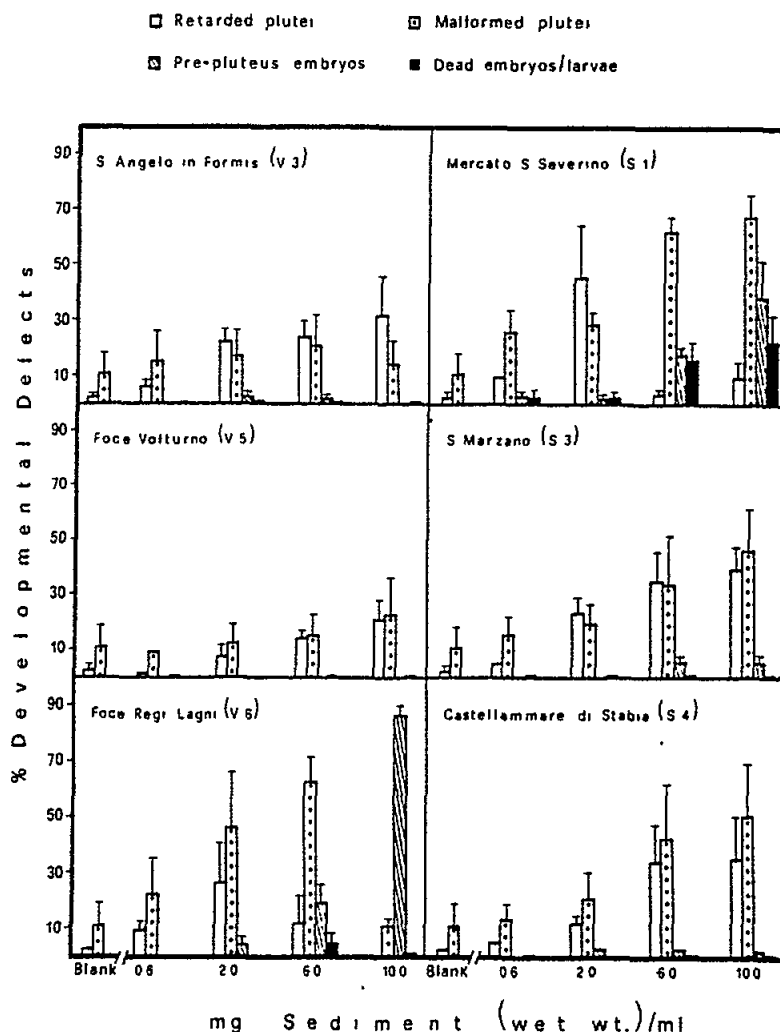


Fig. 2 Developmental toxicity as induced by sediment samples from six selected sites from the Volturno River (V.3 and V.5), the Regi Lagni (V.6), the Solofrana Stream (S.1) and the Sarno River (S.3 and S.4). Triplicate experiment on *P. lividus* embryos

In spite of the relatively scarce toxicity of water samples, the river sediments appeared to act as a pollutant concentrating mechanism. The experiments performed on sediment samples displayed remarkable specificity of their toxicities to either embryos or sperm (from *P. lividus*). The results pointed to high developmental toxicity of the sediment from the Sarno River and the Regi Lagni mouth (but not from the Volturno River mouth). The effects of a realistic amount of sediment on developing embryos were most dramatic at the sites: a) V.6, which receives the wastewaters from the Volturno River lowlands, and b) S.1, which is mainly affected by leather tanning sludge. Lesser effects were detected from the sediment collected at the sites S.3 and S.4 which did, however, demonstrate developmental toxicity. Little, if any developmental toxicity was exerted by the sediment from the Volturno River sites (V.3 and V.5) and from the coastal sites close to the estuaries. The induction of a transmissible damage to the offspring of sediment-pretreated

sperm was exerted by the sediment samples collected at S. Marzano (S.3), Mercato S. Severino (S.1), and the mouth of the Regi Lagni (V.6). The sediment from the Sarno River and Regi Lagni drainage system may be regarded as a potential threat to environmental health, as far as developmental and reproductive toxicity are concerned.

Further research is prompted by the present study. Risks to environmental and human health, associated with the pollution status of the Sarno River and of the Regi Lagni should be investigated. As a means for purifying some major organic contaminants from complex mixtures, "Blue Rayon" (Hayatsu, 1990) is presently utilized in our laboratory with promising results and a wide-scale application of this technique may be suggested. The use of other test systems, analytical information, and new study design are areas for future investigation.

5. CONCLUSIONS

Water samples from the Sarno and the Volturno river, and from the Regi Lagni failed to show any major toxicity to sea urchin sperm or embryos at realistic dilutions. However, the induction of developmental defects in sea urchin embryos, as well as in the offspring of sea urchin sperm exposed to sediment samples from the Sarno River and the Regi Lagni (but not from the Volturno River), may be regarded as an indicator of the poor environmental conditions of these water bodies. Spermotoxic effects were only observed following exposure to sediment samples from some of the Sarno River sites.

6. ACKNOWLEDGEMENTS

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METAL BINDING PROTEINS OF Mytilus galloprovincialis, SIMILAR TO METALLOTHIONEINS, AS A POTENTIAL INDICATOR OF METAL POLLUTION

by

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A B S T R A C T

Bivalvia molluscs of genus Mytilus are generally utilized as indicator organisms for marine pollution control owing to their geographical widespreading as well as a large capacity for accumulation and storage of trace metals. Within the framework of MED-POL (Phase II) research program, the reliability of monitoring concept based on quantitative determination of metallothionein-like proteins (MLP) by application of combined polarographic and spectrophotometric method has been investigated.

Digestive gland has been selected among other tissues (gills, mantle) because it possesses the largest capacity for MLP induction and ability to reflect different concentration levels and duration of exposure to cadmium experimentally added into sea water. Exposure to mixture of other metals (Cu + Pb) in addition to Cd ($0.2 \mu\text{g dm}^{-3}$) at relatively low level resulted in reduction of MLP level as well as of total metal content bound to it when compared by single Cd-exposure.

Observed variations in the MLP content in control mussels unexposed to metals may be attributed to the combined effect of abiotic and biotic factors during their annual reproductive cycle.

Assuming the advantages of the proposed biochemical indicator which are supposed to be in better correlation with bioavailable metal fraction in sea water than the total content of the specific metal in mussels, conceptual and methodological aspects of MLP-using trace metals monitoring were critically evaluated.

1. INTRODUCTION

Goldberg's general concept of the metal pollution monitoring (Goldberg, 1975) involving marine mussels of genus Mytilus (edulis, galloprovincialis, californianus) as indicator organisms has been employed throughout the world.

Mussels are able to synthesize a high level of inducible, low molecular weight, sulfhydryl rich metal-binding proteins similar to metallothionein (MT) in response to elevated concentrations of certain trace metals under laboratory (George et al., 1979) and environmental conditions (Talbot and Magee, 1978; Harrison et al., 1983).

In spite of close physicochemical and functional similarities between MT-like proteins (MLP) of mussels and MTs from mammalian sources, certain distinctions in their aminoacid composition and molecular mass were reported (Viarengo *et al.*, 1984; Frazier *et al.*, 1985). In connection with that, metal-binding stoichiometry has not been precisely defined, thus restricting an application of those quantitative methods which are based upon saturation of known number of metal-binding sites on MLP-s from invertebrate sources. In order to avoid possible underestimation of total MLP level due to determined lower molar proportion of cysteinyl residues as well as observed higher molecular mass of mussels MLP when compared by MT from mammalian sources, our intention was to adapt an electrochemical method essentially based on the Brdicka's (1933) reaction for determination of sulfhydryl-rich proteins sufficiently precise and simple to be applied for routine monitoring of mussels MLP level at coastal locations. For that purpose we examined previously inductive capacity of several tissues for MLP synthesis in order to select those characterized by greatest capacity upon cadmium exposure as well as by functional relationship between measured MLP content and level and duration of metal exposure.

2. MATERIAL AND METHODS

2.1 Mussels

Adult mussels of the mean shell length of 6-9 cm were collected from an aquaculture facility in the Lim Channel, Western Istrian coast, Northern Adriatic. Organisms were kept for a week under laboratory conditions (salinity $37-38 \cdot 10^{-3}$, temperature ranged between 7 and 24°C at different experiments) prior to the exposure to elevated concentration of cadmium (ranged between 0.2 to 1300 $\mu\text{g Cd dm}^{-3}$) added as $\text{CdCl}_2 \cdot n\text{H}_2\text{O}$ into an open continuous-flow sea water system. Control, unexposed, organisms were kept simultaneously in separate basins supplied with running sea water without addition of metal. Mussels for monitoring of seasonal variation of MLP level were sampled every three months at the same location and composite samples of digestive gland were deep-frozen (-20°C) until further processing.

2.2 Isolation of MLP

The soluble phase of the previously separated tissue homogenates was extracted by 0.02 M Tris-HCl buffer, pH 8.6 (3 ml per gram tissue) containing either 1% 2-mercaptoethanol (2-Me) or 1 mM dithiotreitol (DTT) and protease inhibitors (17 mg dm^{-3} PMSF and 3 mg dm^{-3} leupeptin). Ammonium bicarbonate buffer 0.02 M, pH 8.6, containing the same antiproteolytic mixture as well as one of the reductive agents has been alternatively used. The extraction procedure applied was modified according to Olafson *et al.* (1979) and Viarengo *et al.* (1980 and 1984). It was described in detail elsewhere (Pavičić *et al.*, 1985; 1989; Raspor *et al.*, 1987). Following the tissue homogenization using motorized, vibrating teflon pestle with a glass tube cooled in an ice bath, a part of crude homogenate, intended to be polarographically analyzed for the sulfhydryl content later on, was subjected to a heat treatment (70°C ; 10 minutes) in order to denaturate and precipitate interfering high molecular mass proteins. Subsequent centrifugation was performed at 28000 x g for an hour using a Sorwal RC2-B refrigerated centrifuge. The resulting supernatant

was filtered through a medium speed filterpaper. Particle-free preparation was obtained by the filtration through membrane filter (0.2 μm) prior to the application on a chromatographic column.

Initial separation step was provided by the gel-filtration on a Sephadex G-75 column using either 0.02 M Tris-HCl or $\text{NH}_4\text{HCO}_3\text{-NH}_4\text{OH}$ pH 8.6, as an eluant buffer, containing 0.5 mM DTT. When further purification was required, pooled chromatographic fractions corresponding to MTLP maximum were applied to DEAE-cellulose anionic exchange column and eluted by increasing concentration gradient of NH_4HCO_3 buffer (20-400 mM) containing 0.5 mM DTT.

2.3 Analytical methods

Ultraviolet absorbencies at 250 and 280 nm were routinely measured in chromatographic fractions using a Varian DMS 80 or a LKB Ultraspec 413 UV/VIS spectrophotometer. Metal concentrations were determined either by an AAS in both flame and graphite-furnace mode or using polarography (DPASV) on the hanging mercury drop electrode. The electrochemical method for determination of sulfhydryl-containing proteins according to the Brdicka's (1933) reaction was applied in a manner described elsewhere (Raspor *et al.*, 1987). The total protein concentration was determined according to the Bradford's (1976) spectrophotometric method using Coomassie Brilliant Blue reagent. A bovine serum albumin (BSA) or rabbit liver MT (I+II) were utilized as the calibration standards.

3. RESULTS

3.1 Induction capacity of selected tissues for MLP synthesis

In order to select a representative tissue responding to different level of cadmium exposure by a measurable MLP content digestive gland, gills and mantle with gonads were examined. Table 1 summarizes data of the cadmium distribution in different fractions of the selected tissues showing that extraction efficiency of Cd estimated as a proportion of total tissue Cd measured in soluble phase (supernatant) of the digestive gland (68-94%) was significantly higher than that found in two other tissues examined (29-37%). Under different exposure conditions designed as "acute" ($1.3 \mu\text{g Cd ml}^{-1}$; 7 days), "intermediate" ($0.23 \mu\text{g Cd ml}^{-1}$; 22 days) and "chronic" ($0.1 \mu\text{g Cd ml}^{-1}$; 120 days), digestive gland was able to respond by markedly different amount of induced MTLP as presented comparatively on Fig. 1, showing also different cadmium distribution patterns on corresponding elution profiles.

Following acute intoxication, only 20% of Cd determined in soluble phase was associated with the induced MLP fraction and the remaining part was distributed between high molecular mass (HMW) proteins and low molecular mass (LMW) compounds. For comparison, the organisms exposed to a lower concentration levels ("chronic" and "intermediate") the prevailing proportion of soluble Cd 87% and 77%, respectively, was bound to MLP fraction of digestive gland. The results obtained show that the amount of Cd-induced MTLP in a digestive gland of *M. galloprovincialis* was directly proportional to the time of exposure and inversely related to the concentration of Cd in seawater. Similar effect has not been obtained with gills which inductive capacity presented by proportion of soluble Cd associated with MTLP fraction was

Table 1

Cadmium concentration in different fractions of selected tissues of laboratory exposed Mytilus galloprovincialis. (Cd determined in HMW, MLP and LMW fractions is expressed as proportion (%) of the total Cd in the supernatant).

Tissue	Cd	Exposure Days	Cadmium content			Extraction (%)	Cd distribution (%)		
			Tissue (wet weight) ($\mu\text{g g}^{-1}$)	Supernatant ($\mu\text{g ml}^{-1}$)	HMW		MLP	LMW	
Digestive gland	1.3	7	63.7	12.0	23.3	68	19.5	57.1	
"	0.23	22	76.2	14.8	20.0	94	77	3	
"	0.1	120	121.0	28.5	10.5	84	86.8	2.7	
Gills	1.3	7	35.2	2.7	43.4	30	9.9	46.7	
"	0.1	120	32.1	2.8	44.9	37	8.7	46.4	
Mantle with gonads (female)	0.1	120	16.5	1.2	9.2	29	90.8	0	

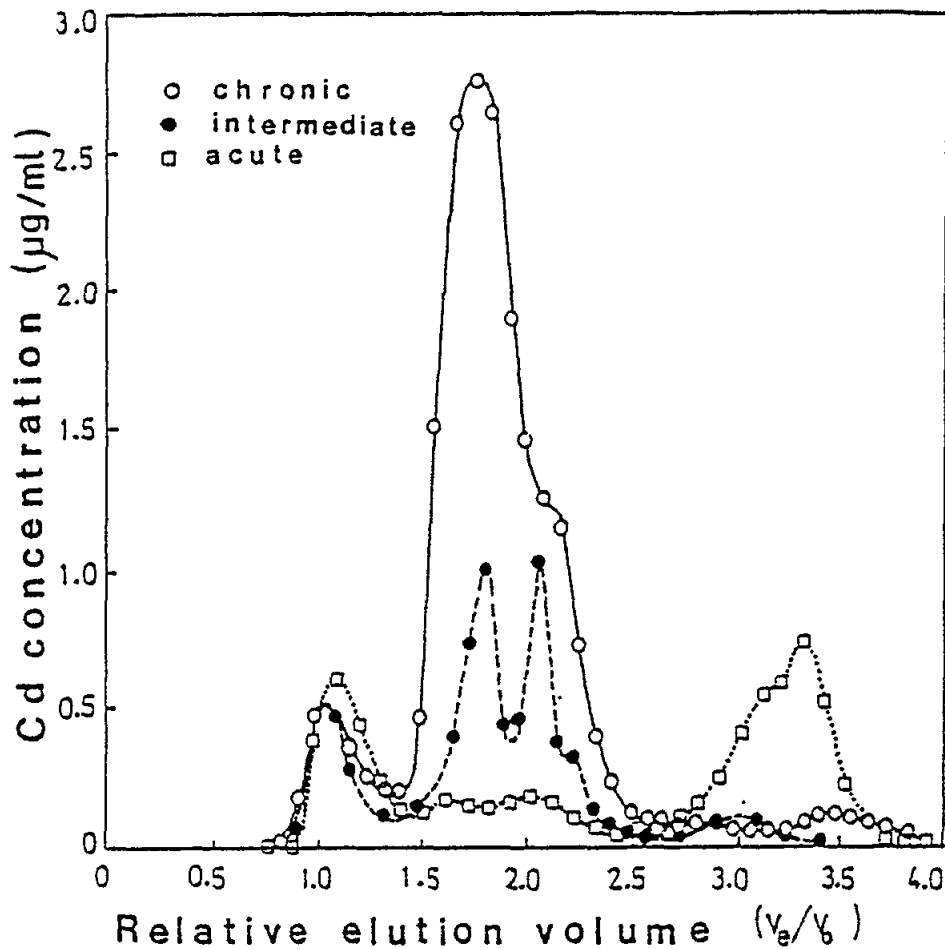


Fig. 1 Cadmium distribution in the soluble phase (27 000 xg) of digestive gland of *M. galloprovincialis* obtained after Sephadex G-75 column chromatography from mussels subjected to different Cd-exposure conditions; chronic ($0.1 \mu\text{g Cd ml}^{-1}$); intermediate ($0.23 \mu\text{g Cd ml}^{-1}$); acute ($1.3 \mu\text{g Cd ml}^{-1}$)

markedly lower (9-10%) in comparison with other tissues examined, irrespectively of exposure conditions. In the mantle with gonads (female) of chronically exposed mussels, cytosolic cadmium was also predominantly associated with MTLF fraction as shown by digestive gland, 87% and 91% respectively, although extraction efficiency of a mantle was significantly lower than that of a digestive gland. The next experiment was performed at the markedly lower exposure level of added cadmium ($0.2 \mu\text{g Cd dm}^{-3}$; denoted as "Cd single" as well as its mixture with some other metals ($0.2 \mu\text{g Cd dm}^{-3} + 2.0 \mu\text{g Cu dm}^{-3} + 1.6 \mu\text{g Pb dm}^{-3}$; denoted as "metal mixture"). Corresponding gel-filtration elution profiles of cadmium distribution determined following 14 days of exposure were presented on Figs. 2 and 3, respectively, as well as sulfhydryl level measured by the polarographic current intensity according to the Brdicka's reaction. A significant distinction observed between amount of cadmium bound to MLP fraction of the control group and each group of metal-exposed mussels, is much more pronounced than the difference between

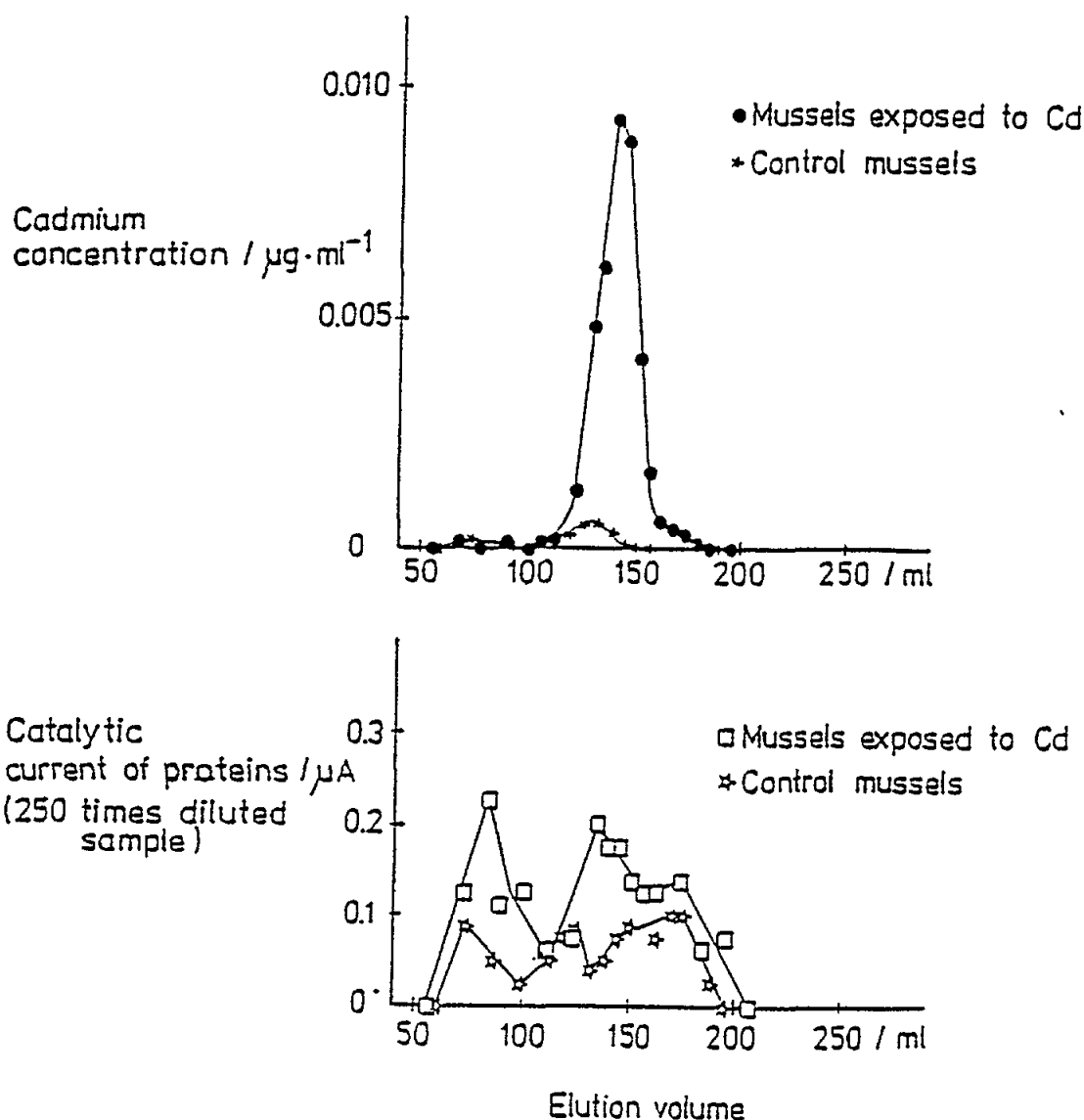


Fig. 2 Sephadex G-75 elution profiles of cadmium and Brdicka's catalytic current distribution in thermally treated digestive gland soluble phase of *M. galloprovincialis*. Mussels exposed to cadmium ($0.2 \text{ ng Cd ml}^{-1}$) for 14 days were presented in comparison with untreated control specimens

their sulfhydryl content. However, the amount of Cd bound to MLP region in elution profile of the single-Cd-exposed group when compared with the control, unexposed mussels (16 x higher), exceeded that of the group subjected to the metal mixture (13 x higher). Induction potential of two other metals (Cu+Pb) added into seawater was not strong enough to increase the total MTLP content of mussels exposed to metal mixture. Comparing the summarized metal content in chromatographic fractions, belonging to MLP region of two differently exposed groups of mussels as presented on Table 2, it may be observed that amounts of Cu and Pb would be comparable or even higher in "Cd-single" group,

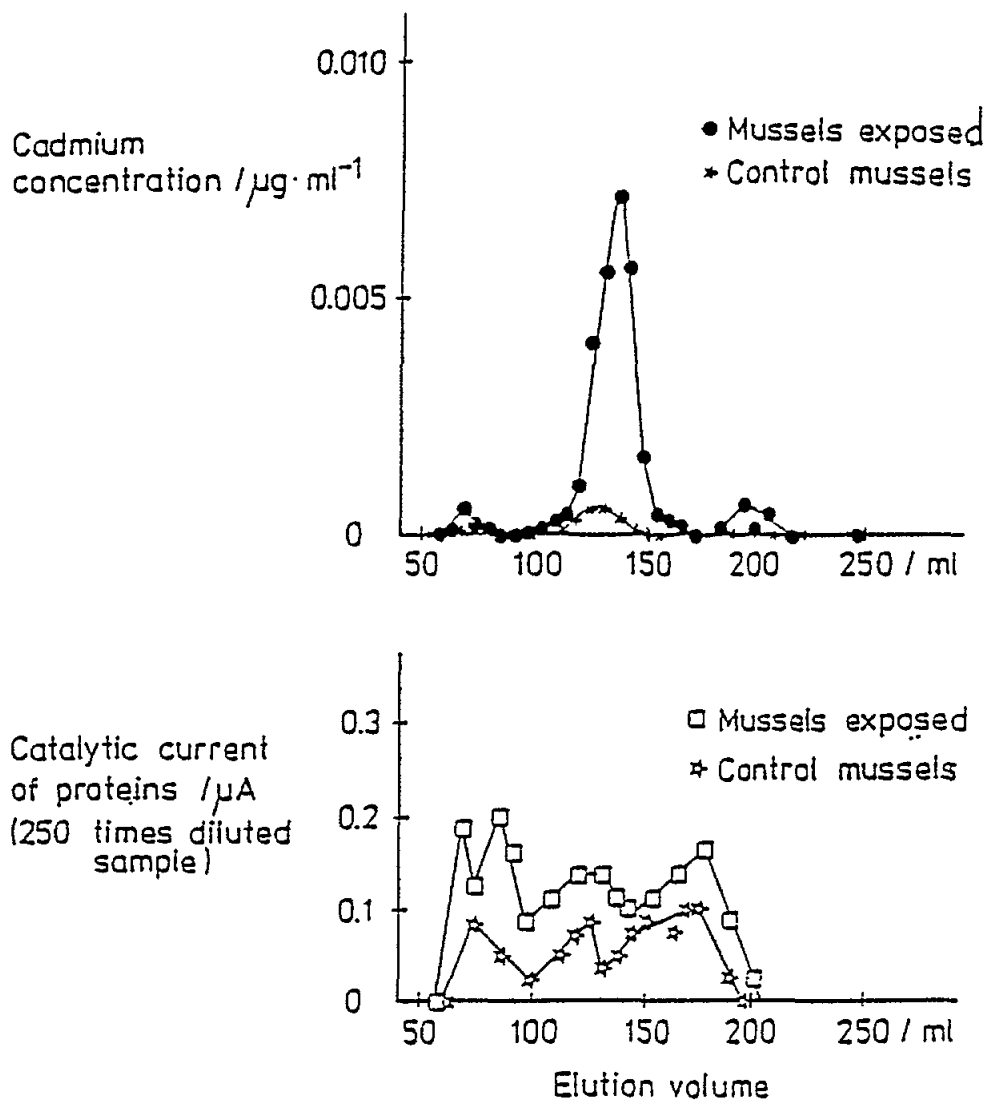


Fig. 3 Sephadex G-75 elution profiles of cadmium and Brdicka's catalytic current distribution in thermally treated digestive gland soluble phase of *M. galloprovincialis*. Mussels exposed to metal mixture ($0.2 \text{ ng Cd ml}^{-1} + 2.0 \text{ ng Cu ml}^{-1} + 1.6 \text{ ng Pb ml}^{-1}$) for 14 days were presented in comparison with untreated control specimens

but that of Zn was several times lower than found in mussels exposed to Cd only. More plausible explanation of that effect would be a possible competition between Cd and other metals for uptake sites via biological membranes than an exchange of loosely bound Zn with Cu or Cd respective to their higher stability constants, because it was not accompanied by an increased content of these metals bound to MLP.

Table 2

Summarized amounts (ng) of Cd, Zn, Cu and Pb determined in chromatographic fractions corresponding to MLP maxima in Sephadex G-75 elution profiles of control and two group of low-metal exposed mussels (Cd-single and metal-mixture). Amounts of MLP and -SH were estimated according to polarographic current (μA) measured using Brdicka's reaction on SH-containing proteins.

METAL	CONTROL	Cd-SINGLE	METAL MIXTURE
	(ng of metal bound to MLP)		
Cd	12	198	153
Zn	>DL	1534	173
Cu	4004	8264	7917
Pb	*	82	67
Total metal (mol 10^{-6})	0.063	0.156	0.129
Pol.cur (μA)	112	196	173
-SH (mol 10^{-6}) ^a	0.259	0.453	0.400
MLP (mol 10^{-6}) ^b	0.016	0.028	0.024

>DL below detection limit

* not measured

^a estimated taking into account $433 \mu\text{A} \mu\text{mol}^{-1}$ -SH

^b estimated taking into account $0.56 \mu\text{A} \mu\text{g}^{-1}$ MLP
(mol. mass 12.6 kDa)

3.2 Quantitative determination of MLP

Quantitation of MLP in *M. galloprovincialis* is based on the determination of the Brdicka's polarographic current intensity in the soluble phase of a heat-denaturated homogenate. Prior to direct determination of the MLP content in samples without fractionation it has been shown that short thermal treatment (70°C; 10 min.) was effective in removing interfering high-molecular weight proteins without significant change of the MLP region as presented on Fig. 4 in comparison with elution profiles of untreated (A) and heat-treated sample (B).

Bearing in mind lower molar proportion of cysteine residues in composition of *Mytilus* MLP when compared with standard mammalian MT, the significant difference might be also expected when measuring polarographic current intensity according to the Brdicka's reaction on sulfhydryl-containing proteins.

Further purification of MLP from a digestive gland has been performed by application of an ion-exchange chromatography (DEAE-cellulose) as shown on Fig. 5, in order to obtain an internal standard for the calibration purpose. Cadmium has been distributed between two well-resolved maxima which may be

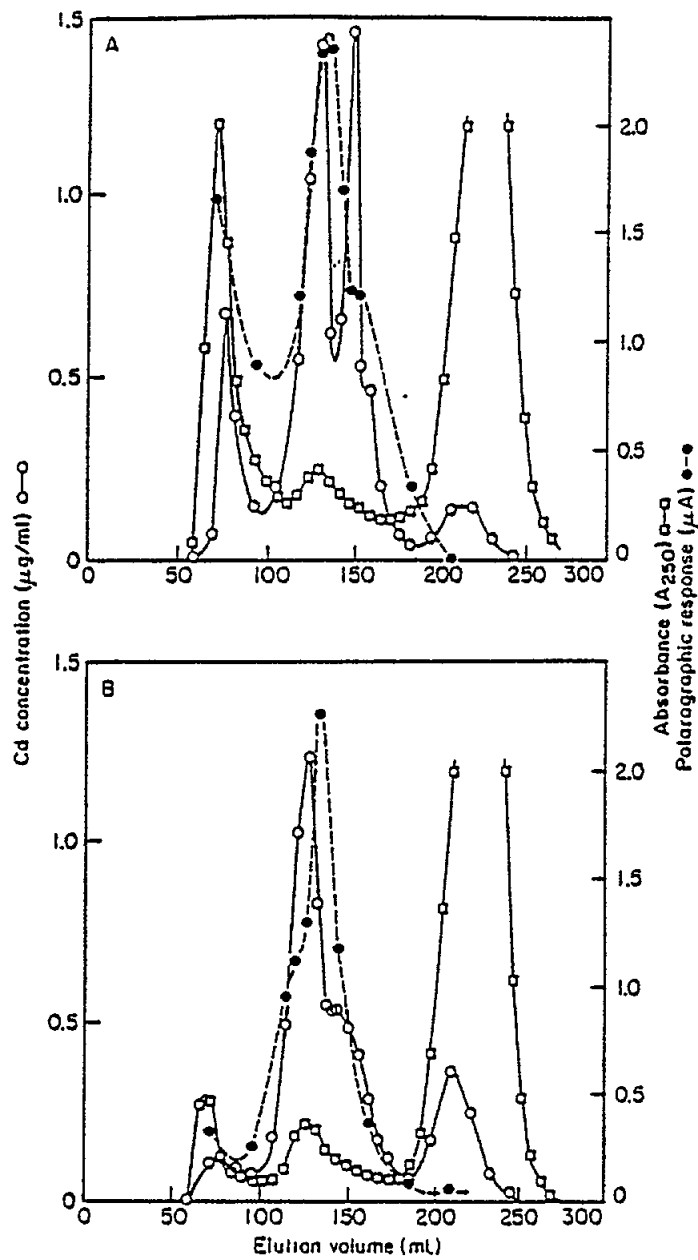


Fig. 4 Cadmium distribution in elution profiles obtained by Sephadex G-75 column chromatography, derived from digestive gland of *M. galloprovincialis* which had been subjected to "intermediate" Cd exposure ($0.23 \mu\text{g Cd ml}^{-1}$; 22 days); UV absorbance at 250 nm and polarographic response according to Brdicka's reaction were also recorded. A/ thermally untreated sample, B/ homogenate subjected to heat treatment (70°C ; 10 min)

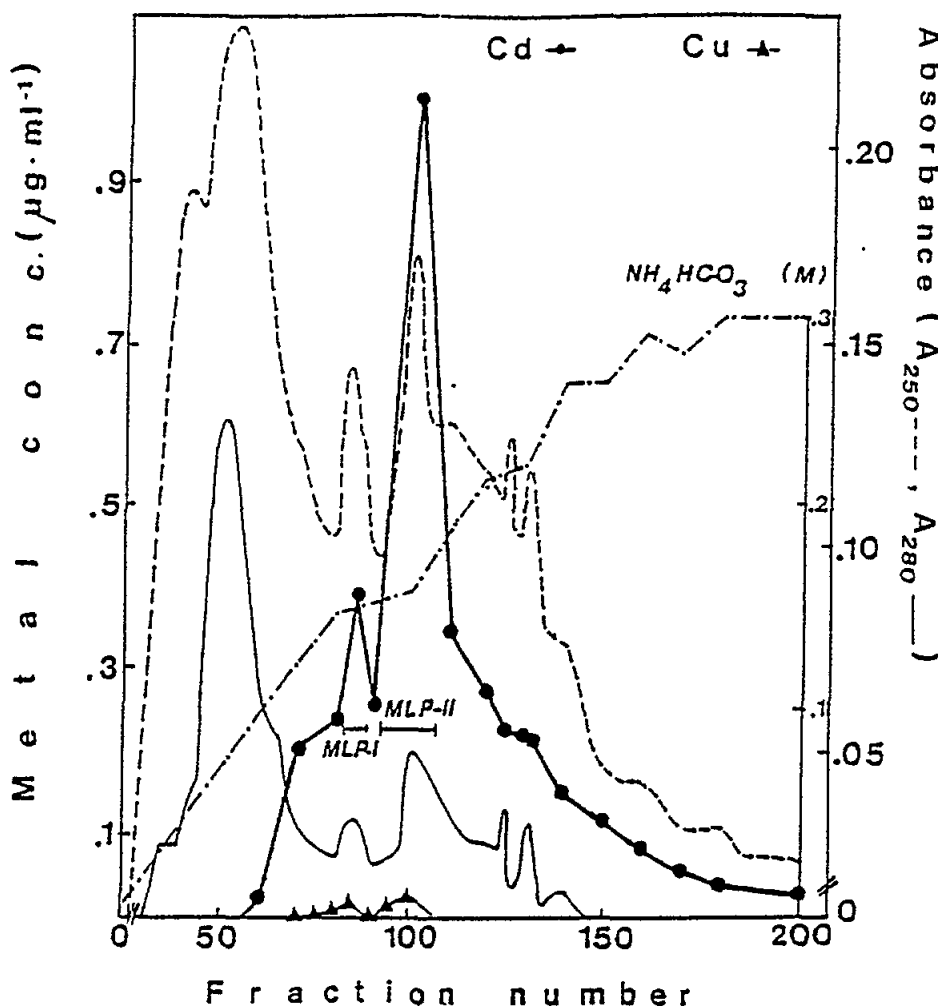


Fig. 5 Ion exchange (DEAE-cellulose) profile showing distributions of metal concentrations (Cd, Cu) in addition to characteristic absorbencies at 250 and 280 nm. Isolated MLP-I and MLP-II components from digestive gland of Cd-exposed mussels ($0.23 \mu\text{g Cd ml}^{-1}$; 22 days) were eluted by application of NH_4HCO_3 concentration gradient (0.02-0.4M) controlled by conductivity measurements

considered as two MLP isoformes designed as MLP-I and MLP-II according to their chromatographic behaviour and spectral characteristics. High sulfhydryl content has also been proved according to the Brdicka's reaction as presented by calibration lines on Fig. 6A indicating considerably higher response per mass of protein of electrophoretically homogenous MLP-II fraction (molar mass 12.6 kDa). Evidently, each of the isolated MLP components from mussels digestive gland has a markedly lower sulfhydryl content in comparison with the mammalian MT standard from rabbit liver being also presented by linear regression line (Fig. 6B) as functional relationship between polarographic current intensity and number of cysteinyl residues per molecule of three different SH-containing compounds as reported by Thompson and Cosson (1984).

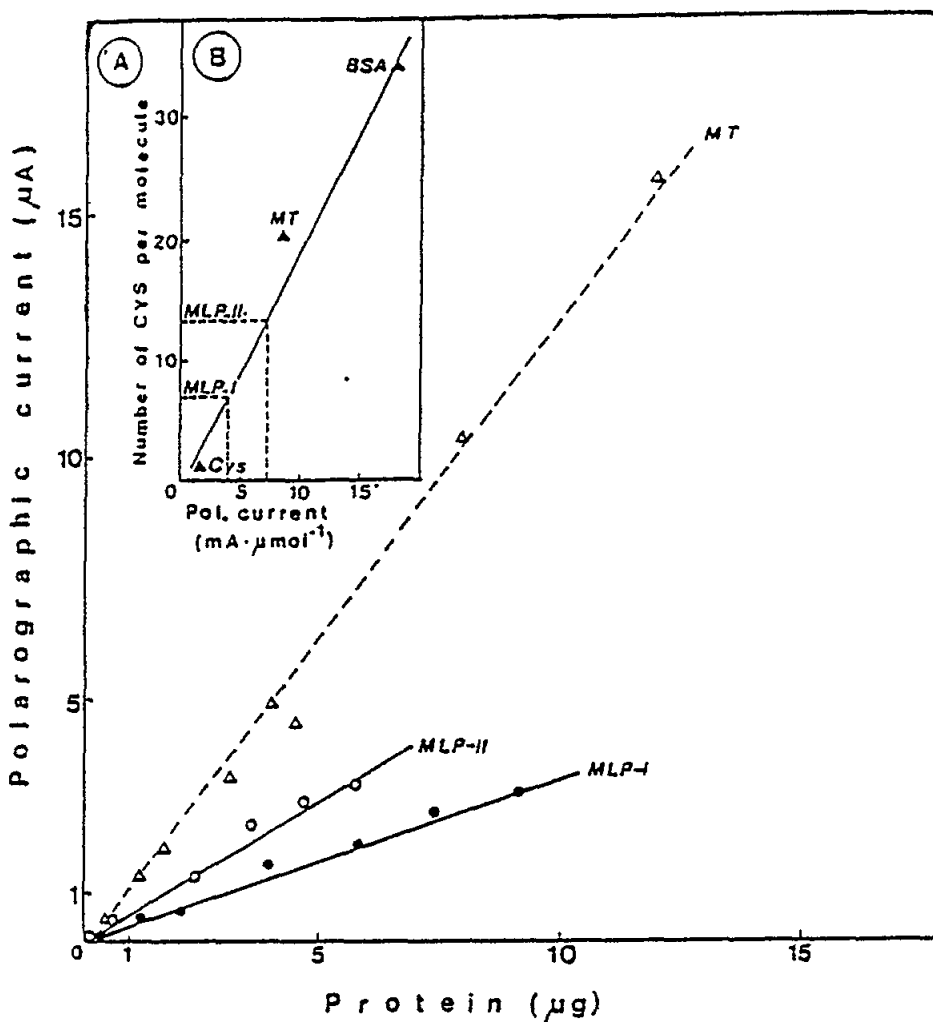


Fig. 6 Polarographic quantitation of MLP using purified MLP-II fraction from digestive gland as an internal calibration standard. A/ Functional relationship between polarographic current of Brdicka's reaction and mass of protein, determined spectrophotometrically according to Bradford (1976) in two isolated MLP fractions of *M. galloprovincialis* and that of commercially prepared MT from rabbit liver (isoformes (I + II)). B/ Dependence of polarographic current intensity on number of cysteinyl residues (in parenthesis) per molecule of SH-containing compounds, showing linear relationship; CYS(1)-cysteine, MT(20)-metallothionein from rabbit liver and BSA(34)-bovine serum albumin

Utilizing purified MLP-II fraction from a digestive gland of *M. galloprovincialis* as the calibration standard, $0.56 \mu\text{A } \mu\text{g}^{-1}$ of MLP has been used for polarographic determination of MLP content in tissue of mussels.

3.3 Monitoring of MLP level within annual reproductive cycle of mussels

The tissue MLP level has been monitored in digestive gland of mussels sampled at intervals of approximately three months during their annual reproductive cycle from the same location (Lim Channel). The results are presented in Table 3, in terms of $\mu\text{A mg}^{-1}$ of soluble protein (C/P ratio) and also expressed as mg MLP g^{-1} of tissue wet weight. A variance coefficient (V_c) of the total soluble protein concentration has been considerably larger than that of polarographic response, 38% and 18%, respectively.

The difference of proteins may be ascribed to seasonal variations rather than to an error of the extraction procedure because of a good agreement found between two replicates of the same sample. The observed variations of C/P ratio between different samples are statistically insignificant at the 0.05 probability level when a t-test is applied. The f-value based on the variance ratio between repetitive preparation of the sample and different samplings during an annual cycle even considerably high has not been found as statistically significant possibly due to a small number of sample preparations.

Table 3

Measurements of sulfhydryl-containing proteins according to Brdicka's polarographic current intensity expressed per total protein content in soluble phase of M. galloprovincialis digestive gland homogenate (C/P ratio) as well as total MLP content per tissue wet weight. Mussels were sampled four times during their annual cycle from the same location (Lim Channel, North Adriatic). Mean shell lengths were also presented.

Sampling date	Shell lgth (mean \pm s.d.)	Pol.cur. ($\mu\text{A ml}^{-1}$) C	Protein (mg ml^{-1}) P	Ratio ($\mu\text{A mg}^{-1}$) C/P	MLP (mg g^{-1}) wet wt.
05.04.90	8.2 \pm 0.5	4.8	0.56	8.6	2.6
14.08.90	6.9 \pm 0.6	4.0	0.68	5.9	2.2
15.11.90	7.4 \pm 0.6	3.7	0.26	14.2	2.0
01.02.91	7.1 \pm 0.8	3.1	0.39	7.9	1.7
	mean:	3.9	0.47	9.2	2.1
	\pm s.d.:	\pm 0.7	\pm 0.18	\pm 3.6	\pm 0.4
	V_c (%):	17.9	38.3	39.1	19.0

4. DISCUSSION

This study has been undertaken in order to find out if induction potential of Cd itself and in combination with some other metals should be used as a suitable indication of mussels exposure to elevated concentrations of trace metals in coastal waters. For that purpose, a digestive gland of M. galloprovincialis has been selected among some other tissues as the most suitable according to the highest extraction efficiency and induction capacity for MLP synthesis which would enable discrimination of different metal exposure conditions including those at a very low concentration level. A

polarographic method for determination of sulfhydryl-containing proteins according to the Brdicka's catalytic reaction has been proposed for the detection of MT and similar proteins because of several advantages; relative simplicity, high sensitivity, low detection level, which may be very useful for large-scale screening in monitoring programs. A sample representing the soluble phase of a tissue homogenate (considered as postlysosomal fraction) would be directly analyzed without being subjected to a time-consuming, conventional liquid chromatography. In order to consider the reliability of trace metals monitoring program using MLP as a potential indicator (Pavičić *et al.*, 1987), several problems have arisen which could be classified into two main aspects; methodological and conceptual. Both of them were experienced during our experimental work or interpretation of the results obtained.

4.1 Methodological problems

The preliminary preparation of the sample, especially homogenization, is the most critical step prior to the analysis. Unequal homogenization of various samples may be consequenced by different extraction efficiency necessarily contributing to the high dispersion of results. In order to control an extraction step, the repetitive homogenization of the same sample is required, and determination of metal concentrations in crude homogenate and supernatant is recommended. Presence of some interfering high-molecular weight SH-containing proteins may introduce a significant error (estimated between 30-50%) particularly in a low-level metal exposed and control samples. Our results proved that significant reduction of HMW maxima occurred when thermal treatment was applied. The addition of a reductive agent (2-mercaptoethanol or dithiotreitol) in order to prevent MLP polymerization caused by intermolecular S-S linkage would markedly improve resolution between HMW and MLP peaks enabling an efficient removal of HMW proteins if small size gel-filtration is utilized, Sephadex G-75 column. The problem associated with the application of a proper calibration standard for quantitative determination of MLP from invertebrate species has been recognized in several reports (Thompson and Cosson, 1984; Wong and Rainbow, 1985) assuming specific characteristics of non-mammalian MTs. Having in mind that MLP from mussels contains a significantly lower molar proportion of cysteinyl residues (Viarengo *et al.*, 1984; Frazier *et al.*, 1985; Roesijadi, 1986; Pavičić *et al.*, submitted for publication) the quantitative method applied in this study, based on the response of tiolic protein, would require an appropriate calibration standard, preferably isolated from tissues of *Mytilus sp.* Recently, the polarographic quantitation of MLP content in whole soft part of *M. edulis* using rabbit liver MT as a calibration standard has been reported for the first time (Bebiano and Langston, 1991). As mentioned before, our results clearly indicate that the polarographic signal of a mammalian MT was nearly two times higher than that of mussels, which would necessarily underestimate their MLP content. Data reported for the digestive gland of the control group (unexposed mussels), (8 mg g^{-1}) expressed on the dry mass basis, were slightly lower in comparison with our results ($2.1 \pm 0.4 \text{ mg g}^{-1}$) when the conversion factor of 5, related to wet/dry mass ratio, was used. In comparison with quantitative data obtained by the application of the immunoassay method (Roesijadi and Morris, 1988), the results of the polarographic quantitation were generally higher than those measured by alternative methods including Cd-saturation method as reported by Onosaka and Cherian (1982). In our opinion, it may be mostly attributed to interfering SH-containing proteins of a HMW pool as being documented by the presence of a measurable amount of tiolic HMW proteins in elution profile, as observed also in study of Bebianno and Langston (1991), in spite of the heat-treatment application.

4.2 Conceptual problems

These problems originate from as yet unclarified biological role of MT and relating proteins with regard to transport and accumulation processes in bivalvae molluscs. Application of the strictly additive approach using results based on a single-metal exposure under laboratory conditions may implicate considerable deviation when extrapolated to complex situation in marine environment where trace metals mostly participate as a mixture of both essential and non-essential in various combinations and concentrations. At present, the inducibility of few metals in mussels has been examined (Cd, Cu, Hg,) showing unequal potential concerning MLP synthesis although not being applied in the mixture at as low concentration level as presented in this study. Having in mind that competitive interactions of different metal ions, present in sea water may occur at different organization levels, i.e. at sites of metal uptake through an outer body membrane of an organism or some particular organ/tissue which may alter accumulation and redistribution of metals among tissues, cellular constituents and particularly on binding sites of MLP molecule. With regard to different induction potential of specific metal ions, such redistributions may initiate an unpredictable and unrealistic response when correlated with trace metals of sea water composition.

Interestingly, our results dealing with induction of MLP by metal mixture (Raspor and Pavičić, in press) could be consistent with observed reduction of total MLP content in comparison with effect of single Cd. Although the difference in total MLP level between single Cd and applied in the mixture with other metals has not been found statistically significant, it may suggest competitive interaction between Cd/Zn or Cu/Cd at binding sites of the MLP molecule and possibly at higher organization level, as reported in some marine and freshwater species of bivalves (Jackim et al., 1977; Hemelraad et al., 1987). In contrast, co-accumulation of certain metals by M. edulis and Macoma baltica has been recorded in the mixture (Kaitala, 1988), while a deviation from the additive model by M. edulis has not even been observed (Phillips, 1976). Similar effects on metal redistributions were observed in the selected tissues of M. edulis following the exposure to copper (Harrison et al., 1983) being affected by the sea water concentration of the metal added. Furthermore, the effect of biotic factors on different rates of growth would seriously influence concentrations of metals accumulated in the soft part of M. galloprovincialis at different coastal locations (Martincic, personal communication) as well as fluctuation of accumulated level of several metals within reproductive cycle of M. edulis (Coimbra and Carraca, 1990).

Considering effects of various biotic factors on fluctuation of MLP content, a possible influence of corticosteroid hormones, previously documented by mammals, as well as high requirement for essential metal ions, particularly zinc in rapidly growing tissues accompanied by the increased synthesis of MT (Brady, 1982) should not be neglected. In connection with that, the presence of progesterone during redevelopment stages of M. edulis has been recently recorded (Coimbra and Carraca, 1990). Our results showing higher MLP concentrations as well as of total soluble proteins during the spring-summer than the autumn-winter season are indicative for fluctuations of the baseline MLP level in the range $\pm 20\%$. At present, it is unclear whether variations of constitutive MLP level could be ascribed exclusively to biotic factor or possibly to their combinations with abiotic factors, particularly associated with progressive decrease of the water temperature decrease during the autumn-winter season which may generally alter biosynthetic processes.

Better understanding of trace metals metabolism in mussels would contribute to more realistic explanation and evaluation of interfering biogenic and possibly other factors upon MLP induction and yield more conclusive evaluation dealing with applicability of the proposed pollution indicator.

5. CONCLUSIONS

Digestive gland of M. galloprovincialis has been selected as the most appropriate tissue for monitoring of MLP level according to the highest induction capacity and extraction efficiency. The proposed tissue is capable of detecting the presence of cadmium experimentally added into sea water responding by synthesis of distinguishing MLP content according to different exposure conditions.

The results obtained confirmed that the Brdicka's reaction for determination of sulfhydryl-containing proteins by DP polarography can serve as a routine method for a large-scale screening of the MLP content in environmentally exposed populations of mussels. Gel-chromatography techniques in conjunction with the detection of low metal concentrations could be used as a complementary method to study the binding affinities and metal interactions in samples of special interest, previously selected by a preliminary screening. The determined baseline level of MLP in digestive gland of control mussels sampled from the Lim Channel varied between 1.7 and 2.6 mg g⁻¹ on the wet weight basis. The recorded MLP fluctuations within the annual reproductive cycle may be ascribed to the joint effect of biotic and abiotic factors. A combined effect of the mixture of three metals (Cd + Cu + Pb) on MLP induction in mussels subjected to relatively low concentration level from the aspect of the coastal pollution, was lower than that induced by a single-cadmium exposure which may be attributed to competitive interactions of metals for both, uptake and MLP binding sites as well as to different inducibility of the specific metal. Beside certain methodological improvements dealing with extraction and precipitation of interfering proteins prior to quantitative determination, an application of a proper internal calibration standard is recommended in order to avoid underestimation of the MLP content, due to significantly lower molar proportion of cysteine residues in comparison with MTs from mammalian sources. A comparison with a more specific, demanding, and expensive immunoassay method might be provided as well as an application of some additional biological parameters which might lead us to a better understanding and control of MLP variations during reproductive cycle of mussels.

6. ACKNOWLEDGEMENTS

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REFEREE'S COMMENT

The authors indicate that there are differences in the levels of MLP fraction in Cd exposed animals compared to metal mixture exposure. This difference does not look statistically different in the figures and in Table 2 the amount of metals bound (except for Zn) are remarkably similar. The authors spend a great deal of time attempting to explain this difference and make it part of the conclusions. I am not sure that the difference is real or that it warrants this amount of discussion and importance.

AUTHOR'S RESPONSE TO REFEREE'S COMMENT

We agree that observed differences in total metal as well as the MLP content between two low-level metal exposed groups of mussels, denoted as Cd-single and metal mixture, are beyond statistical significance. That would not be in agreement with general idea of monitoring that summation of single metal effects on MLP induction would be equal to the combined effect of metals applied in the mixture. We tried to suggest explanation of that phenomenon on the basis of different induction potential as well as by competitive interactions between metal ions for uptake and binding sites, previously reported in bivalve molluscs.

LINDANE EFFECTS ON THE GROWTH, SIZE AND COMPOSITION OF TWO
MARINE UNICELLULAR ALGAE, Monochrysis lutheri (Droop)
AND Phaeodactylum tricornutum (Bohlin)

by

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A B S T R A C T

The effect of high concentrations of lindane (isomer gamma-hexachlorocyclohexane) on the growth (changes in concentration), size (volume) and composition (C, N and pigments) of two marine unicellular algae, Monochrysis lutheri (Droop) and Phaeodactylum tricornutum (Bohlin) has been studied as preliminary work to determine those characteristics of the algae that better respond to the pesticide and can be used afterwards to check the effect of low concentrations of this and other pesticides on the algae.

Of the parameters studied, growth rate, C, N and phaeopigment content seem to be the most sensitive to the action of lindane, thus being good candidates for algal bioassays.

1. INTRODUCTION

Marine microalgae, being at the base of marine food chains, constitute an essential part of marine ecosystems, so any substance that interferes with their normal life will alter the functioning of entire ecosystems.

Therefore, bioassays with microalgae can be considered as highly significant in relation to their predictive capacity for wide ecosystem effects.

The aim of this study was to determine the effect of high levels of lindane on several characteristics of two unicellular algae, the pelagic Monochrysis lutheri and the benthic Phaeodactylum tricornutum, in order to select for bioassays those parameters that should show a better response (strong, clear and repetitive) to the pesticide and could be considered for future work to check the effect of toxic substances on the algae at lower, more realistic concentrations.

The characteristics initially selected to measure the toxic effects of lindane were cell growth, cell size and cell composition (carbon, nitrogen and photosynthetic pigments). Changes in these parameters were followed in cultures of the algae inoculated with two concentrations of lindane, at 1 and 10 (saturating) ppm each.

2. MATERIAL AND METHODS

The species of algae used were the Bacillariophyceae Phaeodactylum tricornutum (Bohlin) and the Chrysophyceae Monochrysis lutheri (Droop). Cultures of these algae were grown in artificial sea water, enriched with "f/2" medium (Guillard and Ryther, 1962).

Experiments were conducted under constant light in an isothermic room at $15 \pm 0.5^\circ\text{C}$, using 250 ml Erlenmeyer flasks containing 200 ml of algal culture, at an initial concentration of about $6,000 \text{ cells ml}^{-1}$.

Two types of lindane were used: technical lindane, with 99.5% active material (SANDOZ, S.A.E.), and wettable powder, with 60% lindane (SANDOZ, S.A.E.). To enhance its solubility in water, both types of lindane were first diluted in acetone before addition to the Erlenmeyer flasks at concentrations of 1 and 10 (saturating) ppm. Three replicates were prepared for each concentration and pesticide formulation. Controls with and without acetone were also used but no significant differences were found between the two controls.

The concentration and size of the algae in the Erlenmeyer flasks were monitored at 24 hours intervals using a Coulter Counter TA II. A Neubauer haemocytometer was also used for counting cells in the experiments with the wettable powder, in order to distinguish clearly between algae and powder (the size of the two overlaps in the electronic counting). After a week of growth, an inoculum of each flask was re-inoculated in fresh medium at the same conditions, and growth and size were monitored for another week.

The growth (exponential) was quantified using the equation,

$$K = (\ln N - \ln N_0) / t$$

where K is the growth coefficient, N_0 and N the cell numbers at the beginning and at the end of the period and t is the length of period (24 hours).

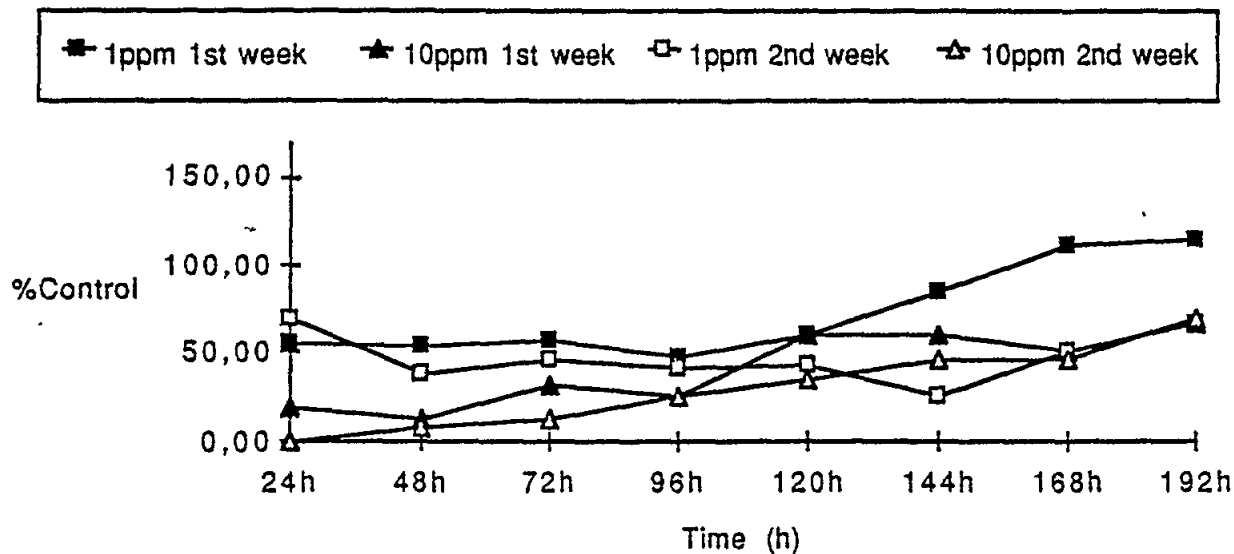
Photosynthetic pigments were quantified following standard spectrophotometric procedures (Parsons *et al.*, 1984) and carbon and nitrogen content were determined with a CN analyzer (Carlo Erba Strum. DP 200), after filtering the algae through Whatman GF/C glass fiber filters. Samples of algae for these measurements were taken from the initial culture, and at the end of the first and second week of exposure.

Data were analysed using an ANOVA computer programme (Abacus STATVIEW for the Macintosh SE) that determined significant differences (at 95% level) between treatment (kind of algae, lindane level and formulation and time of exposure) on the basis of the Scheffe F-test and the Fisher PLSD test. The experiments were designed to fulfill the requirements of these tests.

3. RESULTS

Figures 1 and 2 represent the growth of the algae exposed to 0, 1 and 10 ppm of both technical and wettable lindane. ANOVA analysis indicated a significant effect of both formulations of lindane on the growth of both

A- Technical lindane



B-Wettable lindane

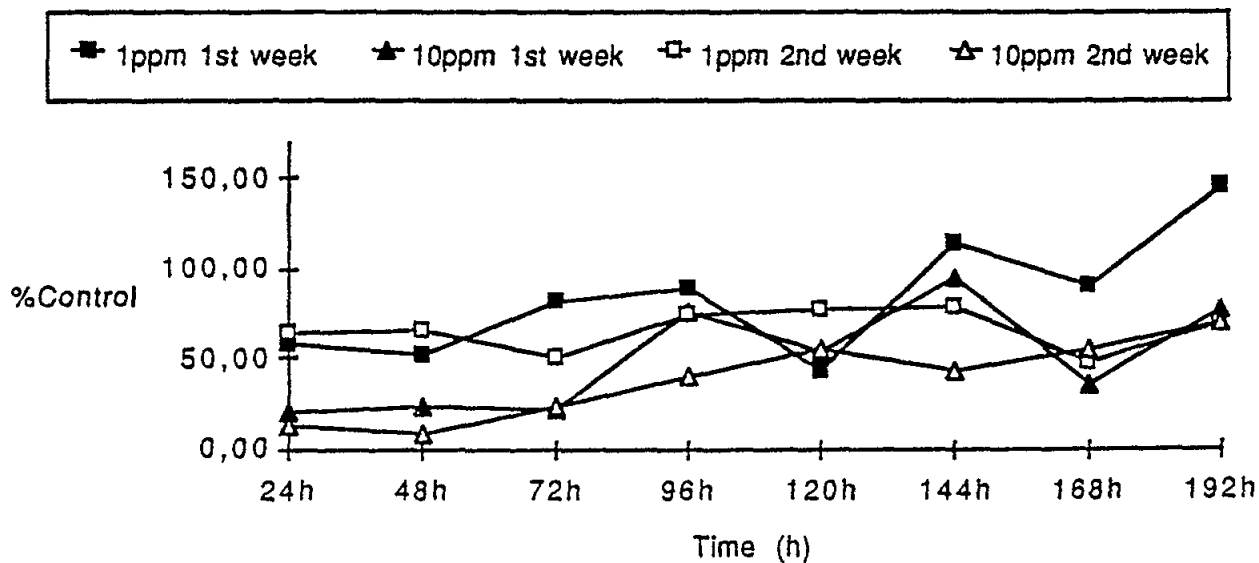


Fig. 1 Growth coefficient (k) of *Monochrysis lutheri* exposed to technical and wettable lindane, as percentages of controls

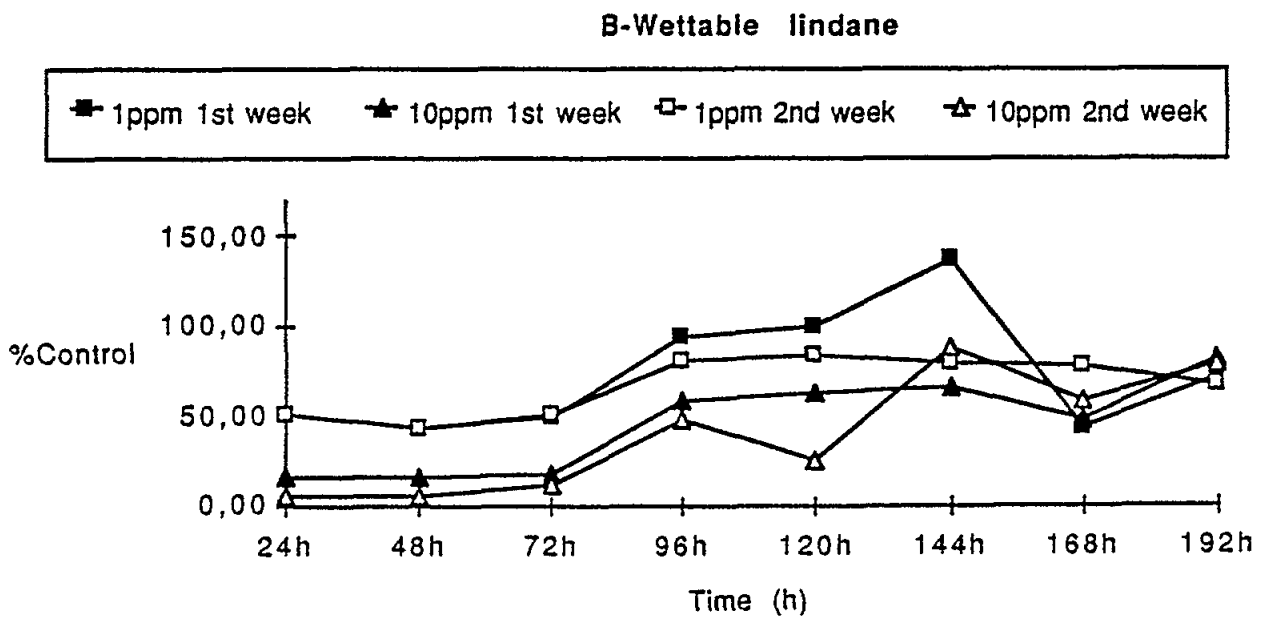
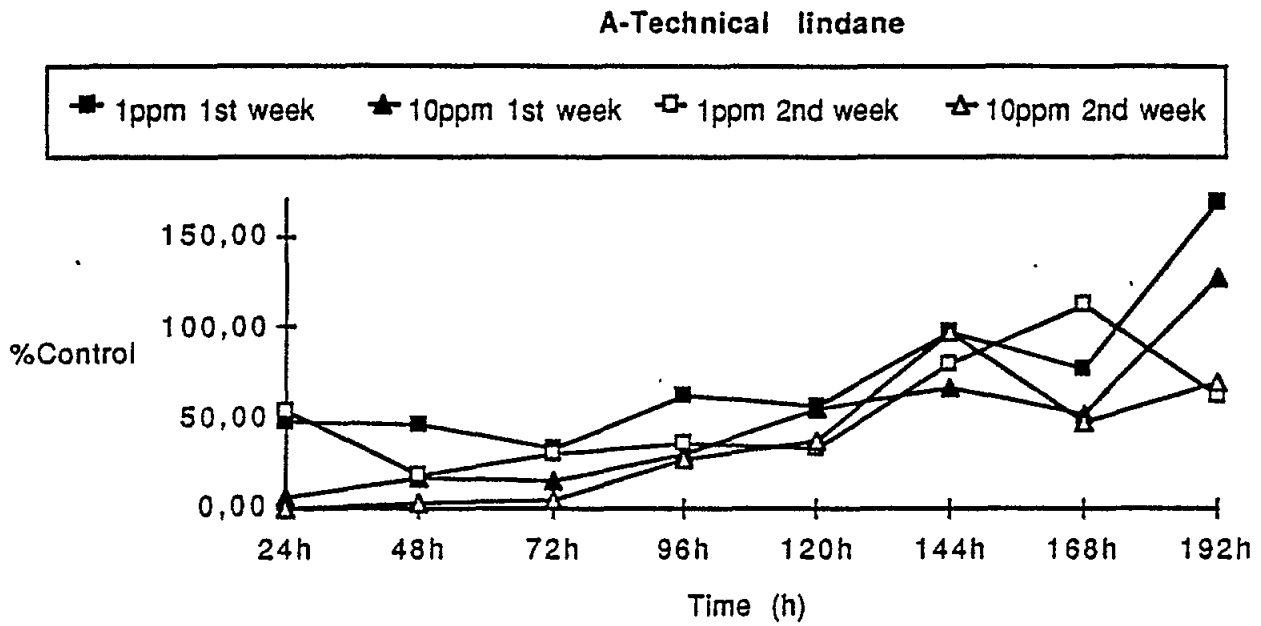


Fig. 2 Growth coefficient (k) of *Phaeodactylum tricornutum* exposed to technical and wettable lindane, as percentage of controls

species of algae, at the two concentration tested. The effect of technical lindane on the growth of both algae was significantly bigger than that of the wettable powder ($P < 0.05$), and the reduction of growth was more pronounced for P. tricornutum than for M. lutheri ($P < 0.05$) and greater in the second week of exposure than in the first week ($P < 0.05$).

It is clear from Figs 1 and 2 that the main reduction in the growth coefficient of algae exposed to lindane (compared to controls) takes place during the first 3-4 days of exposure. The reduction observed in the growth coefficient of M. lutheri after 96 hour exposure to technical lindane ranges between 50-60% at 1 ppm to 75% at 10 ppm, for both the first and second week of exposure. The corresponding values for wettable lindane after 3 days of exposure were 20% and 80% reduction for 1 and 10 ppm respectively, for the first week and higher percentages for the second week (45% and 85% respectively). In P. tricornutum, the observed growth coefficient reduction after 3 days of exposure to technical lindane ranged between 65% at 1 ppm to 90% at 10 ppm for the two weeks. The corresponding values for wettable lindane were 50% and 90%.

In all experiments, a certain recovery in growth was registered after 3-4 days (Figs. 1 and 2). We attribute such recovery to acclimation of the algae to the pesticide, as no significant reduction of pesticide concentration was observed during the experimental run, as determined by gas chromatography of water samples taken at 24-hour intervals in previous experiments (data not shown).

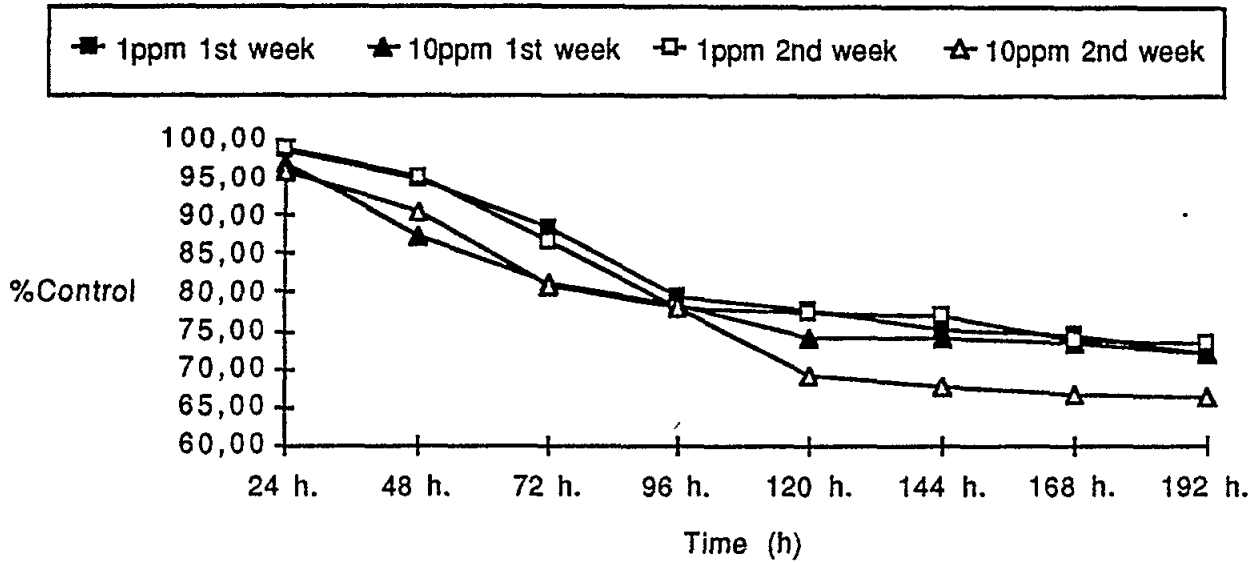
The effect of pesticide on algal size was studied only with technical lindane, given the interference of sizes found with wettable lindane. ANOVA analysis indicated a significant effect of lindane on the size of both species of algae, of similar magnitude to the effect observed on growth. The effect of technical lindane on the size of M. lutheri was greater in the second week of exposure than in the first, and with a direct relationship to concentration. The effect of technical lindane on the size of P. tricornutum was also highly significant. However, no significant differences were found between exposure concentration or between the first and second week of exposure.

The reduction observed in volume for M. lutheri reached values of 20% of the controls after 96 hour exposure, and 25-35% after both weeks of exposure. In P. tricornutum, the reduction observed in volume after 120 hours was approximately 30-35% of controls, reaching 30-40% after both weeks of exposure. These values were not significantly different for 1 and 10 ppm or for the 1st and 2nd week of exposure (Fig. 3).

Algal composition is also affected by lindane. Cellular carbon and nitrogen were below 50% of control values (reduction higher than 50%) after one week of exposure to 1 ppm lindane. Of the pigments studied, phaeopigments were the most sensitive to lindane, with values around 50% of controls after one week of exposure to 1 ppm of technical lindane. Composition was clearly affected by lindane concentration, although values differ clearly, depending on lindane formulation and weeks of exposure.

As in the case of growth, ANOVA analysis shows significant differences for pigments, carbon and nitrogen, related to concentration and pesticide

A-*Monochrysis lutheri*



B- *Phaeodactylum tricornutum*

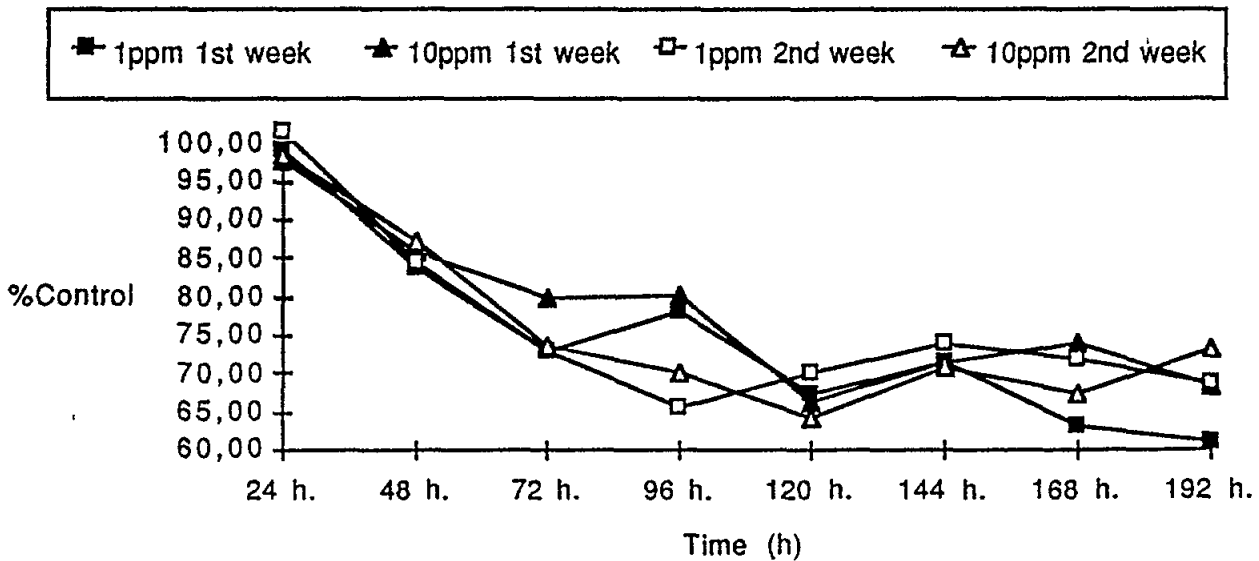


Fig. 3 Mean cell volume of *Monochrysis lutheri* and *Phaeodactylum tricornutum* exposed to technical lindane, as percentages of controls

formulation, algal type and week of exposure. The effect of technical lindane on the cellular composition of both algae was significantly stronger ($P < 0.05$) than the one of the wettable powder ($P < 0.05$), and the reduction of cellular composition was greater for the second week of exposure than for the first week ($P < 0.05$). In Tables 1 and 2, the changes in cellular composition in relation to controls can be observed for each concentration and formulation of lindane, and for each week and algal species.

Table 1

Cellular carbon and nitrogen of *Monochrysis lutheri* and *Phaeodactylum tricornutum* after exposition to different concentrations of technical lindane and wettable lindane.
Mean (M), \pm standard deviation (S.D.),
percent reduction compared with control (%).

Lindane	Week	Concentration	Carbon (%)			Nitrogen (%)		
<i>Monochrysis lutheri</i>								
			M.(pg/cel)	S.D.	%	M.(pg/cel)	S.D.	%
Technical	First	0 ppm	10.23	0.073	0	1.40	0.010	0
		1 ppm	4.33	0.048	58	0.62	0.018	56
		10 ppm	3.17	0.076	69	0.43	0.026	70
	Second	0 ppm	10.16	0.065	0	1.39	0.022	0
		1 ppm	3.29	0.070	68	0.39	0.018	72
		10 ppm	2.40	0.176	76	0.32	0.026	77
Wettable	First	0 ppm	10.28	0.047	0	1.42	0.018	0
		1 ppm	4.92	0.060	52	0.74	0.017	48
		10 ppm	3.56	0.059	65	0.54	0.028	62
	Second	0 ppm	10.27	0.070	0	1.41	0.014	0
		1 ppm	3.86	0.046	62	0.52	0.018	63
		10 ppm	2.93	0.056	71	0.37	0.011	74
<i>Phaeodactylum tricornutum</i>								
			M.(pg/cel)	S.D.	%	M.(pg/cel)	S.D.	%
Technical	First	0 ppm	12.88	0.081	0	2.42	0.066	0
		1 ppm	5.96	0.053	54	1.51	0.041	38
		10 ppm	3.80	0.060	70	0.89	0.027	63
	Second	0 ppm	12.87	0.069	0	2.41	0.060	0
		1 ppm	4.53	0.062	58	1.16	0.031	52
		10 ppm	2.67	0.036	79	0.66	0.014	73
Wettable	First	0 ppm	12.91	0.060	0	2.43	0.068	0
		1 ppm	6.92	0.042	46	2.06	0.035	15
		10 ppm	4.67	0.038	63	1.29	0.033	47
	Second	0 ppm	12.92	0.067	0	2.43	0.066	0
		1 ppm	6.22	0.059	52	1.42	0.020	42
		10 ppm	3.74	0.064	71	0.69	0.012	72

4. DISCUSSION AND CONCLUSIONS

The data presented here support the results of other authors who have studied the drastic effects of pesticides on phytoplankton (Harding and Phillips, 1978; Mayasich *et al.*, 1986), and are also in agreement with those of Ukeles (1962), Moore and Dorward (1968), Borghi *et al.* (1973) and Daste and Neuville (1974), who have also shown reduction of algal growth in the presence of lindane.

Table 2

Cellular pigments of *Monochrysis lutheri* and *Phaeodactylum tricornutum* after exposition to different concentrations of technical lindane and wettable lindane. Mean (M), \pm standard deviation (S.D.), percent reduction compared with control (%).

Lindane	Week	Concentration	Chl. a			Chl. b			Chl. c <i>Monochrysis lutheri</i>			Carotenoids			Phaeopigments		
			M (pg/cell)	SD	%	M (pg/cell)	SD	%	M (pg/cell)	SD	%	M (pg/cell)	SD	%	M (pg/cell)	SD	%
Technical	First	0 ppm	2.30	0.026	0	0.115	0.004	0	0.52	0.018	0	2.45	0.033	0	0.220	0.004	0
		1 ppm	1.85	0.030	20	0.080	0.002	30	0.40	0.015	24	1.73	0.050	30	0.097	0.002	56
		10 ppm	1.38	0.020	40	0.072	0.001	48	0.30	0.015	43	1.28	0.050	48	0.066	0.001	70
	Second	0 ppm	2.30	0.016	0	0.116	0.006	0	0.49	0.023	0	2.45	0.029	0	0.221	0.003	0
		1 ppm	1.61	0.015	30	0.072	0.001	38	0.33	0.012	33	1.47	0.058	40	0.062	0.001	72
		10 ppm	1.15	0.021	50	0.051	0.001	56	0.23	0.010	53	1.05	0.066	57	0.051	0.001	77
Wettable	First	0 ppm	2.30	0.028	0	0.114	0.003	0	0.49	0.046	0	2.46	0.024	0	0.221	0.003	0
		1 ppm	2.02	0.010	12	0.080	0.002	30	0.38	0.020	26	1.55	0.066	37	0.137	0.002	38
		10 ppm	1.50	0.025	35	0.049	0.001	57	0.23	0.015	53	0.89	0.025	64	0.082	0.001	63
	Second	0 ppm	2.29	0.021	0	0.114	0.001	0	0.50	0.015	0	2.45	0.024	0	0.221	0.003	0
		1 ppm	1.73	0.015	25	0.067	0.002	41	0.31	0.010	38	1.13	0.050	54	0.106	0.002	52
		10 ppm	1.29	0.015	44	0.043	0.002	62	0.17	0.010	66	0.56	0.052	77	0.060	0.002	73
Technical	First	0 ppm	1.70	0.018	0	0.053	0.001	0	0.40	0.018	0	2.73	0.021	0	0.259	0.001	0
		1 ppm	1.27	0.015	25	0.040	0.001	25	0.36	0.013	11	2.43	0.052	11	0.135	0.001	48
		10 ppm	0.85	0.031	50	0.030	0.001	43	0.27	0.010	32	1.80	0.038	34	0.098	0.001	62
	Second	0 ppm	1.69	0.019	0	0.053	0.001	0	0.41	0.016	0	2.74	0.024	0	0.260	0.002	0
		1 ppm	1.10	0.010	35	0.035	0.001	34	0.31	0.011	25	2.00	0.050	27	0.096	0.001	63
		10 ppm	0.67	0.021	60	0.025	0.001	53	0.23	0.010	45	1.45	0.038	47	0.068	0.001	74
Wettable	0 ppm	1.70	0.030	0	0.053	0.002	0	0.41	0.018	0	2.73	0.024	0	0.260	0.002	0	
	1 ppm	1.34	0.015	21	0.041	0.001	23	0.34	0.019	17	2.27	0.025	17	0.221	0.001	15	
	10 ppm	0.93	0.020	45	0.027	0.001	49	0.23	0.014	44	1.81	0.063	34	0.136	0.001	47	
Second	0 ppm	1.69	0.023	0	0.054	0.003	0	0.42	0.018	0	2.66	0.134	0	0.261	0.002	0	
	1 ppm	1.18	0.015	30	0.034	0.001	37	0.28	0.010	34	1.86	0.038	30	0.151	0.001	42	
		10 ppm	0.75	0.031	55	0.022	0.001	59	0.17	0.015	69	1.13	0.038	57	0.073	0.001	72

Our results indicate a clear effect of lindane on the growth, size and composition of the two unicellular algae tested. It is possible to distinguish between lindane formulation and concentration for the effects registered on the algae. It is also possible to distinguish between the effects on M. lutheri and on P. tricorutum. During the second week of exposure, the reduction in growth and size and the alteration of composition was greater than during the first. However, a recovery in growth coefficient and size of the algae was registered after the first 3-4 days of exposure in both weeks.

Of the effects measured, the most important responses were obtained for growth coefficient and composition of the algae. For these parameters, it is possible to distinguish between treatments not only for lindane concentrations, but also for formulation, time of exposure and alga employed. Cellular size of the algae, in contrast showed a weaker response.

Algal composition showed better responses to lindane for carbon, nitrogen and phaeopigment content per cell. Chlorophylls and carotenoids also showed important but lower reductions.

Therefore, carbon, nitrogen and phaeopigment content seem to be the most valuable parameters to be used in monitoring the effects of lindane on microalgae. More work is needed to generalize these conclusions to other pesticides.

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BIOLOGICAL EFFECTS OF CONTAMINATED WATER TESTED BY
MARINE BIVALVE EMBRYO-BIOASSAY

by

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A B S T R A C T

In bivalves, embryonic development is very sensitive to reduced water quality. The D larvae stage is reached in a short time after fertilization : 48 h for Mytilus edulis, 24 h for Crassostrea gigas.

Fertilization is carried out in the water to be bioassayed, at a density of about 50000 eggs per litre container and the suspension maintained at the optimum temperature for the species used. The total toxicity of the tested water is evaluated on the basis of the percentage developmental success of D larvae stage compared with that of a control sea water.

Results of tests on urban waste water from a biological treatment plant show that oysters and mussels have a similar sensitivity.

Bioassays with an increasing concentration of water or extract of sediment from the river Seine show evidence of contamination in this area. The same test has been used to assess changes in toxicity of Gyrodinium aureolum cultures.

The marine bivalve embryo-bioassay is not difficult to initiate and could be used as an "indicator" of the general toxicity of a river, extracts of sediment, or urban or industrial sewage before discharge into the environment.

1. INTRODUCTION

Chemical measurements in water, sediment or the organism's tissues alone are not enough to determine the combined effects of environmental contamination on organisms, neither are measures of community structure (Chapman and Long, 1983). To monitor water quality, Woelke (1972) proposed the use of embryos and larvae of Crassostrea gigas.

Following the studies of Bourne et al. (1981), Nelson et al. (1983), Long and Buchman (1989) and Thain (1991) we used a marine bivalve embryo-bioassay with the Japanese oyster (Crassostrea gigas) and the mussel (Mytilus edulis) to evaluate the toxicity of: an urban sewage, a dinoflagellate culture (Gyrodinium aureolum), a water and a sediment elutriate from the river Seine (France).

2. MATERIALS AND METHODS

2.1. Seawater tested

2.1.1 Reference sea water

Reference sea water was taken from the IFREMER hatchery (Argenton) or from the bay of Brest (chart 1). The sea water was used immediately after filtration through a 0.2 μm sterile millipore filter.

2.1.2 Test sample

The urban waste water studied came from the biological treatment plant at Morlaix (chart 1). It was added to the reference sea water (1 to 10%) and the salinity was adjusted to 30 with distilled water. The urban sewage sample was treated but not chlorinated.

The dinoflagellate culture ($>2.10^6$ cell l^{-1}) was either tested whole, or after filtration through a 0.2 μm filter or after detoxification through C18 and Florisil cartridges in order to remove respectively lipophilic compounds and more polar compounds like Gyrodinium aureolum exotoxins (Gentien et al., 1991).

The water from the Seine estuary near Honfleur (chart 1) was added to the reference sea water in order to produce increasing concentrations from 2.5 to 20%. The salinity was adjusted to 30 and the test water was filtered through a 0.2 μm filter.

The sediment elutriate was made with 100 g of sediment sampled on the seashore near Honfleur and mixed with reference sea water. Concentrations of 5 to 100 $g\ l^{-1}$ were tested.

2.3 Experimental protocol

Adult oysters or mussels were collected from natural populations during the spawning season or from the hatchery where they were conditioned to spawn. Ripe bivalves were induced to spawn by thermal stimulation (Loosanoff and Davis, 1963; His and Robert, 1986). Eggs filtered through a 100 μm filter (to remove debris) were retained on a 32 μm filter. The gametes were then transferred in reference sea water, counted and aliquots put in test samples to give a density of 50000 eggs l^{-1} . After filtration through 100 μm filter, 1.5 $ml\ l^{-1}$ of concentrated sperm was added to the test samples simultaneously with the eggs.

Only one female and one male were used and the fertilization was carried out in the test samples; this was because some pollutants can have a direct action on the gametes (His and Robert, 1980).

After fertilization, control and test samples (two to five replicate 30 ml aliquots in polystyrene vials) were incubated without aeration or light at 24°C during 24 hours for oysters and at 20°C during 48 hours for mussels. At the end of the incubation, the larvae were examined immediately or preserved with buffered formalin for future counting.

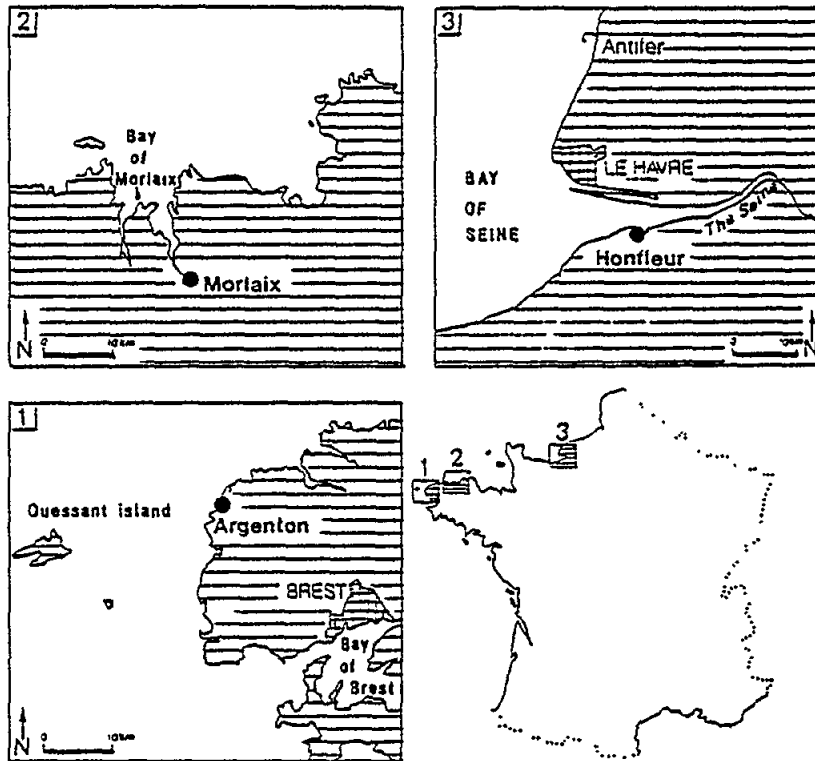


Chart 1 Areas from where water and sediment were sampled.
· Reference sea water : Argenton and bay of Brest.
· Urban sewage : Morlaix.
· Water and sediment from the Seine : Honfleur.

The measure of water quality is evaluated by counting the number of abnormal embryos in test samples compared to those in control (about 800 to 1500 larvae), using Abbott's formula (APHA, 1980).

$$\text{Percent Net Abnormality (PNA)} = \frac{\% \text{ test abnormality} - \% \text{ control abnormality}}{100 - \% \text{ control abnormality}} \times 100$$

A larvae is 'normal' when the two valves have a regular D shape and if the mantle can be wholly withdrawn into the valves.

Bioassays are valid only when at least 80% of larvae develop normally in the control tests.

3. RESULTS

3.1 Effect of waste water

The urban waste water was sampled in the afternoon and stored in a refrigerator (0 to + 4°C) without light for up to ten days. Reference sea water was added to obtain test samples with 1 to 10% of urban sewage.

Results of bioassays carried out at the beginning of June 1989, on mussel and oyster, with waste water filtered through 0.2 µm or 100 µm filter show (Fig. 1) that biological effect was similar for Crassostrea gigas and Mytilus edulis embryos when the waste water was 0.2 µm filtered. Moreover, in this experiment the PNA is greater when the urban sewage was only 100 µm filtered; the additional toxicity may be linked to the suspended matter present.

The same test conducted at the end of June on oyster embryos show that urban sewage is more toxic (experiment 2, Fig. 2) : PNA 100% is reached with 7.5% of waste water against 10% at the beginning of June (experiment 1, Fig. 2).

At the end of June (Fig. 3), different filtrates of urban sewage did not show a significant variation of toxicity. Here, the toxicity was not linked to suspended matter but could have been due to anionic detergents which are important (Lassus et al., 1990). Note that the toxic effect begins at 2% of urban waste water; this is a concentration that can be obtained in the environment (Salomon et al., 1990).

3.2 Effect of Gyrodinium aureolum culture

In these bioassays, the reference sea water came from the bay of Brest and the tests were conducted in daylight (20°C) on mussel. In October, a semi-continuous automated culture system was used to deplete the phosphorus in the culture medium (Gentien et al., 1991).

Three experiments (Table 1) show that this dinoflagellate toxicity resulted from two different processes :

- a) a toxicity by proximity or contact, e.g. more than 87% abnormalities with whole cultures,
- b) a toxicity by production of dissolved compounds which was enhanced by phosphorus depletion of the cell, e.g. 3.5% abnormality in November, but PNA about 40% in October (c.f. Gentien et al., 1991).

3.3 Effect of water from the river Seine

Water sampled in the river Seine at low tide, in May 1988, was stored one month at - 20°C before tests.

Figure 4 gives results of oyster embryo-bioassays; there was no mortality but PNA was more than 30% when there was only 7.5% water from the Seine. A PNA of 50% was reached with 10% of river Seine water; this is not a high dilution in such an estuary.

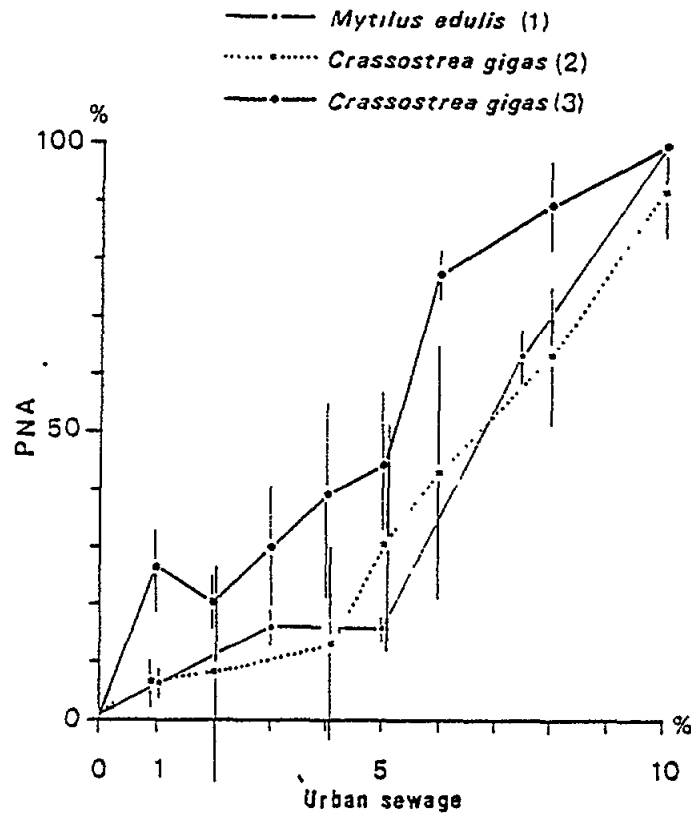


Fig. 1 Experiments conducted at the beginning of June 1989 on *Mytilus edulis* and *Crassostrea gigas* embryos with urban sewage from Morlaix, filtered through 0.2 μm filter (1,2) or 100 μm filter (3) Percent Net Abnormality : PNA = $\bar{x} \pm$ standard deviation

Table 1

Percent Net Abnormality (PNA) in three embryo-bioassays on mussel, conducted with whole culture of *Gyrodinium aureolum*, filtered or detoxified culture medium ($>2.10^6$ cell l^{-1}).

Date	11.09.90	24.10.90	02.11.90
Whole culture	87.5	89.3	100
Filtered culture medium	-	39.8	3.5
Detoxified culture medium	-	5.4	3.5

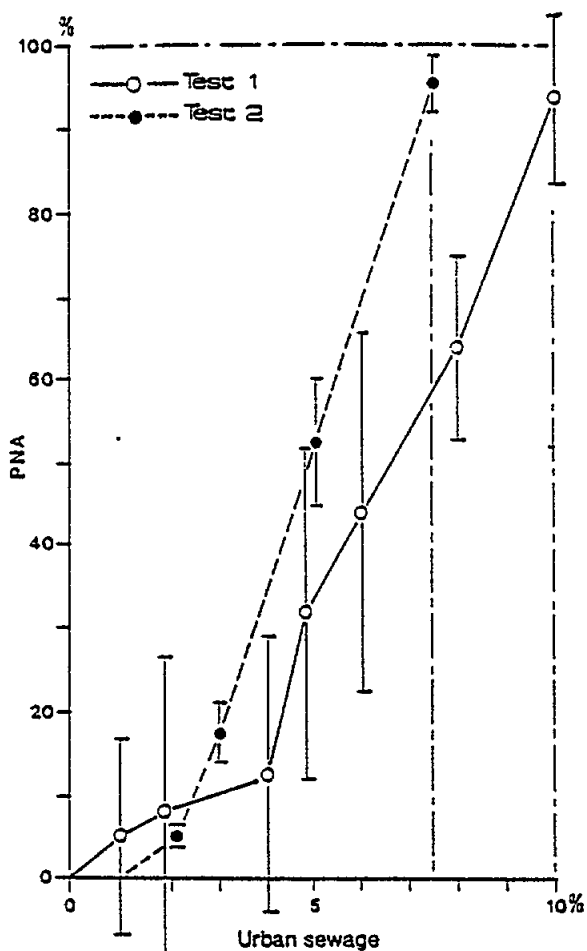


Fig. 2

Bioassays on Crassostrea gigas with 0.2 μm filtered urban sewage

1 - at the beginning of June 1989 (Curve 2 from Fig. 1)

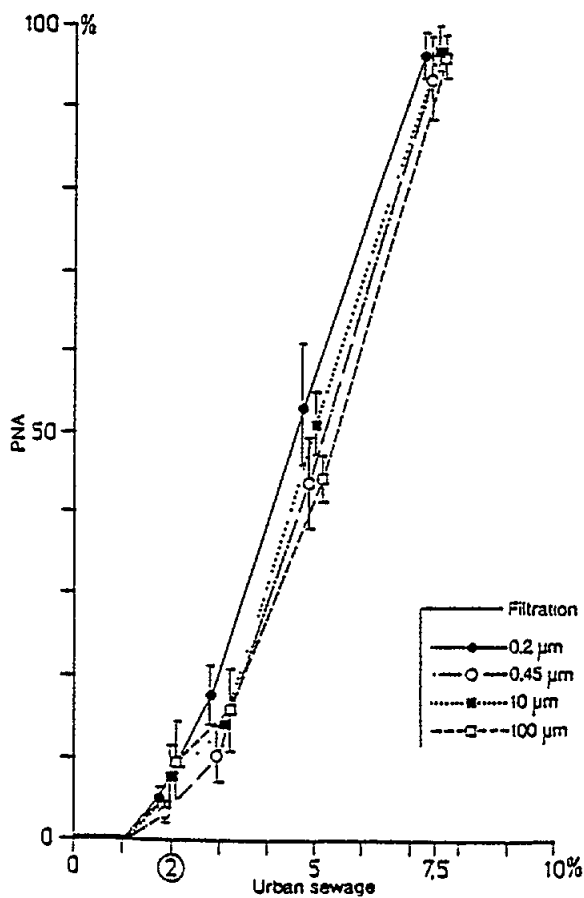
2 - at the end of June 1989

Percent Net Abnormality :
 $\text{PNA} = \bar{x} \pm \text{standard deviation}$

Fig. 3

Effect of filtration rate of urban sewage in a Crassostrea gigas embryo-bioassay.

Percent Net Abnormality :
 $\text{PNA} = \bar{x} \pm \text{standard deviation}$



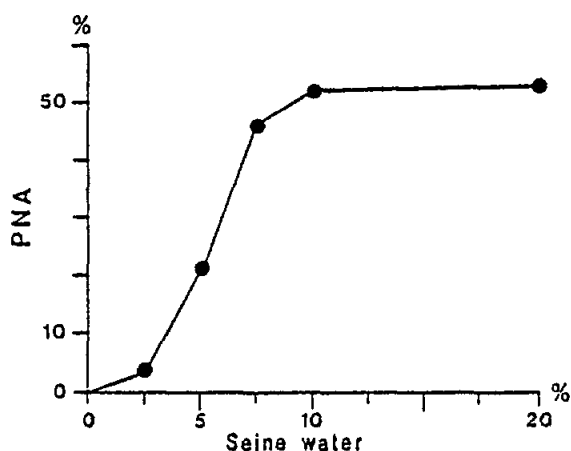


Fig. 4 Effect of water from the river Seine on oyster embryo-bioassay. PNA = Percent Net Abnormality (about 800 to 1500 larvae)

This effect is probably due to the pollution of the river Seine by industrial, urban and agricultural sewages (IFREMER, 1986).

3.4 Effect of sediment elutriate

Sediment samples taken from the seashore at low tide, in May 1988, were stored one month at -20°C before bioassays.

In these tests, 100 g of sediment was mixed with reference sea water and after decantation (1 hour), the elutriate was filtered ($0.2\ \mu\text{m}$) and added to reference sea water in order to obtain equivalent concentrations of 5 to 100 g sediment l^{-1} . Bioassays were conducted on oyster and mussel in June 1988; the two species gave a similar response to sediment elutriate. A PNA of 50% is reached at a concentration of about 35 g l^{-1} (Fig. 5).

4. CONCLUSION

Results show that bioassays with Crassostrea gigas and Mytilus edulis give a similar response in relation to urban sewage from Morlaix or sediment elutriate from Honfleur. However, as embryo development takes 24 hours for C. gigas against 48 hours for M. edulis, tests with oyster could be considered more sensitive.

These experiments show that, according to Woelke (1972), Bourne *et al.* (1981), Long and Buchman (1989) and Thain (1991), such embryo-bioassays could be used to monitor toxicity of waste water, biotoxin of phytoplankton culture, river or sediment. But it will be interesting to standardize the treatment of sediment before use in tests.

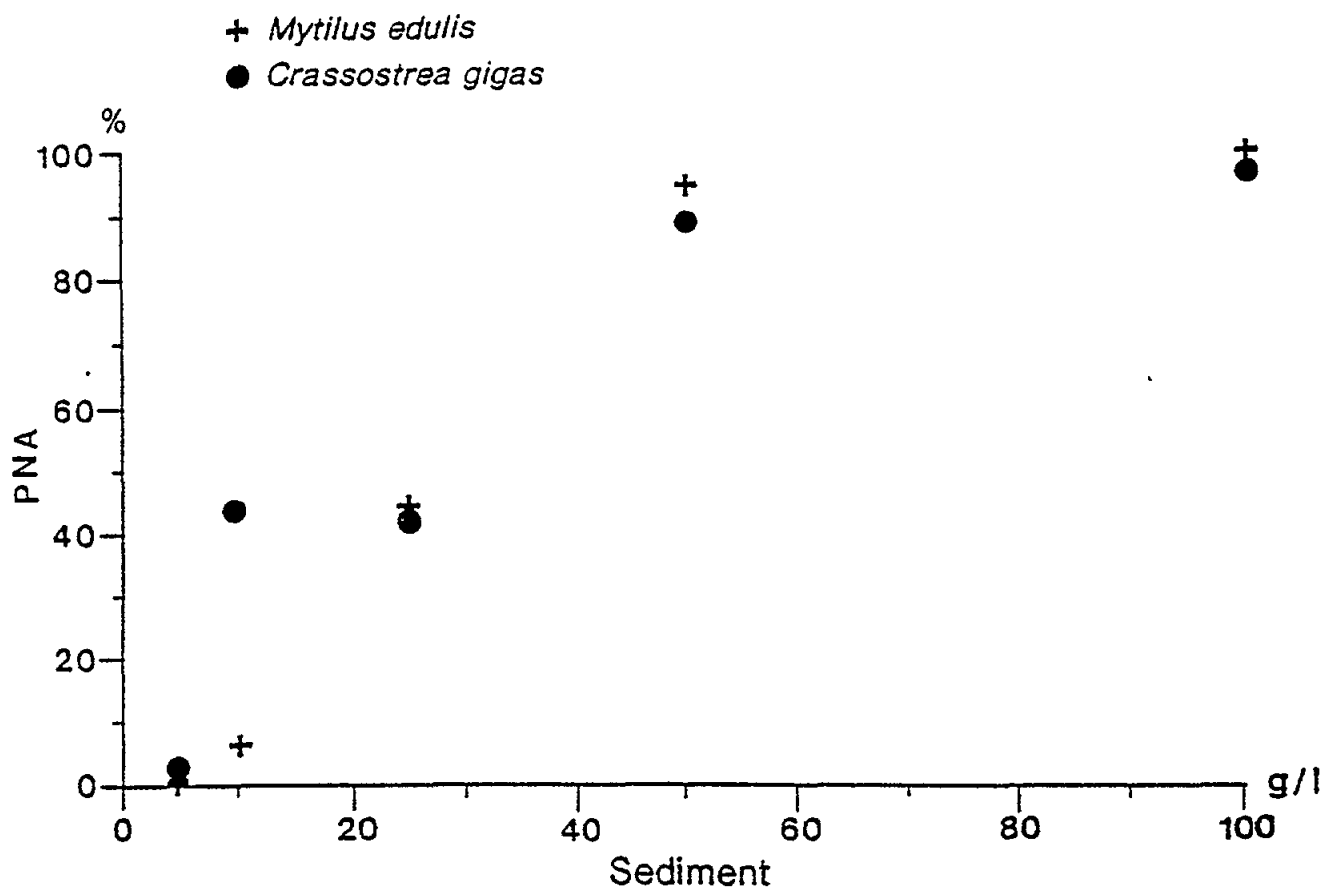


Fig. 5 Effect of sediment elutriate on embryo-bioassays with oyster or mussel. PNA = Percent Net Abnormality (about 800 to 1500 larvae)

This test conforms to the criteria of Stebbing *et al.* (1980); moreover, it is easy to set up, relatively quick and provides an inexpensive indicator of environmental contamination levels.

Its value is enhanced when fertilization of the gametes is carried out in the water to be bioassayed ; in fact it is at the beginning of development that the phenomena of growth are the most sensitive (Pavillon, 1983).

However, it is necessary to be conscious of limits of such a bioassay. Even if it is a good indicator of potential environmental toxicity, it cannot be used to predict the impact on ecosystems. The use of a battery of tests, on organisms from different ecosystem's levels, would be required to make such predictions of the potential biological effects of pollutants on natural populations and communities.

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EFFECTS OF HERBICIDES ON THE GROWTH
OF MARINE PHYTOPLANKTON

by

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A B S T R A C T

The effect of two herbicides; 2,4-D and Trifluralin on the growth of two marine phytoplankton species, Phaeodactylum tricornatum and Skeletonema costatum has been studied. The growth of P. tricornatum was stimulated by 1 and 5 ppm of the herbicides while it was inhibited at higher test concentrations. During the 9 day recovery period, the division rate of P. tricornatum decreased at 1 and 5 ppm of 2,4-D and Trifluralin as did the control while it increased at the other test concentrations even at 30 ppm.

The effect of 2,4-D on the growth of S. costatum was the same as that observed for P. tricornatum. However, the effect of Trifluralin was completely different: this herbicide inhibited the growth of S. costatum at all the concentrations (0.05-1.0 ppm) tested.

1. INTRODUCTION

Pesticide use has increased greatly in recent years and there has long been concern that these compounds could have wider environmental effects.

Among the pesticides, herbicides have been used widely in agriculture and have entered the marine environment either directly or indirectly, where they may affect non-target organisms.

The annual usage of herbicides in the Cukurova region (south of Turkey) ranged between 300 to 400 tons (Ünsal, in press). Of these herbicides, Trifluralin was most commonly used in cotton fields and 2,4-D in cereal fields in order to control unwanted vegetation (Özgür, Personal communication).

Although, there have been many investigations (Wurster, 1968; Fisher, 1974; Powers et al., 1977; Ünsal and Kideys, 1987) of the toxic effects of various insecticides on the growth of phytoplankton, only a few studies have been conducted on the effects of herbicides on these unicellular organisms which form the foundation of the marine food web (Sullivan et al., 1981; Wong and Chang, 1988). Therefore, two herbicides, 2,4-D and Trifluralin which have been widely used in the Cukurova region, were chosen as the test material for this study.

The aim of this study was to evaluate the impact of these herbicides on two marine phytoplankton species; Phaeodactylum tricornatum and Skeletonema costatum.

2. MATERIAL AND METHODS

Two marine phytoplankton species, Phaeodactylum tricornatum and Skeletonema costatum, which were obtained from the axenic stock cultures in our laboratory, were used in this study. The culture conditions have been described in a previous study (Ünsal and Kideyş, 1987).

Trifluralin and 2,4-D were purchased in emulsifiable concentrated forms from commercial sources in İçel (Turkey). The manufacturer's description of Trifluralin is "Trifilin E.C.", active ingredient 480 g l⁻¹ Trifluralin. The proper chemical name is α,α,α -trifloro-2,6 dinitro-N,N-dipropyl-P-toluidine. The manufacturer's name of 2,4-D was "Weed Killer D", active ingredient 500 g l⁻¹ dimethyl amine salt of 2,4 dichlorophenoxyacetic acid.

The working solutions of herbicides were prepared by dilution with sterile distilled water. Different concentrations (1, 5, 10, 15, 20, 25, and 30 ppm) of Trifluralin and 2,4-D were added to 250 ml Erlenmayer flasks containing 50 ml of a culture medium of P. tricornatum enriched with Provasoli nutrient medium (Provasoli *et al.*, 1957). Based on the results of preliminary tests, S. costatum was exposed to the same concentrations of 2,4-D but only to 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ppm of Trifluralin. Three replicates were used at each test concentration and for each control. Experiments were repeated three times. After 96 h of treatment the phytoplankton was fixed with formalin and counted under the microscope. The results were analysed statistically by Student Newman-Keuls Test (Reish and Oshida, 1986). After the toxicity test, experiments continued for 9 additional days in fresh test medium without the toxicant, in order to determine the recovery of the contaminated cells according to the method described in UNEP/FAO/IAEA (1989).

The growth rate (k) of the phytoplankton, during the recovery period, in the test medium and in the control was calculated by the following equation:

$$k = \frac{\log_2 \frac{N_2}{N_1}}{t}$$

where N_1 and N_2 are the numbers of cells at the beginning and at the end of the recovery period and t is its duration (9 days).

3. RESULTS AND DISCUSSION

Two marine phytoplankton species, Phaeodactylum tricornatum and Skeletonema costatum were exposed to different concentrations of Trifluralin and 2,4-D. The mean reduction in cell numbers showed that the effects of the herbicides tested were species dependent (Tables 1 and 2).

Table 1

The mean cell number \pm S.D. of Phaeodactylum tricornatum per ml of test medium after 96 h.

Test Conc. (ppm)	2,4-D				Trifluralin			
	n	Mean	SD	Mean % change comp. W/Cont	n	Mean	SD	Mean % change comp. W/Cont
Contr.	9	224400	89837	-	9	187000	40454	-
1	9	359040	56473	+60.0	9	325795	67631	+74.2
5	9	316653	7785	+41.0	9	203206	21266	+ 8.7
10	9	144613	60112	-35.5	9	128712	33660	-31.2
15	9	73553	21914	-67.2	9	69813	13485	-62.6
20	9	33660	7480	-85.0	9	36539	2159	-80.5
25	9	26180	3740	-88.3	9	12051	720	-93.5
30	9	22440	7480	-90.0	9	11220	5713	-94.0

Table 2

The mean cell number \pm S.D. of Skeletonema costatum per ml of test medium after 96 h.

Test Conc. (ppm)	2,4-D				Test Conc. (ppm)	Trifluralin			
	n	Mean	SD	Mean % change comp. W/cont.		n	Mean	SD	Mean % change comp. W/Cont
Contr.	9	683173	96813	-	Contr.	9	264605	4675	-
1	9	1176853	259986	+72.3	0.05	9	84441	11220	-68.1
5	9	1259133	136360	+84.3	0.1	9	49770	23874	-81.2
10	9	1011046	132879	+48.0	0.2	9	31582	6150	-88.1
15	9	285487	54498	-43.7	0.4	9	24795	2505	-90.6
20	9	259307	43347	-55.2	0.6	9	21193	3740	-91.9
25	9	98487	24902	-74.3	0.8	9	19946	2150	-92.9
30	9	69813	7785	-92.0	1.0	9	18700	2493	-92.9

The growth of P. tricornatum was stimulated by 1 and 5 ppm of 2,4-D and Trifluralin (Table 1). This stimulation was statistically significant ($P < 0.05$) at the 1 ppm level but insignificant at the 5 ppm level (Tables 3 and 4). Boyle (1980) showed that the photosynthetic uptake of ^{14}C by natural phytoplankton was stimulated by treatment with 2 mg l^{-1} 2,4-D DMA. Poorman (1973, cf. in Boyle, 1980) reported enhancement of algal growth at 1, 5 and 10 mg l^{-1} 2,4-D during 24 h by comparison with a control culture.

Table 3

Comparison of test concentrations with control by Student
Newman-Keuls Test (at $P < 0.05$).
(Pollutant: 2,4-D; Species: P. tricornatum).

Comparison	Q	P	$Q^{0.05, 16p}$	Conclusion
Control- 1 ppm	5.273	2	3.00	Different
Control- 5 ppm	3.624	3	3.65	Not different
Control- 10 ppm	3.140	4	4.05	Not different
Control- 15 ppm	5.927	5	4.34	Different
Control- 20 ppm	7.484	6	4.56	Different
Control- 25 ppm	7.785	7	4.74	Different
Control- 30 ppm	7.942	8	4.90	Different

Table 4

Comparison of test concentrations with control by Student
Newman-Keuls Test (at $P < 0.05$).
(Pollutant: Trifluralin; Species: P. tricornatum).

Comparison	Q	P	$Q^{0.05, 16p}$	Conclusion
Control- 1 ppm	7.574	2	3.00	Different
Control- 5 ppm	0.894	3	3.65	Not different
Control- 10 ppm	3.886	4	4.05	Not different
Control- 15 ppm	6.332	5	4.34	Different
Control- 20 ppm	8.249	6	4.56	Different
Control- 25 ppm	9.600	7	4.74	Different
Control- 30 ppm	9.308	8	4.90	Different

The other test concentrations (10, 15, 20, 25 and 30 ppm) inhibited the growth of P. tricornatum (Fig. 1). This inhibition was not significant at the 10 ppm level of both pollutants but significant ($P < 0.05$) at 15, 20, 25 and 30 ppm levels (Tables 3 and 4). Wong and Chang (1988) found also that algal cultures (Chlamydomonas reinhardtii) treated with 1 ppm of 2,4-D showed increases in their chlorophyll- α contents over that of the control. However, 10 ppm 2,4-D did not affect algal photosynthesis though at high concentrations (20 and 40 ppm) it inhibited growth, chlorophyll- α synthesis and photosynthesis of the algal cells.

The response of S. costatum to herbicides was different from that of P. tricornatum; 2,4-D had a stimulating effect on this species at 1, 5 and 10 ppm while it inhibited growth at higher concentrations (15, 20, 25 and 30 ppm) (Fig. 2).

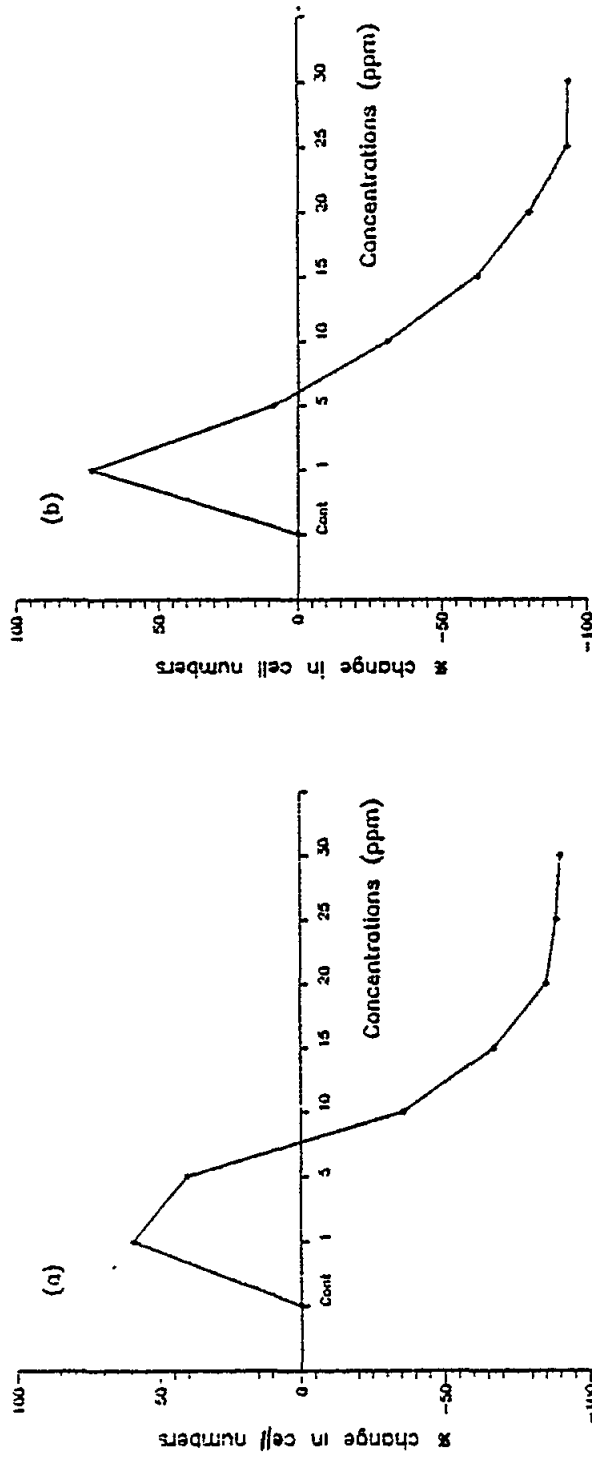


Fig. 1 Mean percent change, compared to the control, of the cell numbers of Paedactylum tricornatum exposed to 2,4-D (a) and Trifluralin (b) for 96 h

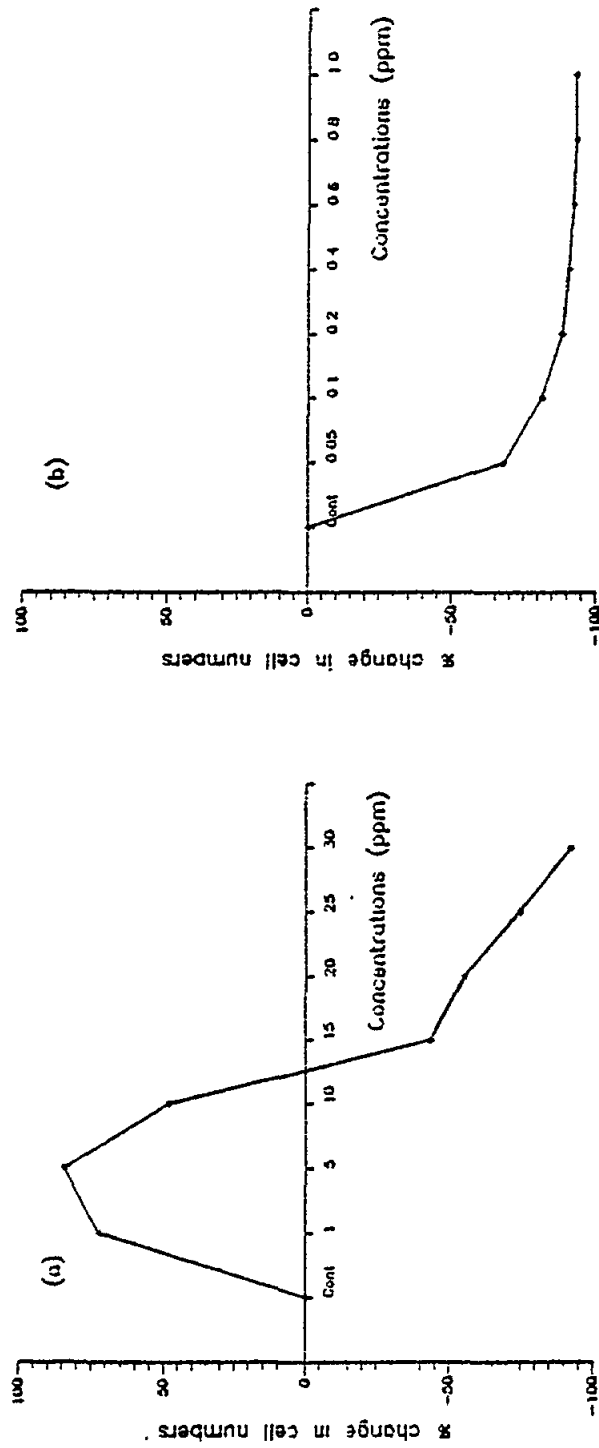


Fig. 2 Mean percent change, compared to the control, of the cell numbers of *Skeletonema costatum* exposed to 2,4-D (a) and Trifluralin (b) for 96 h

A contrasting result was obtained for S. costatum grown in Trifluralin-containing medium; this herbicide was much more toxic to S. costatum than it was to P. tricornatum. For example, the lowest test concentration of Trifluralin (1 ppm) had a stimulating effect on the growth of P. tricornatum while it completely inhibited the growth of S. costatum (Table 2).

The results of Student Newman-Keuls test obtained for S. costatum showed that, all test concentrations gave results which were significantly different from those of the control (Tables 5 and 6). This difference was due to the stimulating effect of 2,4-D at 1, 5 and 10 ppm and to its inhibiting effect at other test concentrations.

In the case of Trifluralin, the difference between control and test concentrations resulted from the inhibitory effect of this pollutant at all test concentrations.

Table 5

Comparison of test concentrations with control by Student Newman-Keuls Test (at $P < 0.05$).
(Pollutant: 2,4-D; Species: S. costatum).

Comparison	Q	P	$Q^{0.05, 16p}$	Conclusion
Control- 1 ppm	7.017	2	3.00	Different
Control- 5 ppm	8.187	3	3.65	Different
Control- 10 ppm	4.619	4	4.05	Different
Control- 15 ppm	5.652	5	4.34	Different
Control- 20 ppm	6.022	6	4.56	Different
Control- 25 ppm	8.310	7	4.74	Different
Control- 30 ppm	8.718	8	4.90	Different

Table 6

Comparison of test concentrations with control by Student Newman-Keuls Test (at $P < 0.05$).
(Pollutant: Trifluralin; Species: S. costatum).

Comparison	Q	P	$Q^{0.05, 16p}$	Conclusion
Control- 0.05ppm	31.32	2	3.00	Different
Control- 0.1 ppm	37.41	3	3.65	Different
Control- 0.2 ppm	40.55	4	4.05	Different
Control- 0.4 ppm	41.71	5	4.34	Different
Control- 0.6 ppm	42.34	6	4.56	Different
Control- 0.8 ppm	42.52	7	4.90	Different
Control- 1.0 ppm	42.75	8	4.90	Different

After 96 h of exposure to 1 and 5 ppm of 2,4-D and Trifluralin, the specific growth rate (division per day) of P. tricornatum was higher than the control but growth decreased markedly in the presence of 10 to 30 ppm of 2,4-D and Trifluralin (Table 7).

The growth rates of S. costatum showed also the stimulating and inhibiting effect of 2,4-D on this species. As it can be seen from Table 7, the division rate of S. costatum exposed to different concentrations of 2,4-D for 96 h was higher at all test concentrations than that of P. tricornatum exposed also to the same concentrations of the same pollutant.

Table 7

Growth rate (division per day) of P. tricornatum and S. costatum grown in different concentrations of 2,4-D and Trifluralin.

Species	Pollutants	Concen. (ppm)	96 h	9 day
<u>P. tricornatum</u>	2,4-D	Control	1.12	1.05
		1	1.29	1.07
		5	1.24	1.06
		10	0.96	1.05
		15	0.71	0.99
		20	0.43	0.91
		25	0.35	0.86
		30	0.29	0.80
	Trifluralin	Control	1.05	0.79
		1	1.25	0.84
		5	1.08	0.96
		10	0.92	0.97
		15	0.70	0.97
		30	0.04	0.75
<u>S. costatum</u>	2,4-D	Control	1.52	
		1	1.72	
		5	1.74	
		10	1.66	
		15	1.21	
		20	1.17	
		25	0.83	
		30	0.70	
	Trifluralin	Control	1.18	
		0.05	0.76	
		0.1	0.58	
		0.2	0.41	
		0.4	0.32	
		1.0	0.22	

During the recovery period (9 days), the division rate decreased at the two lowest concentrations (1 and 5 ppm) of 2,4-D and Trifluralin as it did in the controls; it increased considerably in the presence of the higher concentrations (10, 15, 20, 25 and 30 ppm) (Table 7). This may be due to the fact that, the herbicides had accumulated in the phytoplankton cells at high concentrations during the 96 h. experiments and had a stimulating effect throughout the recovery period.

Trifluralin also stimulated the growth of P. tricornatum at low concentrations, but higher concentrations inhibited growth. This herbicide inhibited the growth of S. costatum at very low concentrations.

In conclusion, the effects of the two herbicides tested were species dependent. 2,4-D had a stimulating effect at low concentrations but it inhibited the growth of both phytoplankton species at high concentrations.

The effect of Trifluralin on P. tricornatum was similar to that observed for 2,4-D. However, it was an inhibitant for S. costatum at all test concentrations.

More experiments should be conducted including the investigation of more phytoplankton species in order to be able to evaluate the effects of herbicides on marine phytoplankton.

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EFFECTS OF SUBLETHAL CONCENTRATIONS OF ZINC, CHROMIUM AND
COPPER ON THE MARINE COPEPODS Tisbe holothuriae
AND Acartia clausi

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A B S T R A C T

The effects of sublethal concentrations of zinc, chromium and copper on the mortality, the fertility, the longevity, the food and oxygen consumption of the marine harpacticoid copepod Tisbe holothuriae (benthic) and Acartia clausi (planktonic) are discussed.

Our experiments led to the following. After prolonged exposure, the percentage of animals producing egg-sacs decreases significantly from generation to generation. The mortality of non-exposed animals, fed with contaminated food, increases proportionally with the exposure concentration. Longevity, fertility and food consumption demonstrate a progressive decrease with increasing concentrations, while respiration rates increase.

The influence of low sublethal concentrations, on the pollution "adapted" population is less pronounced than on the "non-adapted" one.

1. INTRODUCTION

The purpose of this paper is to present the effects of sublethal concentrations of zinc, chromium and copper on the mortality, the fertility, the longevity, the food and the oxygen consumption of the marine copepods Tisbe holothuriae and Acartia clausi.

Heavy metals are considered to be among the most harmful aquatic pollutants. Their toxicity to marine organisms has been found to vary not only between, but also within species. This has been attributed to various factors: temperature, salinity (Vernberg et al., 1974; Verriopoulos, 1980), population density (Verriopoulos and Moraitou-Apostolopoulou, 1981), life stage (Calabrese et al., 1973, 1977; Calabrese and Nelson, 1974; Verriopoulos and Moraitou-Apostolopoulou, 1982).

Sublethal doses affect physiological functions and behaviour without causing direct death. After exposure, a postponement or even inhibition of growth, of various marine animals, has been mentioned by Calabrese et al. (1973), Saliba and Ahsanullah (1973) and Benijts-Clauss and Benijts (1975).

Zinc toxicity is related to the inhibition of enzymic reactions of crustaceans. It affects respiration and osmoregulation (Chen and Slinn, 1980; Bittar et al., 1982; Haya et al., 1983); reduces the regeneration rate (Weis, 1980); and decreases larval growth rate (Benijts-Clauss and Benijts, 1975), longevity, body length, reproduction (Winner, 1981), and the sex-ratio (Lalande and Pinel-Alloul, 1984).

Hexavalent chromium affects reproduction (Biesinger and Christensen, 1972; Oshida and Reish, 1975), feeding activity (Capuzzo and Sasner, 1977) and respiratory rates (Raymont and Shields, 1964).

Copper usually increases the respiration rates of marine organisms (Raymont and Shields, 1964; O'Hara, 1971); reduces egg production; decreases the feeding rate (Reeve et al., 1977; Moraitou-Apostolopoulou and Verriopoulos, 1979); produces changes in the blood (Christensen et al., 1972) and inhibits immunity (Roales and Perlmutter, 1977).

2. MATERIALS AND METHODS

The test animals were collected from two areas in the Saronikos Gulf: a "polluted area" in Elefsis bay and a "non-polluted area" near the Fleves islands.

All experiments were run in constant temperature rooms under the following experimental conditions: Salinity 38; food for Acartia clausi (Exuviella baltica, Skeletonema costatum, Nitzschia closterium and Chaetoceros danicus) for Tisbe holothuriae (Germaline and Ulva lactuca contaminated with different concentrations of Zn); Ulva collected from a non contaminated area was exposed for 48 hours to the following concentrations of zinc 10; 50; 100; 200; 500 and 1000 ppm; photoperiod for Tisbe holothuriae 12 hrs dark - 12 hrs light, for Acartia clausi 24 hrs dark; pollutants, concentrations, experimental temperatures and the lethal concentrations are shown in Table 1.

LC50 values for Acartia and Tisbe had been determined in previous studies, with the same experimental conditions.

Statistical analysis of the results was performed by the paired t-test.

In the case of Tisbe holothuriae, after egg hatching, the parental copepods were placed in other containers filled with freshly prepared solutions. As soon as the females of the F1 generation matured and their first egg-sac appeared, 10-20 of them were placed individually in glass containers filled with 50 ml of toxicant solution.

During the experiments, every 24 hrs: a) all containers were examined under the binocular microscope and dead copepods were removed in order to observe the mortality of the various generations at the different concentrations; b) test animals were supplied with food and c) the toxic solutions in the containers were changed.

For Acartia clausi, the ingestion rate was estimated by haematocytometer, 24 hrs after the addition of food. Oxygen consumption was measured by polarography.

In the longevity and the fecundity observations, for each concentration and for each of the two populations, 20 mature females were placed individually in glass containers filled with 50 ml of solution.

Table 1

Experimental conditions. (*) Animals from polluted area.
(**) Animals from non-polluted area.

Concentrations are: a) Nominal for Ulva
b) as measured at the beginning of the experiment
for Tisbe and Acartia.

Species	Pollutants	Concentrations ppm	Temperature °C	LC50 (48h) ppm
<u>Tisbe holothuriae*</u>	ZnSO ₄ .7H ₂ O	0.000	18 ± 0.5	0.713 Zn
		0.007		
0.010				
0.070				
	Na ₂ CrO ₄	0.000	14 ± 0.5	14.370 Cr
		0.500		
		1.000		
		2.000		
<u>Ulva lactuca</u> (contaminated food)	ZnSO ₄ .7H ₂ O	0.0	18 ± 0.5	
		10.0		
		50.0		
		100.0		
		200.0		
		500.0		
1000.0				
<u>Acartia clausi *</u> <u>Acartia clausi **</u>	CuSO ₄ .5H ₂ O	0.000	14 ± 0.5	0.080 Cu
		0.001		0.034 Cu
		0.005		
		0.010		

The feeding and the oxygen consumption experiments were run in 500 ml Erlenmeyer flasks containing 10 adult females of Acartia clausi. At zero time, the concentration of phytoplankton fluctuated from 48000 to 51000 cells ml⁻¹.

3. RESULTS

3.1 Tisbe holothuriae

Table 2 shows the average percentage mortality for each generation (F1, F2, F3 and F4) as well as the mortality of nauplii (N) and copepodites (C) caused by the different concentrations of Zn. The highest mortality was observed for the F1 generation at a concentration of 0.07 ppm, and for the F4 generation at a concentration of 0.01 ppm, the latter mortality being slightly lower.

Table 3 shows the percentage of animals producing egg-sacs (fertility), for each generation, at the different sublethal concentrations

of Zn. A statistically significant (99%) decrease of the % of animals with egg-sacs is observed with each increase of the sublethal concentration up to 0.07 ppm Zn.

Table 2

Total mortality [%] for each generation of Tisbe holothuriae (F1...F4) at different concentrations of Zn and percentage mortalities (%) in relation to the total mortality for nauplii (N) and copepodites (C).

	0.07 ppm Zn	0.01 ppm Zn	0.007 ppm Zn	Control
F1	↗79.2 (N) [7.6] ↘20.8 (C)	↗85.7 (N) [1.2] ↘14.3 (C)	↗72.9 (N) [1.7] ↘27.1 (C)	↗51.6 (N) [0.8] ↘48.3 (C)
F2		↗81.8 (N) [1.0] ↘18.2 (C)	↗67.5 (N) [0.8] ↘32.5 (C)	↗40.1 (N) [0.7] ↘59.9 (C)
F3		↗88.8 (N) [1.3] ↘11.2 (C)	↗52.2 (N) [1.5] ↘47.8 (C)	↗41.5 (N) [0.6] ↘58.5 (C)
F4		↗85.4 (N) [6.5] ↘14.6 (C)	↗64.3 (N) [1.0] ↘35.7 (C)	↗50.0 (N) [0.5] ↘50.0 (C)

Table 3

Percentage of Tisbe holothuriae producing egg-sacs per day at the different sublethal concentrations of Zn, for each generation (F1, F2 and F3).

		Egg-sacs					
	ppm Zn	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
F1	(0.070)	3.6	2.1	0.4			
F1	(0.010)	25.3	19.5	18.8	11.7	4.5	1.3
F1	(0.007)	35.2	35.0	31.2	25.6	14.8	3.7
F1	Control	41.9	36.6	28.5	26.8	14.3	8.9
F2	(0.010)	19.6	5.6	3.4	2.1	0.7	
F2	(0.007)	15.4	10.9	6.4	4.4	2.1	1.4
F2	Control	21.5	20.6	19.4	15.3	8.7	6.3
F3	(0.010)	6.6	4.7	2.8	1.1		
F3	(0.007)	24.2	16.9	12.1	5.6		
F3	Control	21.9	21.9	20.7	16.5	12.2	10.9

After 3 days exposure to a Zn concentration of 0.07 ppm only 0.38% of the F1 generation produced egg-sacs. With a Zn concentration of 0.01 ppm a statistically significant (99%) decrease of the % of the population producing egg-sacs is observed, from generation to generation. On the other hand there is a non-statistically significant difference in fertility between the control and the 0.007 ppm concentration of Zn.

Tisbe holothuriae fed with zinc contaminated Ulva lactuca presented a mortality which increased proportionally to the degree of contamination of Ulva lactuca as well as with time (Table 4).

Table 5, shows the fertility (egg-sacs/female), the time between successive sacs (in days), the abortion of egg-sacs, the hatching (offspring/female) for the F2 generation and the maturation time (in days) for the different concentrations of Cr.

Table 4

Mortality (%) in 48 hrs of Tisbe holothuriae fed with contaminated Ulva lactuca for different exposure periods.

ppm of Zn	days of exposure	mortality %
10	2	0
	5	5
	7	47
50	2	17
	5	60
	7	100
100	2	52
	5	60
	7	100
200, 500, 1000	2	90
	5	100

Table 5

Tisbe holthuriae population dynamics (fertility, time between egg-sacs, abortion, hatching and maturation time) after exposure to different concentrations of Cr.

Cr ppm	Fertility egg-sacs/female	time between egg-sacs (days)	Abortion %	Hatching offspring/female	Maturation (days)
Control	5.8	4.0	36.9	110.2	12
0.5	3.5	4.5	41.0	52.8	20
1.0	2.5	4.0	89.0	44.1	-
2.0	1.4	3.4	97.0	6.6	-

In comparison to the control animals all chromium concentrations reduce the longevity of F2 females. Very pronounced differences in the longevity of the F3 generation have been observed between the tested concentrations. Not one F3 Tisbe holothuriae survived longer than 4 days at a concentration of 2 ppm of Cr and the development of eggs did not pass the first naupliar stage. Table 6 presents the survival of F2 and F3 generations at the tested chromium concentrations.

Table 6

Survival (%) of F2 and F3 generations of Tisbe holothuriae exposed to different Cr concentrations.

Survival %					
ppm Cr	Day 5	Day 10	Day 15	Day 20	Generation
Control	95	90	80	70	F2
0.50	93	87	75	68	F2
1.00	82	60	20	0	F2
2.00	87	37	0		F2
0.50	96	62	25	12	F3
1.00	25	12	0		F3
2.00	0				F3

3.2 Acartia clausi

There is a statistically significant (95%) decrease in survival with increasing Cu concentration. The sensitivity of Acartia clausi living in the non-polluted area is more pronounced (statistically significant) than that from the polluted area (Table 7).

Table 8 shows the fecundity (number of eggs/copepod on the 4th day), the ingestion rate (cells per copepod in 24hrs) and the oxygen consumption ($\mu\text{l O}_2$ in 20 hrs) of the female Acartia clausi, from the polluted and non-polluted areas, exposed to the different sublethal concentrations of copper.

The fecundity of the "polluted" Acartia clausi was higher than that of the "non-polluted" one. There is no egg production in 0.01 ppm Cu for the "non-polluted" animals. There is a progressive decrease in the egg production with increasing concentrations of Cu, for the "non-polluted" animals and there is a statistically significant difference for the "polluted" animals.

A reduction in food consumption was observed for the "non-polluted" Acartia clausi, between the controls and 0.050 ppm of Cu. For the "polluted" copepods, there is no difference between controls and 0.001 ppm and a sharp decrease was observed at 0.005 ppm Cu. In the control, both populations show a similar ingestion rate.

Concerning respiration, all tested concentrations produce increases in oxygen consumption. Furthermore, the "polluted" population showed higher respiratory rates than the "non-polluted" one.

Table 7

Survival (%) per day of female Acartia clausi from polluted and non-polluted areas exposed to different concentrations of Cu.

ppm Cu	Survival %										(days)
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	
Control	92	80	80	75	70	70	65	60	55	50	polluted
0.0010	80	80	75	70	60	55	45	35	25	15	polluted
0.0025	75	75	75	70	65	60	45	30	15	10	polluted
0.0100	75	70	65	45	40	40	30	15	10	0	polluted
Control	90	80	80	80	65	55	45	40	35	35	non-poll
0.0010	90	72	60	50	30	25	15	0			non-poll
0.0050	85	75	65	35	25	20	10	0			non-poll
0.0100	85	60	15	0							non-poll

Table 8

Effects of Cu on the fecundity, the ingestion rate and the oxygen consumption of the female Acartia clausi from polluted and non-polluted area.

ppm Cu	Ingestion rate (cells ingested per 24h)		Fecundity (eggs per copepod on day 4)		Oxygen consumption (μ l O ₂ consumed in 20h)	
	Poll	non-Poll	Poll	non-Poll	Poll	non-Poll
Control	25600	25550	5.25	3.12	0.010	0.006
0.0010	24950	14440	6.00	1.00	0.018	0.009
0.0025			7.06			
0.0050	12290	3065		0.28	0.022	0.019
0.0100			5.69	0.00		

4. DISCUSSION

The exposure of four subsequent generations of Tisbe holothuriae to three concentrations of Zn (0.07, 0.01 and 0.007 ppm) shows that the toxicity of Zn increases with concentration and prolonged exposure.

The results show that exposure to 0.07 ppm Zn (1/10 of the 48h LC50) does not lead to high population mortality for the first generation but that the copepods cannot produce a second generation; this is due mainly to the small number of egg-sacs (up to 3) and the small % of animals producing egg-sacs. The % of animals producing egg-sacs decreases significantly from generation to generation.

The observed results are due, firstly to the progressive decrease of the % of animals producing egg-sacs, as the concentration of zinc increases from 0.007 to 0.07 ppm Zn; secondly, to the increased mortality of the nauplii as compared to the copepodites and thirdly, to the capacity of the organisms to support certain sublethal concentrations of Zn. Alaysed-Danet *et al.* (1979) attributed the decrease of the developmental rate of Artemia salina exposed to zinc, to the disturbance of the amylase and trypsin systems. According to Bittar *et al.* (1982), zinc stops the catalytic action of the cyclic AMPkinase on the sodium flux of the muscle of Balanus nubilis.

For the 0.07 and 0.01 ppm concentrations of Zn, a disequilibrium between the "adaptation-detoxification" rate and the growth rate is observed; this is why population size decreases with time, from generation to generation.

When non-exposed Tisbe holothuriae were fed with zinc contaminated Ulva, the medium was probably contaminated with Zn from food. In aquatic plants, losses of heavy metals into the surrounding water occurs over the general body surface by diffusion or in association with secretions. However, the ingestion of contaminated food must be the main cause of Tisbe holothuriae mortality. We may assume that, during exposure, Ulva lactuca had absorbed significant quantity of Zn (Bryan, 1974). In most cases the uptake of the heavy metals by aquatic plants seems to be a passive process. The concentration of metals in marine plants tends to reflect those of the water because marine plants are not able to regulate heavy metals.

Mortality of Tisbe holothuriae rises with increasing contamination of Ulva lactuca with Zn. Experimental work on the plaice Pleuronectes platessa (Pentreath, 1973; 1976) and on the euphasiid Meganyctiphanes norvegica (Small *et al.* 1973; Benayoun *et al.*, 1974) has shown that, even at the earliest stages, food is the main source of most metallic contaminants and their radionuclides including, iron, cobalt, zinc, manganese, methyl mercury, cadmium and silver.

All tested Cr concentrations affect the population dynamics of Tisbe. The longevity of both F2 and F3 generations decreased after exposure to Cr and the survival time became progressively shorter with increasing chromium concentrations.

The nauplii of the F3 generation were much more sensitive than the F2 ovigerous females: at 2 ppm their survival time was about 1/15 of that of the control animals. Due to the increased sensitivity of embryonic stages, even at low pollutant concentrations which do not significantly affect adult animals, a population may be destroyed.

Another effect of Cr, on the population density of a species, is the inhibition of sexual maturation or the prolongation of the developmental period. Only very few Tisbe of the F3 generation, which survived at the 0.5 ppm concentration, arrived at maturity and coupled. Chromium also affects sexual maturation or prolongs the developmental period thereby influencing population density. The first ovigerous sac of these females appeared on the 20th day and in the control animals on the 12th day.

Longevity, fertility and food consumption shows a progressive decrease with increasing copper concentrations, while respiration rates increase.

The influence of the low copper concentrations, on the pollution "adapted" population of Acartia clausi, is less pronounced. According to Bryan and Hummerstone (1971), Brown (1976) and Bradshaw (1970), there is a genetic adaptation. This adaptation seems to develop separately for each metal, depending on its presence in the specific environment.

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