MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

FINAL REPORTS ON RESEARCH PROJECTS (ACTIVITY G)

RAPPORTS FINAUX SUR LES PROJETS DE RECHERCHE (ACTIVITE G)

MAP Technical Reports Series No. 48

UNEP
Athens, 1991

This volume is the forty-eighth 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 quarante-huitième 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.

INTRODUCTION

The United Nations Environment Programme (UNEP) convened an Intergovernmental Meeting on the Protection of the Mediterranean (Barcelona, 28 January - 4 February 1975), which was attended by representatives of 16 States bordering the Mediterranean Sea. The meeting discussed the various measures necessary for the prevention and control of pollution of the Mediterranean Sea, and concluded by adopting an Action Plan consisting of three substantive components:

- Integrated planning of the development and management of the resources of the Mediterranean Basin (management component);
- Co-ordinated programme for research, monitoring, exchange of information, assessment of the state of pollution and protection measures (assessment component);
- Framework convention and related protocols with their technical annexes for the protection of the Mediterranean environment (legal component).

All components of the Action Plan are inter-dependent and provide a framework for comprehensive action to promote both the protection and the continued development of the Mediterranean ecoregion. No component is an end in itself. The Action Plan is intended to assist the Mediterranean Governments in formulating their national policies related to the continuous development and protection of the Mediterranean area and to improve their ability to identify various options for alternative patterns of development and to make choices for appropriate allocations of resources.

MED POL - Phase I (1976-1980)

The Co-ordinated Mediterranean Research and Monitoring Programme (MED POL) was approved as the assessment (scientific/technical) component of the Action Plan.

The general objectives of its pilot phase (MED POL - Phase I), which evolved through a series of expert and intergovernmental meetings, were:

- to formulate and carry out a co-ordinated pollution monitoring and research programme taking into account the goals of the Mediterranean Action Plan and the capabilities of the Mediterranean research centres to participate in it;
- to assist national research centres in developing their capabilities to participate in the programme;
- to analyse the sources, amounts, levels, pathways, trends and effects of pollutants relevant to the Mediterranean Sea;

- to provide the scientific/technical information needed by the Governments of the Mediterranean States and the EEC for the negotiation and implementation of the Convention for the Protection of the Mediterranean Sea against Pollution and its related protocols.

- to build up consistent time-series of data on the sources, pathways, levels and effects of pollutants in the Mediterranean Sea and thus to contribute to the scientific knowledge of the Mediterranean Sea.MED POL - Phase I initially consisted of seven pilot projects (MED POL I - VII), which were later expanded by additional six pilot projects (MED POL VIII - XIII), some of which remained in a conceptual stage only.

MED POL - Phase I was implemented in the period from 1975 to 1980. The large number of national research centres designated by their Governments to participate in MED POL (83 research centres from 15 Mediterranean States and the EEC), the diversity of the programme and its geographical coverage, the impressive number of Mediterranean scientists and technicians (about 200) and the number of co-operating agencies and supporting organizations involved in it, qualifies MED POL as certainly one of the largest and most complex co-operative scientific programmes with a specific and well-defined aim ever undertaken in the Mediterranean basin.

The overall co-ordination and guidance for MED POL - Phase I was provided by UNEP, acting as the secretariat of the Mediterranean Action Plan (MAP). Co-operating specialized United Nations Agencies (ECE, UNIDO, FAO, UNESCO, WHO, WMO, IAEA, IOC) were responsible for the technical implementation and day-to-day co-ordination of the work of national research centres participating in the pilot projects.

MED POL - Phase II (1981-1990)

The Intergovernmental Review Meeting of Mediterranean Coastal States and First Meeting of the Contracting Parties to the Convention for the Protection of the Mediterranean Sea against Pollution, and its related protocols (Geneva, 5-10 February 1979), having examined the status of MED POL - Phase I, recommended that during the 1979/80 biennium a Long-term pollution monitoring and research programme should be formulated.

Based on the recommendations made at various expert and intergovernmental meetings, a draft Long-term (1981-1990) Programme for Pollution Monitoring and Research in the Mediterranean (MED POL - Phase II) was formulated by the Secretariat of the Barcelona Convention (UNEP), in co-operation with the United Nations Agencies which were responsible for the technical implementation of MED POL - Phase I, and it was formally approved by the Second Meeting of the Contracting Parties of the Mediterranean Sea against pollution and its related protocols and Intergovernmental Review Meeting of Mediterranean Coastal States of the Action Plan held in Cannes, 2-7 March 1981.

The general long-term objectives of MED POL - Phase II were to further the goals of the Barcelona Convention by assisting the Parties to prevent, abate and combat pollution of the Mediterranean Sea Area and to protect and enhance the marine environment of the Area. The specific objectives were designed to provide, on a continuous basis, the Parties to the Barcelona Convention and its related protocols with:

- information required for the implementation of the Convention and the protocols;

- indicators and evaluation of the effectiveness of the pollution prevention measures taken under the Convention and the protocols;

- scientific information which may lead to eventual revisions and amendments of the relevant provisions of the Convention and the protocols and for the formulation of additional protocols;

- information which could be used in formulating environmentally sound national, bilateral and multilateral management decisions essential for the continuous socio-economic development of the Mediterranean region on a sustainable basis;

- periodic assessment of the state of pollution of the Mediterranean Sea.

The monitoring of, and research on, pollutants affecting the Mediterranean marine environment reflects primarily the immediate and long-term requirements of the Barcelona Convention and its protocols, but also takes into account factors needed for the understanding of the relationship between the socio-economic development of the region and the pollution of the Mediterranean Sea.

For this purpose, monitoring was organized on several levels:

- monitoring of sources of pollution providing information on the type and amount of pollutants released directly into the environment;

- monitoring of nearshore areas, including estuaries, under the direct influence of pollutants from identifiable primary (outfalls, discharge and coastal dumping points) or secondary (rivers) sources;

- monitoring of offshore areas (reference areas) providing information on the general trends in the level of pollution in the Mediterranean;

- monitoring of the transport of pollutants to the Mediterranean through the atmosphere, providing additional information on the pollution load reaching the Mediterranean Sea.

Research and study topics included initially in the MED POL - Phase II were:

- development of sampling and analytical techniques for monitoring the sources and levels of pollutants. Testing and harmonization of these methods at the Mediterranean scale and their formulation as reference methods. Priority will be given to the substance listed in the annexes of the Protocol for the prevention of pollution of the Mediterranean Sea by dumping from ship and aircraft and the Protocol for the protection of the Mediterranean Sea against pollution from land-based sources (activity A);

- development of reporting formats required according to the Dumping, Emergency and Land-Based Sources Protocols (activity B);

- formulation of the scientific rationale for the environmental quality criteria to be used in the development of emission standards, standards of use or guidelines for substaces listed in annexes I and II of the Land-Based Sources Protocol in accordance with Articles 5, 6 and 7 of that Protocol (activity C);

- epidemiological studies related to the confirmation (or eventual revision) of the proposed environmental quality criteria (standards of use) for bathing waters, shellfish-growing waters and edible marine organisms (activity D);

- development of proposals for guidelines and criteria governing the application of the Land-Based Sources Protocol, as requested in Article 7 of that Protocol (activity E);

- research on oceanographic processes, with particular emphasis on surface circulation and vertical transport. Needed for the understanding of the distribution of pollutants through the Mediterranean and for the development of contingency plans for cases of emergency (activity F);

- research on the toxicity, persistence, bioaccumulation, carcinogenicity and mutagenicity of selected substances listed in annexes of the Land-Based Sources Protocol and the Dumping Protocol (activity G);

- research on eutrophication and concomitant plankton blooms. Needed to assess the feasibility of alleviating the consequences and damage from such recurring blooms (activity H);

- study of ecosystem modifications in areas influenced by pollutants, and in areas where ecosystem modifications are caused by large-scale coastal or inland engineering activity (activity I);

- effects of thermal discharges on marine and coastal ecosystems, including the study of associated effects (activity J);

- biogeochemical cycle of specific pollutants, particularly those relevant to human health (mercury, lead, survival of pathogens in the Mediterranean Sea, etc.) (activity K);

- study of pollutant-transfer processes (i) at river/sea and air/sea interface, (ii) by sedimentation and (iii) through the straits linking the Mediterranean with other seas (activity L);

The Contracting Parties at their 6th ordinary meeting (Athens, October 1989) agreed to:

(a) Re-orient the research activities within MED POL in order to generate information which will also be useful for the technical implementation of the LBS protocol in addition to supporting monitoring activities;

(b) replace as from 1990 research activities A-L by the following five new research areas:

Research area I - Characterization and measurement

This area will include projects which cover the characterization (identification of chemical or microbiological components) and measurement development and testing of methodologies of specified contaminants;

Research area II - Transport and dispersion

This area will include projects which aim at improving the understanding of the physical, chemical and biological mechanisms that transport potential pollutants from their sources to their ultimate repositories. Typical topics will be atmospheric transport and deposition, water movements and mixing, transport of contaminants by sedimentation and their incorporation in biogeochemical cycles. Priority will be given to the provision of quantitative information ultimately useful for modelling the system and contributing to regional assessments;

Research area III - Effects

This area will include projects relevant to the effects of selected contaminants, listed in Annexes I and II of the LBS and Dumping protocols, to marine organisms, communities and ecosystems or man and human populations. Priority will be given to effects and techniques providing information useful for establishing environmental quality criteria;

Research area IV - Fates/Environmental transformation

This area will include projects studying the fate of contaminants (including microorganisms) in the marine environment such as persistence or survival, degradation, transformation, bioaccumulation etc. but excluding transport and dispersion which is dealt in area II;

Research area V - Prevention and control

This area will include projects dealing with the determination of the factors affecting the efficiency of waste treatment and disposal methods under specific local conditions as well as the development of environmental quality criteria and common measures for pollution abatement;

(c) define target contaminants or toher variables at periodic intervals depending on the progress of implementation of the LBS protocol;

(d) select project proposals on the basis of their intrinsic scientific validity, their Mediterranean specificity, and encourage whenever possible bilateral and multilateral projects among Mediterranean countries from the north and the south of the basin.

As in MED POL - Phase I, the overall co-ordination and guidance for MED POL - Phase II is provided by UNEP as the secretariat of the Mediterranean Action Plan (MAP). Co-operating specialized United Nations Agencies (FAO, UNESCO, WHO, WMO, IAEA, IOC) are responsible for the technical implementation and day-to-day co-ordination of the work of national centres participating in monitoring and research.

The present volume includes final reports on research projects implemented under Activity G. Final editing and compilation of this volume was done by Mr. G.P. Gabrielides, FAO Senior Fishery Officer (Marine Pollution) while Ms V. Papapanagiotou, FAO Secretary, was responsible for the typing.

INTRODUCTION

Le Programme des Nations Unies pour l'environnement (PNUE) a convoqué une réunion intergouvernementale sur la protection de la Méditerranée (Barcelone, 28 janvier - 4 février 1975) à laquelle ont pris part des représentants de 16 Etats riverains de la mer Méditerranée. La réunion a examiné les diverses mesures nécessaires à la prévention et à la lutte antipollution en mer Méditerranée, et elle s'est conclue sur l'adoption d'un Plan d'action comportant trois éléments fondamentaux:

- Planification intégrée du développemet et de la gestion des ressources du bassin méditerranéen (élément "gestion");

- Programme coordonné de surveillance continue, de recherche, d'échange de renseignements et d'évaluation de l'état de la pollution et des mesures de protection (élément "évaluation");

- Convention cadre et protocoles y relatifs avec leurs annexes techniques pour la protection du milieu méditerranéen (élément juridique).

Tous les éléments du Plan d'action étaient interdépendants et fournissaient le cadre d'une action d'ensemble en vue de promouvoir, tant la protection que le développement continue de l'écorégion méditerranéenne. Aucun élément ne constituait une fin à lui seul. Le Plan d'action était destiné à aider les gouvernements méditerranéens à formuler leurs politiques nationales en matière de développement continu et de protection de zone de la Méditerranée et à accroître leur faculté d'identifier les diverses options s'offrant pour les schémas de développement, d'arrêter leurs choix et d'y affecter les ressources appropriées.

MED POL - Phase I (1976 - 1980)

Le programme coordonné de surveillance continue et de recherche en matière de pollution de la Méditerranée (MED POL) a été approuvé au titre de l'élément "évaluation" (scientifique/technique) du Plan d'action.

Sa phase pilote (MED POL - Phase I) avait les objectifs généraux ci-dessous, élaborés au cours d'une série de réunions d'experts et de réunions intergouvernementales:

- formuler et exécuter un programme coordonné de surveillance continue et de recherche en matière de pollution en tenant compte des buts du Plan d'action pour la Méditerranée et de l'aptitude des centres de recherche méditerranéens à y participer;

- aider les centres de recherche nationaux à se rendre plus aptes à cette participation;

- étudier les sources, l'étendue, le degré, les parcours, les tendances et les effets des polluants affectant la mer Méditerranée;

- fournir l'information scientifique et technique nécessaire aux gouvernements des pays méditerranéens et à la Communauté économique européenne pour négocier et mettre en oeuvre la Convention pour la protection de la mer Méditerranée contre la pollution et les protocoles y relatifs;

- constituer des séries chronologiques cohérentes de données sur les sources, les cheminements, les degrés et les effets des polluants de la mer Méditerranée et contribuer par là à la connaissance scientifique de cette mer.

La Phase I du MED POL comportait à l'origine sept projets pilotes (MED POL I - VII) auxquels sont venus ultérieurement s'ajouter six autres (MED POL VIII - XIII) dont certains n'en sont restés qu'au stade de la conception.

La Phase I du MED POL a été mise en oeuvre au cours de la période 1975 - 1980. Le grand nombre de centres de recherche nationaux désignés par leurs gouvernements pour participer au MED POL (83 centres de recherche de 15 Etats méditerranéens et de la CEE), la diversité du programme et sa couverture géographique, l'effectif impressionnant de scientifiques et techniciens méditerranéens (environ 200) ainsi que la quantité d'organismes coopérants et d'organisations d'appui qui y étaient engagés permettent sans conteste de caractériser le MED POL comme l'un des programmes de coopération scientifique les plus vastes et les plus complexes, comportant un objectif spécifique et bien défini, qui ai jamais été entrepris dans le bassin mediterranéen.

La coordination et la direction générales de MED POL - Phase I ont été assurées par le PNUE, faisant fonction de secrétariat du Plan d'action pour la Méditerranée (PAM). Les organismes spécialisés coopérants des Nations Unies (CEE - Commission économique pour l'Europe, ONUDI, FAO, UNESCO, OMS, OMM, AIEA, COI) étaient chargés de l'exécution technique et de la coordination quotidienne des travaux des centres de recherche nationaux participant aux projets pilotes.

MED POL - Phase II (1981 - 1990)

La réunion intergouvernementale des Etats riverains de la Méditerranée chargés d'évaluer l'état d'avancement du Plan d'action et première réunion des Parties contractantes à la Convention pour la protection de la mer Méditerranée contre la pollution et aux protocoles y relatifs (Genève, 5-10 février 1979), ayant examiné la situation de la Phase I du MED POL, a recommandé que, durant la période biennale 1979 - 80, soit formulé un programme à long terme de surveillance continue et de recherche en matière de pollution.

Sur la base des recommandations énoncées lors des diverses réunions d'experts et réunions intergouvernementales, un projet de programme à long terme (1981 - 1990) de surveillance continue et de recherche en matière de pollution (MED POL - Phase II) a été formulé par le secrétariat de la Convention de Barcelone (PNUE), en coopération avec les organismes des Nations Unies chargés de l'exécution technique de MED POL - Phase I, et il a été officiellement approuvé lors de la deuxième réunion des Parties contractantes à la Convention pour la protection de la mer Méditerranée contre la pollution et aux Protocoles y relatifs et réunion intergouvernementale des Etats riverains de la mer Méditerranée chargée d'évaluer l'état d'avancement du Plan d'action, qui s'est tenue à Cannes du 2 au 7 mars 1981.

L'objectif général à long terme de la Phase II du MED POL était de concourir à la réalisation des objectifs de la Convention de Barcelone en aidant les Parties contractantes à prévenir, réduire et combattre la pollution dans la zone de la mer Méditerranée ainsi qu'à protéger et améliorer le milieu marin dans cette zone. Les objectifs particuliers étaient de fournir constamment aux Parties contractantes à la Convention de Barcelone et aux Protocoles y relatifs:

- les renseignements dont elles avaient besoin pour appliquer la Convention et les protocoles;

- des indications et une évaluation de l'efficacité des mesures prises pour prévenir la pollution en application de la Convention et des protocoles;

- des renseignements scientifiques qui pourraient servir à réviser et modifier les dispositions pertinentes de la Convention et des protocoles et à rédiger des protocoles additionnels;

- des informations qui pourraient servir à formuler sur les plans national, bilatéral et multilatéral, les décisions de gestion, respectueuses de l'evnironnement, qui seraient indispensables à la poursuite du développement socio-économique de la région méditerranéenne;

- une évaluation périodique de l'état de pollution de la mer Méditerranée.

La surveillance continue des polluants affectant le milieu marin de la Méditerranée ainsi que la recherche menée à leur sujet répondent en premier lieu aux prescriptions immédiates et à long terme de la Convention de Barcelona et des protocoles y relatifs, mais elles tiennent également compte des facteurs requis pour la compréhension des relations existant entre le développement socio-économique de la région et la pollution de la mer Méditerranée.

A cette fin, la surveillance continue était organisée à plusieurs niveaux:

- surveillance continue des sources de pollution fournissant des renseignements sur la nature et la quantité des polluants directement libérés dans l'environnement;

- surveillance continue des zones situées à proximite du littoral, y compris les estuaires, et qui sont sous l'influence directe de polluants émis par des sources identifiables primaires (émissaires, rejets et sites côtiers d'immersion) ou secondaires (cours d'eau);

- surveillance continue des zones du large (zones de référence) fournissant des renseignements sur les tendances générales du niveau de pollution en Méditerranée;

- surveillance continue du transfert des polluants à la Méditerranée par voie atmosphérique, fournissant des renseignements supplémentaires sur la charge polluante qui atteint la Méditerranée.

Les sujets de recherche et d'étude inclus initialement dans MED POL - Phase II étaient les suivants:

- mise au point de techniques d'échantillonnage et d'analyse pour la surveillance des sources et des niveaux de pollution. Essai et harmonisation de ces méthodes à l'échelle méditerranéenne, et formulation de méthodes de référence. Substances figurant sur les listes de priorité des protocoles sur les opérations d'immersion et sur la pollution d'origine tellurique (activité A);

- mise au point de la présentation type des rapports à soumettre en application des protocoles relatifs à l'immersion, à la pollution résultant de situations critiques et à la pollution d'origine tellurique, (activité B);

- élaboration des fondements scientifiques des critères de qualité de l'environnement qui serviront à définir des normes d'émission, des normes d'usage ou des directives concernant les substances énumérées dans les annexes I et II du protocole relatif à la pollution d'origine tellurique, conformément aux articles 5, 6 et 7 de ce protocole (activité C);

- études épidémiologiques relatives à la confirmation (ou révision éventuelle) des critères de la qualité de l'environnement (normes d'usage) proposés pour les eaux servant à la baignade, à la culture de coquillages et à l'élevage d'autres organismes marins comestibles (activité D);

- mise au point de projets de directives et de critères régissant l'application du protocole relatif à la pollution d'origine tellurique, conformément à l'article 7 de ce protocole (activité E);

- recherches sur les processus océaniques, et particulièrement sur la circulation en surface et les déplacements verticaux. Cette information est nécessaire à la connaissance de la répartition des polluants en Méditerranée et à la mise au point de plans pour parer aux situations critiques (activité F);

- recherches sur la toxicité, la persistance, la bioaccumulation et le caractère cancérigène et mutagène de certaines substances énumérées dans les annexes du protocole relatif à la pollution d'origine tellurique et du protocole relatif aux opérations d'immersion (activité G);

- recherches sur l'eutrophisation et les floraisons de plancton qui l'accompagnent. Cette information est nécessaire pour évaluer la possibilité de prévenir les effets et les dégâts causés par ces floraisons périodiques (activité H);

- étude des modifications de l'écosystème dans les zones soumises à l'influence des polluants et dans celles où ces modifications sont dues à d'importantes activités industrielles sur la côte ou à l'intérieur des terres (activité I);

- effets des pollutions thermiques sur les écosystèmes marins et côtiers, y compris l'étude des effets connexes (activité J);

- cycle biogéochimique de certains polluants intéressant particulièrement la santé (mercure, plomb, survie des organismes pathogènes dans la mer Méditerranée, etc.) (activité K);

- étude des processus de transfert des polluants (i) aux points de contact entre les cours d'eau et la mer et entre l'air et la mer, (ii) par sédimentation et (iii) à travers les détroits qui relient la Méditerranée aux mers voisines (activité L).

Les Parties contractantes au cours de leur sixième réunion ordinaire ont convenu de:

(a) réorienter les activités de recherche menées dans le cadre du MED POL en sorte qu'elles engendrent des informations qui soient également utiles pour l'application technique du Protocole tellurique, en plus de l'appui apporté aux activités de surveillance continue;

(b) à compter de 1990, remplacer les activités A à L par les cinq nouveaux domaines de recherche ci-après:

Domaine de recherche I - Caractérisation et dosage

Ce domaine englobera des projets de recherche en matière de caractérisation (identification de constituants chimiques ou microbiologiques) et de dosage (mise au point et éssai de méthodes) de contaminants donnés;

Domaine de recherche II - Transfert et dispersion

Ce domaine englobera des projets visant à approfondir notre connaissance des mécanismes physiques, chimiques et biologiques qui véhiculent les polluants potentiels de leurs sources à leurs dépôts ultimes. Les sujets étudiés porteront notamment sur le transfert et le dépôt atmosphériques, les mouvements et le brassage des eaux, le transfert des contaminants par sédimentation et leur incorporation dans les cycles biogéochimiques. Priorité sera accordée à l'obtention de données quantitatives servant, en dernier ressort, à la modélisation des systèmes et à l'établissement des évaluations régionales;

Domaine de recherche III - Effets

Ce domaine englobera des projets relatifs aux effets de certains contaminants énumérés aux annexes I et II du Protocole tellurique et du Protocole relatif aux situations critiques: effets sur les organismes, les communautés et les écosystèmes marins, effets chez l'homme et parmi les populations humaines. Priorité sera accordée aux effets et techniques fournissant des données utiles pour établir les critères de qualité du milieu;

Domaine de recherche IV - Destinées/transformation dans l'environnement

Ce domaine englobera des projets portant sur l'étude de la destinée des polluants (micro-organismes y compris), dans le milieu marin, et notamment sur la persistance et la survie, la dégradation, la transformation et la bio-accumulation, etc., mais non sur le transfert et la dispersion qui sont traités dans le domaine II;

Domaine de recherche V - Prévention et lutte antipollution

Ce domaine englobera des projets traitant de la détermination des facteurs conditionnant l'efficacité des méthodes d'épuration et d'élimination des déchets sous des conditions locales spécifiques ainsi que de l'établissement de critères de qualité du milieu et de mesures communes de réduction de la pollution;

(c) définir des contaminants cibles ou d'autres variables à des intervalles périodiques en fonction de l'état de l'avancement de l'application du Protocole tellurique;

(d) choisir les propositions de projet sur la base de leur valeur scientifique intrinsèque, leur spécificité méditerranéenne et, chaque fois que possible, encourager les projets bilatéraux et multilatéraux entre les pays méditerranéens du nord et du sud du bassin.

Comme lors de la Phase I du MED POL, la coordination et la direction générales de la Phase II étaient assurées par le PNUE, par l'intermédiaire du secrétariat du Plan d'action pour la Méditerranée (PAM). Les organismes spécialisés coopérants des Nations Unies (FAO, UNESCO, OMS, OMM, AIEA, COI) étaient chargés de l'exécution technique et de la coordination quotidienne des travaux des centres de recherche nationaux participant au programme de surveillance continue et de recherche.

Le présent volume comprend les rapports finaux sur les projets de recherche exécutés au titre de l'activité G. La préparation, l'édition et la compilation de ce volume ont été assurées par M. G.P. Gabrielides, FAO Fonctionnaire Principal des Pêches (Pollution Marine), et Mlle V. Papapanagiotou, Sécretaire FAO était chargée de la dactylographie.

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by

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A B S T R A C T

This study was initiated to evaluate the changes in plasma enzyme activity in marine fish after acute exposure to pollutants. Adult grey mullets Mugil auratus were used as test organisms. Acute liver injury was experimentally induced with high carbon tetrachloride and phenol doses. Carbon tetrachloride was used as a model hepatotoxic agent while phenol was chosen for comparison as a general protoplasmatic poison as well as a possible pollutant of the marine environment. Lactic dehydrogenase (LDH), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were selected as relevant enzymes for evaluation of liver intoxication. Dose dependent increases of all three enzymes in plasma were observed promptly after injection of the toxicants. This study showed also that the toxic effect on the normally low plasma GPT activity was relatively higher than on plasma GOT, but the general response of GOT was of longer duration. The electrophoretic patterns of GOT from different mullet tissues, and the organ/tissue distribution of both GOT and GPT suggested 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 effect. However, the increased LDH activity in fish plasma cannot be considered as an indicator of the hepatotoxic effect because the electrophoretic separation indicated that the increased plasma LDH activity was generated by the presence of a substantial amount of LDH_5 isozyme fraction, the origin of which could be assigned to the very large LDH_5 pool from the white muscle tissue.

1. INTRODUCTION

The measurement of serum or plasma enzyme activity is a helpful diagnostic tool in pathological, toxicological and general clinical testing but, until recently, there were few attempts to introduce these techniques into aquatic toxicology studies (Racicot et al., 1975; D'Apollonia and Anderson, 1977; Casillas and Ames, 1983, Casillas et al., 1986; Mehrle and Mayer, 1980; Weiser and Hinterleitner, 1980). As with mammals it has been proved that after functional damage to the tissues and organs of fish some specific cellular enzymes were leaking into the blood where they could be detected (Bouck, 1966; Bouck et al., 1975). In mammalian toxicology, on the basis of the changed plasma enzyme patterns, one can accurately recognize the origin of single enzymes and estimate the functional state of the organ(s) or tissue(s) involved. In principle, it would be very attractive to use the same approach in fish but there are difficulties.

The wide and coarse homeostatic regulation in poikilothermic fish is expressed with a pronounced variability and with a broader range of "normal" values. In practice, the capture stress, anesthesia and different blood sampling methods can dramatically alter the "normal" plasma patterns in fish. Other problems are encountered in the analytical approach and the necessity for species specific standardization of methods has been recognized.

It was our intention to check whether the presence of some cellular enzymes in plasma of a marine fish could be used as indicators of intoxication as well as they have been used in mammalian toxicology. Carbon tetrachloride was used as a model hepatotoxic agent, while phenol was chosen for comparison as a general protoplasmatic poison as well as a possible pollutant of the marine environment. LDH, GOT and GPT were selected as relevant enzymes for evaluation of liver intoxication.

2. MATERIALS AND METHODS

2.1 Experimental animals

Adult grey mullets (Mugil auratus Risso) of average weight 191 ± 25 g were used as test animals. Fish were acclimated for 2 weeks in 250 l aerated basins with a continuous flow of sea water (salinity 37.2 ± $0.4 \cdot 10^{-3}$, temperature 20 ± 0.5°C). They were fed daily to satiation and the remaining food was removed.

2.2 Exposure to toxicant

Ten grey mullets per group were injected intraperitonealy (i.p.) with one of the following: 1 and 2 ml CCl_4 kg^{-1} or 200 mg of phenol kg^{-1} body weight. Phenol was first dissolved in distilled water, while CCl_4 was injected without dilution.

Blood samples were taken during the next 24 hrs, at 6 hrs intervals, by cardiac puncture. Blood sampling and i.p. injections were performed without the use of anesthetics. Heparin was used as anticoagulant. Blood was kept iced and plasma was immediately separated with a refrigerated centrifuge (10 min at 2000 x g).

2.3 Analytical methods

The activities of LDH (EC 1.1.1.27), GOT (EC 2.6.1.1) and GPT (EC 2.6.1.2) were measured by a NADH-linked spectrophotometric method (Bergmeyer and Bernt, 1974a, 1974b, 1974c) with a modification to optimize the assay conditions for grey mullets (Krajnovic-Ozretic and Ozretic, 1987). The activity of the same enzymes was also measured in the extracts of liver, heart, kidneys, white and red muscle and gills. Tissue samples were homogenized with a Polytron grinder in 10 parts of a cold 0.2 M Na-phosphate buffer (pH 7.4) in 20% glycerol with 5 mM mercaptoethanol. The homogenates were centrifuged at 17000 g for 30 min. and the supernatant immediately used for enzyme assay. For plasma, the enzyme activity was calculated in relation to the unit volume (ml) or to the unit weight (g) for body tissues and it was expressed as International Units (U). Biuret reaction was used to determine total proteins (Wieschselbaum, 1946).

LDH electrophoresis was carried out on cellulose acetate strips using 75 mM barbital buffer at pH 8.6 and the separation was achieved in 50 min. at 200 V. The fractions were made visible during incubation with lactate, NAD, MTT and phenazene methosulphate for reduction of tetrazolium to formazen, using the method described by Burlina et al., 1979. After electrophoresis GOT bands were visualized by the methods of Ojala and Konttinen (1978). The color intensity of the electrophoretic bands was evaluated with a Gilford Auto Scanner at 540 nm.

3. RESULTS AND DISCUSSION

The activity changes of LDH, GOT and GPT in plasma of mullets injected with CCl_4 are presented in Fig. 1. When a single dose of 1 ml CCl_4 kg^{-1} was applied the maximal activity for all 3 enzymes in plasma was measured 6 hrs after the injection. Within 24 hrs the activity had fallen towards preinjection levels. This observation suggests the presence of a rapid regeneration mechanism in mullets for relatively low doses of CCl_4. In the group injected with a single dose of 2 ml CCl_4 kg, 12 hrs after injection the enzyme in plasma was increased by nearly 2 orders of magnitude and even up to 24 hrs GOT and GPT were still increasing. LDH started to decrease at 12 hrs post injection. During this time interval GOT activity enhanced about 180 times, GPT activity 465 times, and LDH 44 times with respect to the original preinjection levels. After 8 days the activity of GOT was still high, nearly 10 times higher than in the control, while the activity of LDH and GPT dropped to preinjection levels.

The i.p. injection of phenol resulted also in increased plasma transaminase and LDH activities (Fig. 2). GOT and LDH activity increased ($p<0.01$) at the lowest phenol concentration (20 mg kg^{-1}), the response being proportional to the dose injected. On the other hand, GPT activity was affected only by the highest phenol concentration used (200 mg kg^{-1}). The maximal activity for both transaminases was reached 2 days after the i.p. injections. At that time, GOT activity had increased about 115 times and GPT activity nearly 190 times compared with preinjection values. GPT activity quickly returned to preinjection values, whereas the activity of GOT decreased slowly, with the 200 mg phenol kg^{-1} group still remaining 80 times higher than preinjection values after 8 days. The maximal activity of LDH was reached 24 hrs after i.p. injection, earlier than transaminases. At that time LDH activity had increased about 86 times. The decrease of LDH activity has also been observed earlier at 48 hrs after i.p. injection when LDH activity in the group treated with the lowest phenol concentration dropped to the level of the control value.

To examine the specific distribution of enzymes in some organs and tissues in relation to the plasma enzyme pattern the activity of LDH, GOT and GPT was measured, and the average values are compared in Table I. The highest activity and total content of LDH were found in the white muscle tissue (926 U g^{-1} w.w. and 92% of total activity), while the activity of GOT was higher in the liver, heart and red muscle (39.8, 41.8 and 61.3 U g^{-1} w.w.). As in other vertebrates, the liver of mullet was characterized by the highest specific activity (17.7 U g^{-1}), and by the highest content of the whole body GPT (52.7%). The activity of all 3 enzymes was the lowest in the plasma, for GPT very near to the detection limits.

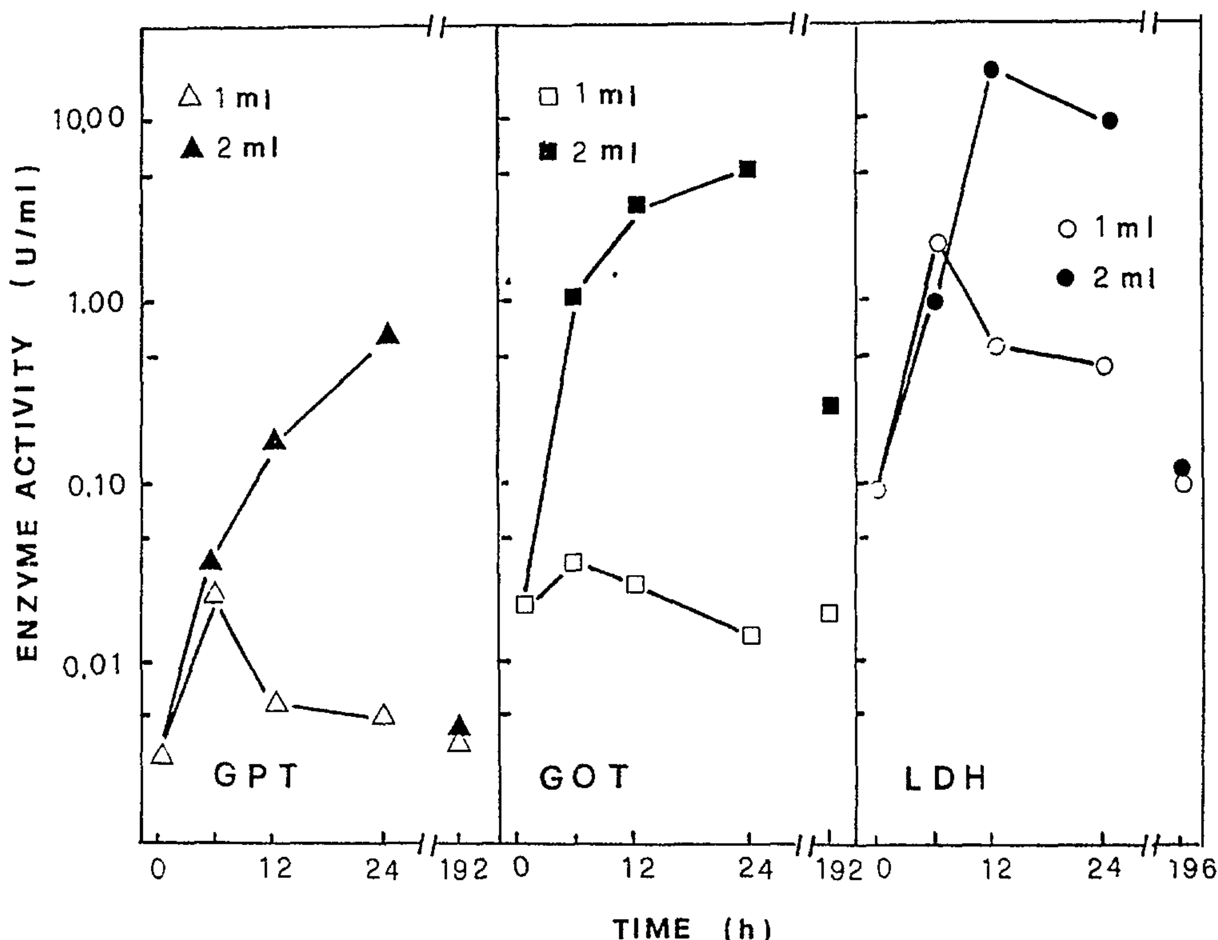

Fig. 1 Mugil auratus. Activity of (▲) GPT, (■) GOT and (●) LDH in cardiac-sampled plasma from mullet after single injection of 1ml and 2ml CCl_4 kg^{-1} wet wt

The electrophoretic separation of plasma and various tissue extracts permitted the identification of the relative distribution of GOT and LDH. Electrophoretic and histochemical detection of GPT was not satisfactory. In the injected fish the zymograms showed two, well separated anodic GOT isozymes (Figs 3 and 4). The fast-moving zone was designated as GOT-1 and belonged to the cytoplasmatic (supernatant) enzyme fraction. The second zone contained a single band of the slower-moving (GOT-2) isozyme. In non-injected fish only a single weak band of GOT-1 was observed in the plasma, corresponding to the very low cytoplasmatic enzyme activity in fish before injection. After the fish were injected either with phenol or CCl_4 the zymograms were substantially changed: 24 hrs after injection the plasma GOT-1 band was greatly increased, and a weak GOT-2 band appeared (Fig. 3a). After 100 days the plasma from mullets injected with 200 mg phenol kg^{-1} retained still a very high GOT activity, and in the zymograms behind the GOT-1 intensive band, two additional but weak bands were observed, while the mitochondrial GOT-2 fraction had disappeared (Fig. 3b). The additional GOT-1 bands resembled the bands of the same mobility and intensity that appeared in the liver zymograms of the same fish (Fig. 3b).

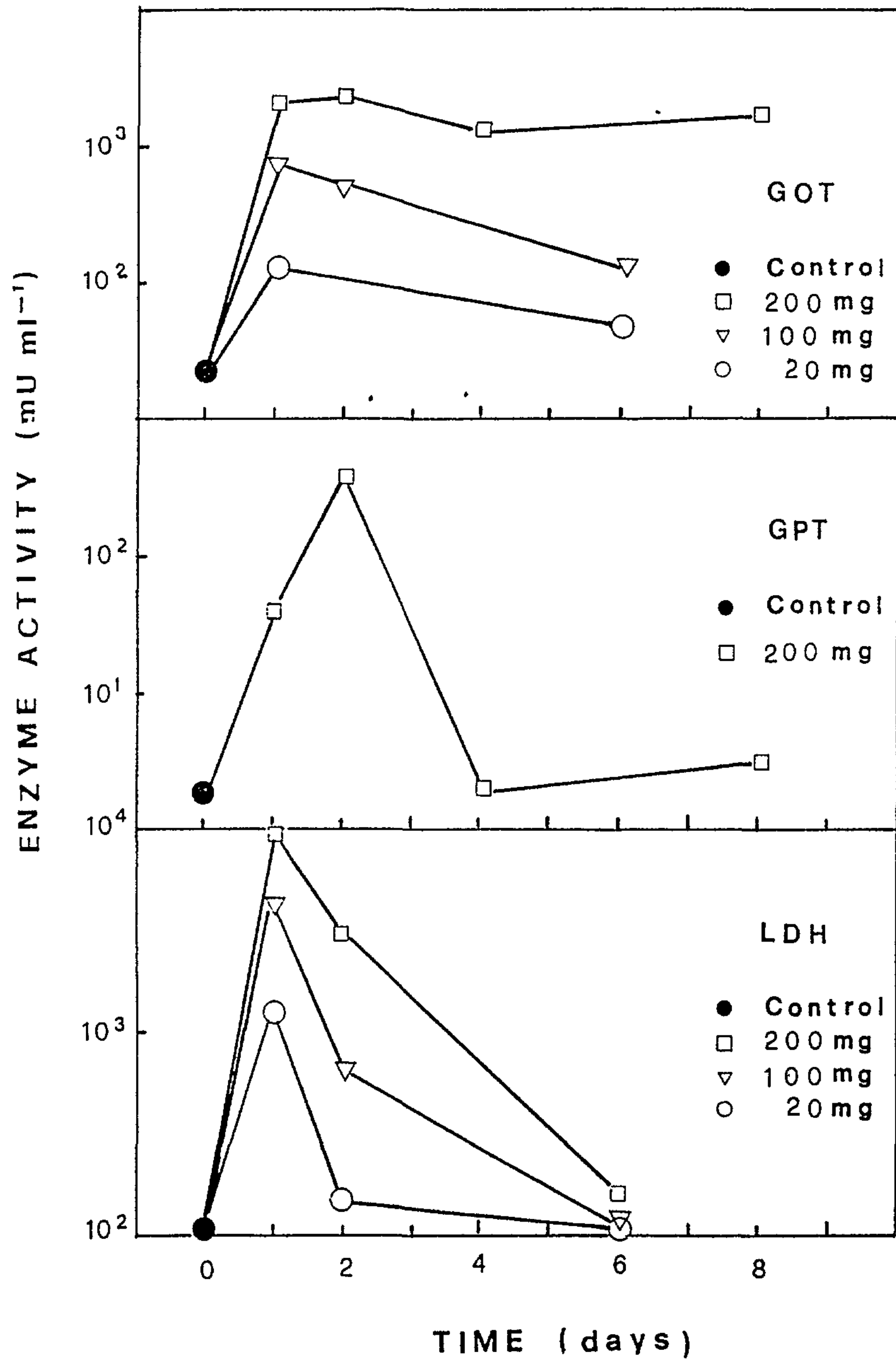

Fig. 2 Mugil auratus. Activity of *GOT*, *GPT* and *LDH* in the plasma of mullets injected with 20, 100 and 200 mg phenol kg^{-1} compared with the preinjection level (control)

Table I

Mugil auratus. Distribution of LDH, GOT, GPT and GLDH in mullet tissues (U g^{-1} wet wt) and in cardiac-sampled plasma (U l^{-1}). Each value represents the mean ± Sd (N=60).

	White muscle	Red muscle	Liver	Kidney	Heart	Gill filaments	Plasma
LDH	926±84	417±76	8±6	69±23	482±21	81±12	110±23
GOT	13.9±4.8	61.3±22.2	39.8±18.1	18.2±5.66	41.8±14.4	10.9±4.5	19.6±11.6
GPT	0.45±0.17	4.4±2.7	17.7±4.4	4.3±1.9	0.50±0.25	0.27±0.12	1.95±0.23

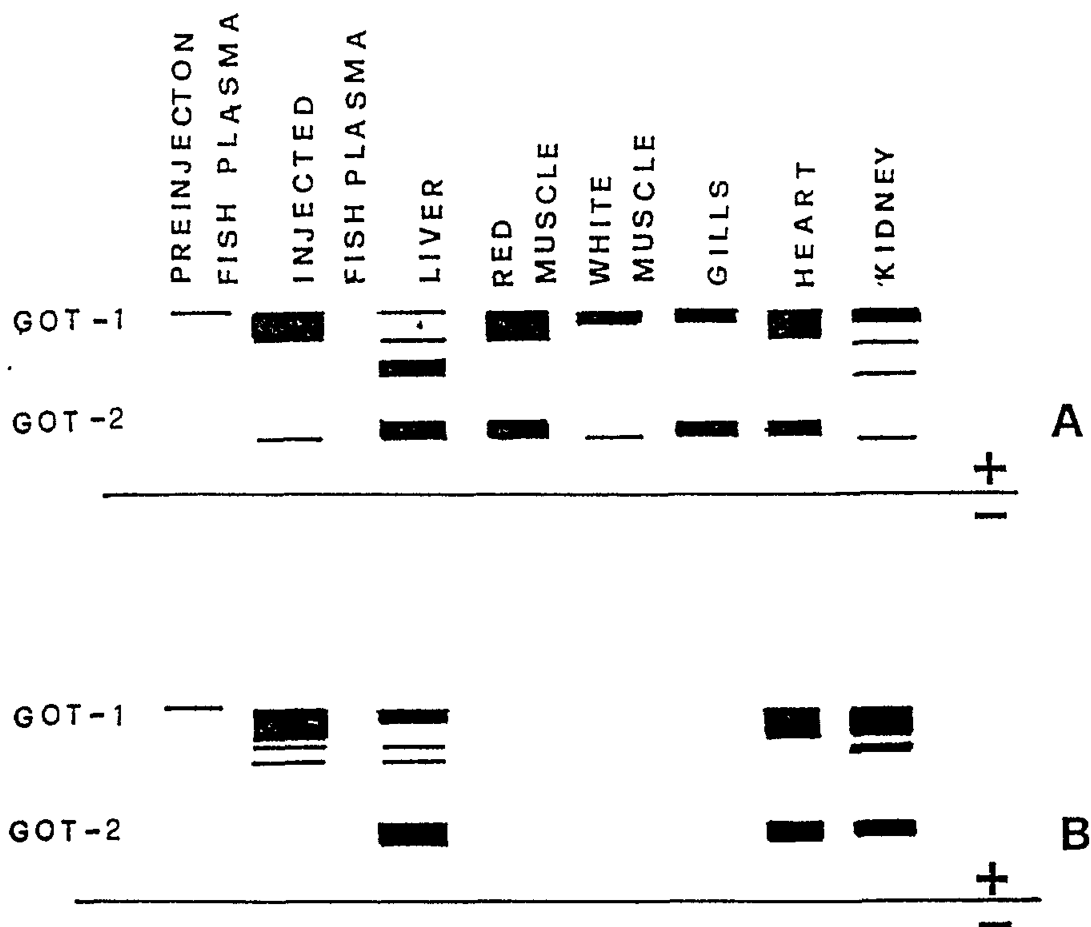

Fig. 3 Mugil auratus. Zymograms of GOT from plasma and tissue extracts. A. Samples prepared 24 hrs after i.p. injection of either 2 ml CCl_4 kg^{-1} or 200 mg phenol kg^{-1} (same effect for each toxicant). B. Samples prepared 100 days after i.p. injection of 200 mg phenol kg^{-1}

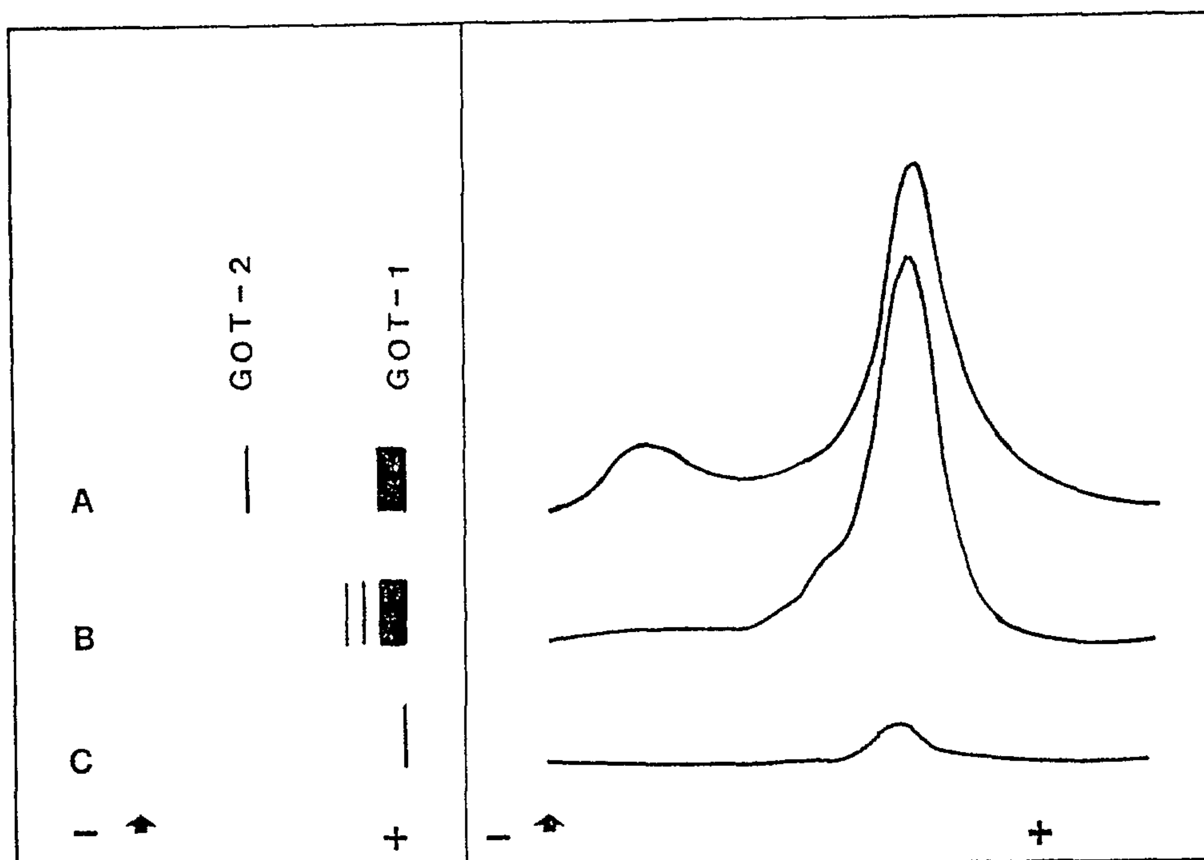

Fig. 4 Mugil auratus. Zymograms and related density diagrams of GOT from plasma of mullet. A. Samples prepared 24 hrs after i.p. injection of either 2 ml CCl_4 kg^{-1} or 200 mg phenol kg^{-1} (same effect for each toxicant). B. Samples prepared 100 days after i.p.injection of 200 mg kg^{-1}. C. Normal plasma.

The increased GOT activity in plasma reflected the intensity of the liver cell damage, and the identification of the GOT isozymes provided additional evidence (Figs 3 and 4). The electrophoretic separation showed that the bulk of the increased plasma GOT activity could be attributed to the cytoplasmatic isozyme fraction. The examination of zymograms from other tissues suggested that the origin of the GOT isozyme fractions in plasma was primarily originated from the liver and kidney, as may be seen in zymograms after partial recovery 100 days later (Fig. 3b). In general, it may be concluded that acute intoxication with either phenol or CCl_4 produced a significant injury of different tissues at the subcellular level, which resulted in leaking of GOT from damaged tissue cells into the blood, where it was detected in substantial amounts in both cytoplasmatic and mitochondrial fractions. Liver and kidney appeared to be the main source of the increased plasma GOT activity. The presence of the GOT-2 isozyme fraction in blood indicated a mitochondrial damage, and the release of the cytosolic GOT-1 isozyme(s) may be attributed to disturbed permeability of the cell membranes. Such mitochondrial damage became detectable after cellular necrosis as has been shown in human acute hepatic diseases by appearance of the mitochondrial isozyme fraction in the blood plasma (Panteghini et al., 1984).

The electrophoretic separation showed that LDH in grey mullet tissue was present in at least 3 isozyme forms (Fig. 5). According to the results of Markert and Faulhaber (1965) and to the LDH_{1-5} nomenclature proposed by Wieme (1974) it was noticed that in mullet heart, red muscle and kidney extracts the highest LDH concentration was pertinent to the LDH_1 isozyme fraction, while the LDH_5 fraction was predominant in liver and particularly in white muscle extracts. In the plasma from the cardiac-sampled blood 2 weak LDH_1 and LDH_5 bands were detected.

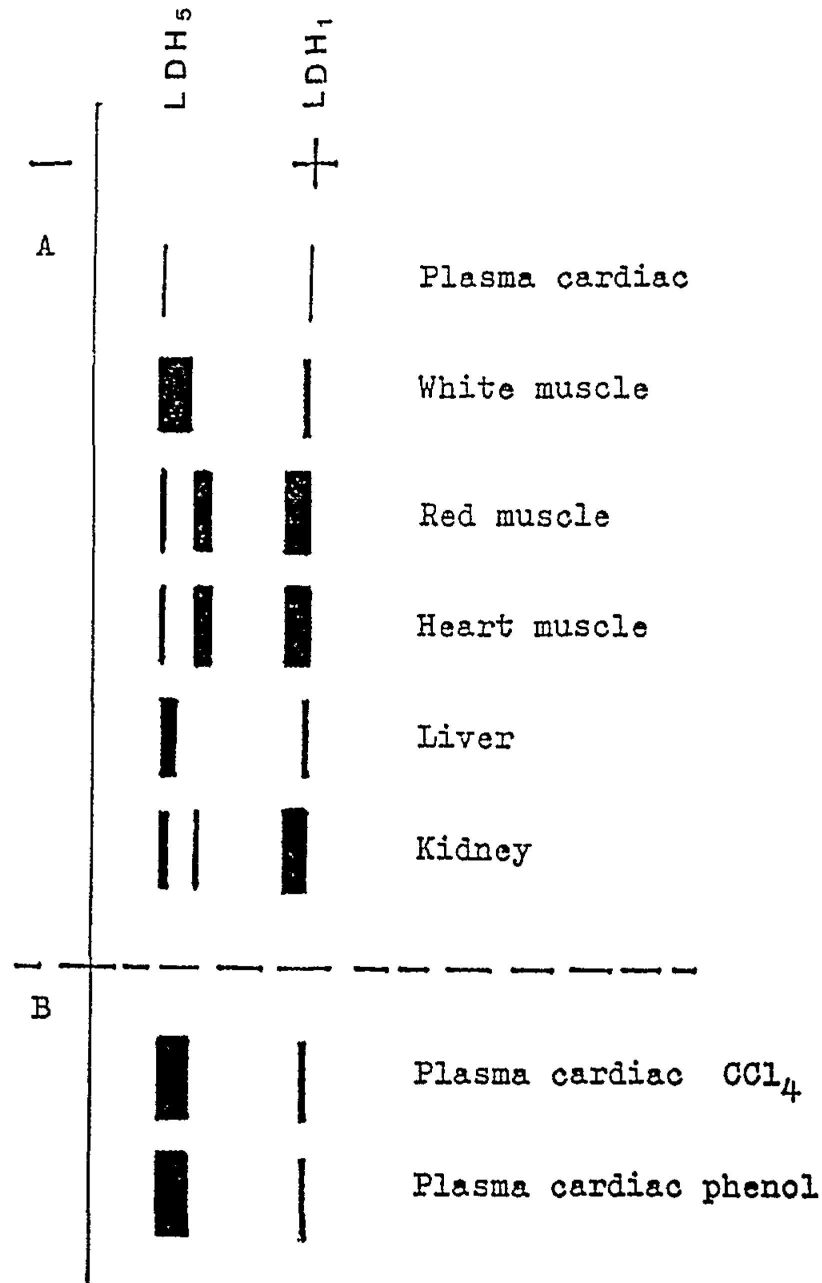

Fig. 5 Mugil auratus. (A) Electrophoretic isozyme fractions of LDH in plasma and tissue extracts. (B) LDH fractions in cardiac sampled plasma from mullets treated with CCl_4 and phenol

In the CCl_4 and phenol treated fish the increased plasma LDH activity was generated by the presence of a substantial amount of the LDH_5 isozyme fraction (Fig. 5), the origin of which could be assigned to the very large LDH_5 pool from the white muscle.

The increased LDH activity in fish plasma could not derive only from the liver because of the low activity and limited biomass of the liver (1.4 to 2% of the total body burden). In fish, substantial amounts of LDH were found within many tissues but the highest specific activity was measured in the white muscle. As it was confirmed by electrophoresis the LDH isozyme in plasma of the injected fish corresponded to the white muscle type. The high LDH specific activity and the large body burden of this tissue suggested that the origin of LDH in plasma was originated as a leaking from the white muscle tissue.

Change in plasma GPT activity was also evident and proportional to the CCl_4 i.p. dose, but only the highest i.p. dose of phenol (200 mg kg^{-1}) produced a significant increase in plasma GPT activity. This reaction was transient: after both CCl_4 or phenol injections, the GPT activity returned to preinjection levels after 8 and 4 days, respectively. This suggests that the increased plasma GPT activity may have resulted from an initial, single leakage of the enzyme from the injured tissue(s), mainly liver, rather than a continuous leakage, as was apparently the case with GOT.

4. CONCLUSIONS

This mullet study demonstrated that changes in plasma enzyme activity can be used as indicators of toxicant stress in marine fish, just as it has been used and successfully applied in mammalian toxicology. Acute, but sublethal poisoning of mullets with either a specific hepatotoxin (CCl_4) or with a general toxicant (phenol) induced rapid, sensitive and dose dependent increase of the normally low LDH, GOT and GPT activity in plasma.

The electrophoretic patterns of GOT from different mullet tissues, and the organ/tissue distribution of both GOT and GPT suggested the liver to be their origin (Krajnovic-Ozretic and Ozretic, 1987). As in mammals, GPT appeared to be a specific "liver-guiding enzyme" than can be used as a sensitive indicator of hepatotoxic effects. However, the increased LDH activity in fish plasma cannot be considered as an indicator of hepatotoxic disfunction. LDH, GOT and GPT are cellular metabolic key enzymes with no evident function in vertebrate plasma. Generally, they were found in small concentrations in plasma, including fish, derived probably from the regular physiological shedding of cells (Schmidt and Schmidt, 1974). Therefore, any detectable increase of their activity in plasma can be used as a reliable indicator of changed metabolic functions or structural damage at the tissue level. Detection methods for these enzymes are simple, rapid and, what is important in fish physiology studies, can be carried out with small blood samples. The determination of the basic physicochemical characteristics and electrophoretic fractionation of isozyme forms provides the opportunity to identify their origin and also permit the estimation of the severity of injury at the tissue/cellular level.

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CADMIUM BIOACCUMULATION AND EFFECTS ON SOLUBLE PEPTIDES, PROTEINS AND ENZYMES IN THE HEPATOPANCREAS OF THE SHRIMP *Callianassa tyrrhena*

by

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ABSTRACT

Callianassa tyrrhena shrimps were exposed to 0.2-0.8 mg Cd l^{-1} of sea water for 2-8 days and hepatopancreas extracts were analyzed.

Cadmium accumulation was dose- and time-dependent with the metal concentration being 7-12 times higher in hepatopancreas than in the total of the rest of body tissues.

Soluble cadmium was found bound to proteins of apparent molecular size >70,000, 15,000 and 7,500; the latter two being the major forms in the presence of 2-mercaptoethanol.

Cadmium caused significant changes in the protein components of tissue extracts observed by electrophoresis and RP-HPLC techniques.

In vitro, cadmium inhibited glutathione S-transferase and esterase activity. In contrast, *in vivo*, cadmium caused a dose dependent increase in activity of the above enzymes. For esterases, this was caused by the differential activation of multiple molecular forms. Cadmium affected differently alkaline phosphatase activity *in vivo* with an increase at low and a decrease at high metal concentrations.

1. INTRODUCTION

Callianassa tyrrhena is an ecologically important and widely distributed shallow water shrimp which has a potential for economic exploitation as it is used as bait for commercially important edible fish (Ahsanullah *et al.*, 1981a). Crustacean hepatopancreas is a vital and major organ in diverse metabolic activities such as synthesis and secretion of digestive enzymes, uptake of nutrients, excretion, lipid and carbohydrate metabolism and storage of inorganic reserves (for a review see Gibson and Barker, 1979). High levels of cadmium have been identified in the hepatopancreas suggesting an important role in the detoxification of this metal (Topping, 1973; Overnell and Trewhella, 1979; Davies *et al.*, 1981; Bjerregaard, 1982; Engel and Brouwer, 1986).

The mechanism of cadmium toxicity is not clear yet, although the toxic effects of cadmium in crustaceans including *Callianassa sp.* are well documented (Ahsanullah *et al.*, 1981a, 1981b). Biochemical parameters studied so far for the effects of cadmium in crustacean species are mainly focused on the synthesis and properties of metal-

binding proteins (Engel and Brouwer, 1986; Lyon et al., 1983; Lerch et al., 1982) with little information on other soluble protein components (Almar et al., 1987; Gould, 1980).

Biochemical responses are likely to be among the first manifestations of excess metal accumulation and possibly the most sensitive indicators of stress. Such early and reliable indicators of pollution are in urgent need for creating standards and guidelines to follow in marine environmental management. With these views in mind the effects of accumulated cadmium to diverse biochemical parameters of protein nature were studied in the hepatopancreas of the shrimp C. tyrrhena.

2. MATERIALS AND METHODS

2.1 Cadmium treatment of animals and tissue extraction

The specimens of the sediment dwelling shrimp C. tyrrhena were collected from Vravrona bay in South Evoikos (Greece). The animals were collected by a method described by Manning (1975) by a pump made from plastic tube (100 cm x 7 cm i.d.), equipped with a plunger. The sucked contents were emptied in a sieve. Medium size shrimps (0.5 to 0.8g) were kept only. Of the two chela, the larger one was cut off to protect shrimps from attacking each other. The animals were transferred to the laboratory where were kept in plastic boxes (11 x 11 x 4 cm), containing 400 ml of synthetic sea water of approximately 38 salinity. Five individuals were kept in each container. The animals were maintained under controlled photoperiods of 12 h light/12 h darkness. The temperature was kept constant at 12 ± 0.5°C. The water of each container was replaced every second day. No food was provided. Dead shrimps were removed twice daily.

Cadmium was added in the form of $CdCl_2$ to make final concentrations of cadmium 0.2, 0.4, 0.6 and 0.8 mg l^{-1}. The metal was added 24 h after bringing the animals to the laboratory. Control animals were kept under the same conditions and time periods without any addition of metal.

Hepatopancreas of control and exposed to cadmium animals was excised from the animals standing on ice and weighed after applying them on filter paper to remove excess liquid. The tissue was homogenized with 2 volumes of chilled 20 mM Tris-HCl buffer, pH 8.0, in a teflon-pestle homogenizer. Pools of at least 30 shrimps were used for each homogenization except where otherwise stated.

2.2 Reversed phase high performance liquid chromatography

Hepatopancreas homogenate from a pool of 12 shrimps was centrifuged in a Beckman/Spinco 152 Microfuge for 2 min at full speed. A volume of 10 μl of the supernatant was mixed with 1 ml of 0.1% trifluoroacetic acid (TFA) solution in water. The supernatant after centrifugation in the Microfuge for 2 min at full speed was passed through one Sep-Pak C18 cartridge (Waters Associates, USA), primed for peptide binding with consecutively forcing 10 ml ethanol, 20 ml water and 20 ml of 0.1% TFA solution through it by using a syringe. The cartridge was washed with 20 ml of 0.1% TFA and bound peptides eluted

with 40% acetonitrile in 0.1% TFA. The sample was lyophilized in a Speed-Vac centrifugal concentrator (Savant model SVC 100). The dry residue was dissolved in 1 ml of 0.1% TFA. An aliquot of 400 μl was mixed with 100 μl of the same solution and the sample was injected in the HPLC system. The system was composed of one Waters Associates HPLC pump model 501 connected to a 4.6 x 250 mm C18 Lichrosorb column (HPLC Technology, U.K.) of 5 μm. A variable wavelength U.V. monitor (LKB model 2151) was set at 220 nm with 0.32 AUFS sensitivity. The starting solution was 0.1% TFA and a stepwise gradient was formed from 6 to 36% acetonitrile by a three-way valve at low pressure. The column was at room temperature and the flow rate was 1 ml min^{-1}.

2.3 Gradient slab polyacrylamide gel electrophoresis and protein staining

Gradient slab polyacrylamide gels (5-20%) were made by the method of Neville (1971) as modified by Egbert et al. (1976) (without SDS and 2-mercaptoethanol) by using a Pharmacia model GM1 gradient mixer. Electrophoresis was carried out in a Shandon model 200 electrophoresis apparatus connected to an LKB model 2177 power supply. Hepatopancreas homogenate was centrifuged in a Beckman ultracentrifuge model L5 equipped with SW Ti rotor at 150,000 x g for 1 h at 4°C. To a small volume of supernatant an equal volume of sample buffer was added and 20 μl of the sample was applied in each well. Electrophoresis was carried out for about 3 h at a constant current of 30 mA at room temperature. It was terminated when bromophenol blue band migrated at a point about 10 cm from the top of the separation gel. After electrophoresis gels were fixed for 30 min with a 20% trichloroacetic acid solution, stained for 24 h with a 0.125% Coomassie Brilliant Blue R solution and destained by a (7:5:88, v:v:v) solution of acetic acid, methanol and water.

2.4 Gel filtration

For gel filtration a column of 150 cm x 2 cm i.d. of Sephadex G-75 equilibrated with 20 mM Tris-HCl buffer, pH 8.0 was used. Hepatopancreas homogenate was centrifuged as mentioned for gradient polyacrylamide gel electrophoresis. Blue dextran (2,000,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), cytochrome c (14,000), aprotinin (6,500) and potassium dichromate (294) were used as molecular size markers.

Aliquots of 25 μl were injected in the Atomic Absorption Spectrophotometer furnace for cadmium measurements.

2.5 Estimation of cadmium levels

Cadmium levels were measured in tissues and in gel filtration fractions in a Perkin-Elmer Atomic Absorption Spectrophotometer model 603 equipped with furnace (Perkin-Elmer model HGA 2100). Digestion of tissues was carried out according to Syversen and Syversen (1975).

2.6 Estimation of enzyme activities

Hepatopancreas homogenate was centrifuged in an International ultracentrifuge model IEC-B/60 equipped with rotor A320 at 80,000 x g for 1 h at 4°C.

Glutathione S-transferase activity was measured according to the method of Habig et al. (1974) at 25°C by monitoring the change in absorption at 340 nm in a Perkin-Elmer model 124 double beam spectrophotometer. In a 1 ml cuvette, 922 μl of 0.1 M phosphate buffer, pH 7.0, 34 μl of 30 mM glutathione solution and 34 μl of a 30 mM 1-chloro-2,4 dinitrobenzene solution were added. An extinction coefficient of 9.6 mM^{-1} cm^{-1} for the latter reagent was used. The reaction was initiated by the addition of 10 μl of the appropriate dilution of the sample.

Alkaline phosphatase activity was measured according to the method of Harris and Coleman (1968) at 30°C by following the absorbance at 405 nm. In a 1 ml cuvette, 657 μl of 0.1 M Tris-HCL buffer, pH 8.0 and 333 μl of a 14.5 mM p-nitrophenyl phosphate solution were added. The reaction was initiated by 10 μl of a suitable dilution of the sample. An extinction coefficient of 18.5 M^{-1} cm^{-1} was used for p-nitrophenol.

Esterase activity was measured according to the method of Mastropaolo and Yourno (1981) at 25°C by following the absorbance at 235 nm. In a 1 ml cuvette, 980 μl of 0.05 M Tris-HCl buffer, pH 7.2 and 10 μl of a 25 mM alpha-naphthyl acetate solution in ethylene monoethyl ether were added. The reaction was initiated by the introduction of 10 μl of a suitable dilution of the sample. A molar extinction coefficient of 23.4 M^{-1} cm^{-1} was used for alpha-naphthol.

For the *in vitro* effect of cadmium on enzyme activity, aliquots of 10 μl of hepatopancreas supernatant were incubated with equal volumes of solutions of cadmium in 0.1 M phosphate buffer pH 7.0 for glutathione S-transferase and 0.05 M Tris-HCl buffer, pH 7.2 for esterases. Incubation lasted 45 min with occasional agitation at room temperature. Controls in the absence of the metal were also prepared.

2.7 Polyacrylamide gel electrophoresis followed by histochemical staining

The samples used for estimation of enzyme activities were also used for electrophoresis. Vertical slab polyacrylamide gel electrophoresis was carried out according to Veini et al. (1986). The concentration of the separating gel was 7.5%. The apparatus was the same as used for gradient polyacrylamide gel electrophoresis. Electrophoresis was carried out for about 3 h at a constant current of 25 mA and terminated when bromophenol blue migrated about 10 cm from the top of the separating gel. Gels were stained for alkaline phosphatase activity to Boyer (1961) and for esterase activity according to the method of Veini et al. (1986).

3. RESULTS

Cadmium accumulation in *C. tyrrhena* hepatopancreas and in the rest part of the body was found to be dependent on both the concentration of cadmium in the surrounding medium (for a fixed time duration of the exposure) (Fig. 1A) and the duration of the exposure (for a fixed concentration of the metal) (Fig. 1B) with an approximately linear increase within the limited concentration (0.2-0.8 mg Cd l^{-1} of water) and time (2-8 days) ranges employed. In both cases the concentration of the metal was 7-12-fold higher in the hepatopancreas than in the

rest of the body. However, when the total amount of Cd in each of the two anatomical compartments (hepatopancreas and rest of the body) was considered, approximately equal values were obtained for the two compartments (Figs. 1C and 1D). At the highest duration and concentration tested (0.8 mg Cd l^{-1} sea water for 8 days) the concentration of the metal in the hepatopancreas was 105.9 ± 29.1 μg Cd g^{-1} wet tissue weight and that of the rest of the body was 12.6 ± 2.7 μg Cd g^{-1} wet weight. Under the same exposure conditions the total cadmium present in hepatopancreas was found to be 6.492 ± 1.955 μg Cd, close to the value found for the rest part of the body (7.179 ± 1.938 μg Cd).

Accumulated Cd appeared to affect the concentration of some of the major soluble components of a hepatopancreas extract as resolved by RP-HPLC (Fig. 2). These components should be mainly of peptidic nature; as peptide bonds absorb at 214 nm, close to the wavelength of 220 nm employed. RP-HPLC is considered a suitable technique for the separation of peptides and small polypeptides not resolved by electrophoretic techniques due to their small size. From 11 major peaks of the control sample (Fig. 2A) eluting from 6 to 36% acetonitrile from a C18 column, 4 of them appeared significantly reduced in Cd-exposed shrimps at 0.8 mg Cd l^{-1} of sea water for 8 days (Fig. 2B) (peaks 2, 3, 6 and 7), one of them appeared increased (peak 1) and one peak appeared as double (peak 11). The RP-HPLC profiles were the same as controls at the lower dose of 0.2 mg Cd l^{-1} of sea water, while at the intermediate concentration of 0.4 mg Cd l^{-1} only a small increase of peaks 5 and 7 was observed (results not shown).

Cadmium was also found to affect some of the higher molecular weight soluble protein components in a crude hepatopancreas extract as observed by one dimension polyacrylamide gel electrophoresis (Fig. 3). By comparison of the electrophoretic profiles of exposed to cadmium (0.6 mg Cd l^{-1} of sea water for 15 days) and control animals, an increase in the staining intensity of 6 bands (Fig. 3, indicated by open tringles up) was observed combined with the appearance of a new minor band (Fig. 3, indicated by closed tringle up) in the exposed to cadmium sample. No significant decrease in the staining intensity was observed in any of the bands in the metal exposed shrimps.

Gel filtration in a Sephadex G-75 column of a hepatopancreas extract showed that all of cadmium appeared at the void volume i.e. bound to protein(s) of 70,000 molecular weight or over (results not shown). However when 10 mM 2-mercaptoethanol and 0.25 mM PMSF were incorporated in the extraction buffer and 2 mM 2-mercaptoethanol in the elution buffer (Fig. 4), a minor peak at the void volume was also observed (peak I) but most of cadmium was found bound to proteins eluting at positions corresponding to molecular sizes of 15,000 (peak II) and 7,500 (peak III). In control animals only traces of cadmium were observed (<5ng Cd ml^{-1}) and these only at the void volume (results not shown).

Cadmium accumulation was also observed to affect the levels of enzymatic activity in three enzyme systems (glutathione S-transferases, alkaline phosphatases and esterases) (Fig. 5). Alkaline phosphatase activity was found to increase to 130% at 0.2 mg Cd l^{-1} for 8 days and decrease thereafter reaching 80% of the control activity level (100%)

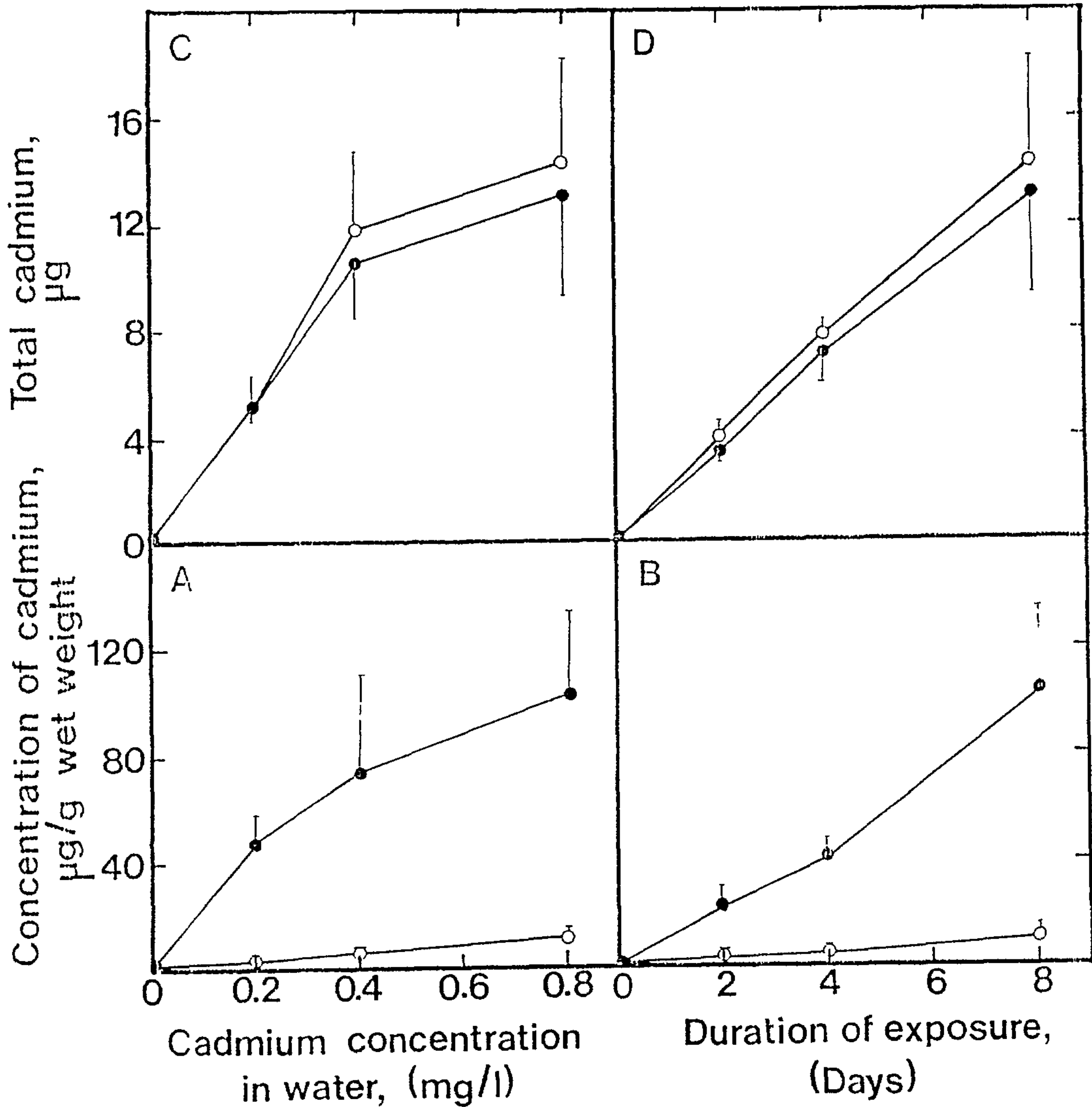

Fig. 1 Accumulation of cadmium in the hepatopancreas and rest of the tissues. A and C, shrimps were exposed to 0.2-0.8 mg Cd 1^{-1} sea water for 8 days while in B and D, shrimps were exposed to 0.8 mg Cd 1^{-1} of sea water for 2-8 days. In A and B bioaccumulated Cd is expressed as µg per g of wet tissue weight while in C and D is expressed as total Cd (µg) in whole tissues. Each point represents the mean value of the analysis of tissues of three shrimps. (●—●), hepatopancreas; (○—○) whole animals minus the hepatopancreas

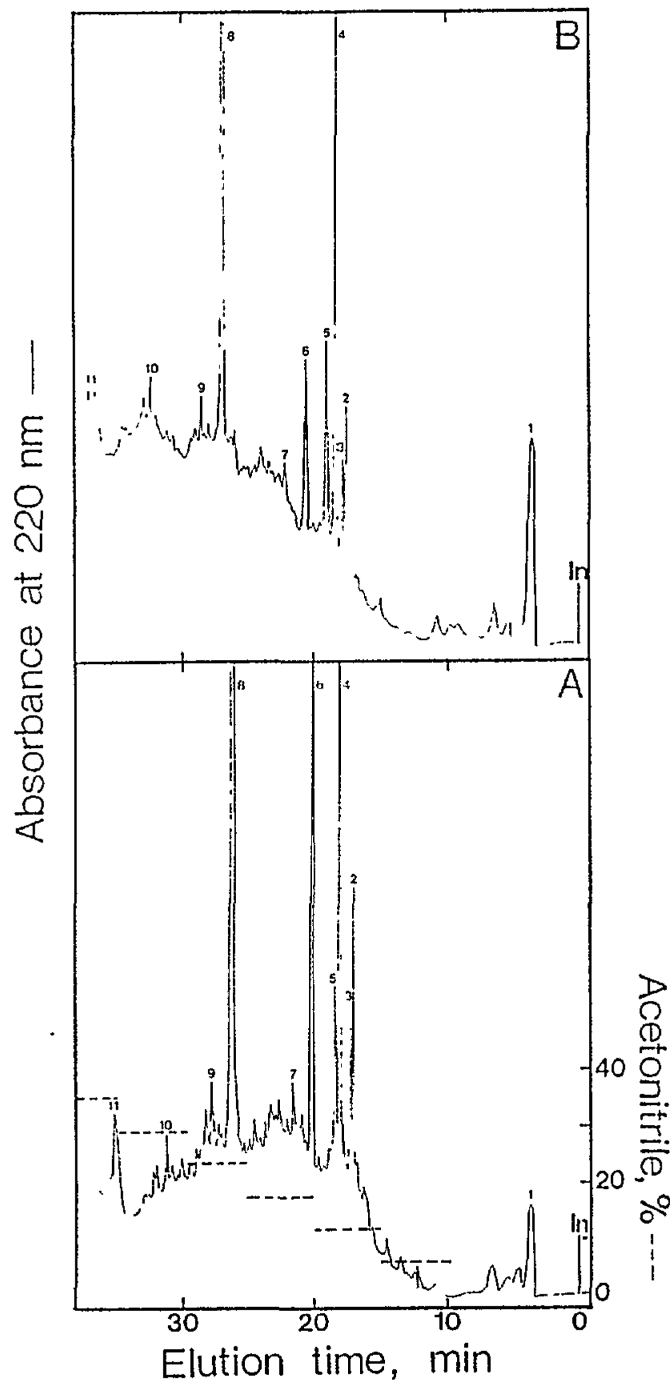

Fig. 2 Separation by reversed phase high performance liquid chromatography of hepatopancreas extract peptides of control (A) and exposed to 0.8 mg Cd l^{-1} of sea water for 8 days shrimps (B). Control shrimps were kept under the same conditions except for the metal. In time of sample injection. Other details as mentioned in the Materials and Methods section

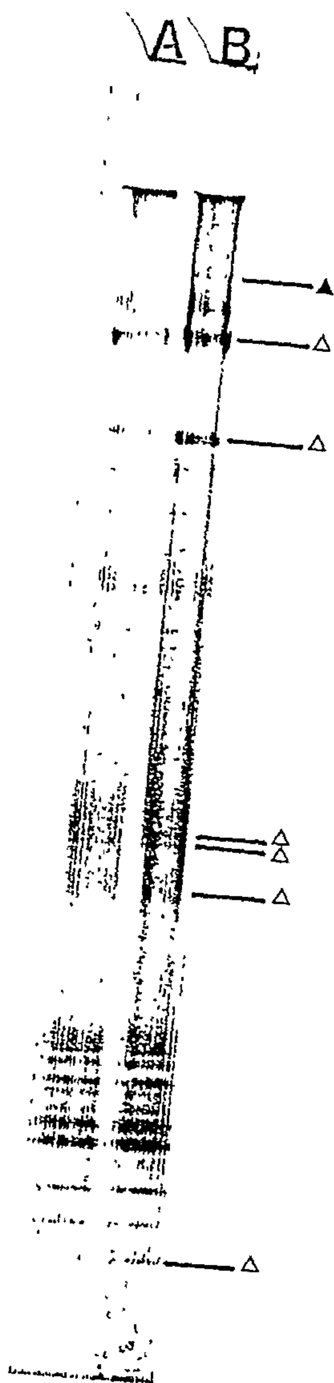

Fig. 3 Gradient polyacrylamide gel electrophoresis of proteins of hepatopancreas extracts of control (A) and exposed to 0.6 mg Cd l^{-1} of sea water for 15 days (B) shrimps

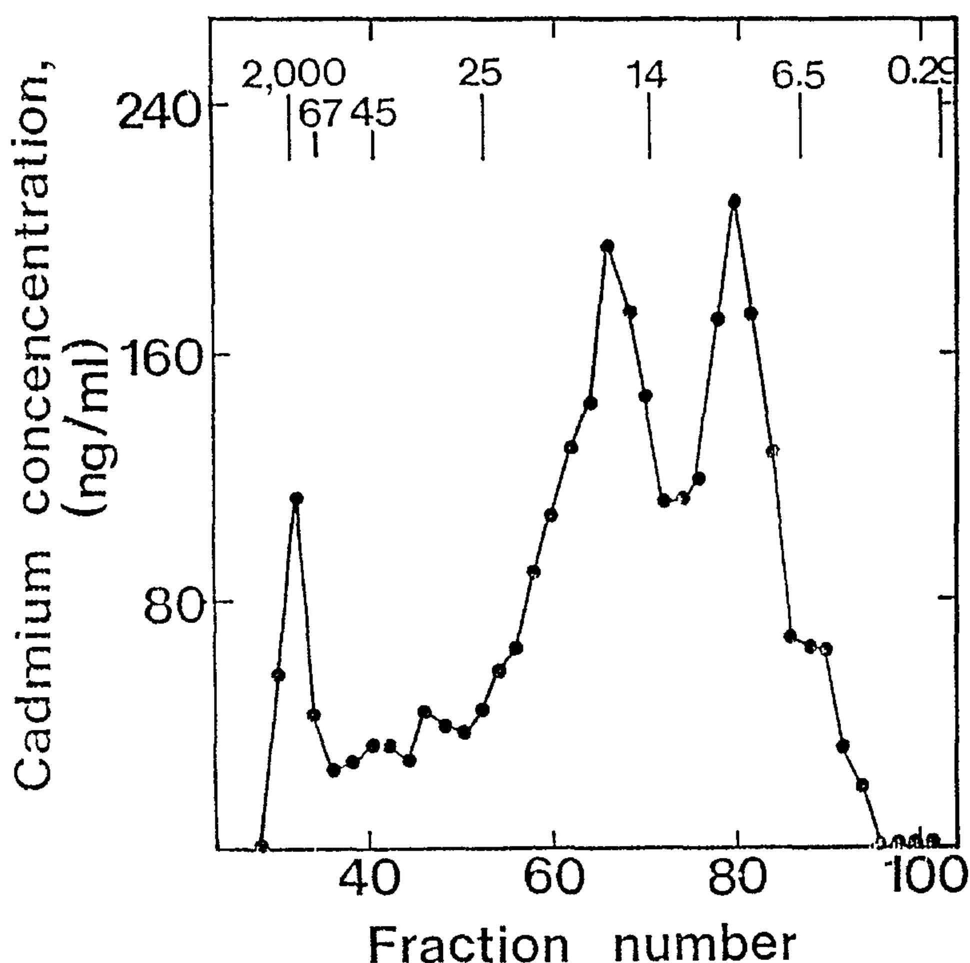

Fig. 4 Sephadex G-75 elution profiles of 1.5 ml of hepatopancreas extracts of shrimps exposed to cadmium. Shrimps were exposed to 0.8 mg Cd l^{-1} of sea water for 6 days and extracts made in the presence of 10 mM 2-mercaptoethanol and 0.25 mM PMSF. The flow rate was 27 ml h^{-1} and 4 ml fractions were collected. The elution positions of the molecular size markers (described in the Materials and Methods section) are indicated by perpendicular bars with molecular sizes indicated in thousands

at 0.8 mg Cd l^{-1} for the same time period. In contrast, glutathione S-transferase and esterase activities were both found to increase gradually reaching 135% and 180% respectively of the control values at 0.8 mg Cd l^{-1}. The in vitro effect of cadmium on glutathione S-transferases and esterases were however the opposite (Fig. 6) i.e. decrease of activity (with 50% of the control activity observed at 0.398 mM and 30 mM of cadmium respectively). The in vitro effect of cadmium on alkaline phosphatase activity was not studied due to precipitation of the metal in the stock solution prepared with the assay buffer (i.e. 0.1 M Tris-HCl, pH 8.0).

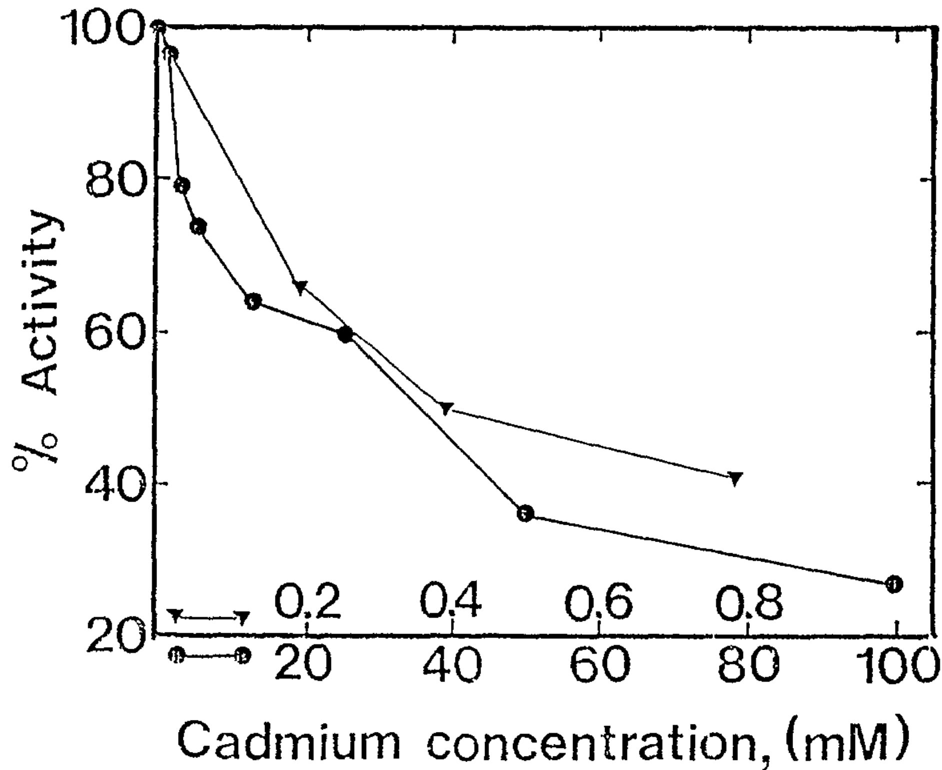

Fig. 5 *In vivo* effects of cadmium on the activity of glutathione S-transferase (▼—▼), alkaline phosphatase (◘—◘) and esterases (●—●). Shrimps were exposed to 0.2, 0.4 and 0.8 mg Cd l^{-1} of sea water for 8 days. Activity corresponding to 100% was 4.686 U ml^{-1}, 0.612 U ml^{-1} and 5.981 U ml^{-1} respectively

Since for both alkaline phosphatases and esterases (but not glutathione S-transferases) there are histochemical methods for enzyme activity staining on polyacrylamide gels, the observed changes in spectrophotometric assay measurements (Figs. 5 and 6) were analyzed for the identification of the underlying changes at the level of multiple molecular forms. Such isoforms were observed for both alkaline phosphatases and esterases in *C. tyrrhena* hepatopancreas (controls in Figs. 7 and 8). Gradual increase in cadmium exposure *in vivo*, caused an increase and eventual decrease in the staining intensity of alkaline phosphatase bands (Fig. 7A) while for esterases an increase in staining intensity of two of the more anodic bands (Fig. 7B, bands 1 and 2) was combined with the appearance of a group of intermediate mobility bands not previously observed in the control samples (Fig. 7B, area I) at 0.8 mg Cd l^{-1} of sea water for 16 days. For esterases *in vitro*, a decrease in the activity of most bands was followed by the concomitant appearance of the new group of bands already observed in the *in vivo* experiments (Fig. 8, lane 2). The bands of this electrophoretic mobility area were found to be of the acetylesterase-like type as they were inhibited by 10^{-4} M eserine (results not shown).

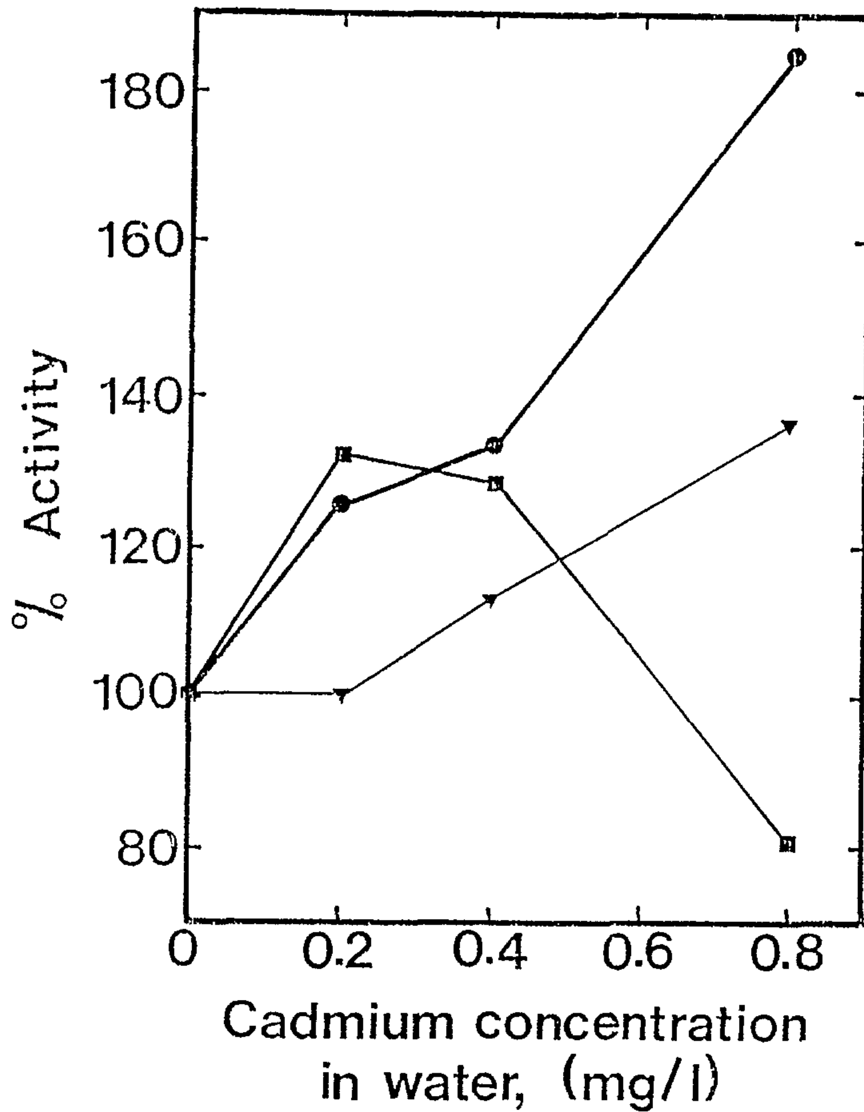

Fig. 6 In vitro effects of cadmium on the activity of glutathione S-transferases (▼—▼) and esterases (●—●). Shrimp extracts were incubated in cadmium concentrations from 0.2 mM to 100 mM

4. DISCUSSION

In the present investigation cadmium was provided through the surrounding medium as this has been reported to be an effective way for cadmium uptake for the related species of C. australiensis (Ahsanullah et al., 1984). Hepatopancreas was chosen for biochemical analysis as it has the highest size than any other crustacean organ in connection with high levels of bioaccumulated cadmium (see introduction). The levels of cadmium found in the hepatopancreas of C. tyrrhena were 7-12 times higher than those in the rest of the body at different exposure conditions to the metal, in agreement with reports on other crustacean

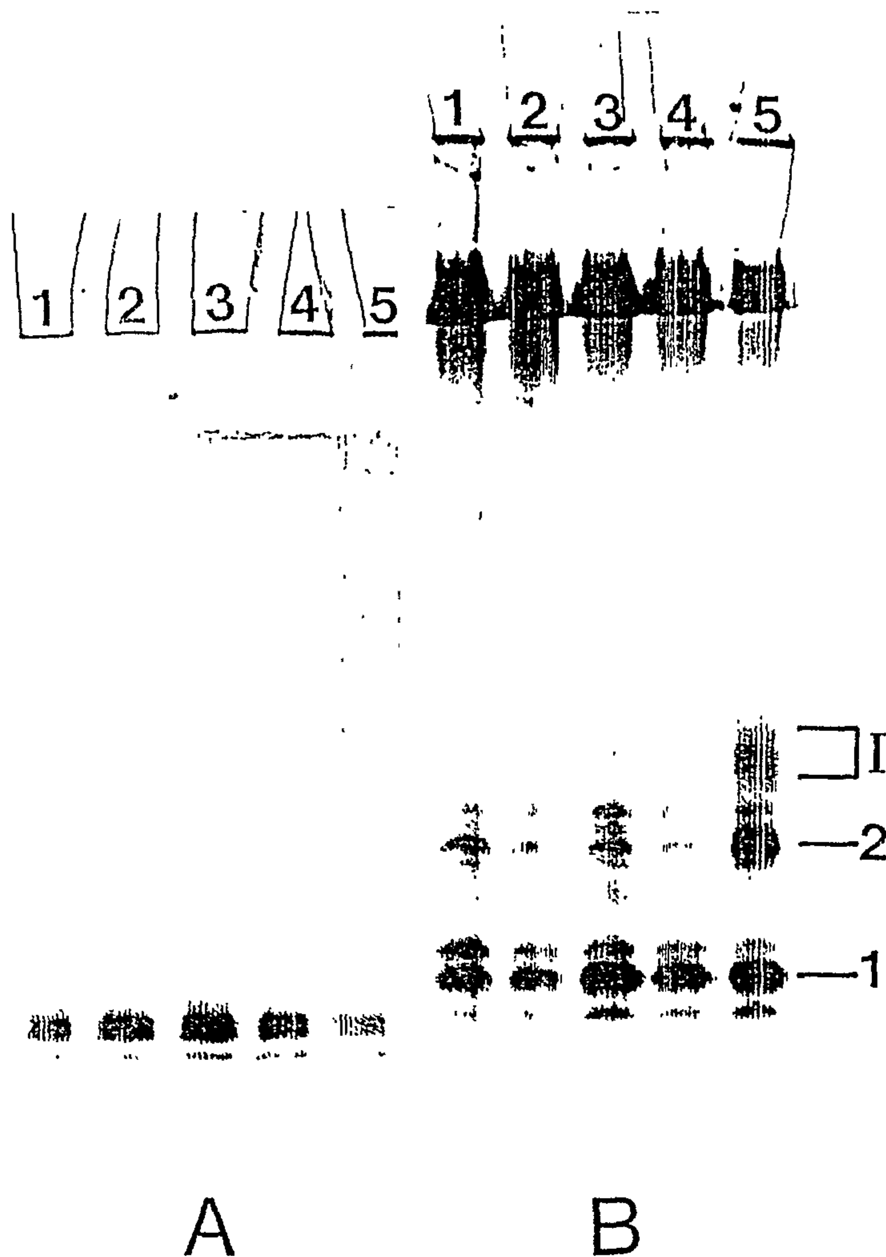

Fig. 7 *In vivo* effect of cadmium on alkaline phosphatase (A) and esterase (B) electrophoretic patterns. Shrimps were exposed for 8 days to 0.2 (lane 2), 0.4 (lane 3) and 0.8 mg Cd l^{-1} of sea water (lane 4) and for 16 days to 0.8 mg Cd l^{-1} of sea water (lane 5). Control animals were kept for 8 days (lane 1). For other indications see the Results section

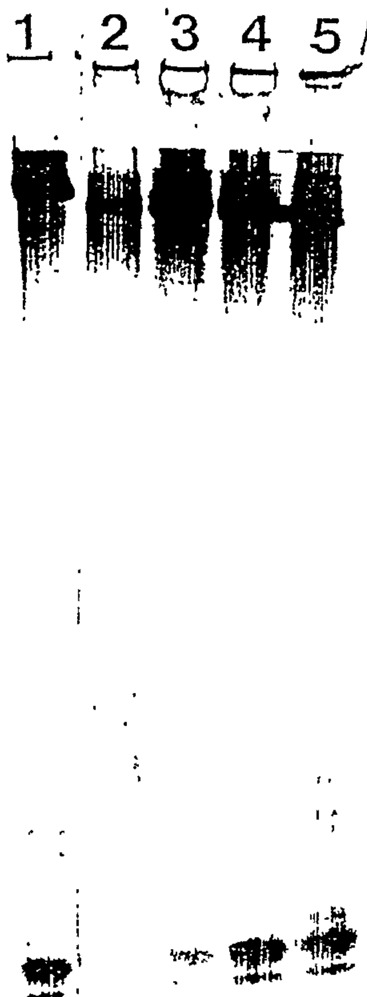

Fig. 8 In vitro effects of cadmium on esterase electrophoretic pattern. Hepatopancreas extracts were incubated with no cadmium (control in lane 1) or with final cadmium concentrations of 33.3 mM (lane 2), 3.33 mM (lane 3), 0.333 mM (lane 4) and 0.033 mM (lane 5)

species (Topping, 1973; Overnell and Trewhella, 1979; Davies et al., 1981; Bjerregaard, 1982; Engel and Brouwer, 1986).

Shrimps were exposed to levels of cadmium of 0.2-0.8 mg Cd l^{-1} of sea water for 2-8 days. These concentrations are approximately one order of magnitude lower than the value of 6.33 mg Cd l^{-1} of sea water reported as the LC_{50} value for 4 days for the related species of C. australiensis (Ahsanullah et al., 1981a) but two orders of magnitude higher than those reported in heavily polluted with cadmium natural environments e.g. 5 µg l-1 in Marseille (Aubert et al., 1980).

However, the range of levels of accumulated cadmium observed in C. tyrrhena hepatopancreas (24 to 106 $\mu g\ g^{-1}$ wet weight) for the above metal concentration and duration conditions, encloses the reported values for the highest accumulated metal in the hepatopancreas of the crustacean Cancer pagurus (50-60 $\mu g\ g^{-1}$ wet weight) in the environment of Orkney Islands (Scotland) (Overnell and Trewhella, 1979; Davies et al., 1981). It is suggested therefore that the biochemical changes observed in C. tyrrhena shrimps exposed to cadmium in the laboratory and discussed below are indicative of the extent and nature of such changes that might be found in crustaceans in polluted with cadmium natural environments.

The observed in C. tyrrhena increase in accumulated cadmium with increasing concentration of cadmium in water and/or duration of exposure is in agreement with what has been observed in other crustaceans such as Palaemon elegans (Papathanassiou and King, 1986; White and Rainbow, 1986), Carcinus maenas (Wright, 1977; Jennings et al., 1979; Amiard et al., 1987), Uca pugilator (O'Hara, 1973) including the related species C. australiensis (Ahsanullah et al., 1981b, 1984).

Soluble cadmium has been found in all crustaceans bound to proteins. However, conflicting results exist as to the nature and size of these metal-binding proteins. The range extends from the well characterized (isolated and sequenced) metallothioneins in the crab Scylla serrata of 57 and 58 residues homologous to the mammalian metallothioneins (Lerch et al., 1982) to the less characterized proteins of 3,600 and 12,000 in the crayfish Austropotamobius pallipes (Lyon et al., 1983) of 11,500 and 27,000 in the crab Carcinus maenas (Rainbow and Scott, 1979) of 9,000 in the crab Callinectes sapidus (Brouwer and Brouwer-Hoexum, 1984), of 30,000 in the lobster Homarus americanus (Engel et al., 1985) and of >68,000, 9,500 and <6,000 (Ray and White, 1981) or >70,000 45,000 and <5,000 (Engel and Brouwer, 1986) for the same species. The observed in C. tyrrhena Cd-binding proteins of 7,500 and 15,000 with the minor form of >70,000 (in the presence of 2-mercaptoethanol), fall within the above range of sizes of cadmium-binding proteins. However, further research is needed for their characterization as metallothionein or non-metallothionein-like. No significant amount of cadmium was found free but only bound to protein components. The fact that in the absence of 2-mercaptoethanol only one high molecular weight cadmium peak (>70,000) was observed may be explained by aggregation through oxidation of the low molecular weight forms, as metallothioneins are known to do (Suzuki and Yamamura, 1980; Minkel et al., 1980; Overnell, 1984).

Cadmium was found to have a significant effect on the expression of major peptides and proteins resolved by reversed phase HPLC and gradient polyacrylamide gel electrophoresis in C. tyrrhena hepatopancreas extracts. RP-HPLC, resolving polypeptides, showed the decrease in concentration of 4 peaks and the increase of one, while polyacrylamide gel electrophoresis, resolving larger polypeptides, showed the increase of the staining intensity of 6 bands as well as the appearance of one new band. One or more of the electrophoretic bands might represent Cd-binding proteins. The induction of protein synthesis in response to cadmium stress has been reported for rat thymic lymphocytes (induction by cadmium of the expression of 2 proteins resolved by 2D-electrophoresis; Maytin and Young, 1983) and

for the fish Mugil cephalus liver and gills (20% increase in total protein level after exposure to high cadmium concentration for a few days; Hilmy et al., 1985).

Glutathione S-transferases are enzymes playing an important role in detoxification of xenobiotics and their reactive metabolites, catalyzing the conjugation of glutathione with electrophilic and potentially alkylating agents which is the first step in mercapturic acid formation (Booth et al., 1961). Glutathione S-transferase activity in the hepatopancreas of C. tyrrhena was found to be observed in vitro is in agreement with similar studies in rat liver glutathione S-transferase (Dierickx, 1982). In contrast, in vivo, a limited increase of 30% was found at 0.8 mg Cd l^{-1} for 8 days. The latter is in agreement with a similar glutathione S-transferase activity increase caused by cadmium in Chinese hamster cell lines (Seagrave et al., 1983). However, other reports have shown a decrease from 40% for the crayfish Procambus clarkii (Almar et al., 1987) to 90% for the plaice Pleuronectes platessa (George and Young 1986) or no effect for rat liver (Pak, 1988). Since in C. tyrrhena the in vitro and in vivo effects are opposite, it is suggested that the enhanced activity observed in vivo might be caused by an increase at the protein level of glutathione S-transferase.

Alkaline phosphatase is a metalloenzyme with an active center containing zinc. This metal might be able to be replaced by cadmium causing enzyme activity inhibition as has been shown for the E. coli enzyme (Applebury et al., 1970; Lazdunski et al., 1969). In the scallop Mizuhopecten yessoensis exposed to cadmium (0.5 mg Cd l^{-1}) an initial inhibition at 14 days was followed by an activation at 30 days and a return to normal levels at 60 days (Evtushenko et al., 1986). Alkaline phosphatase activity in rat kidney brush border increased 2-fold after injections with high loads of cadmium (McEwen Nichols et al., 1981). In C. tyrrhena, in in vivo experiments with increasing cadmium levels in hepatopancreas, an initial increase in alkaline phosphatase activity was followed by a decrease. The changes in the total activity in the in vivo experiments appeared to be equally distributed to the multiple molecular forms of the enzyme resolved by electrophoresis.

The natural substrates and therefore the physiological function of esterases are not known in most cases. However they are identified by their ability to hydrolyse synthetic organic ester bonds. Esterases are widely distributed in animal tissues and consist of a large number of molecular forms (Haites et al., 1972; Salamastrakis and Haritos, 1988). It is the first time to the best of our knowledge that the effects of heavy metals, and cadmium in particular, on total activity and molecular forms of esterases have been studied. We looked upon this enzyme system as Rivière and Kerambrun (1983) had observed a differential inhibition of multiple molecular forms of esterases of the copepod Acartia clausi in a polluted area in Marseille (France). A striking inverse relationship was observed in C. tyrrhena in the in vitro and in vivo effects of cadmium on esterases with total activity decreasing in the former and increasing in the latter. At the level of individual multiple molecular forms, the in vivo increase of total activity was caused by the enhanced activity of two major bands and the appearance of a new group of bands of intermediate mobility. In in vitro experiments, the above two bands disappeared while the new group

of bands appeared again. These results taken together suggest a specific differential increase in the synthesis (and/or decrease in the degradation) of multiple molecular forms of esterases (bands 1 and 2 of Fig. 7) in response to cadmium accumulation in C. tyrrhena together with activation of esterases through interaction with cadmium (group of bands I in Fig. 7).

In conclusion, cadmium accumulation was found to cause qualitative and quantitative changes of soluble protein components in the hepatopancreas of the shrimp C. tyrrhena. Also the molecular targets of cadmium appear to be versatile rather than limited (e.g. to metal-binding proteins). Further investigation in this direction might help in the development of much needed sensitive and reliable bioassays for metal toxicity based on protein expression.

5. ACKNOWLEDGEMENTS

This work was carried out with the support of FAO grants GRE/26-G and GRE/32-G. Abid Ali Thaker was recipient of a fellowship from the Greek Ministry of Education. We are grateful to Dr. M. Economou at the Laboratory of Petrology (Department of Geology, University of Athens) for the use of the Atomic Absorption Spectrophotometer.

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STUDIES ON THE LETHAL AND SUBLETHAL EFFECTS OF CADMIUM ON SOME COMMERCIALLY CULTURED SPECIES OF THE MEDITERRANEAN

by

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1. INTRODUCTION

This report summarizes the research carried out during 1986, 1987 and 1988. Much of the work carried out in the earlier phases of the programme has now been published (Abel and Papoutsoglou, 1986; Papoutsoglou and Abel, 1988), so this work will be presented here only in summary form.

There are three principal phases of investigation:

a) Measurement of the lethal toxicity of cadmium to three fish species and one invertebrate species

b) Study of the sublethal toxicity and toxic effects of cadmium to *Tilapia aurea*

c) Studies on the isolation of metallothioneins from fish exposed to sublethal concentrations of cadmium.

2. LETHAL TOXICITY OF CADMIUM

Using conventional techniques, the lethal toxicity of cadmium to three fish species and one crustacean was estimated. The fish were *Cyprinus carpio*, *Tilapia aurea* and *Mugil cephalus*. The first two were exposed to cadmium in fresh water, and the third in sea water. The crustacean species was *Leander adspersus*.

All lethal toxicity tests were carried out under static conditions with replacement of the test solutions once every 24 hours. The precise experimental conditions and procedures have been described by Abel and Papoutsoglou (1986). The results are summarized in Figures 1 and 2.

Figure 1 shows the toxicity curves for *C. carpio*, *T. aurea* and *M. cephalus*. In the case of *M. cephalus*, it was possible to determine median survival times only at the highest concentrations tested (10 and 20 mg l^{-1}), even though the experiment continued for 40 days. Figure 2 shows the toxicity curves for *C. carpio* and *T. aurea* superimposed on curves drawn to the same scale for other fish, these curves having been obtained from accounts in the published literature. This diagram shows a number of interesting features about the lethal toxicity of cadmium to fish. Firstly, cadmium is a slow-acting poison and lethal threshold concentrations frequently do not occur even in experiments lasting 40

days or more. Inflections in the curves, and segments of curve over which mortality is apparently unrelated to poison concentration, commonly occur. Finally, the curves for different species frequently intersect. This means that the species which appears to be the most, or the least, sensitive to cadmium depends upon the duration of the test. Thus, in a test lasting 2 or 3 days, Noemacheilus barbatulatus is the most sensitive species; on the contrary in a test lasting for 40 days or more, it is the least sensitive. This clearly demonstrates that toxicity tests of short duration (a few days) are likely to produce misleading results. Consequently, there must be great doubt about the validity and usefulness of many published data on the toxicity of pollutants. This point is emphasized because it is not sufficiently widely understood, despite its having been made explicit in numerous examples over the last twenty years (Sprague, 1969, 1970; Abel, 1989).

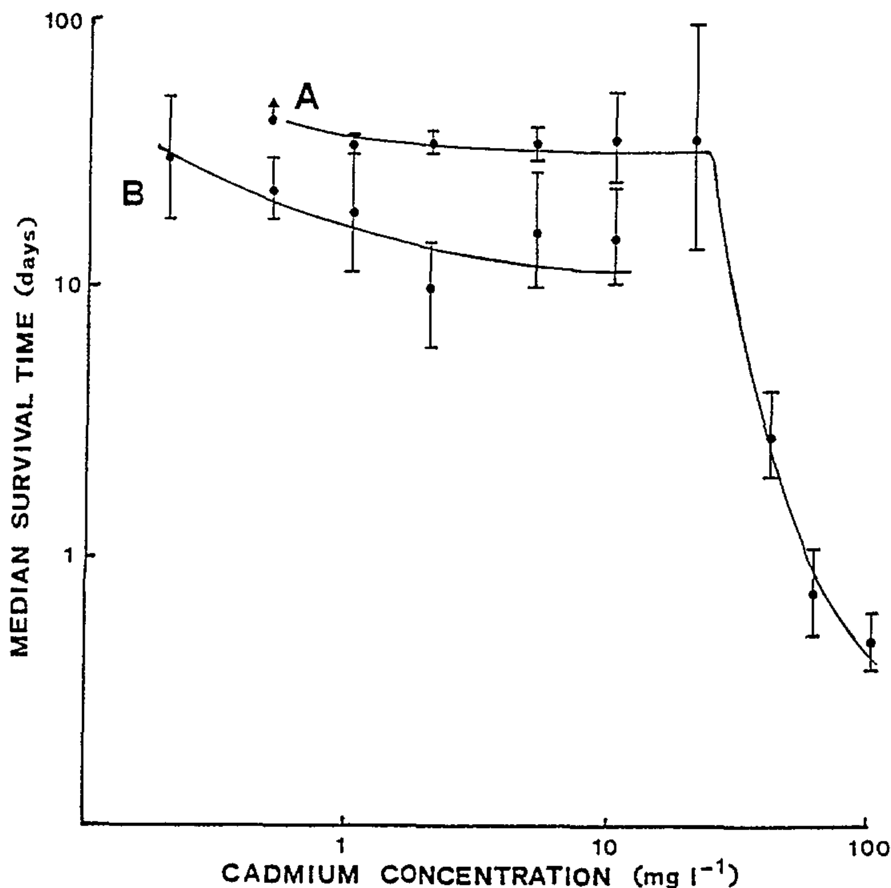

Fig. 1 Toxicity curves for Cyprinus carpio (B), Tilapia aurea (A) and Mugil cephalus (C)

The lethal toxicity of cadmium to the decapod Leander adspersus was measured under similar conditions, in an experiment lasting 40 days over the concentration range 0.5 mg l^{-1} - 20 mg l^{-1}. Mortality was below 50% in all concentrations except the highest two (10 and 20 mg l^{-1}). The 40-day LC_{50} of cadmium to Leander adspersus was approximately 6 mg l^{-1}.

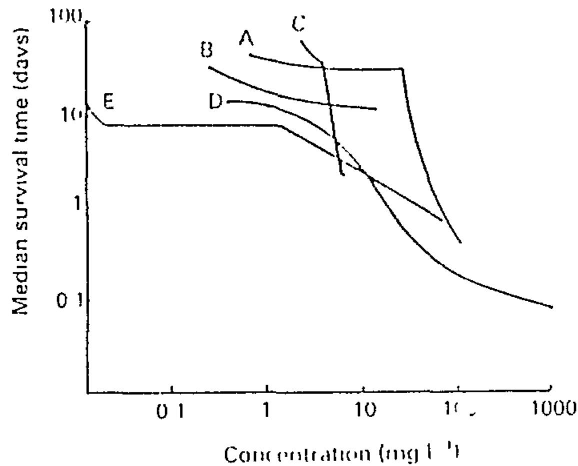

Fig. 2 Toxicity curves for five species exposed to cadmium, drawn to the same scale. A - Tilapia aurea. B - Cyprinus carpio. C- Noemacheilus barbatulatus. D - Gasterosteus aculeatus. E- Salmo gairdneri. For full details, see Abel and Papoutsoglou (1986).

3. SUBLETHAL TOXICITY OF CADMIUM TO TILAPIA AUREA

Study of the sublethal toxicity of pollutants to fish has mainly been confined to North American and North European species, many of them of little commercial importance. Tilapia species are important food fishes in many parts of the world, including the Mediterranean, where they are increasingly important in aquaculture. Relatively little information exists on their susceptibility to pollutants. The study described above demonstrated that in terms of lethal toxicity, Tilapia appears to be among the more resistant species to cadmium (see Fig. 2). It was therefore decided to study the sublethal effects of cadmium on Tilapia aurea.

A full account of this investigation has been given by Papoutsoglou and Abel (1988), so it will be described only briefly here. Juvenile T. aurea (mean weight 3.33 g) were placed in groups of 150 specimens in tanks of 250 litre capacity and maintained for 16 weeks in the following concentrations of cadmium: 0.1, 0.05, 0.02, 0.01 mg l^{-1} and control. Cadmium was administered through an automatic dosing system, and cadmium concentrations were measured daily. The following parameters were monitored: growth rate (weight and length), haematocrit (at 10 weeks and 16 weeks), mean moisture content, fat content, protein content, haemoglobin %, the percentage of damaged erythrocytes, and muscle cadmium concentrations. Full details of the experimental procedures are given in Papoutsoglou and Abel (1988).

The results are summarized in Figures 3 and 4, and in Table I. Cadmium, at the concentrations tested, had no effect on growth rate (Fig. 3), or on any of the variables measured except haematocrit at 10 weeks (Fig. 4) and the muscle concentrations of cadmium at the end of the experiment.

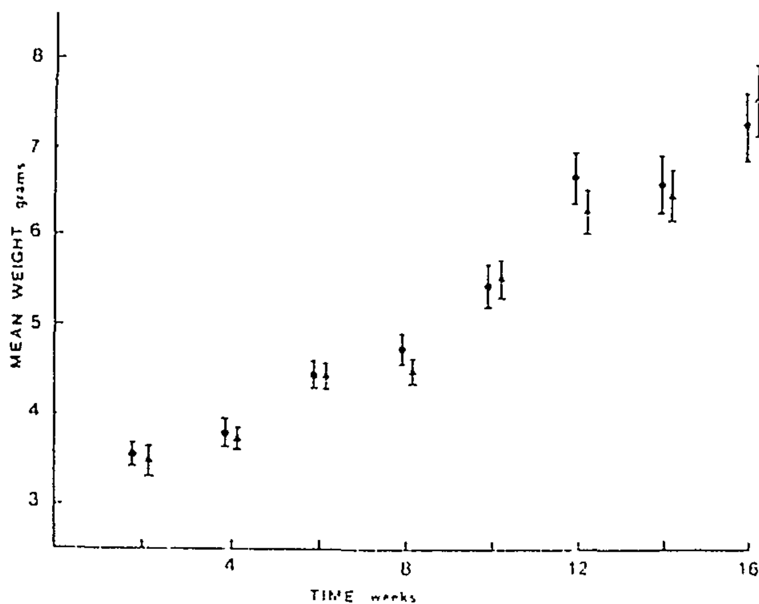

Fig. 3 Growth of control Tilapia aurea (●) and fish exposed to 0.052 mg l^{-1} (▲) cadmium during the sublethal toxicity experiment. No significant difference in mean weights is apparent at any stage of the experiment. Data for intermediate cadmium values have been omitted for clarity. Vertical bars represent 95% confidence limits. For full details, see Papoutsoglou and Abel (1988).

The effects of cadmium on haematocrit are interesting because the results suggest that the initial effect observable at 10 weeks is subsequently abolished, suggesting that the fish undergo some physiological adjustment to the presence of cadmium. In the context of toxicity testing, it again illustrates that the results of an experiment can be seriously influenced by the decision of the experimenter regarding the duration of the experiment. Thus, this decision should never be made on arbitrary grounds.

The significance of the muscle cadmium concentrations is that Tilapia is an important food fish. In most previous studies of cadmium sublethal toxicity and bioaccumulation, cadmium concentrations in muscle have not been measured. This is because most studies have shown that cadmium accumulates more rapidly in other tissues, such as kidney or liver; and the main purpose of the experiments has been to determine the concentration of cadmium which is harmless to the fish. Since Tilapia is a commercial fish species, it is important to consider the muscle concentrations of cadmium, and whether these represent a possible hazard to human health through consumption of contaminated

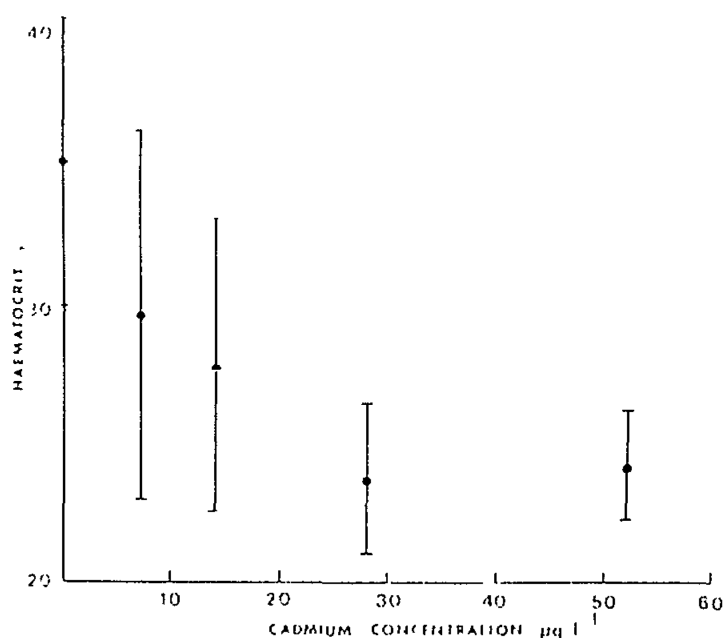

Fig. 4 Haematocrit values (mean and 95% confidence limits) for _Tilapia aurea_ exposed to various levels of cadmium for 10 weeks. Cadmium concentrations shown are measured rather than nominal values. For full details, see Papoutsoglou and Abel (1988).

fish. WHO guidelines for cadmium in foodstuffs tentatively suggest that a concentration of 0.5 mg kg^{-1} is the maximum acceptable level. These data suggest (Table I) that this concentration will be achieved in fish muscle if the fish are exposed to a concentration between 0.014 and 0.028 mg l^{-1}. At these concentrations, it is difficult to identify any serious harmful effect on the fish, but clearly there is a hazard to human consumers of fish flesh.

Taking these considerations into account, it is suggested that the maximum acceptable concentration of cadmium for _T. aurea_ lies between 0.014 and 0.028 mg l^{-1}. This estimate in fact agrees well with estimates based on other fish species, e.g. _Pimephales promelas_ (Pickering and Cast, 1972) and _Lepomis macrochirus_ (Eaton, 1974).

3. STUDIES ON THE ISOLATION OF METALLOTHIONEIN FROM FISH EXPOSED TO SUBLETHAL LEVELS OF CADMIUM

Metallothioneins have attracted much interest in recent years due to their potential use in monitoring the effects of heavy metal pollution. Metallothioneins are proteins of low molecular weight, with a high proportion of sulphydryl groups and a correspondingly high affinity for heavy metals. Numerous studies have shown that they are induced in the tissues of a wide range of vertebrate and invertebrate animals when the animals are challenged with abnormal quantities of heavy metals. They can be identified by passing the supernatant from homogenized and centrifuged tissue through a column chromatograph.

Table I

Values of some variables measured in Tilapia aurea after exposure to various concentrations of cadmium for 16 weeks. Cadmium concentrations are actual, measured values rather than the nominal concentrations referred to in the text. For full details see Papoutsoglou and Abel (1988).

Cd conc^{n} μg L^{-1}	52	28	14	6.8	Control
Mean weight g	7.60 (7.2-8.0)	7.91 (7.40-8.42)	7.33 (7.13-7.53)	7.74 (7.52-7.96)	7.28 (6.68-7.70)
Mean length cm.	7.66 (7.41-7.91)	7.61 (7.33-7.89)	7.52 (7.22-7.82)	7.92 (7.56-8.28)	7.48 (7.18-7.78)
% mortality	4.0	2.7	2.7	4.7	6.7
Mean moisture %	70.1	70.9	70.9	70.1	70.9
Mean fat % dry wt.	22.3	25.4	28.3	24.4	25.5
Mean protein % dry wt.	57.8	57.4	55.5	57.8	57.0
Mean ash % dry wt.	19.9	19.2	19.1	17.8	17.5
Haematocrit %	29.8 (23.5-36.1)	25.7 (20.6-30.8)	34.3 (28.5-40.1)	31.2 (28.8-33.6)	32.0 (26.7-37.3)
% damaged erythrocytes	9.2	3.2	5.0	3.9	1.0
Haemoglobin g 100mL^{-1}	10.29 (9.25-11.33)	9.94 (8.26-9.42)	9.50 (8.58-10.42)	8.48 (7.06-9.90)	10.08 (8.66-11.50)
Muscle Cd mg kg^{-1} net weight	0.92 (0.78-1.10)	0.72 (0.57-0.86)	0.23 (0.20-0.27)	0.12 (0.09-0.14)	0.06 (0.04-0.12)

Fractions containing metallothioneins are those which have the appropriate molecular weight, a higher absorbance at 250 nm than at 280 nm, and which contain high concentrations of heavy metal.

Interest in metallothioneins stems from the fact that since they are induced in the presence of elevated levels of heavy metals, they could possible be used to indicate that the ambient level of organism, either in an experimental situation or in the field. Some examples of the use of metallothioneins in field monitoring are available e.g. Roch et al. (1982). However, it remains to be established whether elevated metallothionein levels can be associated with measurable harm to the fish. Also, although the isolation of metallothioneins is relatively easy in a well-equipped laboratory, existing techniques rely upon the use of relatively large columns which are slow in use, and require relatively large expenditure on consumable and other materials. This provides some impediment to their routine use, especially in areas such as the Mediterranean where resources can be scarce. Large columns, though expensive to operate, provide greater resolution but require relatively large quantities of material for analysis. Small columns require less material, but offer limited resolution.

Therefore the purpose of this phase of the investigation was to attempt to develop methods of isolating metallothioneins on a relatively small scale, offering advantages of speed and economy and leading to the more widespread study of metallothioneins and their applications in the Mediterranean; and secondly, to attempt to use such methods to investigate the induction of metallothionein in Tilapia aurea under experimental conditions, and to associate metallothionein production with conventionally-determined sublethal toxic effects.

3.1 Development of small-scale methods

Conventional techniques for isolating metallothioneins from fish tissues rely upon the use of columns up to 100 cm in length and up to 5 cm diameter.

A series of experiments were carried out using a very small column (30 cm x 1 cm). These experiments were described in detail in the 1986-87 end-of-year report. Attempts were made to isolate metallothioneins from various fish tissues, and from fish tissue homogenates spiked with purified, commercially-available metallothionein. The results indicated that the resolution obtained in such a small column was inadequate.

In a later series of experiments, the size of the column was increased to 50 x 2.5 cm, and it appears that this is the smallest column which is feasible in practice. Some representative results are shown in Figures 5 and 6. Using this column, an attempt was made to investigate the production of metallothioneins in *Tilapia aurea* exposed to cadmium under conditions similar to those of the sublethal toxicity experiment described in section 2 of this report.

3.2 Production of metallothioneins in Tilapia aurea exposed to sublethal concentrations of cadmium

Tilapia aurea were exposed to each of a range of cadmium concentrations under conditions identical to those used in the sublethal toxicity experiment described in section 2 (Papoutsoglou and Abel, 1988), except that the range of concentrations was 0.2, 0.1, 0.05 and 0.02 mg l^{-1}, and control. At the end of the experiment, the fish livers were removed. To determine the presence of metallothioneins, approximately 1g of tissue was homogenized in 1.5 ml of 10 mM tris-acetate buffer at pH 7.4. The buffer contained 0.005 M β-mercapto-ethanol as a reducing agent. The homogenate was centrifuged at 100.000 x g for 1 hour at 4°C. The resulting cytosol was applied to the column, containing Sephadex G-75, and eluted with 0.015M tris-HCl buffer (pH 8.6). Fractions of approximately 1 ml were collected every 15 minutes. All chromatography was carried out at 4°C.

Each fraction was placed in a UV spectrophotometer and its absorbance at 250 nm and 280 nm measured. Subsequently each fraction was tested for the presence of cadmium by atomic absorption spectrophotometry. Fractions containing metallothionein are those which:

a) Have a high ration A_{250}/A_{280}
b) Contain a high level of cadmium
c) Have a molecular weight corresponding to about 12000.

Some typical results are shown in Figures 5 and 6.

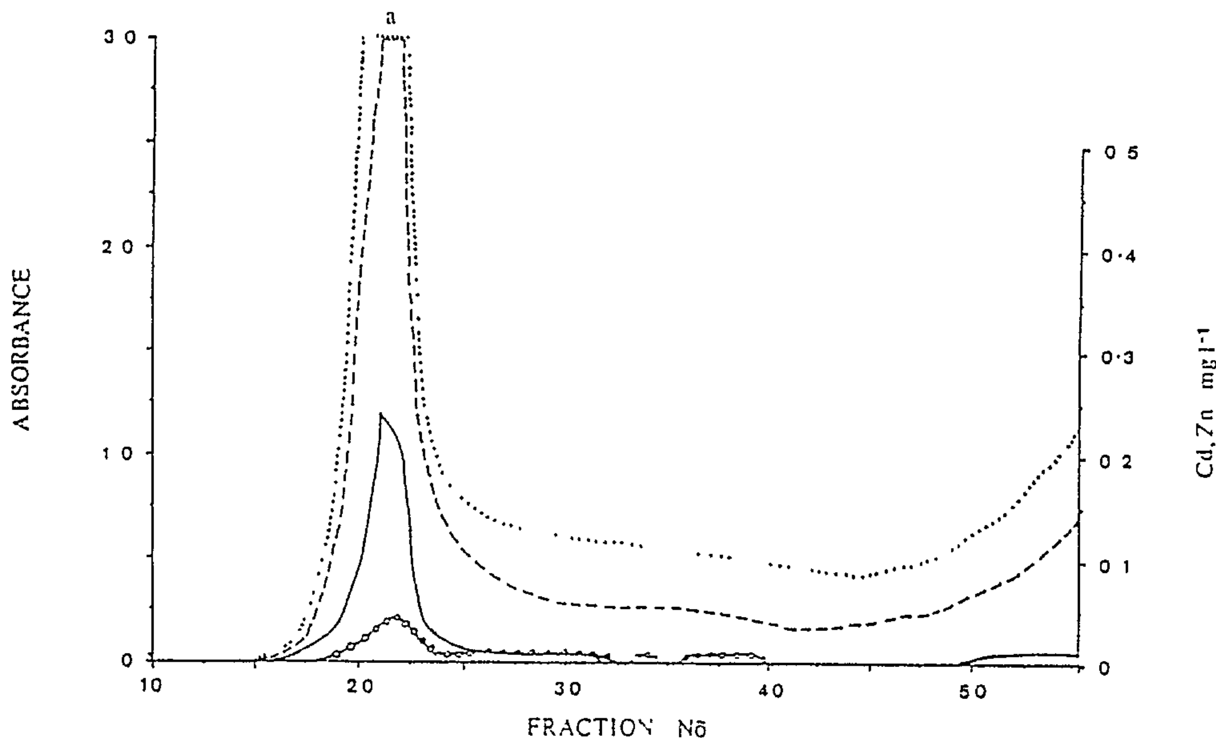

Fig. 5 Sephadex G-75 elution profile of liver extract from control *Tilapia aurea*. The metallothionein peak is marked "a". Key: ---- A_{280}; ····· A_{250}; -o-o-o- cadmium; ———— zinc

Figure 5 shows the elution profile of control fish, and Figure 6 the elution profile of fish exposed to 0.05 mg l^{-1} cadmium. Metallothionein appears to be present even in the control fish, as shown by the high A_{250}, the molecular weight corresponding to about 12000, and the presence of peaks of both zinc and cadmium. This suggests either that the fish were contaminated before the start of the experiment, or that trace amounts of cadmium entered the experimental tanks during the experiment, possibly via airborne dust. In fish exposed experimentally to cadmium (Fig. 6), the cadmium peak is more pronounced, as expected, indicating that metallothionein is induced by exposure to these concentrations of cadmium. However, the presence of prominent zinc peaks suggests either contamination of the experiment with zinc, or possibly reflects the status of zinc as a normal metabolite. These points require further investigation.

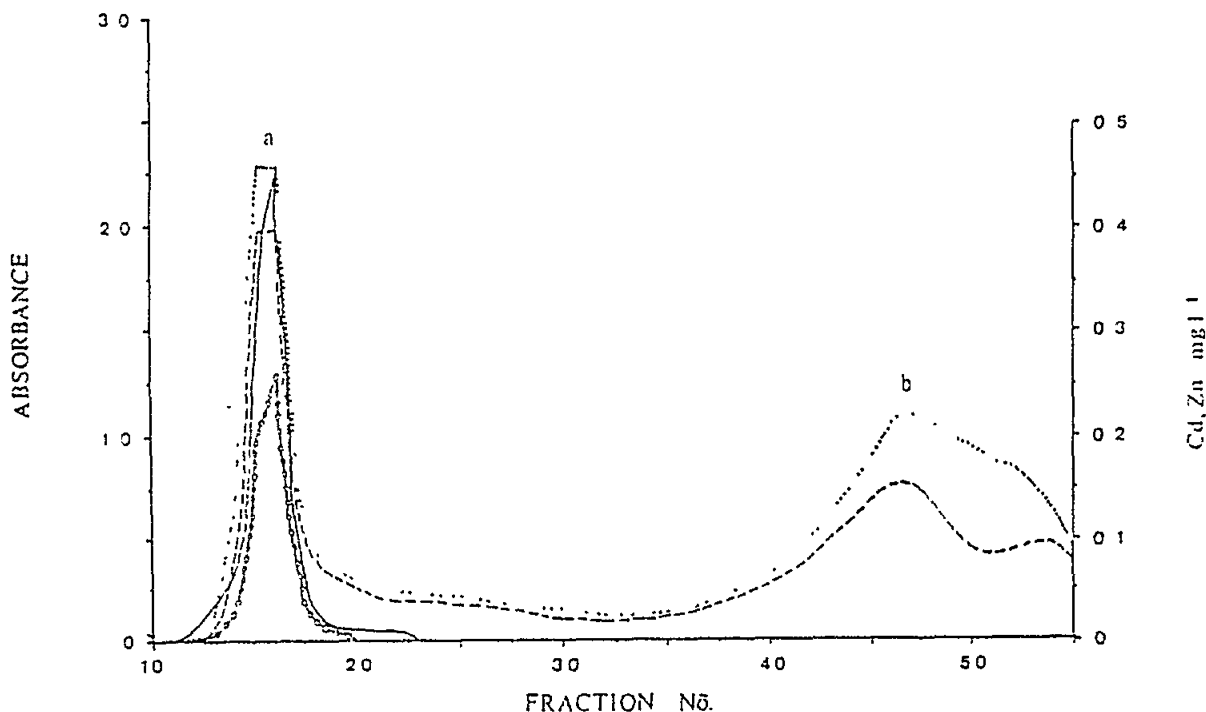

Fig. 6 Sephadex G-75 elution profile of liver extract from T. aurea exposed to 0.05 mg l^{-1} cadmium. The metallothiorein peak is labelled "a". Key: ---- A_{280}; ····· A_{250}; -o-o-o- cadmium; ——— zinc

4. CONCLUSIONS

Measurement of the lethal toxicity of cadmium to three fish species and one representative invertebrate species shows that there are no significant differences between the susceptibility of Mediterranean species and that of species from other parts of the world. Other than this, lethal toxicity tests yield little information which is likely to be directly relevant to marine pollution problems, since the concentrations of cadmium which are lethal are far in excess of those found even in severely polluted water. However, it is relevant to note that the true lethal concentrations are probably much smaller than the total cadmium concentrations recorded here. This is because of the effect of the sea water matrix on the chemical speciation of cadmium. Although total cadmium concentrations in the experimental tanks are known, no information is available on the precise form in which the cadmium is present. It is likely that a

large part of the cadmium is present in insoluble or biologically-unavailable forms, and that the experiments therefore underestimate its toxicity.

Study of the effects of sublethal concentrations of cadmium suggest that the maximum acceptable concentration of cadmium for Tilapia aurea lies between 0.014 and 0.03 mg l^{-1}, again a figure which is comparable to that obtained for several other species by other investigators. In part, this figure is based on consideration of the accumulation of cadmium in the fish muscle, as Tilapia is an important commercial fish.

Investigation of the production of metallothioneins in T. aurea exposed to sublethal levels of cadmium suggests that metallothioneins can be isolated and identified using a much smaller chromatography column than is customarily used, i.e. containing approximately one quarter the volume of Sephadex than is usual. This finding should allow considerable economies of time and cost, and lead to the more widespread use of metallothioneins as routine monitors of heavy metal contamination. Experimentally, it was found that metallothionein production occurs at cadmium concentrations comparable to those found to cause sublethal toxic effect, though further investigation is needed to determine whether there is any threshold cadmium concentration for metallothionein production. The interpretation of the present experiments is made difficult by the fact that metallothioneins were detected even in control fish, and further investigation is needed to establish whether this represents contamination during or before the experiment, or whether it is a feature of the normal heavy metal metabolism in the fish.

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COMPLEX POLLUTION EFFECTS OF TWO HEAVY METALS (MERCURY AND CADMIUM) THE GENETIC STRUCTURE OF POPULATIONS

by

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ABSTRACT

The major objective of this project was to study the effects of the metallic pollutants mercury and cadmium on enzyme systems of marine organisms. The marine organisms observed during this project were the shrimp *Palaemon elegans* and the gastropods *Cerithium scabridum*, *Littorina neritoides* and *Littorina punctata*.

All pollution experiments were carried out through controlled laboratory experimentation. For the two *Littorina* species a survey of natural populations along the Israeli Mediterranean shore was also conducted to compare the frequency of the alleles to that in the laboratory which were found to be sensitive to mercury in non-polluted sites versus a polluted site. The patterns found in the laboratory were confirmed in nature for all the enzymes examined in both species.

For all the species involved in this research we found a specific differential mortality for each type of pollution. The combined effect of mercury and cadmium differed from that of the individual metals and appeared like a specific different pollutant.

Therefore, we concluded that it would be impractical to use an universal critical value for mercury or cadmium pollution. Rather, for each site, both metallic pollutants should be considered in unison. Among the species studied, *C. scabridum* was the most polymorphic and eight loci could be observed. When each of these loci was analyzed separately, four loci showed differential survivorship in at least one of the pollutants. The loci that did not show differential survivorship, displayed when analyzed together, were two four-loci genetic complexes and a two-locus genetic complex showing differential survivorship. We concluded that allozymic polymorphism and multilocus genetic structures respond to the selective pressures imposed by heavy metal pollution, thus providing a possible biological monitor of pollution.

1. INTRODUCTION

The debate whether allelic isozyme variation in natural populations is selectively meaningful (Lewontin, 1974; Wills, 1981) or adaptively neutral (Kimura and Ohta, 1974; Nei, 1975) is currently going on. This problem has both theoretical and practical implications. If neutral, then the abundant allelic isozyme polymorphisms found in natural populations of plants and animals are predominantly of interest

only as genetic markers and can hardly provide the basis for adaptive evolution. However, if this variation is largely adaptive, then it could be exploitable in breeding, meaningful in conservation, and could be used as a detector and monitor of environmental quality.

The physiological stress approach allows us to subject a polymorphic population to stress and determine the differential fitness of allelic isozyme genotypes. During recent years, we have employed artificial pollutants of marine organisms to create a stressful environment in order to amplify any potential differences between allozyme genotypes found in nature.

Pollution effects on population genetic structure by heavy metals and especially by mercury and cadmium, each as a separate pollutant (Nevo et al., 1983), were previously observed on the marine shrimp Palaemon elegans for the enzyme PGM (phosphoglucose isomerase) and on the marine gastropods Monodonta turbinata and M. turbiformis for the enzyme PGI (phosphoglucoisomerase). In these experiments we identified allozyme genotypes that were both tolerant and sensitive to the pollutants.

The present study is a test comparing separate pollutant effects of mercury and cadmium with their interactive effect on the marine gastropods Cerithium scabridum, Littorina punctata and L. neritoides, and on the shrimp Palaemon elegans. We present here evidence indicating that the interactive effects of the two heavy metal pollutants differ from their separate effects.

2. MATERIALS AND METHODS

Cerithium scabridum is a marine gastropod originating from the Red Sea which has colonized the eastern Mediterranean Sea through the Suez Canal. Its zoogeographical distribution is presented by Barash and Danin (1983). Samples consisting of several hundreds of individuals were collected at Akko (North of Haifa Bay), from rocky bottom pools and were introduced into 25 liter sea water aquaria (70 x 30 x 40 cm) at the Institute of Evolution, University of Haifa.

The two Littorina species tend to crawl on the aquaria walls, above the water level. In order to avoid this the several hundreds ofindividuals collected from the rocky shores of Haifa region were placed in quadrangular cages (25 x 25 x 5 cm) made of perspex. These cages were subdivided into smaller interconnected cells (5 x 5 x 5 cm) by a plastic net so that water currents could pass freely through all cells. Each cell held two animals.

The shrimp, Palaemon elegans, is a widespread and abundant species of the marine littoral zone along the Mediterranean Sea coasts. Samples of hundreds of individuals were collected near Mikhmoret, a known unpolluted coast, south of Haifa Bay. The animals were brought to the laboratory and introduced in batches of 50 individuals in cages similar to those described above. Each shrimp was put into its own cell to prevent, as much as possible, aggressive and predatory interactions.

Fresh sea water for the experiments was pumped from 30m depth at the Shikmona National Institute of Oceanography. Conditions in all aquaria were identical (22°C; pH = 8.1; constant aeration and deprivation of food).

The pollutants chosen for these experiments were $HgCl_2$, $CdCl_2$ and a combination of $HgCl_2$+$CdCl_2$. In preliminary experiments, these pollutants were found to cause the highest mortality rate in the shortest time among several pollutants tested (Pb, Zn, Cu).

The duration of the tests (until LD_{50} was achieved) varied depending upon the concentrations of pollutants (Table I). All tests conducted simultaneously were matched with one control. Survival in the controls was always almost 100%; otherwise, all experiments were discarded. The experimental organisms were observed daily, and dead animals were removed and deep frozen (-80°C), as were all survivors at the termination of the tests. For the electrophoretic analysis, whole frozen animals were homogenized and studied by horizontal starch gel electrophoresis (Selander et al., 1971).

Table II presents loci and alleles of the enzymes of Cerithium scabridum analyzed for sensitivity to heavy metal pollution.

For the Littorina, only PGI (phosphoglucose isomerase) in both species and (AP) aminopeptidase in L. neritoides were found suitable for pollution experiments, which depend on the following stringent criteria: a) Loci tested for pollution must be strongly polymorphic, (>10%) in order to detect differential mortality in sample sizes under hundreds of animals. b) the enzyme tested must remain active also in the dead animals, so that the distribution of genotypes can be compared directly between dead and live animals; therefore no error variation is added that would bias the results. c) A high resolution is imperative when scoring the electrophoretic results because the difference in allozymic frequencies between live and dead animals may be small. PGI for both species had 3 alleles (F, M, S) and AP had 4 alleles (F, M+, M, S). The PGM locus of Palaemon elegans consisted of five alleles designated F+, F, M, S and S-.

Samples from 8 populations of L. punctata (252 animals) and of L. neritoides (228 animals) from the same sites along a north-south transect of the Mediterranean sea (from Akhziv in the north to Ashkelon in the south), were tested for allozymic variation at the loci in which allozyme genotypes showed differential viability in mercury pollution laboratory experiments.

3. RESULTS

3.1 Cerithium scabridum

The results for the four enzymes showing differential patterns of heterozygote survivorship are summarized in Table I. The full name of the enzyme and a detailed presentation of the genotypic and allelic frequencies are given in Table III. All rare alleles (which appeared only once) were excluded from Table I. The rare alleles were found in heterozygous conditions in the dead.

Table I

Differential patterns of survivorship of heterozygotes in three types of pollution (Hg, Cd and Hg+Cd), in *Cerithium scabridum*.

Pollutant	Hg	Cd	Hg+Cd
Pollutant Concentration g/liter	0.0010-0.0018	0.004-0.01	0.0012-0.0004
No. of tests	6	6	6
Total			
Live	322	232	196
Dead	340	456	348
Analyzed			
Live	60	72	72
Dead	60	72	72

Proportion of Heterozygotes: Locus:	Hg Live	Hg Dead	Cd Live	Cd Dead	Hg+Cd Live	Hg+Cd Dead
AAT	0.170	0.474**(1)	0.150	0.221	0.212	0.235
ALP	0. 95	0.213	0.250	0.200	0.111	0.286**(1)
G6PD	0. 14	0.137	0.118	0.186*(2)	0.266	0.225
PGI	0.214	0.222	0.113	0.239*(1)	0.220	0.233

* $p<0.05$
** $p<0.005$
(1) By Bailey test
(2) By Sign test

Table II

Loci and alleles of the enzymes of *Cerithium scabridum* analyzed for sensitivity to heavy metal pollution.

Enzyme	Abbreviation	Alleles
Acid phosphatase	ACPH	F, M, S, S-
Alkaline phosphatase	ALP	F+, F, M, S, S-
Aspartate amino transferase	AAT	F+, F-, F, M, S
Esterase	EST	F, M, S
Glycerate dehydrogenase	GLYDH	F, M, S+, S, S-
Glucose-6-phosphate dehydrogenose	G6PGD	F, M+, M, M-, S
Phosphoglucomutase	PGM	F+, F, F-, M+, M, M-,S
Phosphoglucose isomerase	PGI	F+, F, M, S

Table III

Genotype distribution in Cerithium scabridum for the enzymes affected by the pollutants mercury, cadmium and the joint effect of cadmium and mercury when each locus is analyzed separately.

Pollutant		Hg		Cd		Hg+Cd	
Enzyme		Live	Dead	Live	Dead	Live	Dead
	Genotypes						
Aspartic acid	MM	0.509**	0.228	0.825	0.705	0.531	0.500
transaminase	SS	0.321	0.298	0.050	0.000	0.250	0.265
AAT	MS	0.170**	0.456	0.125*	0.273	0.219	0.235
	Alleles						
	M	0.594	0.456	0.889	0.841	0.641	0.617
	S	0.406	0.546	0.111	0.159	0.359	0.383
	Genotypes						
Alkaline	MM	0.318	0.283	0.535	0.333	0.599	0.492
phosphatase	FF	0.273	0.283	0.070	0.282	0.209	0.175
	SS	0.114	0.152	0.093	0.154	0.079	0.048
	MF	0.182	0.087	0.116	0.128	0.041	0.095
	MS	0.114	0.065	0.140	0.103	0.070	0.175
	SF	0.000	0.065	0.023	0.000	0.000	0.016
	Alleles						
	M	0.466	0.358	0.663*	0.449	0.625	0.627
	F	0.364	0.359	0.163*	0.365	0.230	0.226
	S	0.170	0.283	0.174	0.205	0.115	0.145
	Genotypes						
Glucose-6-	MM	0.727	0.824	0.632	0.537	0.540	0.541
phosphate	FF	0.036	0.020	0.103	0.209	0.048	0.115
dehydrogenase	SS	0.036	0.020	0.103	0.104	0.159	0.098
G6PD	MF	0.109	0.020	0.059	0.090	0.063	0.066
	MS	0.091	0.118	0.088	0.015	0.175	0.148
	SF	0.000	0.000	0.015	0.045	0.016	0.033
	Alleles						
	M	0.827	0.892	0.706*	0.564	0.659	0.648
	F	0.091	0.029	0.140	0.264	0.071	0.163
	S	0.082	0.079	0.155	0.171	0.270	0.188
	Genotypes						
Phosphogluco-	MM	0.786	0.824	0.849	0.732	0.780	0.766
isomerase	FF	0.000	0.000	0.014	0.028	0.000	0.000
PGI	FM	0.196	0.123	0.110	0.155	0.169	0.167
	MS	0.018	0.053	0.027	0.085	0.051	0.050
	SF	0.000	0.000	0.000	0.000	0.000	0.017
	Alleles						
	M	0.893	0.912	0.918	0.852	0.890	0.875
	F	0.098	0.061	0.069	0.106	0.085	0.092
	S	0.009	0.027	0.013	0.042	0.020	0.033

* $p<0.05$ by Bailey test
** $p<0.01$ by Bailey test

As can be seen in Table II, in respect to the pollutant influence on the heterozygote distribution, it appears that each type of pollution affects a different specific enzyme. Yet for both AAT and PGI selection based on the heterozygotes display a similar trend in all three pollution treatments, though it is not always significant. When both pollutants are present, selection is based on ALP heterozygotes, unlike the situation when each pollutant is tested alone. Similarly, in cadmium pollution, G6PD heterozygote frequency in the dead fraction is higher, unlike the situation in the other two pollution treatments. In cases of significant differential survivorship of heterozygotes due to pollution - the proportion of heterozygotes was higher in the dead animal fraction.

Table III presents evidence enabling us to specify whether the selection is actually against the heterozygotes or only against a dominant allele. The enzyme G6PD demonstrates in cadmium pollution the occurrence of a resistant allele (M) and a sensitive allele (F). Even though the difference between the frequency of the allele F in the survivors as compared with the dead animals is not statistically significant, each genotype including allele F shows higher sensitivity to cadmium pollution. The enzyme G6PD also demonstrated the specificity of response for each type of pollution. The three treatments show no similar trends in either genotype or allele frequencies. While analyzing each locus by itself, may be profitable one should recall that natural selection may frequently be operating on multilocus structures, or the entire genotype (Franklin and Lewontin, 1970).

Figure 1 presents multilocus genetic complexes including only those enzymes that showed no differential survivorship when individual loci were analyzed.

The comparison between the frequencies of multiloci complexes among the dead and among the live animals, was performed according to the Bailey test (Bailey, 1959).

In mercury pollution the resistant 4 loci complex of ACPH, AAT, GLYDH, PGM was F-, M-, S-, M-. This combination of alleles was more resistant to mercury, irrelevant of the nature of the second allele in each locus, apart from GLYDH, where (S-) includes all (S) bearing genotypes excluding S+S. The same trend, but nonsignificant was observed in cadmium pollution. In the combined pollution this trend is reversed but even though the difference between the frequency of this genetic complex in the fraction of the dead versus the fraction of the life, is huge, it is not statistically significant since the number of animals for whom all four enzymes could be read was small.

The same holds true for the ALP, AAT, GLYDH, PGM complex, where the S-, M-, M-, M- genotypes were more resistant in mercury pollution, but the trend was reversed in cadmium and in the combined pollution. The reversion of this trend, meaning that the complex S-, M-, M-, M-, is more sensitive to cadmium pollution was statistically significant, but for the combined pollution this is not statistically significant, again, due to the small number of animals where all four enzymes could be stained. Therefore, for the influence of the combined pollution two

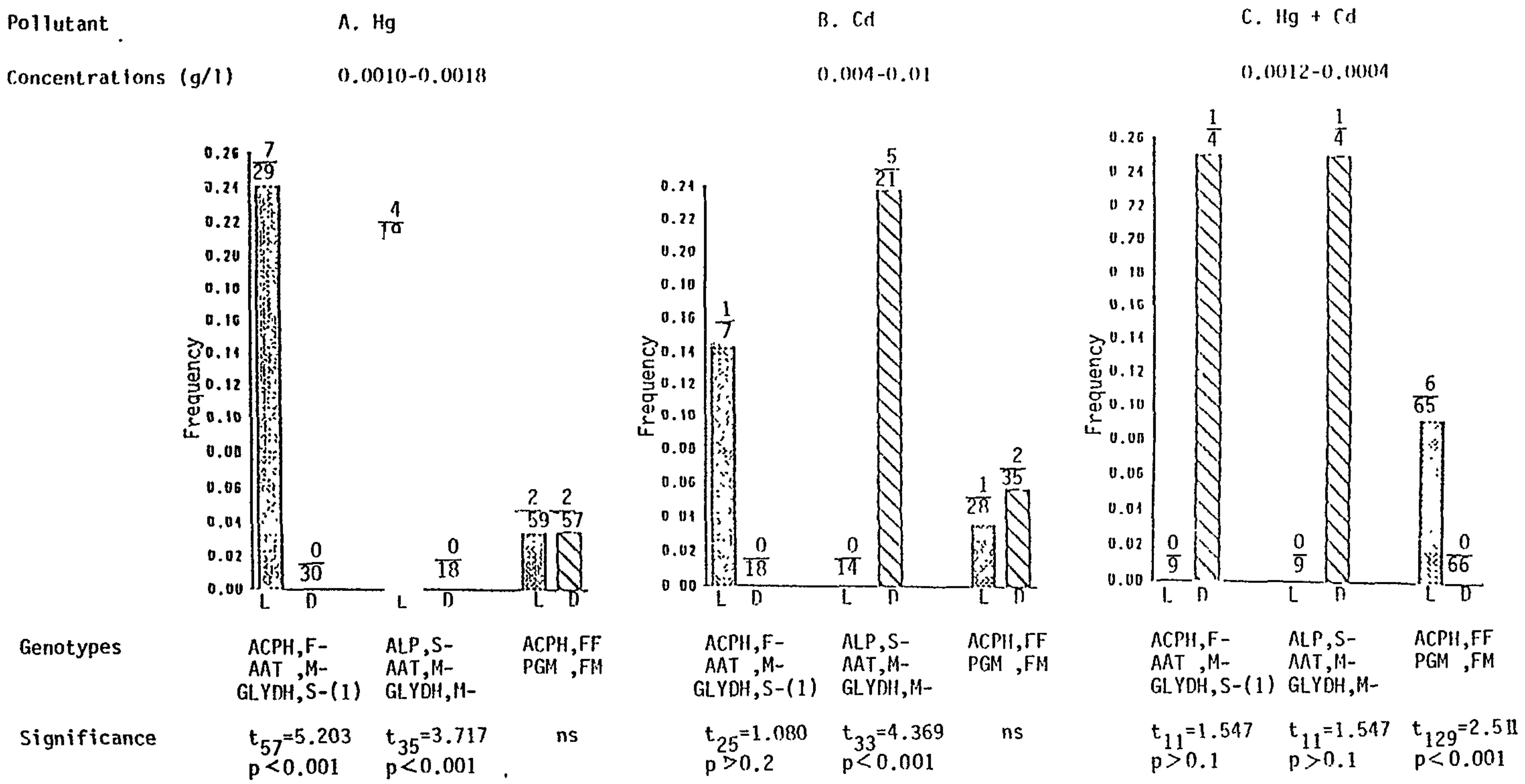

Fig. 1 Differential survivorship of multilocus genotypes of *C. scabridum*

locus complexes were analyzed. The ACPH, PGM complex FF, FM was more resistant to the combined pollution but not for each pollutant separately.

These results demonstrate not only that when single loci seem to be neutral to selection there may be found multilocus co-adapted complexes, but also, as encountered previously in single locus differential survivorship, that each pollutant as well as the combined pollution, influences in a unique and specific way the allozymic polymorphisms represented in this study in multilocus genetic complexes.

3.2 Littorina

3.2.1 Laboratory pollution experiments

Tables IV and V present the frequencies of all the genotypes and alleles in the survivor fraction and in the dead fraction. The results for Littorina punctata are summarized in Table IV. There are general trends for all pollutants as well as a specific influence of the interaction between cadmium and mercury.

3.2.1.1 Patterns specific for combined pollution

The genotype FM showed a unique and significant resistance to the combined influence of Hg+Cd.

While for both Hg and for the Cd treatments the heterozygotes were more sensitive to pollution at the concentrations applied, in the Hg+Cd group there were more heterozygotes in the live than in the dead fraction (but not significantly so).

3.2.1.2 Patterns common to all types of pollution, but varying in significance

The MM genotype was significantly more frequent in the surviving fraction, for the two pollutants, mercury and cadmium, but in the (Hg+Cd) treatment group this tendency was not statistically significant.

The SS genotype was found exclusively in the non-surviving fraction, but only in the Hg+Cd group was this tendency statistically significant.

The MS genotype was more abundant among the dead, but for Hg this tendency was not statistically significant.

The genotype FS was less abundant among the live animals, but this reached statistical significance only in Cd pollution.

The M allele was more frequent among the live animals, but in no separate test was this tendency statistically significant.

The allele S was more frequent among the dead, but this pattern reached statistical significance only in Hg+Cd.

Table IV

Differential patterns of survivorship of allelic isozyme genotypes of PGI L. punctata in three types of pollution (Hg, Cd and Hg+Cd).

Pollutant	Hg		Cd		Hg+Cd	
Pollutant concentration g/liter	0.001-0.030		0.005-0.030		0.001-0.30	
No. of tests	4		5		5	
Analyzed	Live	Dead	Live	Dead	Live	Dead
N=	60	60	58	57	68	68
Allelic isozyme Genotypes						
MM21	.666	.450*.	.656	.410**	.367	.279
FF	.067	.050	.155	.054	.132	.147
SS	.000	.033	.000	.054	.000	.176*
FM	.083	.0183	.017	.071	.398	.221*
MS	.117	.150	.172	.286**	.000	.074*
FS	.067	.133	.000	.125**	.103	.103
Alleles						
M	.766	.617	.750	.578	.566	.427
F	.142	.208	.164	.158	.383	.309
S	.092	.175	.086	.254	.051	.264*
Proportion of heterozygotes	.267	.466*	.190	.526***	.723	.563

by Bailey test (Bailey, 1959)
* $p<0.05$
** $p<0.01$
*** $p<0.001$

In conclusion, considering the PGI system in L. punctata the mercury pollution selects for the genotype MM and against heterozygotes in general; the cadmium pollution selects for the genotype MM and against heterozygotes, (especially against MS and FS). The combined mercury-cadmium pollution selects for the genotype FM and against the genotypes SS, MS, and in general it selects for the S allele.

Table V summarizes the results for Littorina neritoides. The concentration of pollutants was the same as for L. punctata. Table Va presents the results of the enzyme PGI while Table Vb presents the results of the enzyme AP.

Considering the results presented in Table Va, there are general trends for all pollutants, but the statistical significance of these trends varies from one treatment to another.

Table V

Differential patterns of survivorship of allelic isozyme genotypes of Littorina neritoides in three types of pollution (Hg, Cd and Hg+Cd).

a. Phosphoglucose isomerase (PGI)

Pollutant No. of test	Hg 9		Cd 7		Hg+Cd 7	
Analyzed N=sample size	Live 87	Dead 87	Live 70	Dead 68	Live 59	Dead 59
Allelic isozyme Genotypes						
MM	.694	.564	.557	.412	.678	.474*
FF	.059	.092	.100	.029	.051	.085
SS	.000	.034	.029	.074	.034	.119
FM	.212	.241	.257	.382	.169	.136
MS	.035	.069	.043	.044	.068	.135
FS	.000	.000	.014	.059	.000	.051
Alleles						
M	.818	.719	.707	.625	.796	.609*
F	.165	.213	.236	.249	.136	.179
S	.017	.068(1)	.057	.126	.068	.212*
Proportion of hetergozygotes	.253	.310	.314	.485*	.237	.322

* $p<0.05$ by Bailey test (Bailey, 1959)
(1) $p<0.05$ by sign test (Siegel, 1956)

The genotypic frequency of MM and the allelic frequency of M were higher among the live animals, but this trend is significant only in Hg+Cd.

The allelic frequency of S is higher among the dead, but in Cd this is not significant. In Hg+Cd the results are significant according to the Bailey test, in Hg out of nine tests, three tests had a similar allelic value of S for the live and the dead fraction and six tests showed a higher proportion of S in the dead fraction ($p<0.05$ by sign test) (Siegel, 1956).

The proportion of heterozygotes was higher among the dead but only for Cd was this statistically significant.

Table V (cont.)

b. Amino peptidase (AP)

Pollutant No. of tests	Hg 5		Cd 5		Hg+Cd 9	
Analyzed N=	Live 45	Dead 45	Live 43	Dead 47	Live 79	Dead 72
Allelic isozyme Genotypes						
MM	.111	.244	.348	.064	.317	.208
FF	.044	.134	.140	.064	.064	.167
SS	.156	.022*	.140	.192	.076	.069
M+M+	.022	.134*	.047	.064	.102	.153
F+F+	.000	.000	.047	.000	.026	.013
S-S-	.022	.022	.000	.021	.038	.000
FM	.044	.066	.140	.256	.064	.041
MS	.089	.022	.047	.064	.026	.083
M+M	.178	.111	.000	.107*	.128	.222(1)
MS-	.000	.000	.000	.021	.000	.000
F+M	.000	.000	.023	.084	.000	.000
FM+	.044	.067	.000	.021	.089	.041
FS	.156	.067	.023	.021	.000	.013
FS-	.000	.022	.000	.000	.014	.000
F+F	.000	.067	.023	.000	.014	.013
M+S	.134	.022	.000	.000	.014	.013
SS-	.000	.000	.023	.021	.014	.000
F+M+	.000	.000	.000	.000	.014	.027
Alleles						
M	.266	.344	.453	.330	.426	.381*
F	.166	.278	.244	.213	.154	.221
S	.346	.077*	.175	.245	.096	.124*
M+	.200	.234	.047	.128	.225	.291
F+	.000	.034	.070	.042	.040	.033
S-	.022	.033	.011	.042	.059	.000
Proportion of heterozygotes	.644	.444	.278	.617**	.215	.306

* $p<0.05$ by Bailey test (Bailey, 1959)
** $p<0.001$ by Bailey test
(1) $p<0.05$ by sign test (Siegel, 1956)

In conclusion, considering the PGI system in L. neritoides the mercury pollution selects against allele S, the cadmium pollution selects against heterozygotes in general, and the combined pollution selects for the genotype MM and the allele M in general and against the allele S. For both species L. punctata and L. neritoides the combined mercury-cadmium pollution selected against the slowest allele S, while cadmium alone

selected against heterozygotes in general. This pattern of cadmium pollution was also encountered also for two species of Monodonta turbinata and M. turbiformis, and for Cerithium scabridum (Lavie and Nevo, 1986).

Considering Table Vb, the influence of Hg seems to be unique. The only common trend for all treatments is the abundance of the genotype M+M+ and the allele M+ among the dead. But only for Hg is the M+M+ genotype significantly more sensitive. Hg is unique: (a) in selecting for genotype SS and the allele S; (b) in not showing the sensitivity of the M+M genotype. In the combined treatment the frequency of the M+M genotype was higher among the dead in all six tests where it was present. In the Cd treatment group no M+M genotype was encountered in the live fraction, and (c) while in the Cd group there is a highly significant sensitivity of heterozygote genotypes and Hg+Cd shows the same trend but not significantly so, whereas in the Hg treatment group this trend is reversed (not significant).

Summarizing the results for the AP system in L. neritoides the mercury pollution selects for the genotype SS and the allele S, and against the genotype $M^{+}M$. Cadmium pollution selects against the genotype $M^{+}M$ and heterozygotes in general and the combined mercury-cadmium pollution selects for the allele M and against the genotype $M^{+}M$ and the allele S.

3.2.2 Prediction and verification in nature

In order to link between laboratory and nature we compared the gene frequencies of a natural population at a polluted site to natural populations from unpolluted sites. A survey along the Mediterranean Coast has shown that the highest levels of mercury pollution occurred near Akko due to industrial discharge into the Bay (Roth and Hornung, 1977). Specimens of the Monodonta turbinata marine gastropods collected at Akko were found to concentrate mercury at levels of about 20 times higher than animals from the unpolluted Shikmona site (Hornung et al., 1981). For the shrimp Palaemon elegans and for the gastropod Monodonta turbinata the PGM and PGI allozymes genotypes most resistant to mercury pollution in the laboratory, were found in the highest frequencies near the mercury polluted sea site of Akko as predicted (Nevo et al., 1984).

The present study is a second successful attempt to verify laboratory predictions in nature. Here we present evidence for the littoral marine gastropods Littorina punctata and L. neritoides indicating that the alleles most sensitive to mercury pollution in laboratory conditions displayed the lowest frequency at the polluted site of Akko, as expected.

The data presented in Figure 2, indicate that the allele S of PGI in both species and allele M+ of AP in L. neritoides, previously shown to be mercury sensitive in laboratory experiments, display their lowest frequencies at the Akko polluted site. As seven populations in non-polluted sites showed for all three sensitive alleles higher values than in the Akko polluted site - this result is highly significant statistically. The probability to obtain by chance the above mentioned results is $p=(1/8)^3=0.002$.

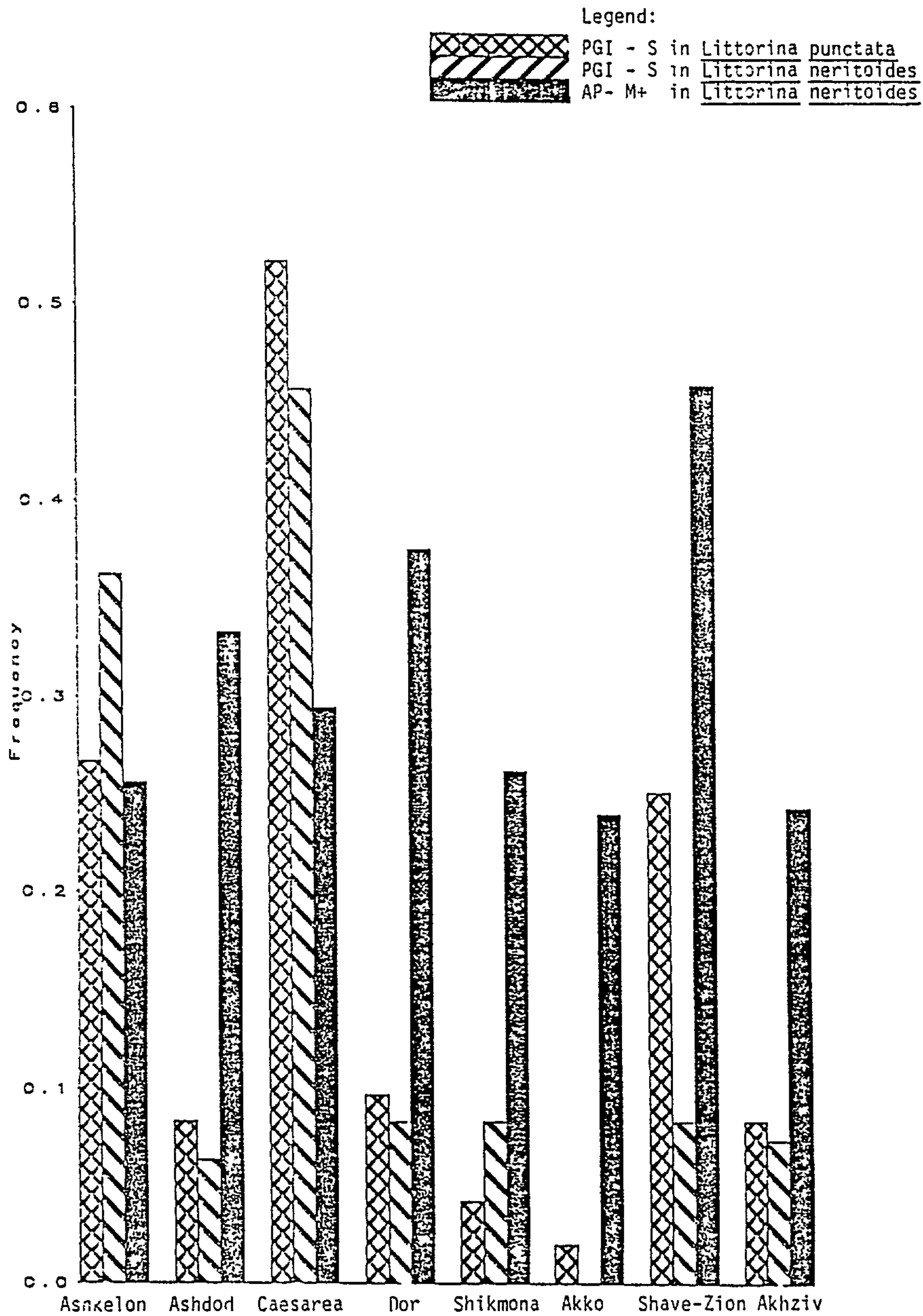

Fig. 2 Frequencies of the mercury sensitive alleles along the Mediterranean shore of Israel, in species of <u>Littorina</u>

3.3 Palaemon elegans

Table VI summarizes the frequency distribution of the common genotypes between live and dead animals in each of the pollutants. Different allozyme genotypes appear to be resistant or sensitive to different pollutants. The genotype MS has a significantly higher proportion among live animals (Nevo et al., 1981a, 1981b) in pollution of 0.2 ppm $HgCl_2$. The same genotype, MS, has a higher proportion among dead animals in pollution of 20 ppm $CdCl_2$ (chi-square = 3.25, 1 d.f. p=0.07). There is no significant difference (p=0.23, chi-square=1.42, 1 d.f.) in the frequency of the MS genotype between live and dead animals in the combined pollution of 0.2 ppm $HgCl_2$+20ppm $CdCl_2$, although it might be slightly sensitive.

The genotype FM was found to be resistant to pollution with 0.2 ppm $HgCl_2$ + 20 ppm $CdCl_2$. The proportion of the FM genotype among live animals is much higher than in dead ones (chi-square = 3.00, 1 d.f. p=0.08). It seems that the FM genotype is slightly favoured in all pollutants (Table VI). There is no statistically significant difference in FM distribution in pollution with either $HgCl_2$ (chi-square=0.04, 1 d.f., p=0.83), or with $CdCl_2$ (chi-square=0.59, 1.d.f., p=0.44).

Table VI

Differential survivorship of PGM allelic isozyme genotypes caused by heavy metal pollutants.

a. Pollution with $HgCl_2$ (0.2 ppm)

Genotype	Proportion of animals	
	Live (N=114)	Dead (N=136)
FF	0.044	0.051
FM	0.202	0.191
FS	0.079	0.140
MM	0.237	0.265
MS*	0.307	0.250
SS	0.088	0.066
others	0.044	0.037
number of experiment	5	

* the difference in proportion between dead and live animals for the MS genotype is not significant in this experiment due to a small sample size but it is in accordance with previous significant results (Nevo et al., 1981a, 1981b).

Table VI (cont.)

b. Pollution with $CdCl_2$ (20 ppm)

Genotype	Proportion of animals Live (N=151)	Dead (N=145)
FF	0.040	0.055
FM	0.185	0.152
FS	0.099	0.090
MM	0.325	0.276
MS	0.219	0.303 @
SS	0.099	0.090
others	0.033	0.034
number of experiments		6

@ p=0.07 (chi-square = 3.25, 1 d.f.)

c. Pollution with the combination of $HgCl_2$+$CdCl_2$ (0.2 ppm and 20 ppm, respectively)

Genotype	Proportion of animals Live (N=153)	Dead (N=145)
FF	0.052	0.028
FM	0.255	0.172 @
FS	0.072	0.062
MM	0.209	0.248
MS	0.248	0.310
SS	0.137	0.103
others	0.026	0.076
number of experiments		6

@ p=0.08 (chi-square = 3.00, 1 d.f.)

4. DISCUSSION

The mercury concentration necessary to obtain the LD_{50} in the experimental time span was in all cases much higher than the concentrations found in mercury polluted sea environments (Roth and Hornung, 1977). Yet, while the effects of pollution may be small in an open sea situation, effects are certainly appreciable in estuaries and coastal water. This is especially true in the case of benthic animals which lie on the sediments, where the concentration of pollutants is higher than in the water column (see reports in ICSEM/UNEP, 1981). These animals accumulate pollutants from seawater and food organisms of seabed sediments to levels as high as 10^4-10^8 times above ambient (Ruivo, 1972; El-Nady, 1986). The concentration range of mercury in our experiments was comparable to the body concentration of animals living in mercury polluted environments.

This laboratory experiment only considered survivorship because none of the investigated species reproduces successfully in our laboratory conditions. In nature, tolerant genotypes may not only live longer, but may also reproduce better in the polluted environment (Nevo _et al._, 1984).

Our results indicate significant differential survivorship of allelic isozyme genotypes, of specific alleles, and of overall heterozygotes in marine organisms when exposed to pollution by mercury and cadmium separately or in combination.

There was not a single enzyme whose isozyme frequencies were not affected by pollution. The only species that enabled us to observe the effects of pollution on a battery of enzymes, was _Cerithium scabridum_. Four loci did not show differential mortality on a single locus analysis. However, our results do demonstrate significant differential survivorship of multilocus genetic complexes of these loci caused by heavy metal pollution with mercury and cadmium separately and in combination.

These results are concordant with the selectionist explanation of the genetic variability and are inconsistent with the neutral theory. When the results were analyzed for each locus separately, we reported that only 4 out of 8 loci showed differential survivorship when exposed to the heavy metal pollution stress. Franklin and Lewontin (1970) criticized the models of population genetics for ignoring the "natural" unit of selection, the genotype, in favour of the gene. In this study we went one step further from single genes towards the genotype, by analyzing multilocus genetic complexes. We found two four-locus and one two-locus adaptive genetic complexes for genes, that when analyzed singly seemed nonadaptive.

Therefore, as we have shown previously for single loci (Nevo _et al._, 1983, 1988), multilocus genetic complexes can be used as biological indicators of pollution. Such direct monitoring may become an indispensable tool, sometimes preferable to the chemical ones used routinely (Toribara _et al._, 1977), as it alerts us to both the short- and long-term genetic changes that populations undergo before their final extermination as a result of pollution.

As encountered earlier for the analysis of single loci, each pollutant has a specific effect on the multilocus genetic complexes.

Along with trends common to all three types of pollution used in this study, each pollutant affects the distribution of allelic isozyme genotypes in a different way. The interaction of mercury and cadmium when combined is not additive. The mixture of mercury and cadmium sometimes acted like an independent pollutant, resembling none of the separate pollutants.

The differential survivorship demonstrated by the various allelic isozyme genotypes supports the view that at least part of the allozyme polymorphism can be subjected to natural selection, though selection on linked genes cannot be eliminated by our experiment.

The selective effects of heavy metal pollution are explicable biochemically (Milstein, 1961). Inhibition of enzyme activity by heavy metal may proceed by incorporation into the enzyme molecule through selective chemical modifications of affinity labelling, metal chelation, noncovalent binding of inhibitors and analogues, and nonspecific associations with complex ions. One of the mechanisms may be responsible for the enhanced sensitivity of specific genotypes. If cadmium and mercury act on different sites of the enzyme, the interaction of both pollutants may cause a specific response to this type of separate pollutants. In addition the specific results obtained for the pollution by both pollutants may be a consequence of the fact that the combined effect of cadmium and mercury operated differently than each pollutant alone. This hypothesis could be tested by radiolabelling the pollutants and tracing their molecular effects (e.g. Powers et al., 1979). The sensitivity of allelic isozyme genotypes to pollution has been reconfirmed in multiple tests (Nevo et al., 1983, 1984). However, our 3 recent tests on the interactive effects of pollutants suggest that in the sea, where multiple pollutants may operate in unison, the effects on genic frequencies may be different than that caused by each pollutant separately. While the precise mechanism involved in this phenomenon remains to be elucidated, the effects should be considered if allelic isozyme frequencies are to be used in genetic monitoring of pollution.

Should criteria for maximum allowable pollutant concentrations be universally established or should local decisions be made based on environmental conditions and pollutants? Our results suggest that the latter approach may be more reasonable when considering the different effects of single vs multiple present pollutants.

5. ACKNOWLEDGEMENTS

We thank Shimon Simson for field and laboratory assistance and wish to extend our deep gratitude for financial support to FAO/UNEP, to the Israeli Discount Bank Chair of Evolutionary Biology, and the Ancell-Teicher Research Foundation for Genetics and Molecular Evolution established by Florence and Theodore Baumritter of New York.

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THE RATE OF MUTAGENIC ACTIVATION OF 2-AMINOFLUORENE BY RAT LIVER MICROSOMES AS MEASURED WITH THE SALMONELLA MUTAGENICITY TEST

by

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ABSTRACT

In this study, the rate of biotransformation of 2-aminofluorene (AF) by rat liver microsomes into the ultimate mutagenic metabolites, which were quantitated using the Salmonella/microsome plate incorporation test, was examined. Mutagenic activation of AF by rat liver microsomes increased constantly with time of incubation up to 20 min and then slowly up to the 100th min of measurement time.

Escherichia coli plasmid transformation test designed to show the effect of mutagenic metabolites on biological inactivation of plasmid pBR322 seems to suggest that at least some of the plasmids were being inactivated within 20 min of incubation period, in which the plasmid DNA, microsomes and AF were kept together at 37 degrees.

In summary, the two forementioned tests seem to be simple and quantitative enough to be used for assaying the metabolic activation of promutagens/procarcinogens with S9 preparations from different species of interest.

1. INTRODUCTION

In the 1940s and 1950s the hypothesis first developed was that many drugs, industrial chemicals, pesticides, pollutants and other materials are not toxic *per se* but elicit their effects only after metabolism to electrophilic species which can modify molecules (Miller and Miller, 1981). The enzyme systems responsible for these metabolic transformations are localized mainly in the liver and majority of these enzymes are inducible (for a review see Guengerich and Liebler, 1985).

Primary aromatic amines, including AF, are a class of carcinogenic compounds to which humans may be exposed from industrial, environmental, and dietry sources. Numerous studies have suggested that N-hydroxylation of these compounds is a necessary step in their activation to electrophiles that covalently bind to DNA, induce mutations, and initiate carcinogenesis (for a review see Radomski, 1979). Several studies exist on the mutagenicity of 2-acetylamino-fluorene (AAF) and N-OH-AF is the ultimate mutagen derived from these compounds (for a more recent paper see Beranek *et al.*, 1982). No further activation of N-OH-AF within the bacterium has been demonstrated and the C-8 deoxyguanosine adduct produced *in vitro* was also found in the bacterial DNA. The formation of this adduct was further shown to have a high correlation with the observed mutagenicity (Beranek *et al.*, 1982).

In view of the above mentioned information it was intended to study the rate of metabolism of AF using the Salmonella/microsomes mutagenicity test (as revised by Maron and Ames, 1983) considering the simplicity and ease of application of this test. In addition, another test (E. coli plasmid transformation) was run to show that mutagenic activation of AF could impair the biological activity of the plasmid DNA with which the activated mutagens would be able to interact directly and as soon as they are formed.

2. MATERIALS AND METHODS

Chemicals, bacteria and animals used for the Salmonella mutagenicity test; induction of enzymes by sodium phenobarbital treatment, preparation of S9 mixture and determination of protein concentration have all been previously described (Bagci, 1989).

Escherichia coli strain HB101 and HB101 harboring the plasmid pBR322 were obtained from Dr. M. Simsek. Lysozyme was from BDH Chemical Ltd., RNase A (pancreatic) from Sigma. Nutrient broth, nutrient agar, Chloramperticol, Tetracycline and L broth were prepared as described by Maniatis et al. (1982).

2.1 Mutagenicity assay with S9 activation

To 10 ml of high S9 mixture (10%), 2 ml of a fresh overnight culture of Salmonella typhimurium tester strain TA98 was added; mixed by vortexing and four 0.2 ml samples were taken and plated for spontaneous reversion. To the remaining solution was added 1.5 ml of AF in dimethylsulfoxide (DMSO). The final concentration of AF is 10 microgram/plate. The solution was vortexed and incubation at 37°C waterbath was initiated. At time intervals 0.2 ml of samples were taken and plated in quadruplicates for His^+ revertants as described (Maron and Ames, 1983).

2.2 Isolation of plasmid pBR322 and transformation of E. coli

Plasmid DNA was prepared by Summerton et al. procedure (1983). E. coli strain HB101 cells were made competent and transformed with mutagen-treated and non-treated plasmid DNAs according to the method by Dagert and Ehrlich (1979).

3. RESULTS

3.1 Optimum AF concentration

In order to find out the optimum AF concentration to be used in metabolic activation assays, increasing amounts of AF were added to an S9 mixture with 10% S9 protein and a plate incorporation test was carried out with the tester strain TA98. The result of this experiment is shown in Figure 1. As Figure 1 shows the number of His^+ revertants obtained with 10 μg of AF was not so much different from that obtained with 40 μg of AF/plate. Thus, the 10 μg/plate concentration was chosen as it was far enough from toxic concentration range.

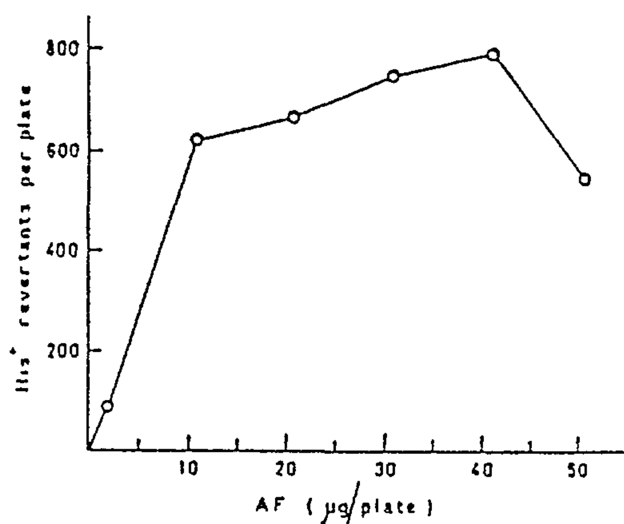

Fig. 1 Concentration dependent mutagenicity of AF with S9 activation. Each value is the mean of two plates

3.2 Determination of the rate of metabolism of AF

Rat liver microsomes, the mutagen and the tester bacteria are incubated together at 37°C for up to 100 min. At time intervals samples were taken and plated for His^+ revertants. The results of this experiment are plotted as the log of number of His^+ revertants/plate vs time of incubation (Fig. 2).

As could be seen from Figure 2, the rate of mutagenic activation of AF was constant almost up to the 20th min but slower afterwards. Similar results were obtained when the test was repeated with a low S9 (2%) mixture (data not shown). Moreover, when the sample were first centrifuged and the pellets containing the tester bacteria were washed to remove the mutagen and S9 mixture constituents before the cells are plated for His^+ revertants, no significant change in the rate curve was observed (data not given).

3.3 Inactivation of biological activity of pBR322

I reasoned that if mutagenic metabolites were produced and they were the cause of increased reversion frequencies to His^+ phenotype, they might also inactivate the plasmid DNA present in the same reaction tube. To test this possibility, the metabolic activation mixture was prepared in the presence of plasmid DNA (but in the absence of the tester bacteria) and incubated at 37°C for 20 min. Then, 5 μl samples were mixed with 0.1 ml of competent E. coli cells and transformation was carried out as described by Dagert and Ehrlich (1979). Controls with non-treated plasmid DNAs and heat-inactivated metabolic activation mixture were also included in this test. The results are given in Table I.

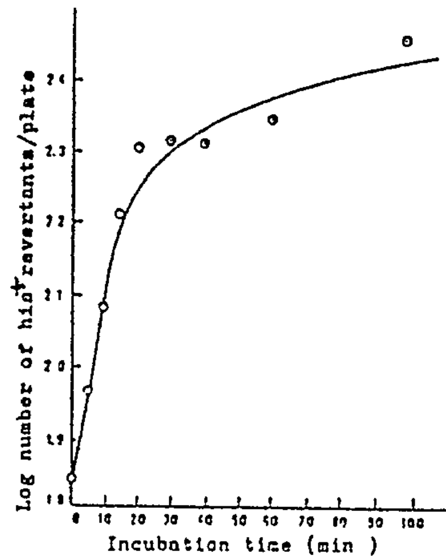

Fig. 2 Effect of incubation time of AF with rat microsomes on the number of His^+ revertants of the strain TA98

Table I

Transformation of E. coli HB101 cells with mutagen-treated and non-treated plasmid DNAs.

Source of plasmid DNA (0.33 µg)	No of transformants/ml
DNA alone (in saline)	5.5 E04
DNA (44 µl) + heat-inactivated S9 mix (350 µl) + AF (50 µl in DMSO)*	1.8 E02
DNA (44 µl) + S9 mix (350 µl) + AF (50 µl in DMSO)*	2.0 E01

* The mixtures are incubated at 37°C for 20 min before the samples are taken for transformation

4. DISCUSSION

In the present study it was shown that AF could be activated to mutagens using the S9 protein obtained from rats in a S. typhimurium assay, presumably by forming N-OH-AF (N-hydroxy-2-aminofluorene). The rate of mutagenic activation was constant for the first 20 min (Fig. 2). Frederick et al. (1982) have shown earlier that rat liver microsomes catalyzed the formation of N-OH-AF from AF at the rate of 1.6 nmol/min/mg protein. They have also found that when the purified porcine liver flavin-containing monooxygenase was incubated with AF and

cofactor-generating system, the accumulation of N-OH-AF was linear for 30 min. In addition, they have used the Salmonella mutagenicity assay (TA98) and found that 10, 25 and 50 μg AF, yielded 190, 700 and 1800 revertants per plate. It is thus clear that our findings are similar to theirs.

On the other hand, Razzouk et al. (1980, 1982), using a modified plate assay in which rat or guinea pig liver microsomes were incubated for various lengths of time with 7-iodo-2-acetylaminofluorene (2-AAIF) together with the suspension of S. typhimurium TA1538, have found that rat microsomes were unable to activate 2-AAIF, while incubation of the same promutagen with guinea pig microsomes produced a significant time-dependant increase in the number of His^+ revertants. They have also studied the effect of incubation time of AF with guinea pig microsomes on the concentration of N-OH-AF and the number of His^+ revertants when the incubation medium is then plated together with Salmonella (TA1538, in the Ames test. They have found that the production of N-OH-AF increased with time of incubation up to 10 min. This metabolite then disappeared from the medium. Similarly, the number of His^+ revertants increased up to 10 min and declined thereafter progressively back to the spontaneous reversion rate. Since in our test, the tester bacteria are included in mutagenic activation system, declined in the number of His^+ revertants was not observed. Contrary to the above result of Razzouk et al. (1982), Figure 2 shows that there was a progressive increase up to the 100th min of incubation time in the number of His^+ revertants. For this reason, we consider our test "mutagenic activation test in the presence of Salmonella tester strain" to be a simple, quantitative and inexpensive test for assaying the metabolic activation of promutagens/procarcinogens with S9 preparations from different species of interest.

Also in this study, effect of mutagenic activation on biological activity of the plasmid pBR322 was examined. Purified plasmid DNA was incubated in the presence of S9 mixture and AF at 37°C for 20 min and then .005 ml of this mixture was used to transform competent E. coli cells. Selection for Ampicillin resistance showed that activity was decreased dramatically when the treated plasmid DNAs were used (Table I). Reduction in the biological activity of the plasmid was so much to be attributed to the formation of DNA adducts alone. One possible cause could be DMSO used to dissolve AF. The DMSO bottle had been kept at room temperature and opened repeatedly and there is claim that the oxidation products of DMSO are very inhibitory to transformation (Maniatis et al., 1982, p. 253).

On the other hand, plasmid DNA treated with AF and active S9 preparation showed still more inactivation than the plasmid DNA treated with heat-inactivated S9 preparation and AF. Inactivation of Ampicillin marker could result from the inhibitory actions of DNA adducts or degradation of plasmid DNA by nucleases present in the S9 mixture. Unfortunately our assay procedure does not allow us to eliminate any of these possibilities. However, assuming that (1) the rate of N-hydroxylation is 1.6 nmol/min/mg, and (2) a trapping efficiency of 0.1% (Frederick et al., 1982) we expect to find in 20 min of incubation time approximately 7.0 E14 N-OH-AF and 7.0 E11 plasmid bound molecules. The number of plasmid DNAs added (0.33 μg) is approximately 7.0 E9. This would give about 100 N-OH-AF per plasmid

molecule. Tang et al. (1982) have shown that more than 150 AF adducts per molecules of 0X174 replicating form(RF) DNA were needed to reduce the plaque-forming frequency to 1% level of that of the non-treated DNA. Since the genome of 0X174 phage is slightly larger than that of pBR322 we would expect that less than 150 adducts per plasmid molecule would be sufficient to reduce the biological activity of the plasmid DNA to a level similar to that observed with 0X174 RF DNA.

With the above reasoning we would be inclined to consider that first possibility that the AF DNA adducts could cause the observed decline in biological activity of the plasmid.

5. ACKNOWLEDGEMENT

The financial support provided through FAO (GFCM) (TUR/5-G) is gratefully acknowledged.

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EFFECT OF HEAVY METALS IONS ON ENZYME ACTIVITY, MORTALITY AND BEHAVIOUR OF THE MEDITERRANEAN WHITE MUSSEL *Donax trunculus*

by

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ABSTRACT

Heavy metals are known to be toxic to living organisms, and affect enzyme activity. We examined the effects of 0.1 ppm, 1 ppm and 10 ppm individual doses of cadmium, mercury, copper, zinc and lead on the activity of cytochrome oxidase, lactate dehydrogenase, malate dehydrogenase, ATPase, alkaline phosphatase and α-amylase of the Mediterranean bivalve mussel *Donax trunculus*. The animals were exposed for 24h to each of the five metal ions and the activities of the six enzymes tested. Cytochrome oxidase was the most sensitive enzyme. α-amylase, ATPase and Alkaline phosphatase were only slightly affected by any ion, therefore the *in vitro* effect after incubation of these enzymes extract with the heavy metals were tested. In the *in vitro* experiments the enzymes were found to be more sensitive than in the *in vivo* experiments.

The enzymes tested were more sensitive to Cd and Zn ions than the other ions, therefore we examined the mortality of *D. trunculus* using different concentrations of each of these two ions. Zn did not kill the animals at concentrations of 0.1 to 1 ppm. At the concentration of 6,6 ppm, 30% of the population died between 96h and 144h after the exposure of the animals to the pollutant. Cd was more potent, and 65% of the population died after 48h; all the animals died after 96h of exposure to 10 ppm Cd.

1. INTRODUCTION

The toxic effects of heavy metals on organisms have received considerable scientific interest in the last decade. Most of this work has been done concerning with Cd, Cu, Hg, Zn and Pb (Martin and Holdich, 1986; Arnac and Lassus, 1985). Heavy metal ions are waste products of many industrial processes and in many places are released into the ocean.

Heavy metal ions in sufficiently high concentration might kill organisms or cause other adverse effects, that changing aquatic community structures. Lethal effects and bioaccumulation of these ions have been widely studied. However, sublethal effects on organisms have received relatively less attention. Widdows (1985) states that sublethal doses of a heavy metal might change the biochemical and cellular performance of the organism, affecting enzymatic activity likewise changing the physiological performance of the organism and reducing its scope for growth. Thorberg (1980) suggested that the activity of glycolytic enzymes should be studied, since it reflects the utilization of energy.

The bivalve Donax trunculus is found along sandy beaches of the Mediterranean Sea. Haifa Bay on the northern part of the coast of Israel supports a dense population of D. trunculus (Neuberger-Cywiak et al., 1990). This area is polluted by effluents from oil refineries, chlor-alkali plants, fertilizer plants and other industrial activities.

Bivalves are known to be heavy metal accumulators (Romeo and Gnassia-Barelli, 1988). Roth and Hornung (1977) showed that in Haifa Bay, the concentration of heavy metals in sediment and in certain organisms, including Donax trunculus, is higher than in samples from an unpolluted area. El-Rayis (1986) pointed out that D. trunculus in relation to other marine organisms is a good accumulator of cadmium. Mytilus edulis was also found to be an accumulator of zinc and cadmium (Phillips, 1977).

The aim of the present study was to examine the effects of three different concentrations of each of five heavy metal ions on the activities of six enzymes in Donax trunculus. Since it is known that heavy metals inhibit a wide range of enzymes (Dixon and Webb, 1964), we choose representative examples of dehydrogenases (lactate and malate dehydrogenase), a respiratory enzyme (cytochrome oxidase), a digestive enzyme (α-amylase) a phosphatase enzyme (alkaline phosphatase) and an ion transport enzyme (ATPase). In the course of the experiments it was found that the most potent metals were Cd and Zn and the effects of these metals on the behaviour and mortality was studied. The acute effects of relatively moderate concentrations of selected metals were examined. These concentrations were higher than those found usually in the locality where the animals occur (Roth and Hornung, 1977), but might be encountered during a given temporal event of pollution.

2. MATERIALS AND METHODS

Specimens of Donax trunculus were collected at Hof Haargaman near Akko (Acre) and kept at 20±1°C in aquaria with circulated sea water. The bottom of the aquaria was covered with a 5 cm layer of carbonate gravel, on top of which a layer of sand, collected in Akko, was placed.

For enzyme preparations, 5 specimens of D. trunculus were placed in a 1 litre beaker with 0.5 l of aerated sea water containing 0.1 ppm, 1 ppm or 10 ppm each of mercury, zinc, copper, cadmium or lead. The animals were kept in the solution for 24h, at 20±1°C. Enzyme activity was estimated and recorded at the end of that period. Each experiment was repeated 5 times.

The crystalline styles of these animals were removed and used for the determination of α-amylase activity. The remainder of the body was used for the determination of the activity of alkaline phosphatase, ATPase, lactate dehydrogenase, malate dehydrogenase and cytochrome oxidase. Enzyme activity was related to 1 mg of protein estimated by the folin phenol reagent (Lowry et al., 1951).

Styles of 5 specimens were removed and homogenized at 4°C in 2 ml of 20 mM phosphate buffer, pH 6.9, containing 6.7 mM NaCl. The suspension was then exposed to an ultrasonic vibrator for 30 sec and centrifugation at 15000 x g for 5 min. α-amylase activity was determined by the method decribed by Bernfeld (1951) and was expressed

in terms of mg glucose released in 8 min by 1 mg of protein. The liberated glucose was measured by 2,5 dinitrosalicylate reagent and was determined spectrophotometrically at 540 nm. The in vivo reaction mixture contained 0.1 ml of crystalline styles extract, 0.4 ml of phosphate buffer and 0.5 ml of 1% soluble starch. In vitro the reaction mixture contained 0.1 ml of crystalline style extract from untreated animals, 0.3 ml of phosphate buffer and 0.1 ml of various concentrations of heavy metals.

Bodies of 5 specimens were removed and homogenized at 4°C in 5 ml of distilled water. The suspension was then centrifuged at 15000 x g for 5 min. The supernatant was used for the enzyme assay.

Alkaline phosphatase determined by method described earlier (Bessy et al., 1946) and was expressed in terms of mmole p-nitrophenyl released in 1 min by 1 mg protein. The in vivo reaction mixture contained 0,5 ml of tris buffer pH 10, 0.5 ml of p-nitrophenyle phosphate and 0.1 ml homogenate. In vitro reaction mixture contained 0.1 ml of homogenate from untreated animals, 0.5 ml tris buffer, 0.5 ml of p-nitrophenyle phosphate and 0.1 ml of various concentrations of heavy metals. The released nitrophenyle was measured spectrophotometrically at 420 nm.

ATPase activity was determined by the Fiske and Subbarow (1925) method and was expressed in terms of µmole inorganic phosphate released in 1 min by 1 mg protein. The released phosphate was measured by molybdate sulfuric acid, by 1 amino 2 naphtol 4 sulfonic acid reagent and was determined spectrophotometrically at 660 nm. The in vivo reaction mixture contained 0.5 ml tris buffer pH 7.5 containing 100 mM Nacl, 20 mM KCl and 5 mM Mgcl2, 0.1 ml ATP and 0.2 ml homogenate. In vitro reaction mixture contained 0.2 ml of homogenate from untreated animals, 0.5 ml tris buffer, 0.1 ml ATP and 0.1 ml of various concentrations of heavy metals.

Lactate dehydrogenase activity was determined as described by Kornberg (1955). The reaction mixture contained 0.1 ml of pyruvate 0.02 M, 0.1 ml of NADH (0.5 mg ml^{-1}), 0.2 ml of tris buffer pH 7.5 and 0.5 ml homogenate. The decrease in pyruvate concentration after 30 min was determined spectrophotometrically at 540 nm by 2.4 dinitrophenyl-hydrazine reagent (Friedemann, 1957).

Malate dehydrogenase activity was estimated as described by Roodyn et al. (1962) by following the reduction of NAD by malate at 340 nm. The reaction mixture contained 2 ml of glycine NaOH buffer pH 9.9; 0.3 ml of 0.18 M sodium-1-malate; 0.3 ml of NAD. The reaction was started by the addition of 0.1 ml of the body extract (1 volume of the suspension was pretreated with 10 volumes of 0.2% tritonX100 in 0.3 M sucrose for 30 min at 0°C before assay).

Cytochrome oxidase was measured by following the oxidation of reduced cytochrome C at 550 nm, as described by Freeman (1965). The results are expressed by activity units. An activity unit is defined as oxidizing cytochrome C at the rate of 1 µmole per minute, per 1 mg protein. The reaction mixture contained 2.6 ml of 17µM reduced cytochrome C in 0.03 M phosphate buffer pH 7.4 and 0.1 ml of the body extract. The examined suspension was the same as that prepared for malate dehydrogenase estimation.

We examined the effects of heavy metal ions on the mortality and the behaviour of D. trunculus at different intervals of up to one week. Individuals of Donax were exposed to different concentrations of Cd and Zn in short term static bioessays. The concentrations of Cd ranged from 0.1 to 10 ppm, while that of Zn from 0.66 to 6.0 ppm. For each metal three sets of experiments, in triplicate, were examined. The behaviour of the animals was examined and noted. Three types of responses were found as described by Bodoy (1976).

unburied (Fig. 1A)
half buried (Fig. 1B)
buried (Fig. 1C)

Death was considered when there was a lack of response when touching the foot, mantle and siphons of unburied animals.

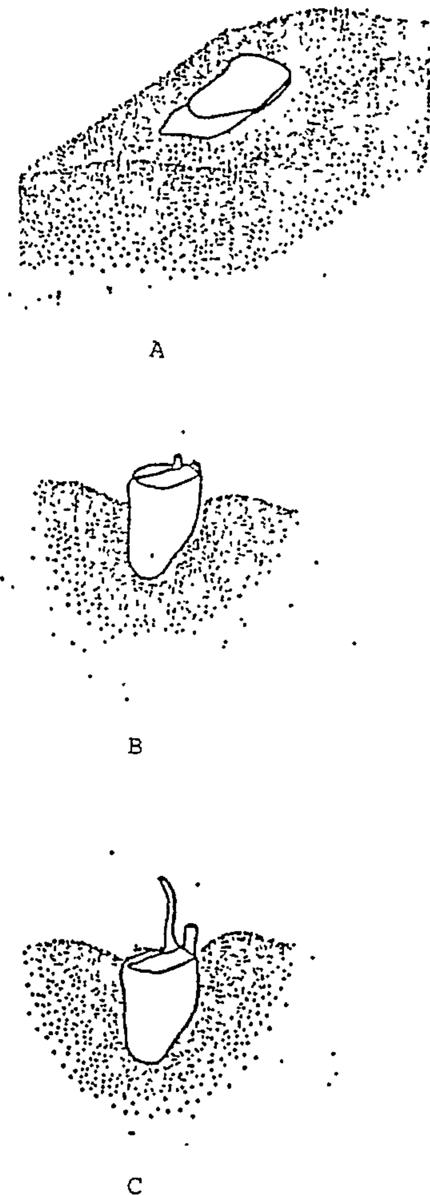

Fig. 1 Behaviour pattern of Donax, redrawn from Bodoy (1976)

3. RESULTS

α-amylase: The presence of the heavy metal ions in the water caused a slight inhibition of this enzyme. The difference in inhibition between the three concentrations studied was very small (Fig. 2A). In the in vitro test Zn completely inhibited the α-amylase activity at the lower concentration (1 ppm), Cd and Cu inhibited about 55%, Hg about 30%. Lead appeared to be the less inhibitor of all metals studied, even at the higher concentration (10 ppm) (Fig. 2B).

ATPase: In the in vivo test the heavy metal ions caused a slight inhibition of the activity of this enzyme (Fig. 3A). In the in vitro test, all the metals except lead inhibited 30% of the ATPase activity at 10 ppm. 10 ppm Zn inhibited 60% of its activity. The Pb effect was very small (Fig. 3B).

Alkaline phosphatase: In the in vitro test, Zn at 10 ppm inhibited about 50% of the activity of this enzyme (Fig. 4A). Cd inhibited about 17% at 10 ppm. The other metal ions caused a slight inhibition of the enzyme (Fig. 4B).

Malate dehydrogenase: The activity was most strongly inhibited by Hg, the inhibition increased from 30% at 1 ppm at 80% by 10 ppm. This enzyme was partially inhibited by the other ions, the exception being Pb, which did not affect the activity (Fig. 5).

Lactate dehydrogenase: 10 ppm and 1 ppm of Cd inhibited 80% and 68% of the activity respectively. With Hg there was 60% inhibition at 10 ppm and 20% by 1 ppm. The other ions, Zn, Cu and Pb, were hardly effective even at the high concentration (Fig. 6).

Cytochrome oxidase: The results obtained for cytochrome oxidase activity showed the same trend as that of lactate dehydrogenase activity (Fig. 7). A strong inhibition by Cd and Hg. With Zn about 45% of the activity was inhibited by 10 ppm. Cu and Pb were less effective.

In the control after that period all animals were buried (Fig. 8A), while with Zn at 0.66 ppm the digging activity lasted for about 96h (Fig. 8B). At 6.6 ppm animals tended to emerge from the sand, death began to be detected after 144h (Fig. 8C).

With Cd, the behavior was totally different. At 0.1 and 1 ppm the behavior pattern was normal (Fig. 9A,B,C), but in higher concentration 10 ppm the organisms penetrate into the sand and remained buried till death (Fig. 9D). Their siphons were very thin and elongated in comparison to normal animals. The siphon stretched on the sand.

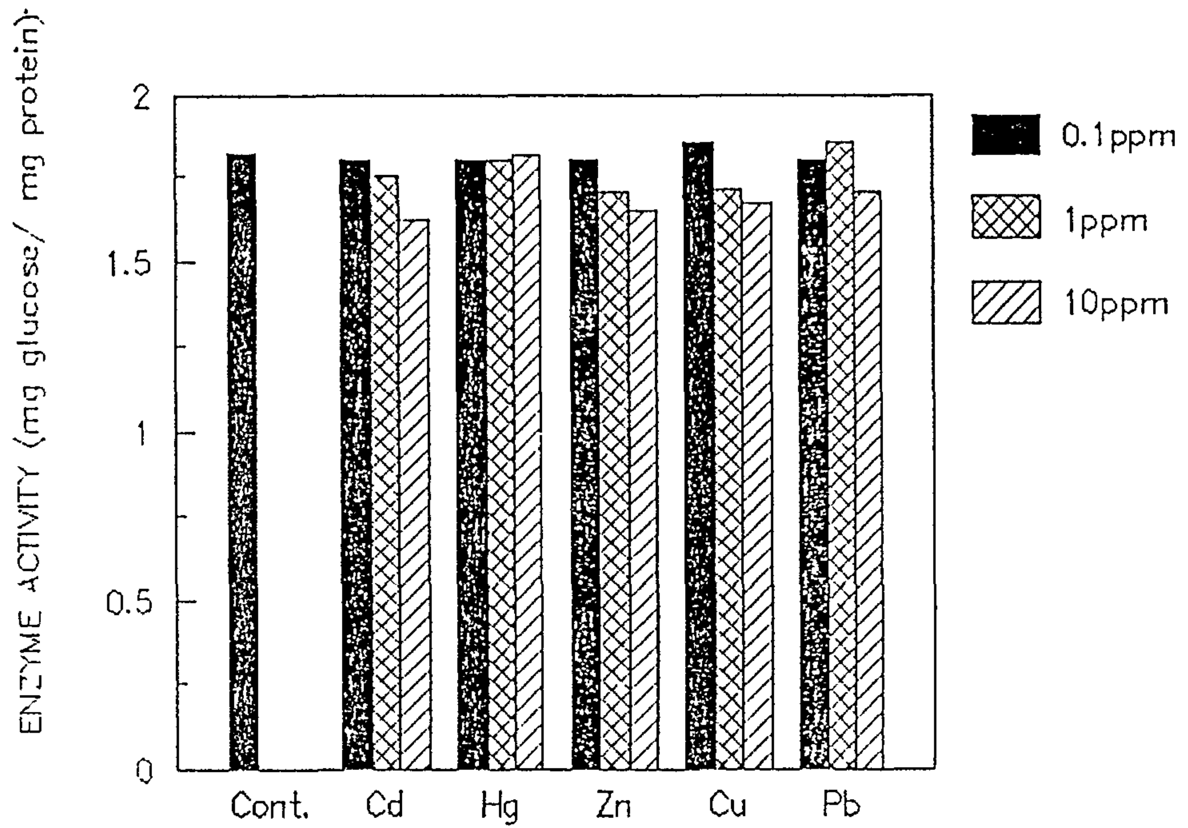

Fig. 2A α-amylase activity (<u>in vivo</u>)

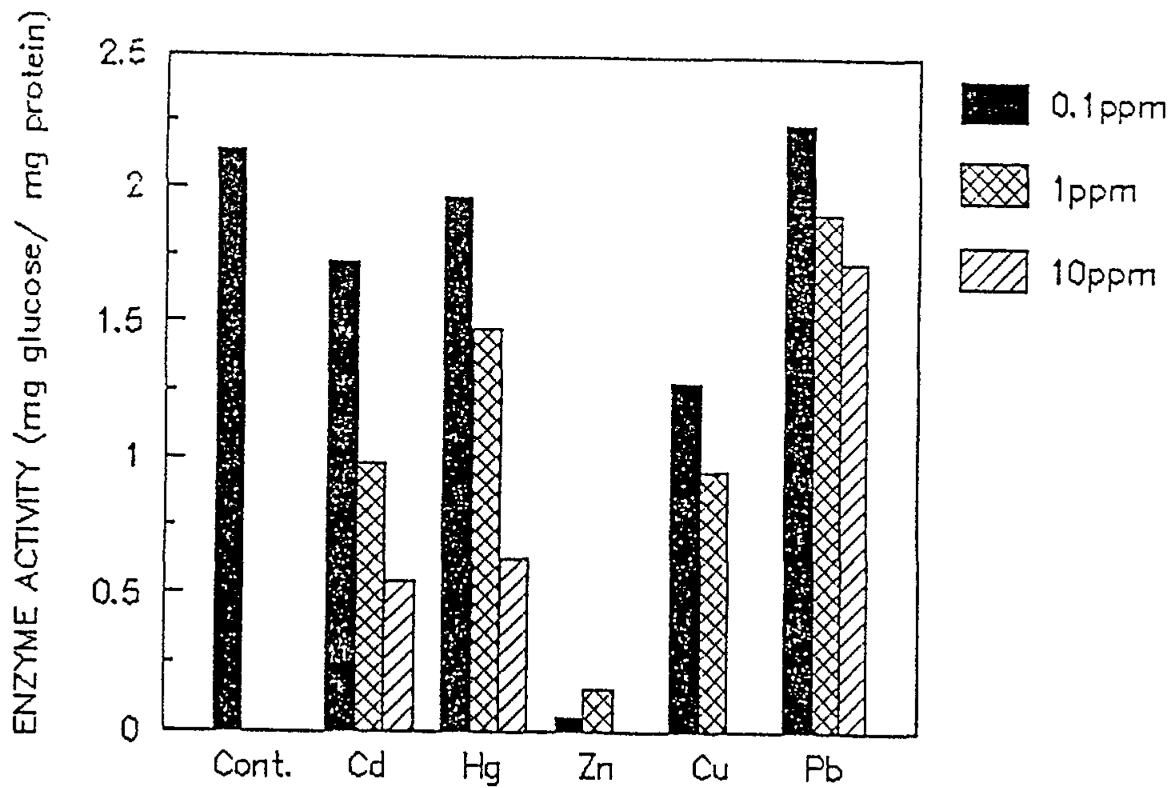

Fig. 2B α-amylase activity (<u>in vitro</u>)

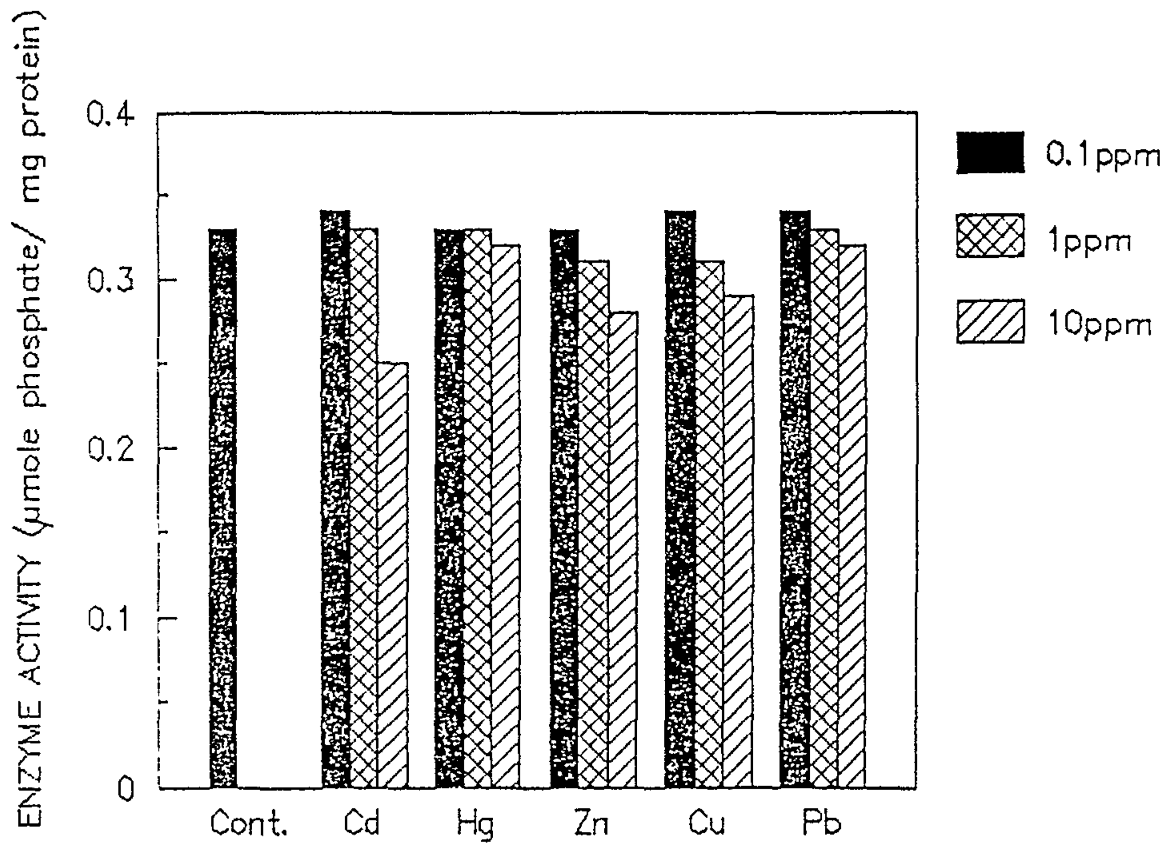

Fig. 3A ATPase activity (in vivo)

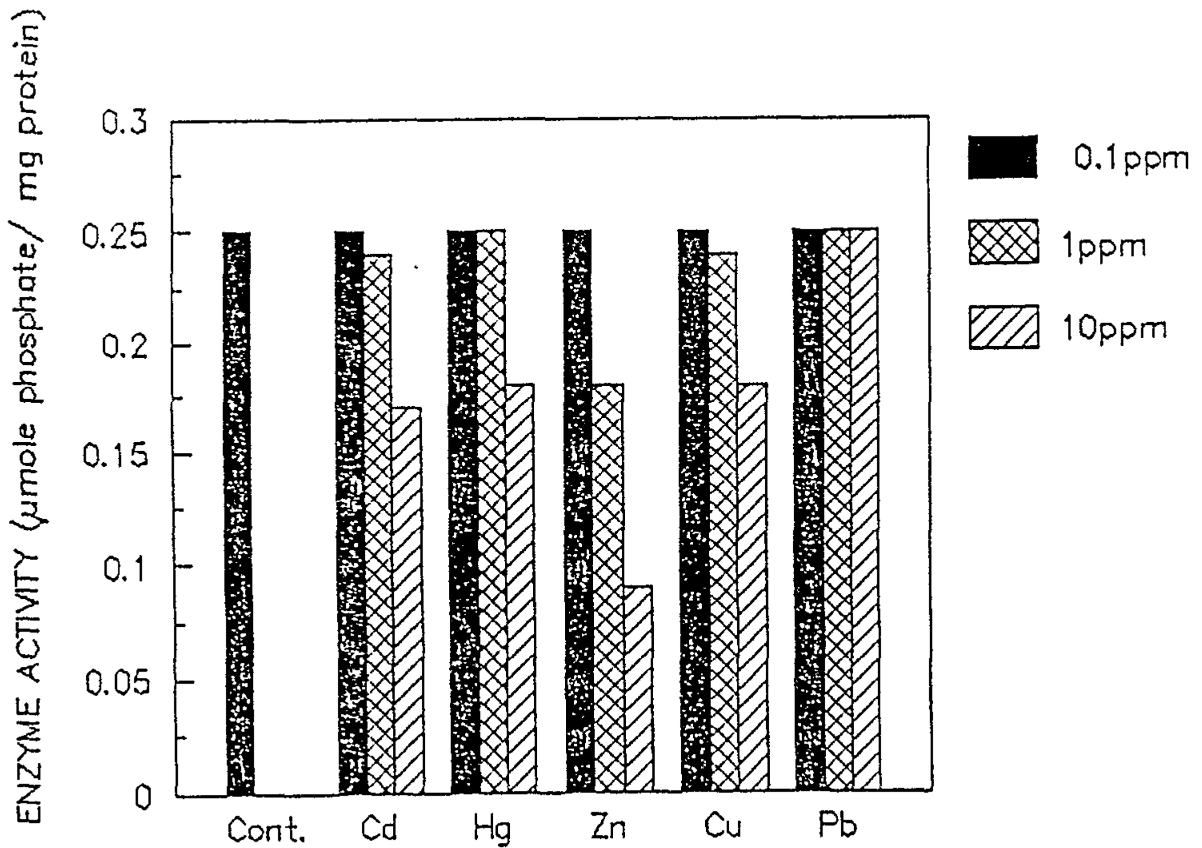

Fig. 3B ATPase activity (in vitro)

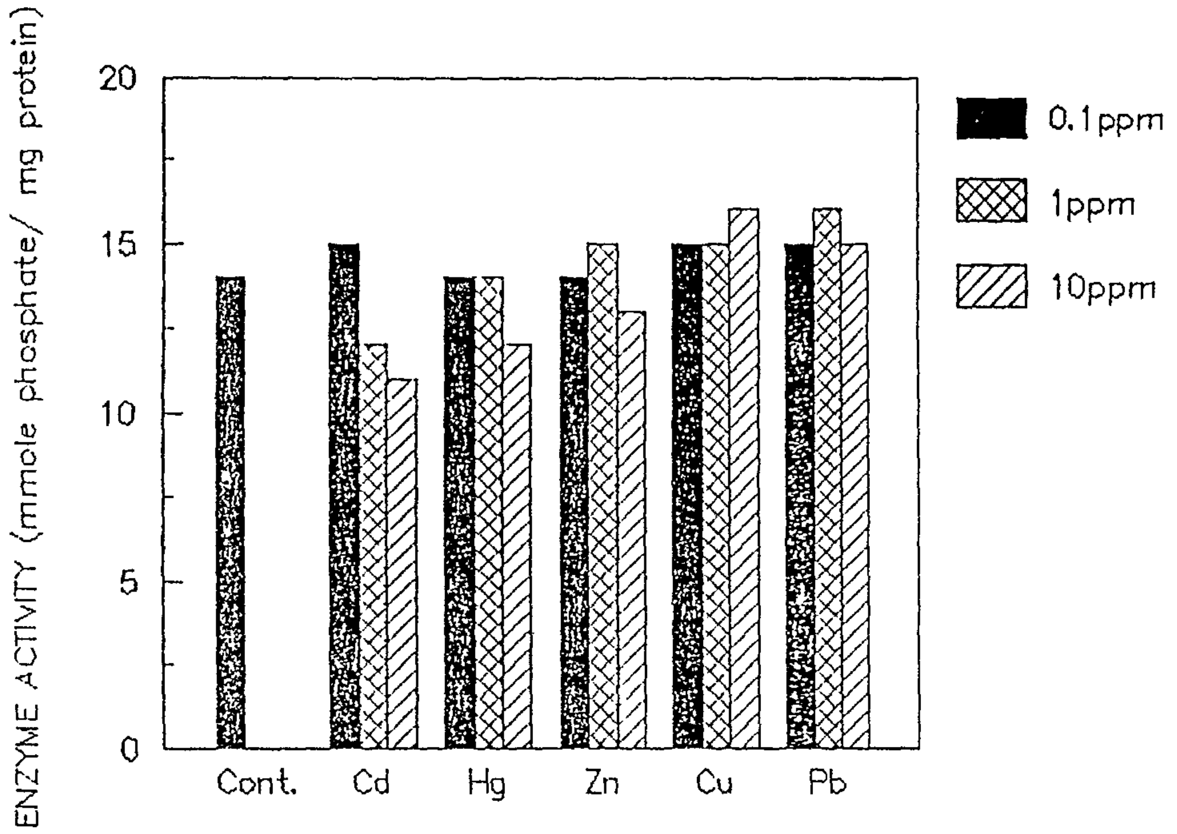

Fig. 4A Alkaline phosphatase activity (*in vivo*)

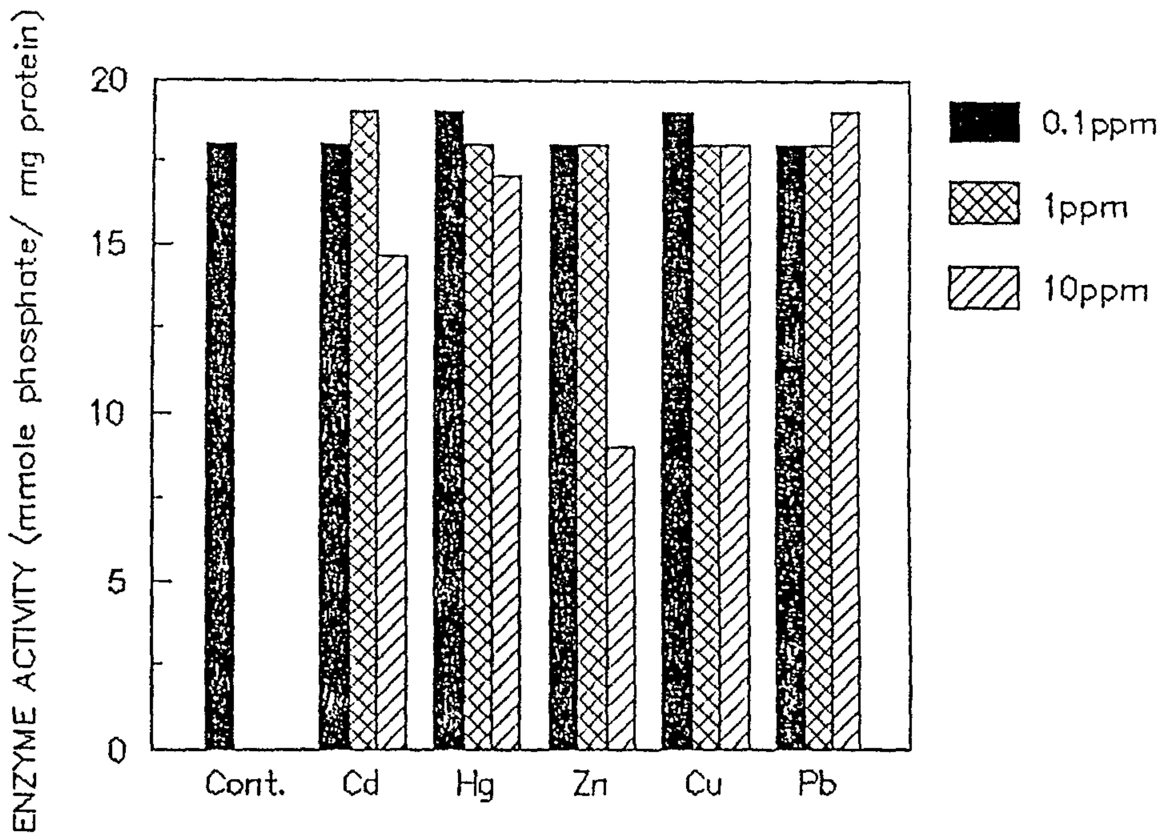

Fig. 4B Alkaline phosphatase activity (*in vitro*)

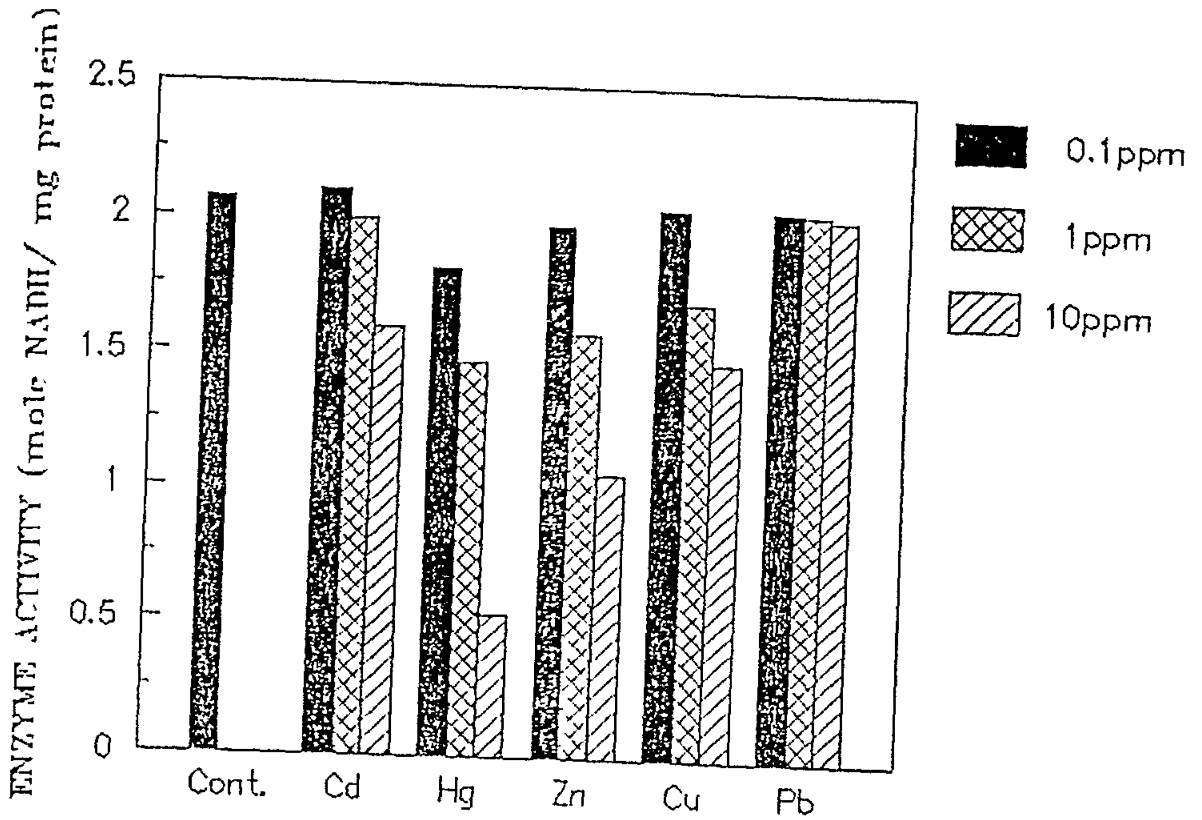

Fig. 5 Malate dehydrogenase activity

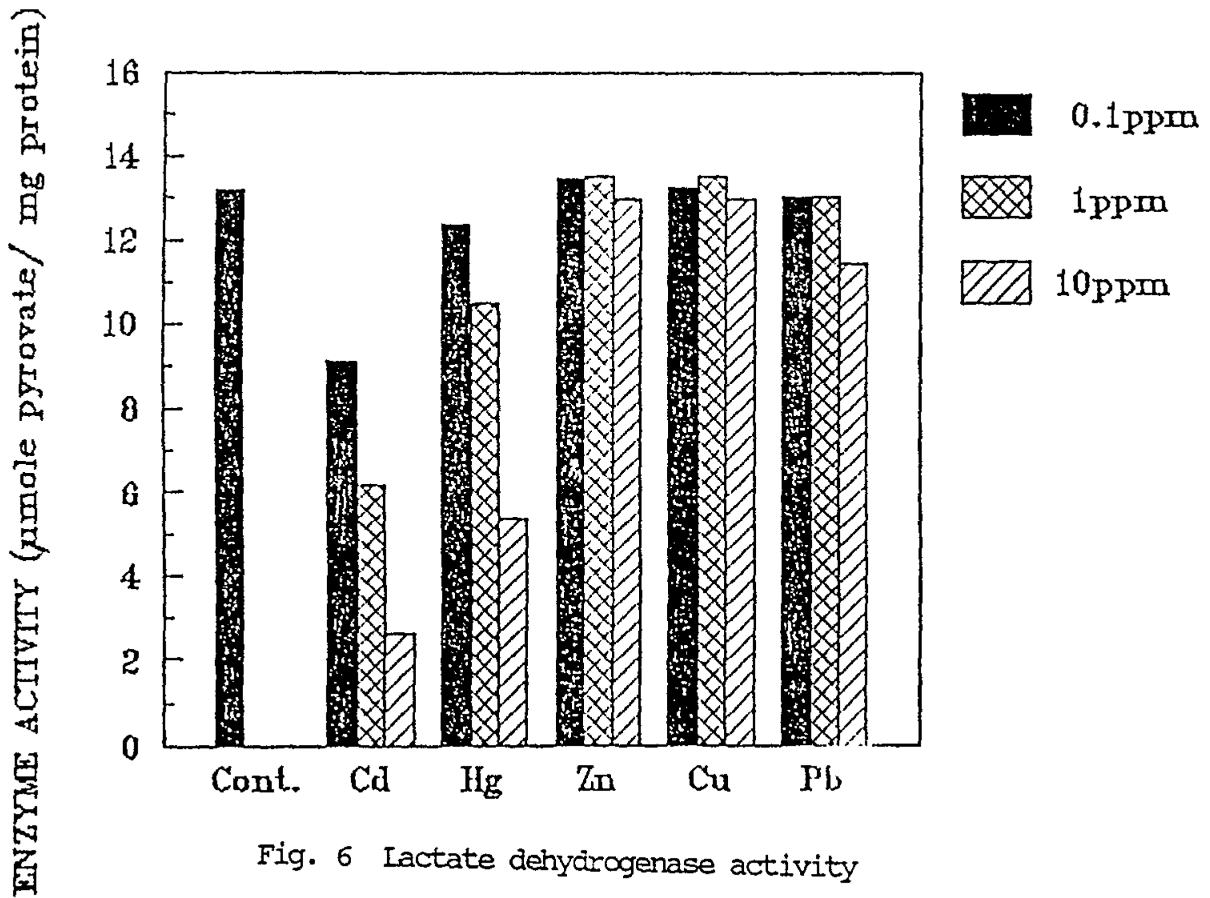

Fig. 6 Lactate dehydrogenase activity

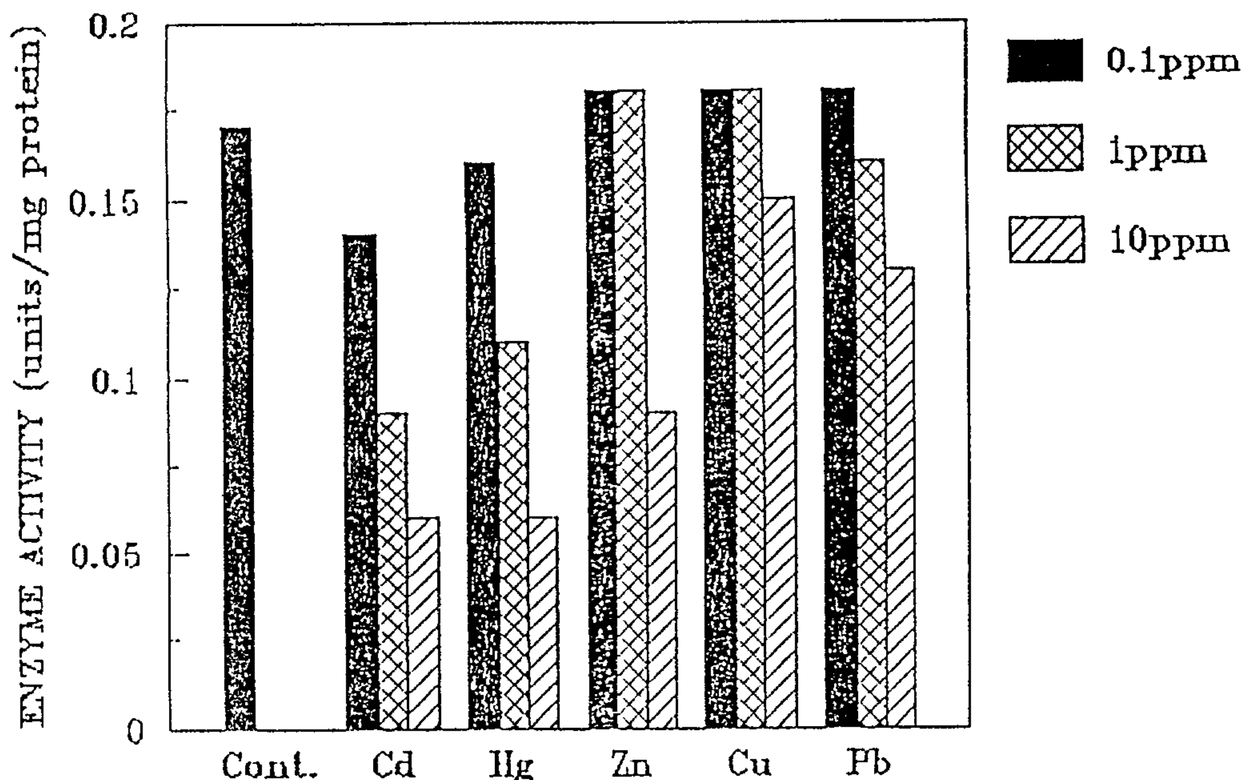

Fig. 7 Cytochrome oxidase activity

4. DISCUSSION

The results demonstrate a wide variation in the acute toxicities of different metals. Enzymes were affected differently in the presence of the ions studied. α-amylase which is an important digestive enzyme of bivalves (Wojtowicz 1972; Seiderer et al., 1982), alkaline phosphatase, which catalyzes of many different esters of phosphoric acid and ATPase enzyme that transports Na and K ions, controls cell volume and causes the activity transport of sugars and amino acids and it consumes more than 30% of the total energy requirement of most cells (Albert et al., 1983). These three important enzymes were only slightly affected by any ion. In the in vitro experiments, these enzymes were found to be much more sensitive than in the in vivo experiments. α-amylase acts primarily in the stomach which contains organic particles that are able to bind metal ions (Simkiss and Taylor, 1981; Howard et al., 1981) and cause them to be unavailable for ineraction with the enzymes. Therefore in the in vivo experiment, the enzyme was actually exposed to lower concentration of heavy metal ions, which did not affect α-amylase activity. Wolfe (1970) found that in Crassostrea virginica nearly all the Zn was bound to soluble high molecular weight protein or to structural protein.

In the in vitro experiments Zn and Cd inhibited the activity of alkaline phosphatase and ATPase enzymes, while in the in vivo test the heavy metal ions caused a slight inhibition only with 10 ppm. Jackim (1974) mentioned that in fish Cu and Pb have differential effects on in

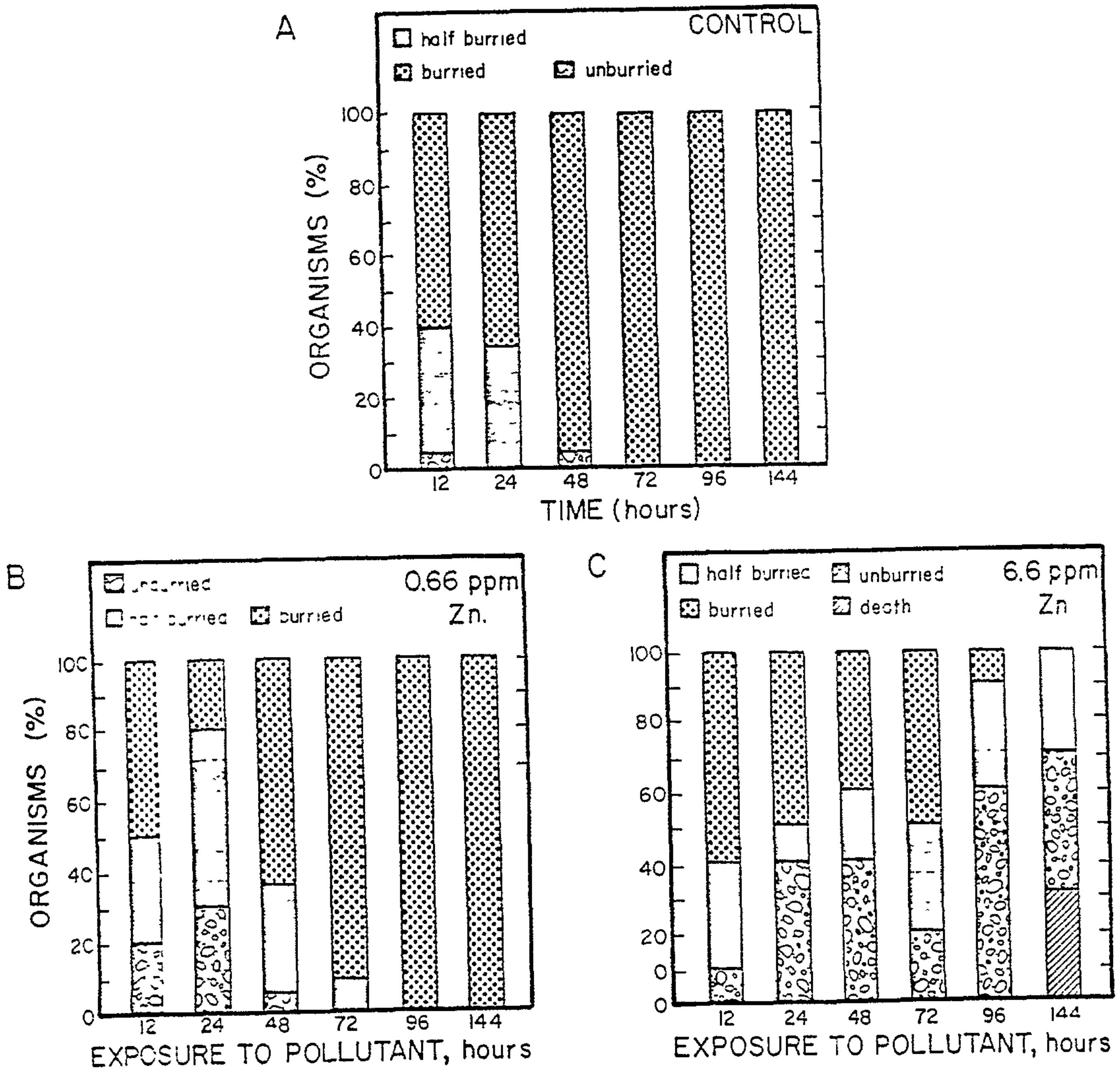

Fig. 8 Behaviour of <u>Donax</u> <u>trunculus</u> on exposure to different concentration of zinc

<u>vitro</u> and <u>in</u> <u>vivo</u> alkaline phosphatase activity. While <u>in</u> <u>vitro</u> studies revealed an inhibition of alkaline phosphatase activity, <u>in</u> <u>vivo</u> assays of the same metals stimulated the activity of this enzyme. In the <u>in</u> <u>vivo</u> experiments ATPase was more sensitive to Zn at 10 ppm than the other metals.

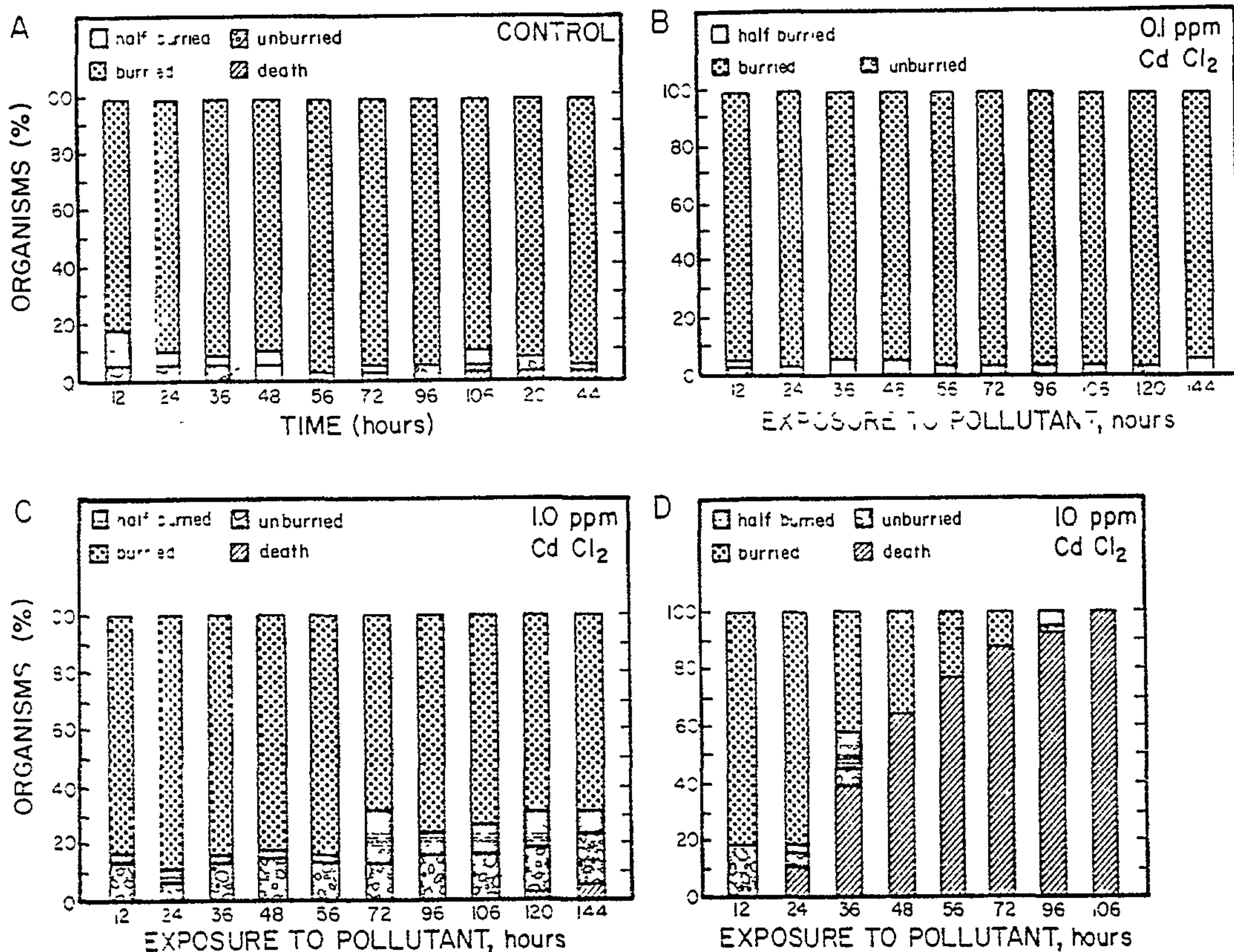

Fig. 9 Behaviour of <u>Donax</u> <u>trunculus</u> on exposure to different concentration of cadmium

In the present study, in the <u>in vivo</u> experiments the other enzymes tested were more sensitive to heavy metal ions than these three enzymes.

It is known that heavy metal ions showing biological activity form metallo structures with proteins by binding to the functional group of proteins. This might cause inhibition or stimulation of enzyme activity (Eichhorn <u>et al.</u>, 1969). Mercury, copper and lead are well known for their ability to combine with sulphydryl groups of proteins (Brown, 1976; Viarengo, 1985). The different response of the enzymes can be explained by a differential binding to functional groups of the proteins.

It is apparent that the respiratory enzyme, Cytochrome oxidase, was the most sensitive to the heavy metal ions studies. Earnshow <u>et al.</u> (1986) pointed out that the motility of mussel's spermatozoa was

slowed down by heavy metals, which is a most logical consequence of respiratory inhibition by these ions. Crespo and Sala (1986) showed that the mitochondria of the chloride cell are targets of zinc contamination, and treatment with Zn caused ultrastructural alteration of the chloride cell of the dogfish Scyliorhinus canicula. They suggested that these alterations are related to an impairment of oxidative phosphorylation.

The activities of lactate dehydrogenase and cytochrome oxidase of Donax were found to be highly inhibited by cadmium and mercury. With zinc and copper there was only a slight inhibition in the activity of the respiratory enzymes. Brown and Newell (1972), working with zinc and copper reported that solution of 500 ppm of copper exerted an inhibitory effect on the oxygen consumption of the whole animal Mytilus edulis. Copper, but not zinc, exerted an inhibition effect on gill tissue respiration. Tort et al. (1982) reported that fish treated with zinc caused an inhibition of gill tissue respiration. Cronin and Flemer (1967) pointed out that chemicals which slow down physiological processes may result in slow down of energy transfer in the community and change the structure of it. Brown (1976) claimed that although a sublethal doses do not kill the animal within a short time, they affect the ability of the organism to respond to the environment and likely will shorten its life.

The results presented here show that inhibition of enzyme activity is exerted by concentrations of heavy metal pollutants at concentrations higher than those found in the area where the animals were collected (Roth and Hornung, 1977; Hornung et al., 1984). Therefore, the present level of pollution does not endanger the population of Donax, but may be encountered in a case of heavy leaks or discharge form the nearby industrial plants. This kind of events, which caused high mortality of animals, were noticed by us near a chemical factory.

We concentrated our testing on the behaviour of Donax in presence of Cd and Zn ions because we found that these ions inhibited the activities of the enzymes more than the other ions were tested. On a typical pattern of burrowing behaviour, first the mussels stay unburied and project their foot outside the valves, begin to touch and test the sand. Typical digging behaviour is then followed, a process with high energy cost which is not always immediately completed and at the end of it, the animals are totally or partly buried. Different behaviour pattern is observed when the environmental conditions are becoming adverse, usually ending with the death of the organism.

Zn at concentrations of 0.1 to 1 ppm did not kill the animals, however the fact that the animals are uncovered may lead to mortality due to other factors like exposure to predation, stranding on the beach encountered a situation when the dying stranded animals piled up on the beach. It might be that these animals were washed ashore by the waves after emergence from the sand as a response to environmental condition. Verriopoulos and Hardouvelis (1988) mentioned that sublethal doses of Zn affect physiological functions as well as the behaviour of marine organisms without causing their death. They found that in the harpacticoid copepod Tisbe holothuriae there is a relationship between mortality and exposure time and Zn concentration. These results resemble those of the present study.

Cd has a different effect on the animals' responses. In normal conditions, when Donax is touched it contract its siphons and foot into the valves and close them very quickly. In the presence of Cd the response was altered. In many cases the valves remained open, the foot and the siphons were extended. The siphons were very thin and long. Closure of the valves was very slow and in many cases there was no synchronization between contractions of the foot and siphons, which cause cutting of the foot or siphons by the valves. These injuries usually cause death of the animals.

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MERCURY BIOACCUMULATION AND EFFECTS ON SOLUBLE PEPTIDES, PROTEINS AND ENZYMES IN THE HEPATOPANCREAS OF THE SHRIMP *Callianassa tyrrhena*

by

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ABSTRACT

Callianassa tyrrhena shrimps were exposed to 0.1-0.4 mg Hg l^{-1} of sea water for up to 15 days and hepatopancreas extracts were analyzed for metal bioaccumulation and protein components.

Soluble mercury was found in proteins of over 70,000 apparent molecular size.

Mercury caused quantitative and qualitative changes in (a) the reversed phase HPLC profile of major soluble polypeptides and (b) the electrophoretic profile of major soluble proteins.

In vitro, mercury inhibited glutathione S-transferase, alkaline phosphatase and esterase activity. *in vivo*, mercury similarly caused a decrease in activity only for alkaline phosphatases in contrast to a significant increase for glutathione S-transferase and esterase activity levels. For the latter enzyme system, a differential activation of its multiple molecular forms was observed in response to mercury exposure.

1. INTRODUCTION

Mercury is widely used in industry and the highest concentrations of this metal (up to 3.6 μg l^{-1} of sea water) in the Mediterranean Sea were reported in coastal waters of localized industrial areas (IRPTC, 1978; Aubert *et al.*, 1980). It is highly toxic and as biochemical responses are likely to be amongst the first manifestations of metal accumulation, attention has been drawn in the effects of mercury on metal-binding proteins (Roesijadi and Hall, 1981; Olafson *et al.*, 1979; Langston and Zhou, 1986; Wagemann *et al.*, 1984; Piotrowski *et al.*, 1974) and enzymes (Sastry and Agrawal, 1979; Iverson *et al.*, 1974; Hilmy *et al.*, 1981; Addya *et al.*, 1984). The toxicity of mercuric ion is generally associated with the high affinity for thiol group of enzymes and other functionally important proteins (Clarkson, 1972; Fang and Fallin, 1976). However the mercury induced change in the biosynthesis of proteins can not be ruled out as in the case of mercury-metallothionein (Bouquegneau *et al.*, 1975; Cherian and Goyer, 1978; Durnam and Palmiter, 1981).

Callianassa tyrrhena is an ecologically important and widely distributed shallow water sediment dwelling shrimp which has a potential for economic exploitation as it is used as bait for commercially important edible fish (Ahsanullah et al., 1984) while hepatopancreas is a vital and major organ in diverse metabolic activities of crustacean species including storage of inorganic reserves and heavy metal accumulation (for a review see Gibson and Barker, 1979). Although several reports exist on the toxicity of mercury in crustacean species (Green et al., 1976; Johnson and Gentile, 1979; Lussier et al., 1985; Papathanassiou, 1986), little is known at the molecular level (Olafson et al., 1979).

It is hoped that changes in tissue protein components measured in terms of protein levels of their activities may be useful as a kind of biochemical autopsy tool for diagnostic sublethal metal poisoning. In this respect the effects of mercury to biochemical parameters of protein nature were studied in the hepatopancreas of the shrimp C. tyrrhena.

2. MATERIALS AND METHODS

2.1 Mercury treatment of animals and tissue extraction

The specimens of the sediment dwelling shrimp Callianassa tyrrhena were collected from Vravrona Bay in South Evoikos. The animals were collected by a method described by Manning (1975) by a pump made from plastic tube (100cm x 7cm i.d.), equipped with a plunger. The sucked contents were emptied in a sieve. Medium weight shrimps (0.5 to 0.8 g) were kept only. Of the two chela, the larger one was cut off to protect the shrimps from attacking each other. The animals were transferred to the laboratory where kept in plastic boxes (11 x 11 x 4cm), containing 400 ml of synthetic sea water of approximately 38 $^{o}/_{oo}$ salinity. Five individuals were kept in each container. The animals were maintained under controlled photoperiods of 12 h light/12h darkness. The temperature was kept constant at 12 ± 0.5°C. The water of each container was replaced every second day. No food was provided. Dead shrimps were removed twice daily.

Mercury was added in the form of $HgCl_2$ to make tinal concentrations of 0.1, 0.2 and 0.4 mg l^{-1} in the sea water. Mercury was added 24h after bringing the animals to the laboratory. Control animals were kept under the same conditions and time periods without any addition of metal.

Hepatopancreas of control and exposed to mercury animals was excised from the animals standing on ice and weighed after applying them on filter paper to remove excess liquid. The tissue was homogenized with 2 volumes of chilled 20 mM Tris-HCl buffer, pH 8.0, in a teflon-pestle homogenizer. Pools of at least 30 shrimps were used for each homogenization except where otherwise stated.

2.2 Reversed phase high performance liquid chromatography

Hepatopancreas homogenate from a pool of 12 shrimps was centrifuged in a Beckman/Spinco 152 Microfuge for 2 min at full speed. A volume of 100 µl of the supernatant was mixed with 1 ml of 0.1%

trifluoroacetic acid (TFA) solution in water. The supernatant after centrifugation in the Microfuge for 2 min at full speed was passed through one Sep-Pak C18 cartridge (Waters Ass., USA), primed for peptide binding with consecutively forcing 10 ml ethanol, 20 ml water and 20 ml of 0.1% TFA solution through it by using a syringe. The cartridge was washed with 20 ml of 0.1% TFA and bound peptides eluted with 40% acetonitrile in 0.1% TFA. The sample was lyophilized in a Speed-Vac centrifugal concentrator (Savant model SVC 100). The dry residue was dissolved in 1 ml of 0.1% TFA. An aliquot of 400 μl was mixed with 100 μl of the same solution and the sample was injected in the HPLC system. The system was composed of one Waters Ass. HPLC pump model 501 connected to a 4.6 x 250 mm C18 Lichrosorb column (HPLC Technology, U.K.) of 5 μm. A variable wavelength U.V. monitor (LKB model 2151) was set at 220 nm with 0.32 AUFS sensitivity. The starting solution was 0.1% TFA and a stepwise gradient was formed from 6 t 36% acetonitrile by a three-way valve at low pressure. The column was at room temperature and the flow rate was 1 ml/min.

2.3 Gradient slab polyacrylamide gel electrophoresis and protein staining

Gradient slab polyacrylamide gels (5-20%) were made by the method of Neville (1971) as modified by Egbert et al. (1976) (without SDS and 2-mercaptoethanol) by a Pharmacia model GM1 gradient mixer. Electrophoresis was carried out in a Shandon model 200 electrophoresis apparatus connected to an LKB model 2177 power supply. Hepatopancreas homogenate was centrifuged in a Beckman ultracentrifuge model L5 equipped with SW Ti rotor at 150,000 x g for 1h at 4°C. To a small volume of supernatant an equal volume of sample buffer was added and 20 μl of sample was applied in each well. Electrophoresis was carried out for about 3h at a constant current of 30 mA at room temperature. It was terminated when bromophenol blue band migrated at a point about 10 cm from the top of the separation gel. After electrophoresis gels were fixed for 30 min with a 20% trichloroacetic acid solution, stained for 24h with a 0.125% Coomassie Brilliant Blue R solution and destained by a (7:5:88, v:v:v) solution of acetic acid, methanol and water.

2.4 Gel filtration

For gel filtration a column of 150cm x 2cm i.d. of Sephadex G-75 equilibrated with 20 mM Tris-HCl buffer, pH 8.0 was used. Hepatopancreas homogenate was centrifuged as mentioned for gradient polyacrylamide gel electrophoresis. Blue dextran (2,000,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), cytochrome c (14,000) and aprotinin (6,500) were used as molecular size markers. Protein concentrations were measured at 280 nm. Whole fractions were analysed for mercury.

2.5 Estimation of mercury levels

Mercury levels were measured in hepatopancreas and in gel filtration fractions using a Varian model 64 vapor generation kit connected to a Varian model AA 157 Atomic Absorption Spectrophotometer. Digestion of tissues was carried out according to the method of Syversen and Syversen (1975).

2.6 Estimation of enzyme activities

Hepatopancreas homogenate was centrifuged in an International ultracentrifuge model IEC-B/60 equipped with rotor A320 at 80,000 x g for 1h at 4°C.

Glutathione S-transferase activity was measured according to the method of Habig *et al.* (1974) at 25°C by monitoring the change in absorption at 340 nm in a Perkin-Elmer model 124 double beam spectrophotometer. In a 1 ml cuvette, 922 μl of 0.1 M phosphate buffer, pH 7.0, 34 μl of a 30 mM glutathione solution and 34 μl of a 30 mM 1-chloro-2,4 dinitrobenzene solution were added. An extinction coefficient of 9.6 mM^{-1} cm^{-1} for the latter reagent was used. The reaction was initiated by the addition of 10 μl of the appropriate dilution of the sample.

Alkaline phosphatase activity was measured according to the method of Harris and Coleman (1968) at 25°C by following the absorbance at 405 nm. In a 1 ml cuvette, 657 μl of 0.1 M Tris-HCl buffer, pH 8.0 and 333 μl of a 14.5 mM p-nitrophenyl phosphate solution were added. The reaction was initiated by 10 μl of a suitable dilution of the sample. An extinction coefficient of 18.5 M^{-1} cm^{-1} was used for p-nitrophenol.

Esterases activity was measured according to the method of Mastropaolo and Yourno (1981) at 30°C by following the absorbance at 235 nm. In a 1 ml cuvette, 980 μl of 0.05 M Tris-HCl buffer, pH 7.2 and 10 μl of a 25 mM alpha-naphthyl acetate solution in ethylene monoethyl ether were added. The reaction was initiated by the introduction of 10 μl of a suitable dilution of the sample. A molar extinction coefficient of 2.34 M^{-1} cm^{-1} was used for alpha-naphthol.

For the *in vitro* effect of mercury on enzyme activity, aliquots of 10 μl of hepatopancreas supernatant were incubated with equal volumes of solutions of mercury in 0.1 M phosphate buffer pH 7.0 for glutathione S-transferase, in 0.1 M Tris-HCl buffer, pH 8.0 for alkaline phosphatase and in 0.05 M Tris-HCl buffer, pH 7.2 for esterases. Incubation lasted 45 min with occasional agitation at room temperature. Controls in the absence of the metal were also prepared.

2.7 Polyacrylamide gel electrophoresis followed by histochemical staining

The samples used for estimation of enzyme activities were also used for electrophoresis. Vertial slab polyacrylamide gel electrophoresis was carried out according to Veini *et al.* (1986). The concentration of the separating gel was 7.5% except for alkaline phosphatase analysis of *in vitro* samples for which case a 7.5% gel was used. The apparatus was the same as used for gradient polyacrylamide gel electrophoresis. Electrophoresis was carried out for about 3h at a constant current of 25 mA and terminated when bromophenol blue migrated about 10cm from the top of the separating gel. Gels were stained for alkaline phosphatase activity according to Boyer (1961) and for esterase activity according to the method of Veini *et al.* (1986).

3. RESULTS AND DISCUSSION

The hepatopancreas of C. tyrrhena appeared to accumulate significant quantities of mercury (Table I) when shrimps were exposed to 0.1-0.4 mg Hg l^{-1} of sea water for 2-6 days, although the number of samples analysed were limited. The levels of concentration of accumulated mercury varied from 3 to 30 $\mu g\ g^{-1}$ wet tissue weight and the total mercury in whole hepatopancreas from 0.1 to 3 μg. Similar values have been reported for the hepatopancreas of another crustacean species, the fiddler crab Uca pugilator (Verneberg and O'Hara, 1972), i.e. 3.6 $\mu g\ g^{-1}$ wet weight for crabs exposed to 0.18 mg Hg l^{-1} for 3 days. Mercury accumulation has also been reported for molluscs in a time and concentration dependent fashion i.e. in Mytilus edulis (Roesijadi, 1982) exposed to 0.005 mg Hg l^{-1} of sea water for 28 days, levels of 130 $\mu g\ g^{-1}$ wet tissue weight were observed in gills and in the mussel Anodonta cygnea (Salanki and Balogh, 1985) exposed to 0.1 μg Hg l^{-1} of sea water from 35 days the levels of mercury in kidney were 250 μg Hg g^{-1} dry weight while after the first week was less than 5 μg Hg g^{-1} dry weight.

Table I

Bioaccumulation of mercury in C. tyrrhena hepatopancreas in relation to duration and concentration of the metal in the sea water.

Duration (Days)	Concentration in sea water (mg l^{-1})	Total metal in hepatopancreas (μg)	Concentration in hepatopancreas ($\mu g\ g^{-1}$ wet weight)
2	0.4	0.328	5.460
		0.430	6.442
4	0.4	2.344	26.909
		2.969	30.018
6	0.4	1.641	26.042
		1.875	31.125
6	0.2	0.703	14.679
		1.647	24.127
6	0.1	0.117	3.271
		0.234	4.389

The accumulated mercury appeared to affect a subset of peptides of hepatopancreas extract as separated by RP-HPLC and monitored at 220 nm, close to the 214 nm wavelength of maximum absorbance of peptide bonds. In comparison to the control chromatogram (Fig. 1A) no significant change was observed at 0.1 mg Hg l^{-1} of sea water exposure for 6 days (results not shown). However, at 0.2 mg Hg l^{-1} of sea water for the same period of exposure a significant increase in peak 10 was observed

with a concomitant dramatic increase in peak 7 (Fig. 1B). These two peaks were further increased in height at the higher concentration of 0.4 mg Hg l^{-1} of sea water (Fig. 1C). Peaks 3 and 5 increased in height only at 0.4 mg Hg l^{-1} of sea water (Fig. 1C). This is the first time that this analytical technique (RP-HPLC) has been successfully employed for the identification of changes in the expression of tissue polypeptidic components in response to the toxic metal accumulation. It is suggested that RP-HPLC in conjunction to electrophoresis resolving higher sized proteins, might provide a useful analysis system for toxicological studies at the molecular level.

Indeed apart from the effects of accumulated mercury on the RP-HPLC profiles in the hepatopancreas extract, a strong disturbance in the protein profile resolved by gradient polyacrylamide gel electrophoresis was also observed. Namely, comparison of the electrophoretic profiles of control and exposed to 0.1 mg Hg l^{-1} of sea water shrimps for a period of 15 days (Fig. 2) showed the appearance in the latter of one new band (indicated with a full triangle up) while 4 other bands disappeared (indicated with full triangles down). Apart from the above qualitative differences, quantitative differences were also observed. The intensity of 5 bands increased (open triangles up) while the intensity of 3 bands decreased (indicated by open triangles down). Induction of protein synthesis and/or reduced degradation in response to heavy metal exposure have been previously reported for cadmium and rat thymic lymphocytes by 2D-electrophoresis (Maytin and Young, 1983) and cadmium and the fish _Mugil cephalus_ (Hilmy _et al._, 1985).

Gel filtration in a Sephadex G-75 column of a hepatopancreas extract showed that all mercury appeared bound to protein(s) eluting at the void volume i.e. of 70,000 or higher molecular weight (Fig. 3). Extraction in the presence of 10 mM 2-mercaptoethanol and 0.25 mM PMSF with 2 mM 2 mercaptoethanol in the elution buffer caused the transfer of all the metal to a peak eluting with potassium dichromate (results not shown). The association of most soluble mercury to high molecular weight protein component(s) instead of thionein-like ones as observed for cadmium, has been reported for the narwhal whale _Monodon monoceros_ (Wagemann _et al._, 1984), the gastropod _Littorina littorea_ (Langston and Zhou, 1986) and the crab _Scylla serrata_ (Olafson _et al._, 1979). In contrast in the mussel _Mytilus edulis_ most of mercury was observed bound to 14,000 apparent molecular weight components as compared to mercury bound to high molecular weight protein(s) (Roesijadi and Hall, 1981).

The _in vivo_, in relation to the _in vitro_ effects of mercury on enzyme systems such as those of glutathione S-transferases, alkaline phosphatases and esterases were studied. In _in vivo_ experiments by spectrophotometric assay measurements of enzyme activity (Fig. 4) alkaline phosphatase activity was found to increase to 140% at 0.1 mg Hg l^{-1} of sea water as compared to 100% of control activity (in shrimps not exposed to mercury), followed by a gradual decrease at higher exposures to the metal (0.2 and 0.4 mg Hg l^{-1} of sea water) finally reaching 70% of the control activity. A different pattern was observed for the other two enzymes (glutathione S-transferases and esterases) i.e. a decrease to 70% at 0.2 mg Hg l^{-1} of sea water followed by a sharp increase to 270% and 170% respectively at 0.4 mg Hg l^{-1} of sea water. In _in vitro_ experiments a gradual decrease in

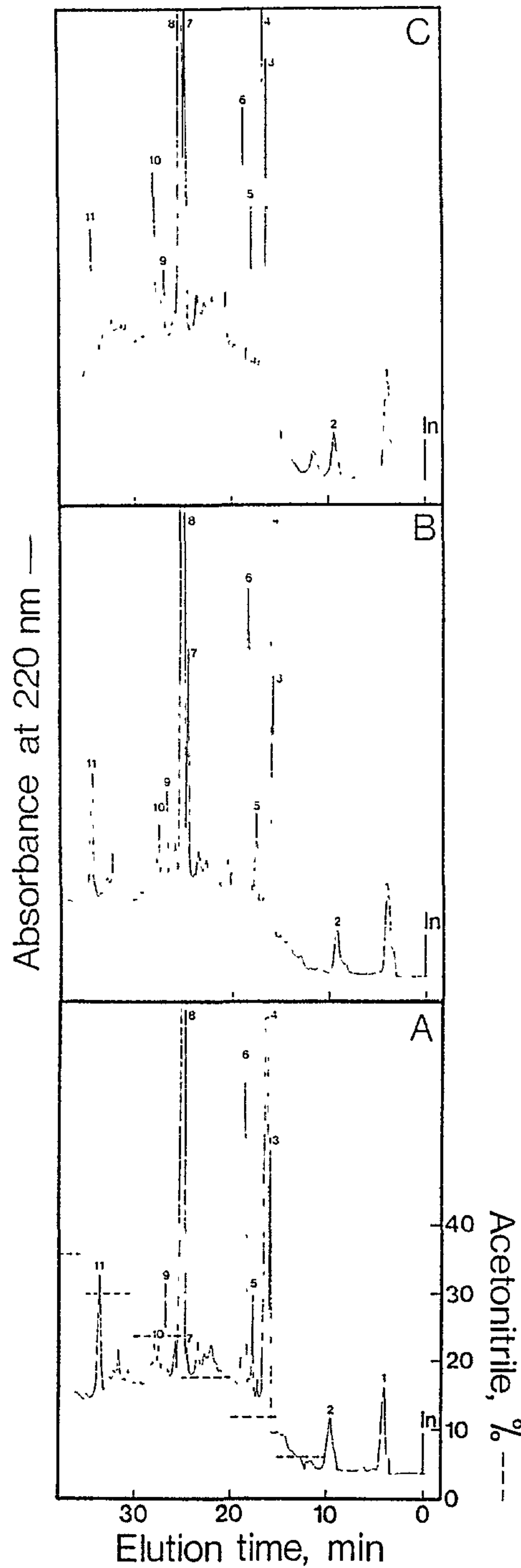

Fig. 1 Separation by reversed phase high performance liquid chromatography of hepatopancreas extract peptides of control (a) and exposed to 0.2 (B) and 0.4 (C) mg Hg l^{-1} of sea water for 6 days. Control shrimps were kept under the same conditions except for the metal. In, time of sample injection. Other details as mentioned in the Materials and Methods section

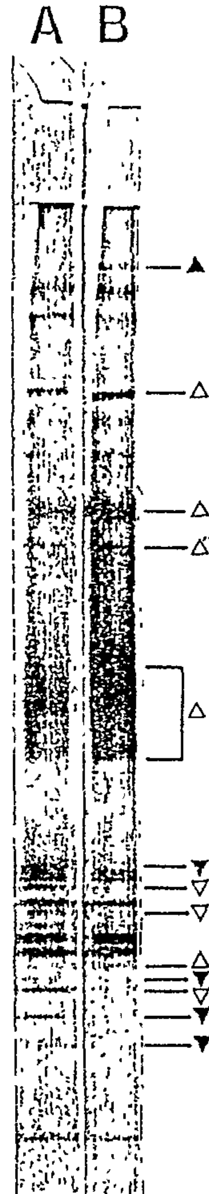

Fig. 2 Gradient polyacrylamide gel electrophoresis of proteins of hepatopancreas extracts of control (a) and exposed to 0.1 mg Hg l^{-1} for 15 days (B) shrimps

activity was found for all three enzyme systems with increasing concentration of mercury in the incubation medium (Fig. 5). Fifty per cent inhibition for glutathione S-transferase, alkaline phosphatase and esterase activity was observed at 0.194 mM, 19.49 mM and 3.12 mM respectively. Therefore the _in vitro_ response of alkaline phosphatases, glutathione S-transferases and esterases were significantly different from the _in vivo_ one. Since for both alkaline phosphatases and esterases (but not for glutathione S-transferases) biochemical methods exist for enzyme activity staining on polyacrylamide gels, we investigated changes in the expression of

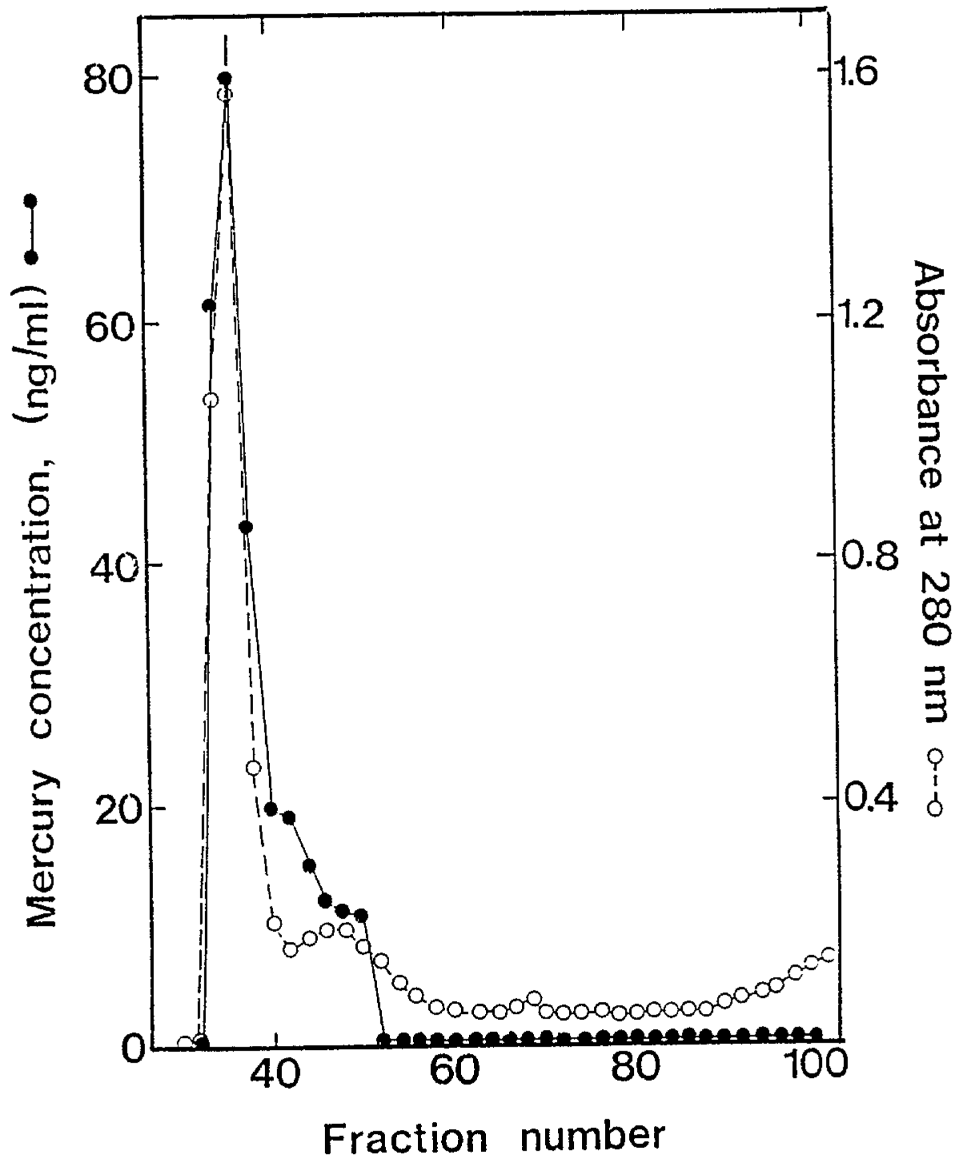

Fig. 3 Sephadex G-75 elution profiles of 1.5 ml of hepatopancreas extract of shrimps exposed to 0.2 mg Hg l^{-1} for 8 days. The flow rate was 25.3 ml h^{-1} and 3.8 ml fractions were collected. The elution positions of dextran blue (void volume) and aprotinin are indicated by perpendicular bars marked by letters a and b respectively

individual molecular forms (Figs. 6 and 7) underlying the observed overall activity changes (Figs. 4 and 5). In the *in vivo* experiments for alkaline phosphatases (Fig. 6) both of the two bands in the control sample were reduced in intensity at high mercury exposure. In the case of esterases (Fig. 6) a reduction in the staining intensity of major bands observed at 0.2 mg Hg l^{-1} of sea water Figure 6B, lane 3 was followed by an increase in intensity of bands 1 and double band 2 (Fig. 6B, lane 4) at higher mercury concentration (0.4 mg Hg l^{-1} of sea water) with the interesting appearance of a new band (indicated by

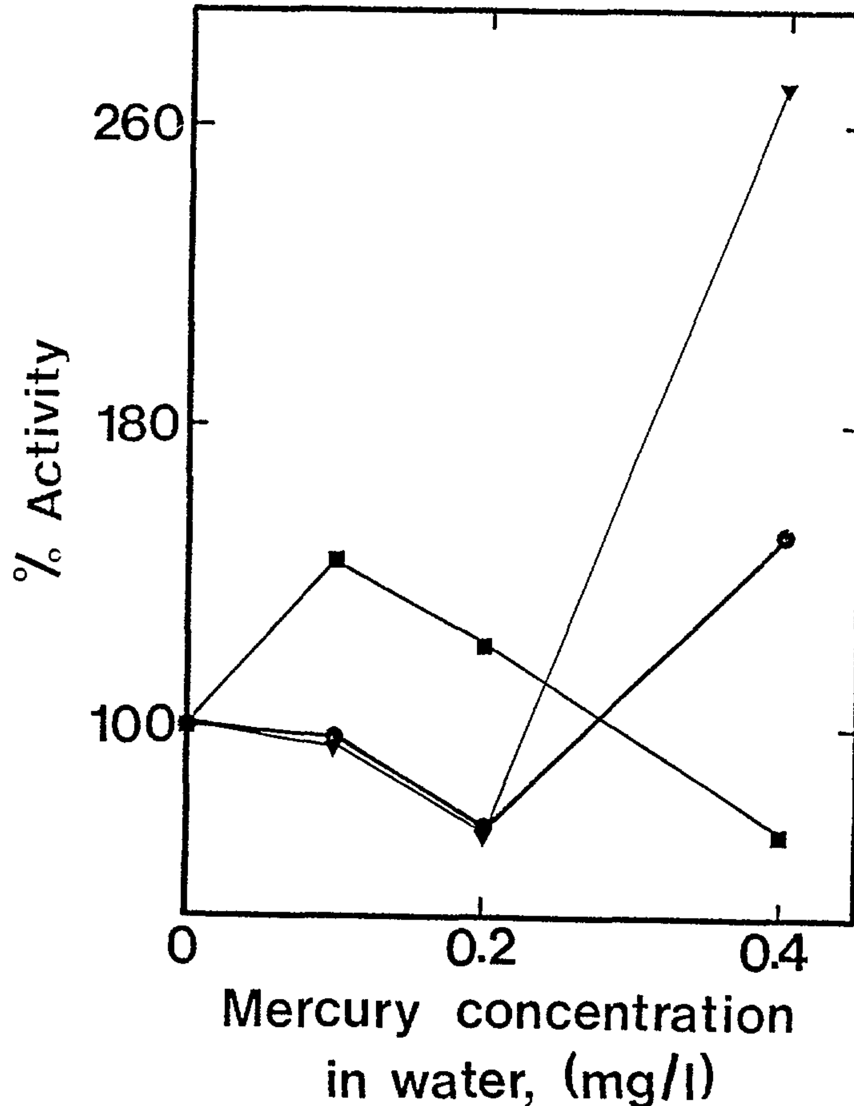

Fig. 4 *In vivo* effects of mercury ont he activity of glutathione S-transferase (▼—▼), alkaline phosphatase (■—■) and esterases (●—●). Shrimps were exposed to 0.1, 0.2 and 0.4 mg Hg l^{-1} for 6 days. One hundred per cent activity corresponded to 4.27 U ml^{-1}, 0.30 U ml^{-1} and 5.98 U ml^{-1} respectively

number 3 in lane 4 of Fig. 6B) of less anodic mobility. In the <u>in vitro</u> exposure of enzymes in tissue extracts to mercury, differentpatterns of multiple molecular forms appeared (Fig. 7) in comparison to those observed in the <u>in vivo</u> experiments (Fig. 6) i.e. for alkaline phosphatases, 2 new bands of less anodic mobility appeared, as compared to control with maximum intensity at 25 mM Hg and with reduced intensity at higher or lower mercury concentrations (50 and 12.5 mM). For esterases (Fig. 7) a gradual decrease in staining intensity of all bands with increasing mercury concentration was observed.

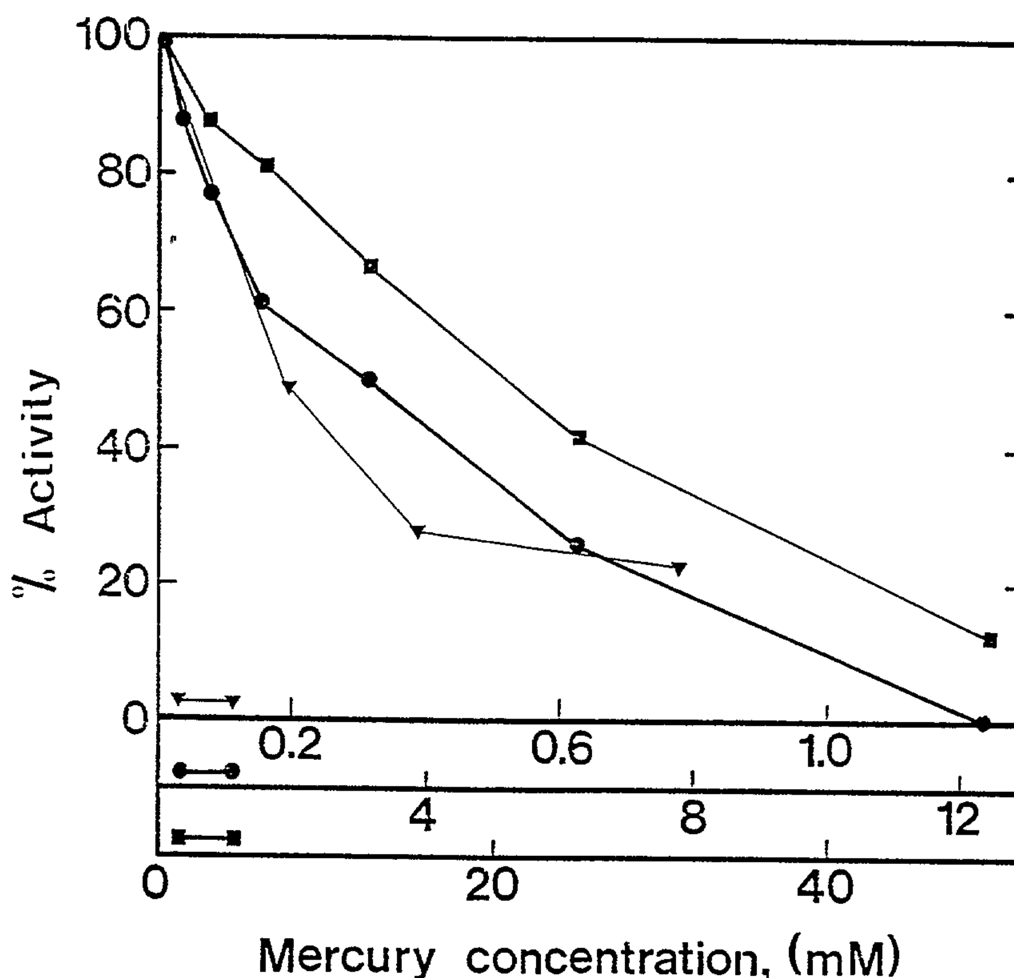

Fig. 5 <u>In vitro</u> effects of mercury o the activity of glutathione S-transferase (▼—▼), alkaline phosphatase (■—■) and esterase (●——●). Shrimp extracts were incubated in final mercury concentrations from 1.56 mM to 50 mM

The <u>in vitro</u> reduction of activity of the three studied enzymes in <u>C. tyrrhena</u> hepatopancreas might be explained by the strong interactions of this metal with the sulphydryl groups of the enzymes and/or their substrates or by the substitution of the endogenous metal

of metalloenzymes by mercury as in the case of alkaline phosphatase. In in vivo experiments, other factors contribute as the changes in synthesis and/or degradation rates of the enzyme due to the metal accumulation in the tissues. In agreement to the observed reduction of alkaline phosphatase in the hepatopancreas of C. tyrrhena a reduction of activity levels in the serum of the fish Aphanius dispar was

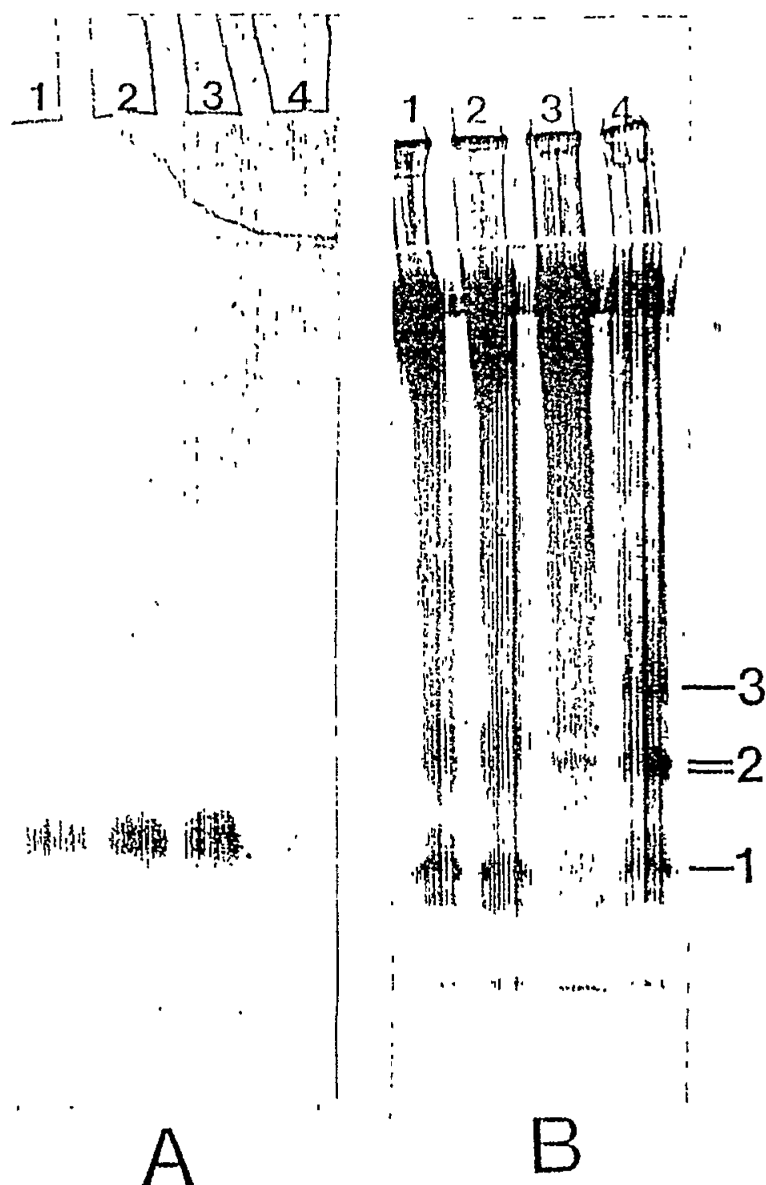

Fig. 6 In vivo effect of mercury on alkaline phosphatase (A) and esterase (B) electrophoretic patterns of hepatopancreas extracts. Shrimps were exposed for 6 days to 0.1 (lane 2), 0.2 (lane 3) and 0.4 (lane 4) mg Hg l^{-1} of sea water. Control animals (lane 1) were treated in the same way without the addition of mercury for 6 days. (For other indications see the Results section).

observed (Hilmy et al., 1981). Decrease in activity levels in response to mercury were also reported for the rat kidney glutathione S-transferase (Addya et al., 1984) in contrast to the significant increase in activity levels for this enzyme observed in C. tyrrhena. It is noted however that only the apparent scarcity of information for the in vivo effect of mercury on enzymes permits a comparison of species as diverse as mammals and crustaceans.

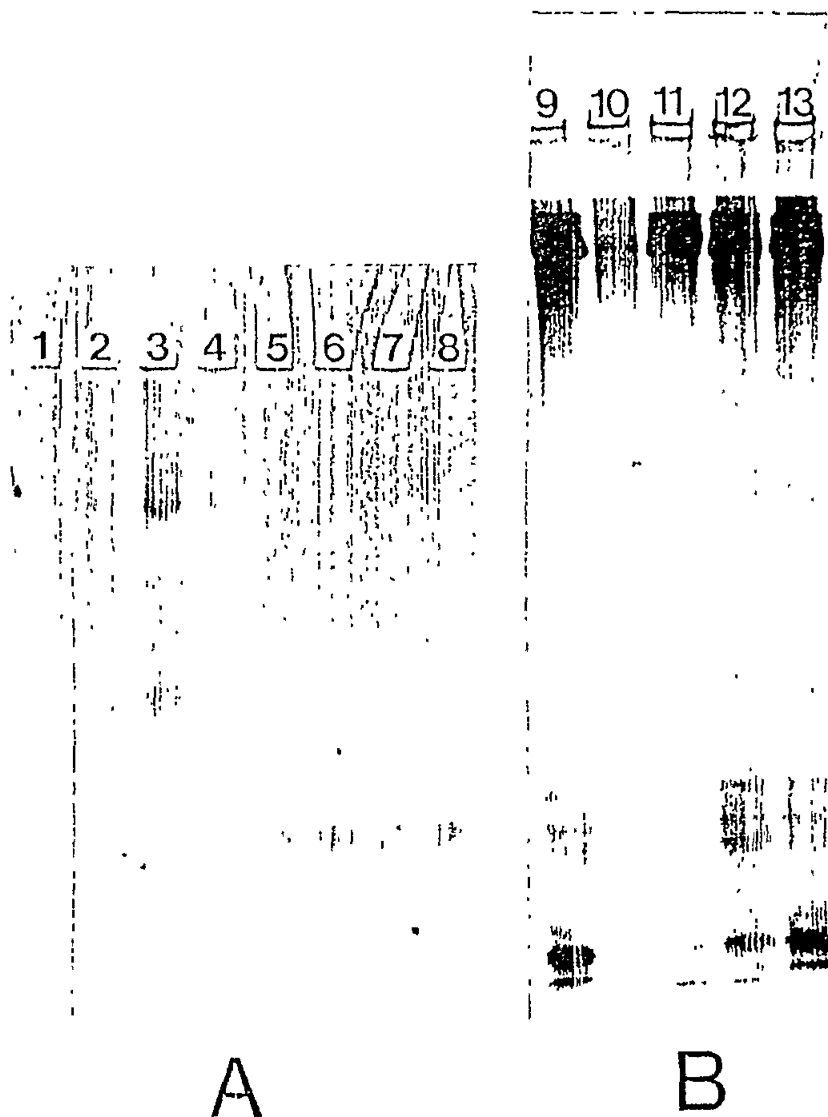

Fig. 7 In vitro effects of mercury on alkaline phosphatase and esterase electrophoretic patterns. Lanes 1-8 were stained for alkaline phosphatase activity and lanes 9-13 for esterase activity. Hepatopancreas extract was incubated with 50 (lane 2), 25 (lane 3), 12.5 (lane 4), 6.25 (lane 5), 3.12 (lane 6), 1.56 (lane 7), 0.78 (lane 8), 33.3 (lane 10), 3.33 (lane 11), 0.33 (lane 12) and 0.033 (lane 13) mM Hg. Controls are in lanes 1 and 9

In conclusion, mercury accumulation was found to cause significant qualitative and quantitative changes to the levels of soluble protein components of the hepatopancreas of the shrimp C. tyrrhena. Further investigation into other protein components and longer exposure periods under lower mercury concentrations is needed for unravelling the protein perturbations caused by heavy metal uptake that might serve as sensitive and probably specific indicators of early stages of toxicity.

4. ACKNOWLEDGEMENTS

This work was carried out with the support of FAO grants GRE/26-G and GRE/32-G. Abid Ali Thaker was recipient of a fellowship from the Greek Ministry of Education. We are grateful to Dr. A.V. Catsiki, National Centre for Marine Research (Ag. Kosmas, 116 04 Hellinikon, Athens) for the mercury measurements.

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ETUDE EXPERIMENTALE DE L'EFFET DU CADMIUM SUR LA FERTILITE DE LA TRUITE ARC-EN-CIEL (Salmo gairdneri)

par

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RESUME

La fertilité de spermatozoïdes (nombre de spermatozoïdes/ovule = 0, 10, 20, 70, 100, 200, 500, 700, 1000, 2000, 5000, 7000, 10000) ayant été exposés, préalablement avant la décondation, à des concentrations de 0, 10, 20, 50, 70, 100, 200 et 500 μg Cd l^{-1}, pendant 5 minutes, a été étudiée. D'autres spermatozoïdes ont été exposés dans les mêmes conditions à une concentration de 10 μg Cd l^{-1} pendant 10 ou 20 minutes.

Le taux de fertilité de 100%, chez les témoins, est obtenu avec un rapport minimum de 200 spermatozoïdes par ovule; le taux de fertilité, pour un temps donné, est inversement proportionnel à la concentration du cadmium.

En présence ou en absence du cadmium, il existe une relation de proportionnalité semi-logarithmique, entre le taux de fertilité et le nombre de spermatozoïdes/ovule, et, pour une concentration de cadmium donnée, la fertilité diminue avec la durée d'exposition.

1. INTRODUCTION

L'étude de la fertilité est l'un des tests les plus simples et les plus rapides, pour l'évaluation de la toxicité de différentes molécules en milieu aquatique. Ce test utilise comme matériel biologique des cellules germinales; il a déjà été utilisé chez l'oursin de mer, (Runnstrom and Hagstrom, 1955; Okubo K. and Okubo T., 1962; Boolitan, 1966; Tyler and Tyler, 1966; Guidice, 1973; Kobayashi, 1971; Hagstrom and Lonning, 1973; Czihak, 1975; Oshida et al., 1981) pour le contrôle de la pollution du milieu aquatique, et pour l'étude de al toxicité des métaux lourds (Hoadley, 1923; McIntyre, 1973; Young and Neleda, 1974; Cleand, 1983), des dérivés pétroliers (Lonning et al., 1975) et des eaux usées (Dinnel et al., 1981). Dans les expérimentations à court terme (quelques minutes), les spermatozoïdes sont exposés à des concentrations inférieures à celles entraînant des mortalités en 96 heures chez les poissons (Dinnel et al., 1982).

Ce test a été appliqué, dans ce travail, chez une espèce carnassière d'eau douce, la truite arc-en-ciel (Salmo gairdneri). Il s'agit d'une espèce intéressante au plan de l'évaluation de la qualité des eaux et des risques écotoxicologiques que les espèces dulscaquicoles, et par la suite, les espèces marines, peuvent affronter

au cours d'une période de leur cycle de développement. Dans ce travail, on a déterminé l'effet de trois paramètres qui sont: la concentration du cadmium, le nombre de spermatozoïdes par ovule, et la durée d'exposition.

Dans le but d'appliquer, éventuellement, ce principe à l'étude de la pollution des eaux douces par des métaux lourds ou d'autres polluants, on a entrepris de l'appliquer à une espèce qui, à notre connaissance, n'a pas encore été bien étudiée, à savoir la truite arc-en-ciel.

2. MATERIELS ET METHODES

2.1 Animaux

On a utilisé des gamètes provenant de truite arc-en-ciel (Salmo gairdneri) agée de trois ans, dont les paramètres de reproduction sont représentés dans le Tableau I. Les animaux provenaient de la Station de Pisciculture et d'Hydrobiologie d'Azrou, et étaient maintenus dans des bacs à flux ouvert d'eau douce; ils ont reçu une alimentation artificielle (Trouw, France).

La méthodologie suivie dans ce travail a été conforme au protocole standard de tests des cellules spermatiques de Dinnel et al. (1982).

2.2 Préparation des gametes

Les animaux ont été soumis au frayage séparé: pour les besoins de l'expérimentation, on a utilisé 6 géniteurs, 3 mâles et 3 femelles. La récolte des oeufs et de la laitance s'est effectuée artificiellement. La laitance est récupérée dans des tubes à col-à-vis, et les oeufs dans des béchers. Les oeufs ont été nettoyés systématiquement, et les spermatozoïdes sont conservés, à l'obscurité, avant la fécondation.

2.3 Comptage des spermatozoïdes

La numération des spermatozoïdes a été faite au microscope (grossissement 400) sur 1ml d'une suspension obtenue en mélangeant, à l'obscurité, 10ml de laitance et 1ml d'acide acétique à 10%, puis dilution avec 50ml d'eau.

Une goutte de la suspension de spermatozoïdes diluée a été introduite dans la cellule de comptage (Hémacytomètre ou cellule de Mallassez). On a déterminé la densité spermatique dans le stock de laitance concentré en appliquant la formule:

$$Y = \frac{D \times S}{C} \times F$$

D : Facteur de dilution
S : Nombre de spermatozoïdes
F : Facteur de dilution de l'hémacytomètre
C : Nombre de carreaux

Tableau I

Paramètres de reproduction des géniteurs utilisées (Salmo gairdneri).

Poissons géniteurs		Poids (g)	longueur (cm)	Gamètes Quantité	Gamètes nombre total	Rapport gonado-somatique	Facteur de Fécondité (1)
Mâles	1	320	32	11 ml	33 10	-	34
	2	330	30,5	8 ml	42,4 10	-	26
	3	450	23	10 ml	66 10	-	20
Femelles	1	330	30	67 g	1005	20,31	3045
	2	360	32,5	65 g	975	19,9	2708
	3	320	28	68 g	1020	21,2	3887

$$(1) = \frac{\text{Volume de la laitance x 1000}}{\text{Poids de l'animal}} \quad \text{ou} \quad \frac{\text{Nombre d'ovules x 1000}}{\text{Poids de l'animal}}$$

2.4 Fécondation

La fécondation a été réalisée dans des tubes à col-à-vis de 20ml. La fécondité de spermatozoïdes (nombre de spermatozoïdes/ovule: 0, 10, 20, 50, 70, 100, 200, 500, 700, 1000, 2000, 5000, 7000, 10000) ayant été préalablement exposés à des concentrations de 0, 10 gCd++ l^{-1} pendant 5, 10 ou 20 mn avant la fécondation a été déterminée. D'autres spermatozoïdes ont été exposés dans les mêmes conditions, à des concentrations de 0, 10, 20, 50, 70, 100, 200 et 500 μg Cd++ l^{-1} pendant 10 ou 20 minutes avant la fécondation.

Deux méthodes ont été utilisées pour mettre en évidence la fécondation. La première est microscopique, elle a consisté à mettre en evidence la formation de la membrane de fécondation; la deuxième méthode est macroscopique, elle est basée sur la persistance de la coloration orange à rosâtre des oeufs. En effet, les oeufs non fécondés sont immédiatement détruits par endosmose, avec apparition de tâches blanchâtres sur le pôle contenant le disque germinatif ou sur la totalité de l'oeuf.

2.5 Méthode de calcul

Toutes les réponses tests (pourcentage d'oeufs non fécondés) sont corrigées par rapport aux réponses normales des contrôles par la formule d'Abbot (Finney, 1971):

$$\frac{\text{\% réponse test - \% réponse contrôle}}{\text{100 - \% réponse contrôle}} \text{ X 100}$$

3. RESULTATS

Lors de la première expérimentation (durée 5 minutes), le nombre minimal de spermatozoïdes par ovule donnant 100% de fertilité chez les témoins est de 200; il est respectivement de 300 et de 1000 lorsque la durée d'expérience est de 10 ou 20 minutes.

Pour les lots expérimentaux, il apparaît que les trois paramètres (rapport spermatozoïdes/ovule, durée d'exposition et concentration de cadmium) sont étroitement liés.

3.1 Effet concentration

Pour le taux de 200 spermatozoïdes/ovule, l'effet de la concentration du Cd (durée d'exposition 5 minutes) sur la fertilité est très prononcé: la fertilité est nulle pour toutes les concentrations supérieures ou égales à 100 μg Cd l^{-1}, elle est supérieure à 50% pour les concentrations inférieures à 50 g Cd l^{-1} (Figs 1 et 2; Tableau II).

A partir du rapport 5000 spermatozoïdes/ovule, le taux de fertilité est supérieur à 50% pour n'importe quelle concentration de cadmium inférieure ou égale à 500 μg Cd l^{-1}; le rapport 10000 semble donner un maximum de fertilité >80% pour n'importe quelle concentration de cadmium.

3.2 Effet durée

L'effet durée a été étudié uniquement pour la concentration de 10 μg Cd l^{-1} (Figs 3,4,5,6,7). Pendant la période active (les 5 premières minutes), on observe un chevauchement de la courbe exprimant l'exposition à 10 μg Cd l^{-1} et celle des témoins: dans.ces conditions, la concentration employée n'affecte pas notablement la fertilité (Figs 5,6).

La durée de séjour des spermatozoïdes avant la fécondation, ayant été préalablement exposés ou non au toxique, avant la fécondation, fait abaisser le taux de fertilité. Par ailleurs, plus la durée d'exposition au cadmium augmente, moins le pourcentage de fertilité est important. Une exposition à 10 μg de Cd pendant 10 minutes, fait chuter la fertilité de 10% alors qu'un séjour de 20 minutes la fait abaisser de 30% (Figs 3,4,6,7). Une relation de proportionnalité semi-logarithmique est enregistrée entre le taux de fertilité (en probit) et le rapport spermatozoïdes/ovule indifféremment de la durée d'exposition et de la concentration du cadmium (Figs 5,6).

4. DISCUSSION

D'une manière générale, le taux de fertilité chez Salmo gairdneri est dit acceptable lorsque sa valeur est supérieure ou égale à 50%.

Dans cette étude, la fertilité maximale est obtenue dans l'intervalle des 5 premières minutes, aussi bien chez les témoins qu chez les lots expérimentaux. En dehors de toute exposition au cadmium, la durée d'activité et le pouvoir de fertilisation des spermatozoïdes dans le milieu extérieur, diminue progressivement.

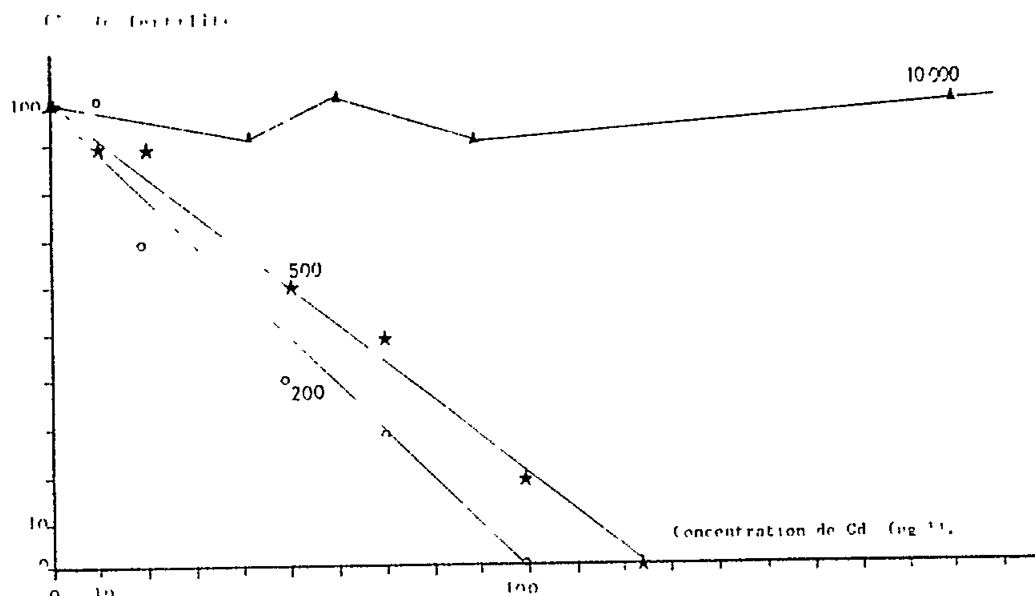

Fig. 1 Evolution du taux de fertilité (%), en fonction de la concentration en cadmium (μg Cd l^{-1}), aux rapports (S/O) 200, 500 et 10000

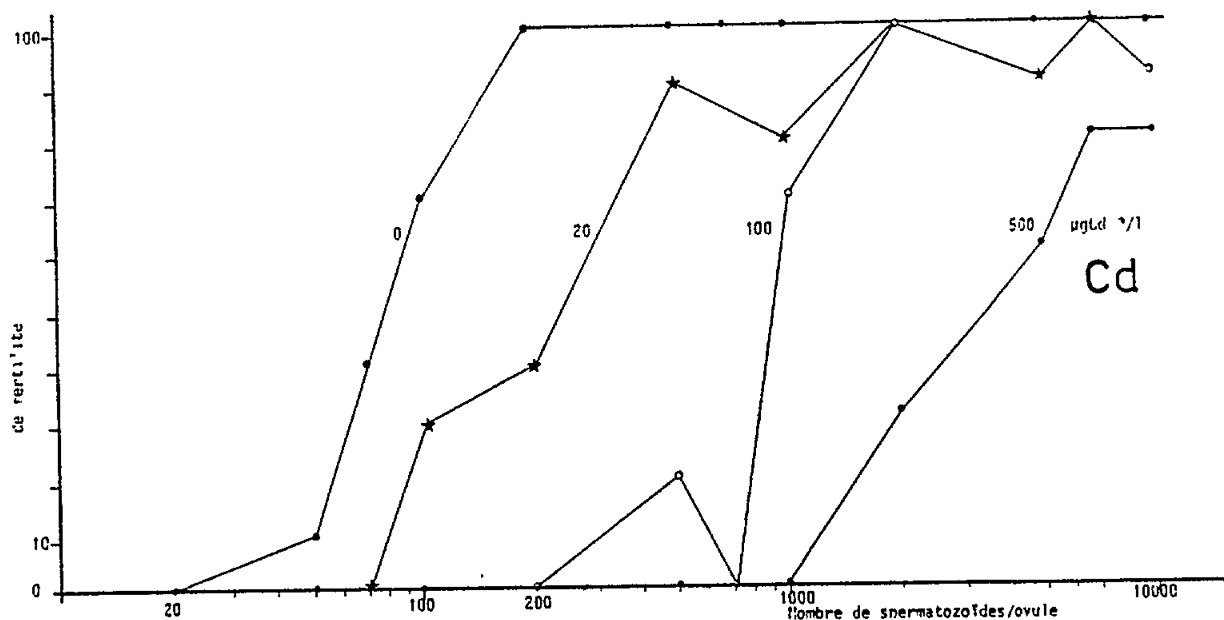

Fig. 2 Evolution du taux (%) de fertilité, en fonction du nombre de spermatozoides par ovule (S/O), et de la concentration en cadmium (μg Cd l^{-1})

Tableau II

Evolution du taux de fertilité (%), en fonction du nombre de spermatozoides par ovule (S/O) et de la concentration (C) en cadmium ($\mu g\ l^{-1}$).

C \ S/O	0	10	20	50	70	100	200	500	700	1000	2000	5000	7000	10000
0	0	0	0	10	40	70	100	100	100	100	100	100	100	100
10	0	0	0	0	35	75	95	90	100	70	100	80	100	100
20	0	0	0	0	0	30	70	90	100	80	100	90	100	100
50	0	0	0	0	10	10	40	60	100	50	90	100	100	90
70	0	0	0	0	0	10	30	50	50	100	70	60	100	100
100	0	0	0	0	0	0	0	20	0	70	100	80	100	90
200	0	0	0	0	0	0	0	0	0	40	50	90	80	100
500	0	0	0	0	0	0	0	0	0	0	30	60	80	80

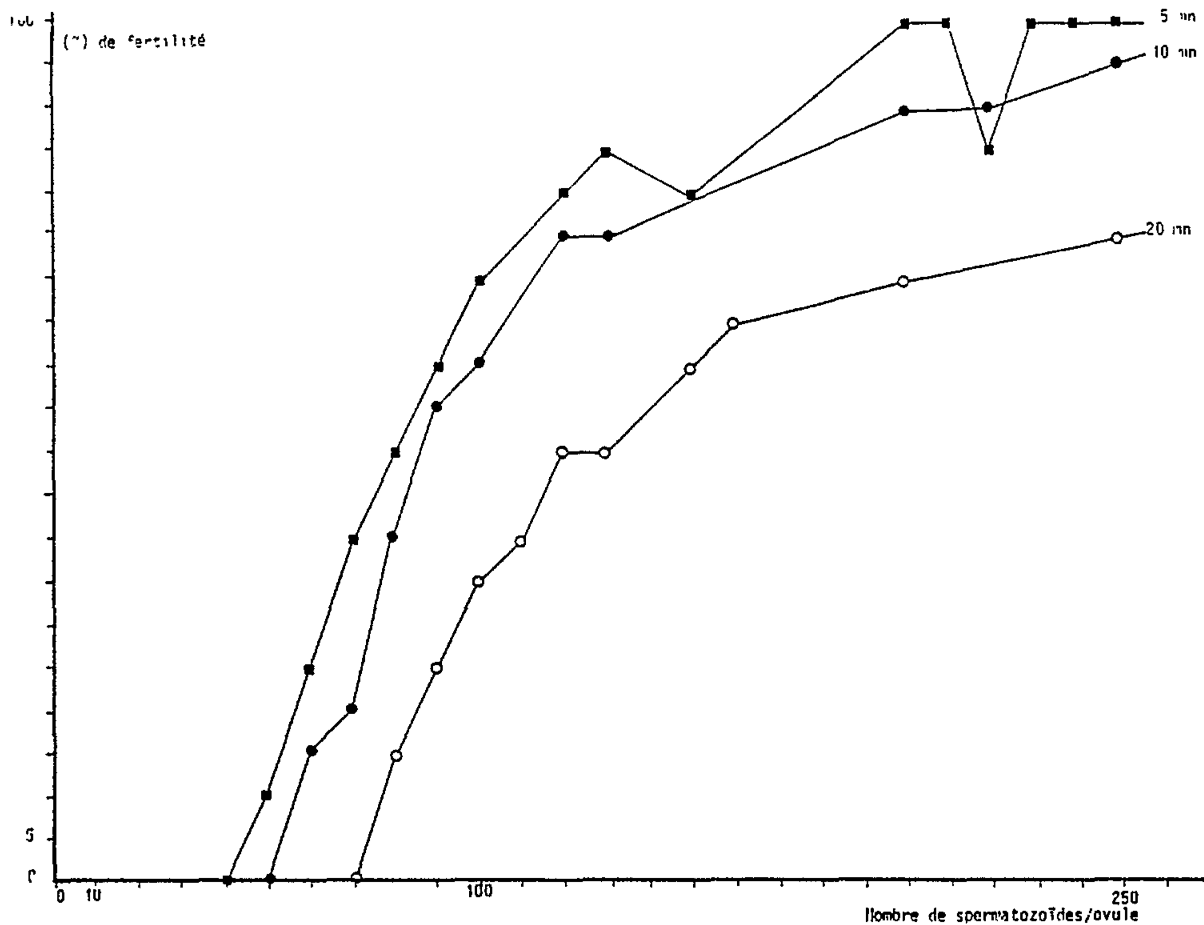

Fig. 3 Evolution du taux de fertilité (%), en fonction du temps de séjour avant la fécondation, et du nombre de spermatozoides par ovule, chez les témoins

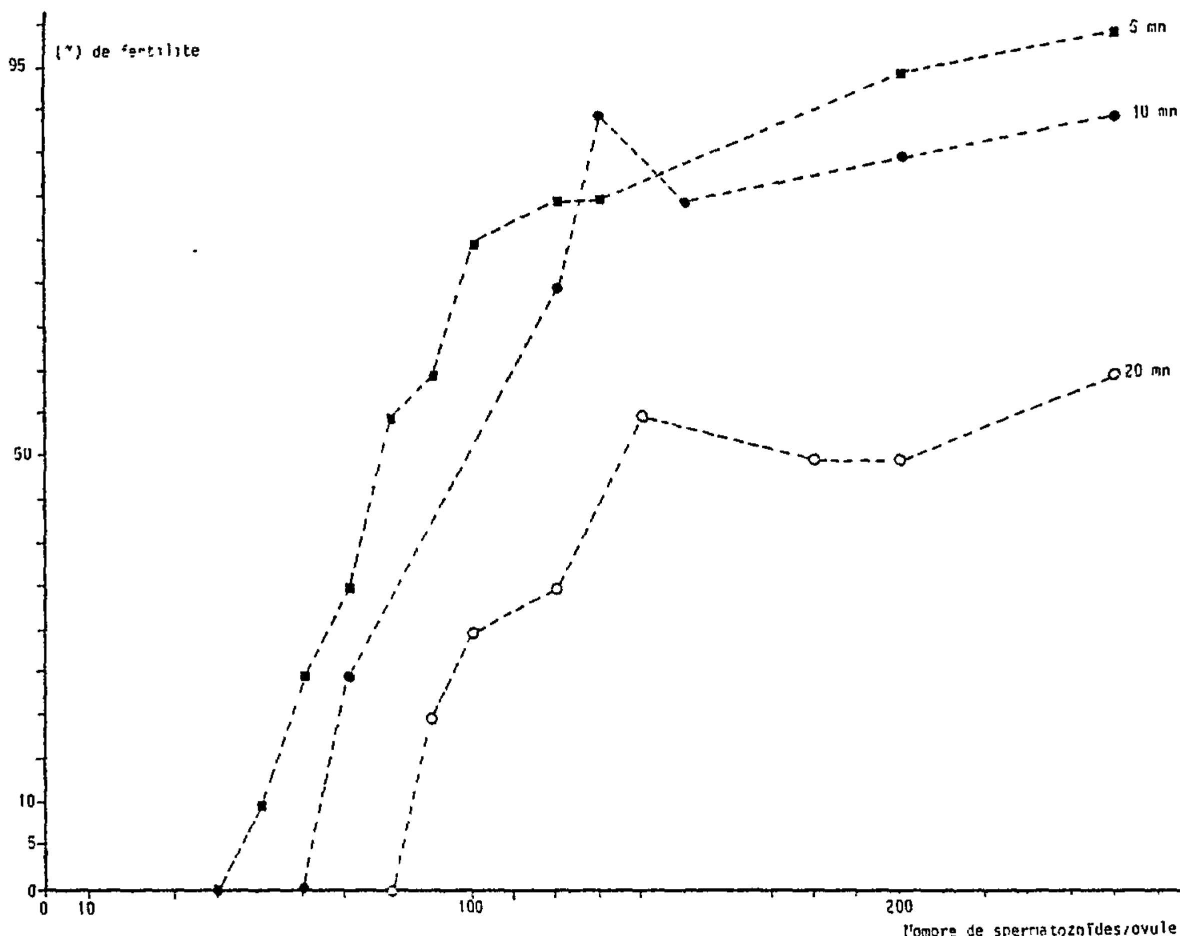

Fig. 4 Evolution du taux de fertilité (%), en fonction du temps de séjour avant la fécondation, et du nombre de spermatozoides par ovule, chez les éssais (10 µg Cd l^{-1})

Ce pouvoir diminue relativement plus rapidement lorsque les spermatozoïdes sont exposés au cadmium. Des observations comparables ont été rapportées, avec le méthylmercure par McIntyre (1978), et Billard et Roubaud (1985) chez Salmo gairdneri, et par Abu et Weis (1987) chez Fundulus heteroclitus, et par Environmental Protection Agency (1972) chez Pimephales promelas. Il est permis de parler dans ces conditions de synergie, entre l'effet toxique du cadmium et la durée d'exposition à ce métal.

On constate en outre que trois facteurs interviennent principalement pour modifier le taux de fertilité; la durée d'exposition, la concentration du métal lourd et le rapport nombre de spermatozoïdes par ovule; ces constatations sont conformes à celles émises par Renzoni (1974), Dinnel et al. (1981, 1982), Oshida et al. (1981), et Pagano et al. (1983): plus la durée d'exposition augmente, plus l'effet du cadmium est prononcé. Donc, le pouvoir de fertilité peut être favorisé soit en diminuant la durée d'exposition et la concentration du cadmium, soit en augmentant le nombre de spermatozoïdes par ovule.

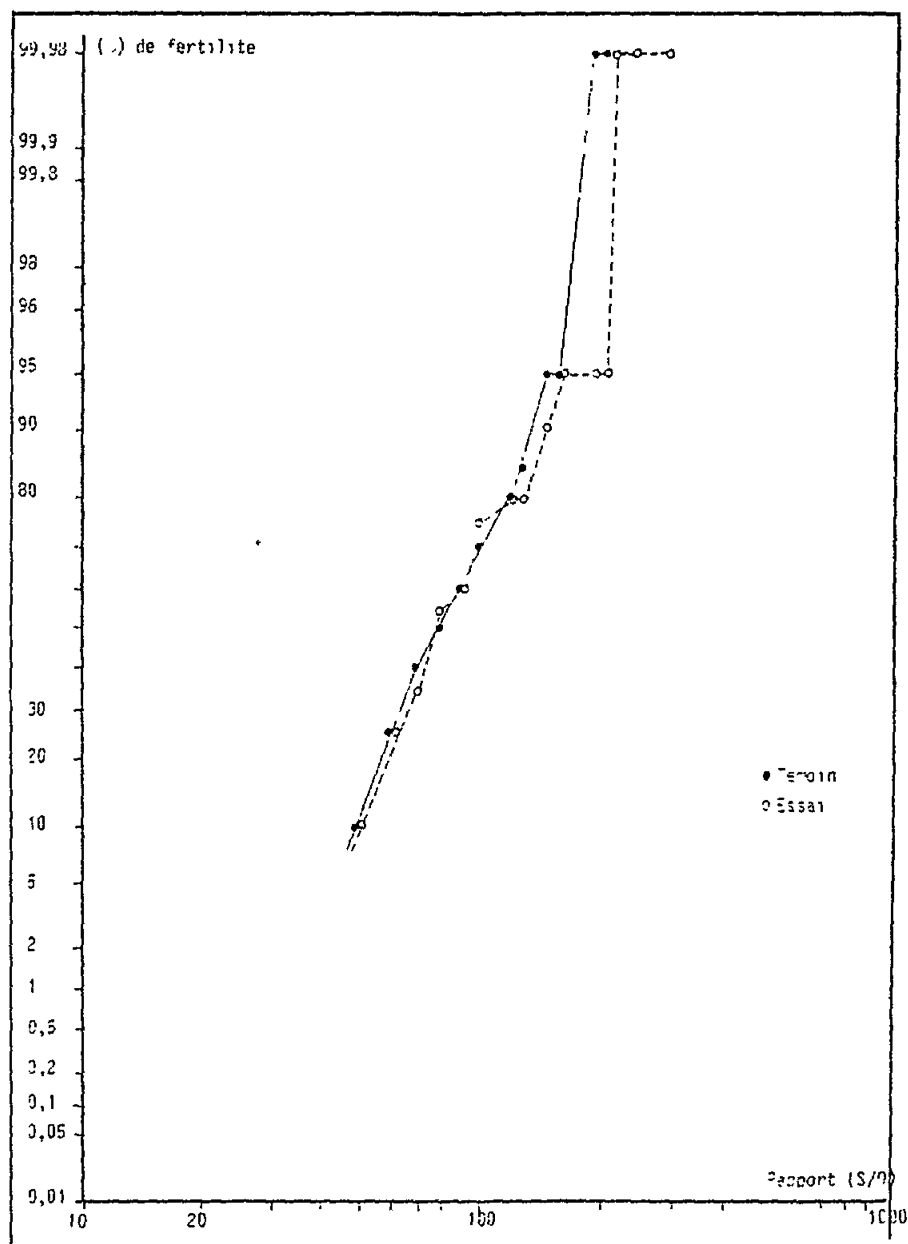

Fig. 5 Evolution du taux de fertilité (%) (probit), en fonction du temps de séjour (5mn) avant la fécondation, et du rapport (S/O) (log.)

A la lumière de ces données, il est permis d'émettre deux hypothèses qui ne s'excluent pas nécessairement: 1) les spermatozoïdes, à l'état d'activité optimale (5 premières minutes), seraient moins sensibles au cadmium; par la suite, la mobilité des spermatozoïdes est davantage affectée par le cadmium. Ceci pourrait être en relation avec le perte d'énergie d'activation, ce qui fait perdre aux spermatozoïdes leur pouvoir métabolique après les 5 premières minutes; 2) d'autres facteurs peuvent être limitants: il s'agit de la période requise pour le contact ovule-spermatozoïde, et du rôle du liquide spermatique qui favorise la fécondation.

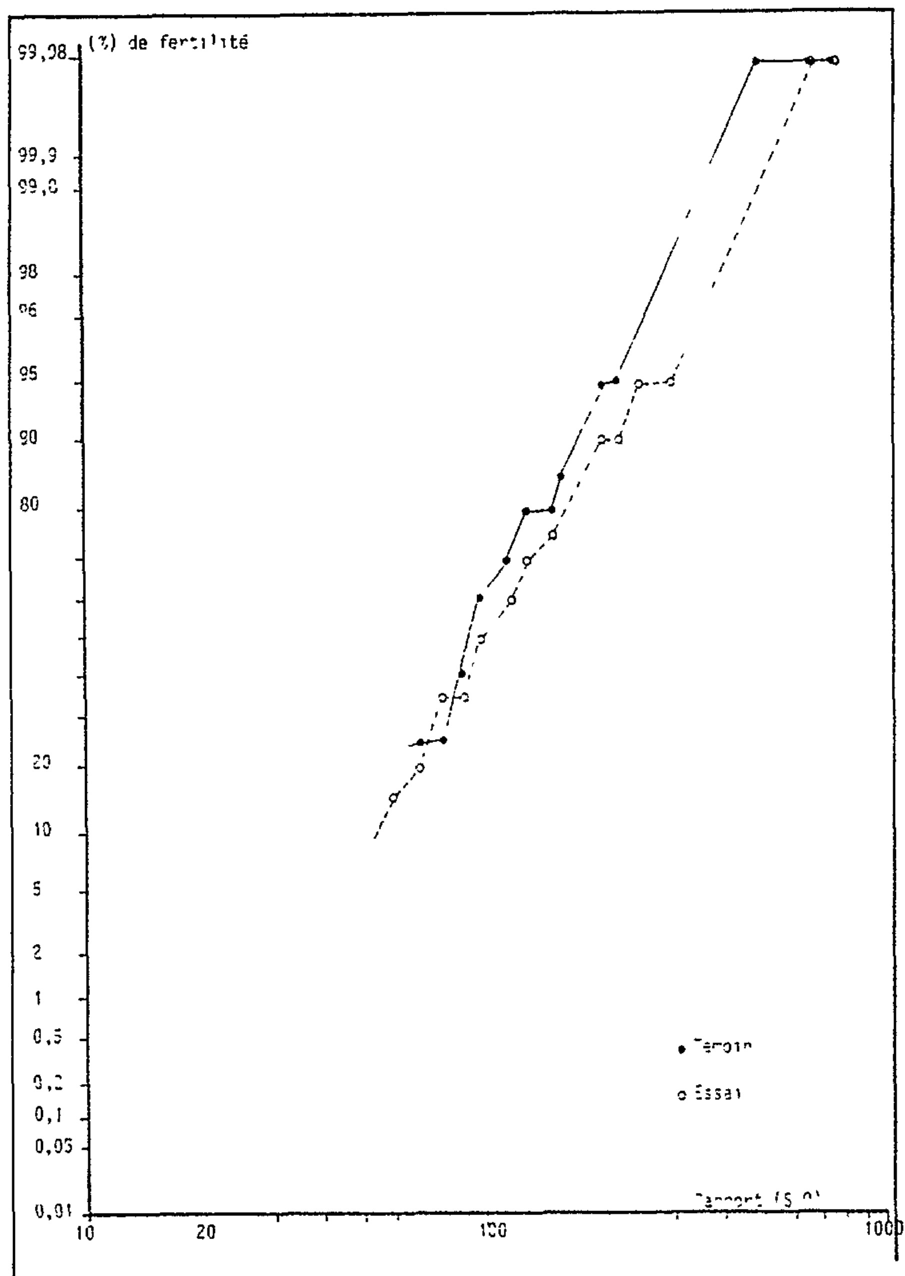

Fig. 6 Evolution du taux de fertilité (%) (probit), en fonction du temps de séjours (10mn) avant la fécondation, et du rapport (S/O) (log.)

5. CONCLUSION

Dans l'étude de la toxicité du cadmium, la fertilité des spermatozoïdes se trouve liée, simultanément, à trois composantes qui sont la concentration du cadmium, la composante biologique qui dégage l'effet rapport (nombre de spermatozoïdes/ovule), et la composante physique qui résulte de la durée d'exposition au cadmium.

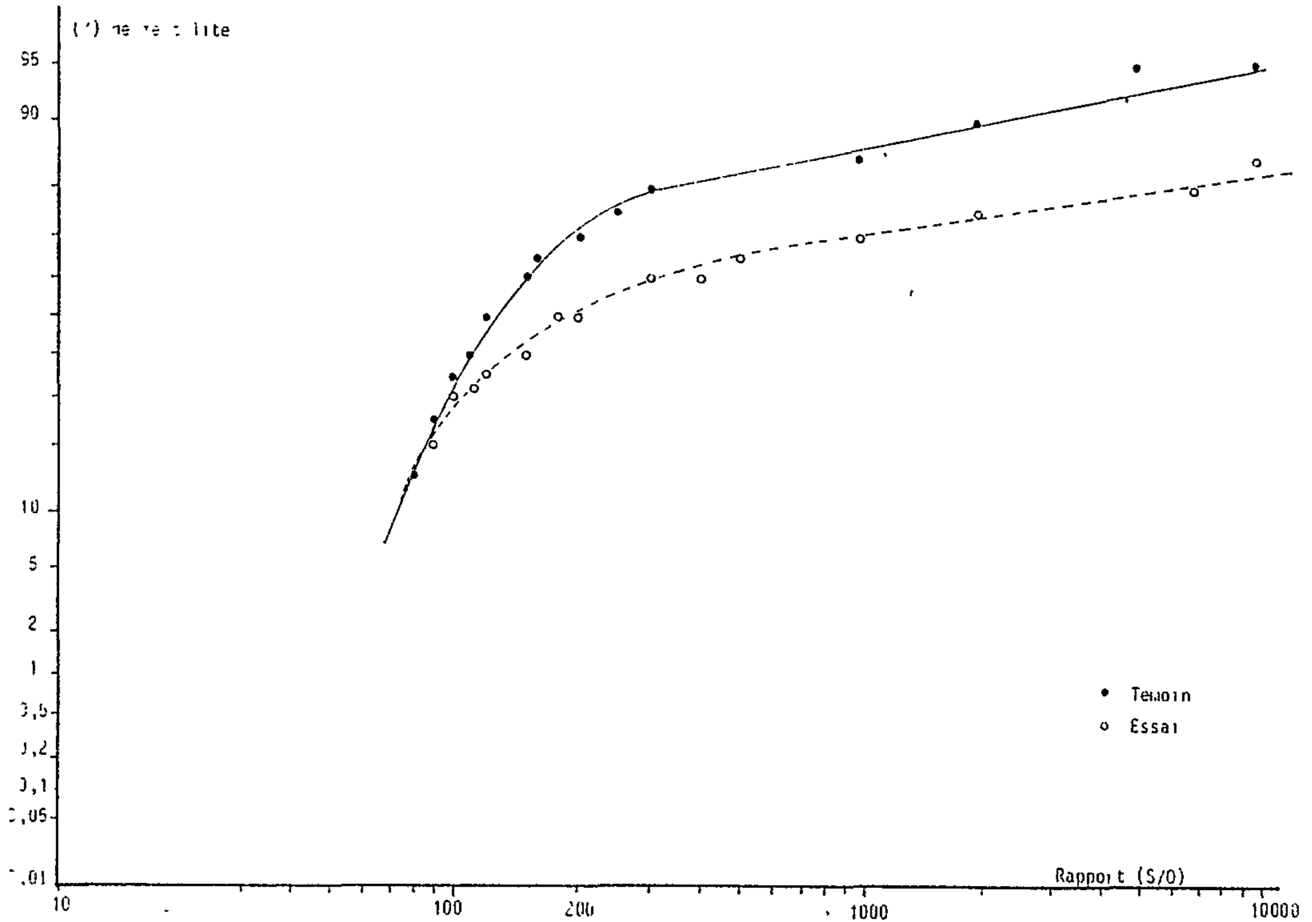

Fig. 7 Evolution du taux de fertilité (%) (probit), en fonction du temps de séjours (20mn) avant la fécondation, et du rapport (S/O) (log.)

Le développement et la finesse de ce test biologique, conduit à une procédure rapide et sensible, qui permet la détection globale d'une large gamme de toxiques dans le milieu aquatique. Cela devant aider dans la prédiction des effets écotoxicologiques et donne une bonne réponse au modèle de contrôle de la qualité des eaux. S'il faut considérer l'impact du polluant dans l'environnement, il ne faut pas, toutefois, négliger l'effet du facteur durée d'exposition qui est considéré comme un important paramètre de l'action des polluants au stade gamète.

En fin de compte, le modèle de pollution par le cadmium étudié ici démontre qu ce test biologique est applicable à Salmo gairdneri; il pourrait être appliqué à des espèces marines.

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Publié et imprimé par:

Plan d'action pour la Méditerranée
Programme des Nations Unies pour l' Environnement

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