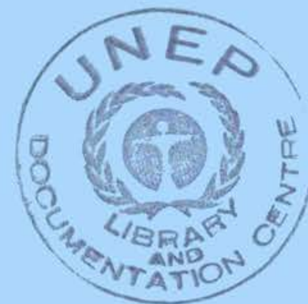
MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

FINAL REPORTS ON RESEARCH PROJECTS DEALING WITH BIOLOGICAL EFFECTS (RESEARCH AREA III)

RAPPORTS FINAUX SUR LES PROJETS DE RECHERCHE RELATIFS AUX EFFETS BIOLOGIQUES (DOMAINE DE RECHERCHE III)



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This volume is the one-hundred and third 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 cent-troisième 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.

PREFACE

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 and 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 and appropriate allocations of resources.

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.

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.

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 contains the final reports on research projects dealing with biological effects (Research Area III). 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.

PREFACE

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 développement 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 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.

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.

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.

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 (Athènes, octobre 1989) 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 relatifs aux effets biologiques (Domaine de Recherche III). 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 Mme V. Papapanagiotou, Secrétaire FAO était chargée de la dactylographie.

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by

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ABSTRACT

Benzo(a)pyrene [B(a)P] treatment of gilthead seabream, 25 mg kg^{-1}, i.p. for five consecutive days, did not cause any significant changes in ethylmorphine N-demethylase and aniline 4-hydroxylase activities of liver microsomes. The same treatment did not alter the liver microsomal cytochrome b5 content, NADH-cytochrome b5 reductase and NADPH-cytochrome P450 reductase activities. However, benzo(a)pyrene treatment caused 2-3-fold increase in 7-ethoxyresorufin O-deethylase (7-EROD) activity of gilthead seabream liver microsomes. Although, upon treatment, total cytochrome P450 content of liver microsomes increased about 1.7-fold in 1990 fall, no such increase was observed in spring 1991. However, a new cytochrome P450 with an apparent M_r of 58,000 was observed on SDS-PAGE of liver microsomes obtained from benzo(a)pyrene treated gilthead seabream. Besides, in vitro addition of 0.2×10^{-6} M benzo(a)pyrene to the incubation mixture inhibited 7-ethoxyresorufin O-deethylase activity by 93%. Gilthead seabream liver microsomal 7-ethoxyresorufin O-deethylase activity was characterized with respect to substrate concentration, amount of enzyme, type of buffer used, incubation period and temperature.

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

1. INTRODUCTION

The sea is a repository for a number of foreign compounds (xenobiotics) which occur as an environmental pollutants. As a result of high technological and industrial development, the aquatic environment is becoming increasingly threatened by an alarming number of chemicals including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAH), dioxins and alkyltin compounds.

Biotransformation of relatively insoluble organic chemicals to more water soluble compounds is a requisite for their detoxification and excretion.

The fist step in biotransformation is usually the oxidative step, catalyzed by the microsomal cytochrome P450 dependent mixed function oxidase (MFO) system (also called monooxygenases, drug metabolizing enzymes). This "Phase I" metabolism is usually followed by "Phase II" in which oxygenated groups of xenobiotics are conjugated with glutathione, sulfate or glucuronate by different families of transferase enzymes. Thus, the resulting polar and water soluble end product can be excreted from the organism through bile or urine (Nebert and Gonzalez, 1987).

In addition, cytochrome P450 dependent mixed-function oxidation is also responsible for the activation of foreign chemicals to the reactive intermediates that ultimately results in toxicity, carcinogenicity and mutagenicity (Conney and Burns, 1972; Pelkonen and Nebert, 1982). The degree of detoxification versus toxification of these compounds depends on which metabolic pathway predominates. Thus, it would be important to identify the factors (homeostatic and environmental) influencing the direction of metabolism of these compounds.

Fish populations living in highly polluted areas often have high incidences of gross pathological lesions and neoplasms associated with elevated levels of toxic chemicals in the sediments (Payne et al., 1987). Malins et al. (1985) reported high levels of neoplasms in fish collected from a creosote (mixture of petroleum products) polluted Puget Sound, U.S.A.. Kocan et al. (1985) observed that much of the cellular toxicity associated with the extracts of sediments from Puget Sound, require metabolic activation. DNA isolated from neoplastic nodules of hepatic tissues of English sole exposed to creosote pollution in Puget Sound was shown to contain modified guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy Gua) (Malins et al., 1990).

Benzo(a)pyrene, a member of PAHs, is also present in petroleum products and in waste materials of industry, and has both mutagenic and carcinogenic properties (Conney and Burns, 1972; Heidelberger, 1973). Induction of microsomal MFO activities (B(a)P hydroxylase and 7-EROD activities) in response to PAH treatment are observed in fish (Stegeman and Kloepper-Sams, 1987; Goksøyr and Förlin, 1992). Many laboratory experiments have been carried out to demonstrate the elevated levels of MFO activities in fish in association with petroleum hydrocarbons, PAHs, and PCBs.

In 1977, Bend et al. studied the effect of administration of 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the hepatic microsomal MFO activities of little skate (Raja erinacea). They found that B(a)P hydroxylase activity of skate liver microsomes was increased when skate was treated twice with 3-MC (50 mg kg^{-1} body weight), orally. Similar results were also obtained with TCDD. B(a)P hydroxylase activity of little skate hepatic microsomes was markedly increased (about 15- to 18-fold) after administration of two separate doses of TCDD (4.5 μg kg^{-1} body weight) intraperitonially. However, they observed no significant changes in aniline hydroxylase, 7-ethoxycoumarin O-deethylase (7-ECOD), and cytochrome P450 content of the fish liver microsomes with TCDD treatment.

In 1981, Law and Addison studied the effect of 10 different chlorinated environmental contaminants on brook trout (Salvelinus fontinalis) hepatic MFOs. They fed the fish with a gelatine capsule containing oil-fortified test compounds 3 times at 2-day intervals. They found a 2-fold increase in B(a)P hydroxylase and 2 to 3-fold increase in 7-ECOD activities of Aroclor 5460 fed fish. Induction of hepatic B(a)P hydroxylase activities of flatfishes, Citharicthys sordidas and Citharicthys stigmaeus by crude oil and PCB ingestion were demonstrated by Spies et al. (1982). They fed the fish with seep-oil-augmented squid and Aroclor 1254 augmented squid, separately. There was a significant increase in hepatic B(a)P hydroxylase activities of fish fed with either oil or PCB. However, no significant differences were observed between control and oil-fed fish in their B(a)P hydroxylase activities when oil was administrated at low doses. Moreover, they found that more crude oil was required than PCB to induce equivalent MFO responses by fish.

In another study, coho salmon (Oncorhyncus kisutch) were exposed to water soluble fraction of the crude oil using a flow-through apparatus. Hepatic B(a)P hydroxylase activity of the coho salmon was increased about more than 3-fold upon exposure of maximum 30 days. It was shown that the persistence of enzyme induction was dependent on the length and concentration of exposure (Collodi et al., 1984). Later, Förlin et al. (1985) showed the induction of 7-EROD activity of fourhorn sculpin (Myoxocephalus quadricornis) upon exposure to a pulp bleach plant effluent for 9 months. The increase of hepatic microsomal 7-EROD activity was about 5-fold. They suggested that the PAH inducers which are responsible for this induction might be present in the effluent.

Induction of specific cytochrome P4501A1 isozyme in response to PAH treatment is observed both in mammals and in fish (Nebert and Gonzalez, 1987; Stegeman and Kloepper-Sams, 1987; Stegeman et al., 1990; Goksøyr and Förlin, 1992).

Recently, we have characterized the liver microsomal MFO system of gilthead seabream (Arınç and Şen, 1993) an economically valuable marine fish inhabiting lagoons along the Mediterranean and Atlantic Coasts of Europe (Funkenstein et al., 1990).

The purpose of this study is to investigate the in vivo effect of B(a)P treatment on liver microsomal MFO components and enzymatic activities of gilthead seabream. Since it is known that 7-EROD is associated with cytochrome P4501A1, in this work, first of all, 7-EROD activity of gilthead seabream liver microsomes is characterized.

2. MATERIALS AND METHODS

2.1 Chemicals

Resorufin was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, USA. N, N, N', N'-tetramethylethylene diamine (TEMED) and ammonium

persulfate were purchased from Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey, USA. p-Aminophenol (pAP), formaldehyde, **β**-mercaptoethanol, potassium ferricyanide, and sodium dodecyl sulfate (SDS) were obtained from Fluka A.G., Buchs S.G., Switzerland. Ethylene diamine tetra acetic acid disodium salt (EDTA), dimethylsulfoxide (DMSO), yeast D-glucose-6-phosphate dehydrogenase, glycine, methanol, sodium dithionite were purchased from E. Merck, Darmstadt, Germany.

Acrylamide, N, N'-methylene bisacrylamide (BIS), benzo(a)pyrene [B(a)P], bovine serum albumin (BSA), bovine liver catalase, cimetidine, 3**α**, 7**α**, 12**α**-trihydroxycholanic acid, sodium salt (cholic acid, sodium salt), coomassie brilliant blue R, egg albumin, 7-ethoxyresorufin (7-ER), D-glucose-6-phosphate monosodium salt, bovine liver L-glutamate dehydrogenase (type IV), glycerol, N-2-hydroxyethyl-piperazine-N'-2, ethane sulfonic acid (HEPES), 3-methylcholantrane (3-MC), nicotinamide adenine dinucleotide, reduced form (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), 2-amino-2(hydroxymethyl)-1, 3-propandiol (trizma base) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA. All the other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2 Fish

The fish used in this study, gilthead seabream (Sparus aurata), were obtained from the fish farm of Pinar Sea Products ltd., Ildir, Çeşme, İzmir, Turkey. The fish were grown in special hatches until they reached to the required weight and then were taken into adaptation tanks to be inured to sea conditions. Finally, they were transferred to the cages, placed in the Aegean Sea. The fish used in this research weighed 250±25 g and were about 24 months old. The total body length of the fish was 23.3±1.5 cm. The values are averages of 20 fish and given as Mean±SD.

2.2.1 Benzo(a)pyrene treatment

Fish were transferred from their cages settled in the sea into two-ton tanks equipped with circulating filtered seawater at 21±0.5°C three days before starting the experiment. Fish were fed with a commercially available pellet containing dried proteins, carbohydrates, some vitamins and feeding was stopped 12 hours before the first injection. However, the fish were fed every other day during the experiments. The treatment solution was prepared first by dissolving 625 mg B(a)P in 15 ml of DMSO by immersing in a water bath at 60°C. Then, the solution was completed to final volume of 50 ml with corn oil, the ratio of DMSO to corn oil was 3:7, and the final concentration of B(a)P was 12.5 mg ml^{-1}. During the treatment, fish were held gently with fish strainer and 0.5 ml of this solution containing 6.25 mg B(a)P was administered to each fish once daily, intraperitonially for five consecutive days (25 mg B(a)P per kg body weight per day). Some of the control fish were treated with DMSO-corn oil mixture (3:7, v/v). No other treatment was done to anesthesize the fish for handling. Fish were killed about 18-20 hours after the last treatment. There was no

significant difference in liver microsomal MFO components and activities when liver enzymes from untreated or DMSO:corn oil treated fish were used. Accordingly, most of the data for control fish in these studies were from untreated fish.

2.2.2 Preparation of gilthead seabream liver microsomes

Gilthead seabream liver microsomes were prepared by differential centrifugation as described by Arınç and Adalı (1983) with slight modifications. The fish were killed by decapitation. The livers, each weighing 2.5 to 4.5 g, were removed immediately. The gall bladders were removed carefully to avoid the spillage of its content which is known to be inhibitory to MFO activities. The livers were then frozen by putting into liquid nitrogen. Freshly frozen fish livers were transported in liquid nitrogen from İzmir to university laboratory in Ankara, about 450 miles away.

About three to ten fish livers were used for preparation of microsomes. In the laboratory, the livers were taken from liquid nitrogen and thawed, and the connective and fatty tissues were removed. All subsequent steps were carried out at 0 to 4°C. Then, they were washed with distilled water and with 1.15% KCl solution several times. After draining and blotting on a filter paper, tissues were weighed to the nearest 0.1 g and were cut into small pieces. The resulting tissue mince was homogenized in 1.15% KCl solution containing 1 mM EDTA, by using Potter-Elvehjem glass homogenizer packed in crushed ice, coupled motor driven Teflon pestle at 2400 rpm. The volume of the homogenization solution used was equal to 2.5 times the weight of the livers.

The homogenate was centrifuged at 10,500 rpm (13,300 g) by using Sorvall RC-2B Automatic Refrigerated Centrifuge, Ivan Sorvall Inc., Newton, Connecticut, 06740 USA with SS-34 rotor for 40 minutes to remove cell debris, nuclei and mitochondria. The supernatant fraction containing endoplasmic reticulum and other soluble fraction of the cell was filtered through double layers of cheese cloth in a Buchner funnel while avoiding the loose pellet.

The microsomes were sedimented from the supernatant solution by centrifugation at 45,000 rpm (133,573 g) for seventy minutes using a Beckman 50 Ti rotor in Beckman L-2 65B ultracentrifuge (Spinco Division of Beckman Instruments. Palo Alto, California, 94304, USA). Time, required for microsomal sedimentation of fish liver samples, were increased from 50 minutes to 70 minutes. The supernatant fraction was discarded. The softly packed microsomal pellet was suspended in 1.15% KCl solution containing 1 mM EDTA and resedimented by ultracentrifugation at 133,573 g for 60 minutes. The supernatant fraction was discarded again.

The washed microsomal pellet was resuspended in 10% glycerol containing 1 mM EDTA. For each gram of liver, 0.5 ml of suspension solution was used. The glycerol concentration in suspension solution was reduced from 25% to 10% because in the enzyme assays, during the centrifugal separation of denatured proteins, the presence of glycerol concentration higher than 10%, caused poor sedimentation. In order to get a homogenous microsomal suspension, resuspended microsomes were homogenized manually using the Teflon-glass homogenizer.

Microsomal suspensions containing approximately 20 to 30 mg protein per milliliter were gassed with nitrogen in eppendorf tubes and stored in liquid nitrogen for the mixed-function oxidase enzymatic assays.

2.3 Analytical procedures

2.3.1 Protein determinations

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

2.3.2 Determination of cytochrome P450

Cytochrome P450 concentrations were determined by the method of Omura and Sato (1964a,b) using Hitachi 220A Double-beam recording spectrophotometer (Hitachi 220A, Hitachi ltd. Tokyo, Japan) with cuvettes of 1.0 cm light path length. Aliquots of 0.5 to 1.0 ml microsomes containing 10 to 30 mg protein were diluted to 5.0 ml with 0.1 M potassium phosphate buffer, pH 7.7 containing 30% glycerol and 1 mM EDTA, and cholate was added to get 1% cholate in the final concentration, and then placed both in sample and reference cuvettes of spectrophotometer. Carbon monoxide was bubbled through the sample in sample cuvette for about 20 seconds and the samples in both cuvettes were reduced by the addition of a pinch of sodium dithionite, $Na_2S_2O_4$. Then, carbon monoxide was bubbled through the reduced sample in sample cuvette for about 40 seconds more and the CO-difference spectrum was recorded with about 3-minutes intervals.

The cytochrome P450 amount was calculated using an extinction coefficient of 91 mM^{-1} cm^{-1}. The absorbance values used in calculation was the difference between the absorbance at 450 nm and 490 nm in the CO-induced difference spectrum of dithionite-reduced samples (Omura and Sato, 1964a,b).

2.3.3 Determination of cytochrome b5

Cytochrome b5 concentrations of gilthead seabream liver microsomes were determined according to the method of Nishibayashi and Sato (1968). An aliquot of 0.5 ml microsomes containing approximately 10 mg protein were diluted to 5.0 ml with 0.1 M potassium phosphate buffer containing 30% glycerol and 1 mM EDTA and cholate was added to obtain 1% cholate in the final concentration. Then, sample was divided into reference and sample cuvettes of spectrophotometer equally. The sample in sample cuvette was reduced by the addition of a pinch of solid sodium dithionite and dithionite-reduced minus oxidized difference spectrum was recorded immediately using Hitachi 220A Double-beam recording spectrophotometer with cuvettes of 1.0 cm light path length.

The concentration of cytochrome b5 was estimated from the initial dithionite-reduced minus oxidized difference spectrum using an extinction coefficient of 185 $mM^{-1}.cm^{-1}$ for the difference in absorption between 424 nm and 410 nm.

2.3.4 Determination of NADPH-dependent cytochrome P450 reductase activity

NADPH-dependent cytochrome P450 reductase activity was measured spectrophotometrically, according to the procedure of Masters et al. (1967) except that the reaction was carried out in 0.3 M potassium phosphate buffer, pH 7.7 at room temperature. The assay was dependent on the measurement of the rate of reduction of an artificial substrate, cytochrome c, at 550 nm.

The reaction mixture contained 0.7 ml of cytochrome c (1.1 mg cytochrome c per ml in 0.3 M potassium phosphate buffer, pH 7.7), 0.025 ml of freshly prepared 3.3 mg per ml NADPH solution and appropriate concentration of microsomal enzyme (0.01 to 0.10 ml) in 0.3 M potassium phosphate buffer, pH 7.7. The reaction was initiated by the addition of NADPH and followed for a few minutes at 550 nm at room temperature using Hitachi 220 A double beam spectrophotometer with cuvettes of 1.0 cm light path. The enzyme activities were calculated using the extinction coefficient of 19.6 mM^{-1} cm^{-1} for the difference in absorbance between reduced minus oxidized form of cytochrome c at 550 nm as described by Yonetani (1965).

One unit of reductase is defined as the amount of enzyme catalyzing the reduction of one nmole of cytochrome c per minute under the conditions described above.

2.3.5 Determination of NADH-cytochrome b5 reductase activity

The NADH-cytochrome b5 reductase activity in microsomes was determined by the method of Strittmatter and Velick (1957) in which ferricyanide acted as an electron acceptor. The assay was based upon the measurement of reduction of potassium ferricyanide at 420 nm.

The reaction mixture contained 0.9 ml of 0.1 M potassium phosphate buffer, pH 7.5, 0.04 ml of 3 mM NADH, 0.04 ml of 5 mM potassium ferricyanide, and appropriate concentration of microsomal enzyme in a final volume of 1.0 ml.

The reaction was started by the addition of NADH and the reduction of ferricyanide was followed by recording the absorbance decrease at 420 nm using Spectronic 21 spectrophotometer, equipped with an OmniScribe series D5000 recorder (Bausch & Laumb, Houston Instruments, Texas, USA) at 25°C. Ferricyanide was also reduced with NADH chemically, without the enzyme. The background value was subsequently subtracted from the rate of enzymatic reaction. The enzyme activity was calculated using the extinction coefficient of 1.02 $mM^{-1}.cm^{-1}$ for the difference in absorbance between reduced minus oxidized form of ferricyanide at 420 nm.

One unit of enzyme activity is defined as the amount of enzyme causing the reduction of 1.0 μmole of potassium ferricyanide per minute under the conditions described above.

2.3.6 Determination of aniline 4-hydroxylase activity

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

The reaction mixture contained 100 mM HEPES buffer, pH 7.6, 10 mM aniline, 6 mg microsomal protein, and 0.5 mM NADPH generating system consisting of 0.5 units of glucose-6-phosphate dehydrogenase, 2.5 mM glucose-6-phosphate, 2.5 mM $MgCl_2$, 14.6 mM HEPES buffer, pH 7.8 and 0.5 mM $NADP^+$ in a final volume of 1.0 ml. The test tube containing the generating system then was incubated at 37°C for 5 minutes. One unit of glucose-6-phosphate dehydrogenase is defined as the amount of enzyme reducing 1 μmole of $NADP^+$ in one minute at 25°C.

p-Aminophenol solution was used as standard. Since it is sensitive to light, each time freshly prepared 0.5 mM pAP solution was used and kept in the dark. Standards at four different pAP concentrations (2.5, 5.0, 12.5, and 25.0 nmoles) containing aniline and other incubation constituents were run under the same conditions as for reaction mixture.

The reaction was started by the addition of 0.15 ml of NADPH generating system to microsomal incubation mixtures and to zero time blanks to which 0.5 ml of 20% trichloroacetic acid (TCA) was added before addition of cofactor and was carried out at 25°C for 25 minutes under the air with moderate shaking in water bath (Nüve Instruments Ltd., 06440 Ankara, Turkey). At the end of the incubation time, the reaction was stopped by the addition of 0.5 ml of 20% TCA. Denatured proteins were removed by centrifugation at 12,100 g for 40 minutes, using Sorvall RC-2B automatically refrigerated centrifuge by using SS-34 rotor. When some difficulties were encountered for the sedimentation of denatured fish microsomal proteins, centrifugation was repeated to ensure removal of denatured microsomal proteins completely.

Finally, 1.0 ml aliquots were mixed with 0.5 ml 20% sodium carbonate, Na_2CO_3, and with 0.5 ml of 0.4 N NaOH containing 4% phenol. The mixture was incubated at 37°C for 30 minutes. Then, the intensity of blue color developed was measured at 630 nm using Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). A standard curve of pAP was constructed and the amount of pAP formed was calculated from standard graph.

2.3.7 Determination of ethylmorphine N-demethylase activity

Ethylmorphine N-demethylase activity was determined by measuring the quantity of formaldehyde formed according to the method of Nash (1953), as modified by Cochin and Axelrod (1959). The assay conditions optimized for liver microsomal ethylmorphine N-demethylase by Arınç (1985) were also used for determination of ethylmorphine N-demethylase activity of fish liver microsomes with slight modifications.

A typical assay mixture contained 100 mM HEPES buffer, pH 7.7, 15 mM ethylmorphine-HCl, 4 mg microsomal protein and 0.5 mM NADPH generating system in a final volume of 1.0 ml. NADPH generating system was composed of 2.5 mM $MgCl_2$, 14.6 mM HEPES buffer, pH 7.8, 0.5 mM $NADP^+$, and 0.5 units of glucose-6-phosphate dehydrogenase.

A 0.5 mM freshly prepared formaldehyde solution was used as standard. Standards at four concentrations (12.5, 25.0, 50.0, and 100.0 nmoles) were prepared and were made up to 1.0 ml with distilled water and were run under the same conditions.

The reaction was initiated by the addition of 0.15 ml of NADPH generating system to incubation mixtures and to zero time blanks to which 1 ml of 0.75 N perchloric acid was added before the cofactor and was carried out at 25°C under the air with constant, moderate shaking in a shaking water bath. After exact period of 15 minutes, enzymatic reaction was stopped by the addition of 1.0 ml of 0.75 N perchloric acid solution. Denatured proteins were spin down by centrifugation at 12,100 g for 40 minutes, using Sorvall RC-2B automatically refrigerated centrifuge by using SS-34 rotor, and 1.2 ml supernatant was transferred into eppendorf tubes and recentrifuged at 13,000 rpm for 25 minutes using micro centrifuge, for complete removal of denatured microsomal proteins.

Finally, 1.0 ml aliquots were mixed with Nash reagent (prepared by the addition of 0.4 ml of acetylacetone just before use to 100 ml solution containing 30.8 gm ammonium acetate and 0.6 ml of glacial acetic acid). The mixture was incubated 10 minutes at 50°C. The intensity of yellow color developed was measured at 412 nm using Shimadzu UV-1201 spectrophotometer. A standard calibration curve was constructed and used for calculation of enzyme activities.

2.3.8 Determination of 7-ethoxyresorufin O-deethylase activity

The gilthead seabream liver microsomal 7-ethoxyresorufin 0-deethylase (7-EROD) activity was measured spectrophotometrically by combining and modifying the methods described by Pohl and Fouts (1980) and Klotz et al. (1984). This activity was determined by measuring the intensity of the pink color at 572 nm, which was produced by the conversion of 7-ethoxyresorufin into resorufin.

Assay conditions for the gilthead seabream liver microsomes were optimized as described in "Results". Typical optimized assay mixture contained 100 mM potassium phosphate buffer, pH 7.8, 100 mM NaCl, 1.2 mg BSA, 1.5 μM 7-ethoxyresorufin, 0.2 mg microsomal protein, and 0.5 mM NADPH generating system in a final volume of 1 ml. A 0.5 mM stock solution of 7-ER was prepared by dissolving 1 mg 7-ER (as obtained in its commercial bottle) in 8.32 ml of DMSO and were kept at 0-4°C. Daily solutions for enzymatic assays were prepared by diluting a 0.1 ml of stock solution to 5.0 ml with 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1 M NaCl. The reaction was initiated by the addition of 0.15 ml of NADPH generating system to microsomal incubation mixture and to zero time blanks to which

2.0 ml of methanol were added before the addition of NADPH generating system and reaction mixtures were incubated at 25°C for 10 minutes. At the end of incubation time, the reaction was stopped by the addition of 2 ml methanol. Precipitated proteins were centrifuged down at 10,000 rpm (12,100 g) for 40 minutes. Finally, 1.0 ml of aliquots were transferred into small, clean test tubes and the intensity of developed pink color was measured at 572 nm against reagent blank containing no microsomal protein on Shimadzu UV-1201 spectrophotometer.

Enzyme activities were calculated using the extinction coefficient of 73.0 mM^{-1} cm^{-1} for the absorbance at 572 nm as described by Klotz et al. (1984). One unit of enzyme activity is defined as the amount of enzyme converting 1.0 nmole 7-ethoxyresorufin into resorufin per minute under the described conditions.

2.3.9 SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of anionic detergent, sodium dodecyl sulfate (SDS), was performed on 3% stacking gel and 7.5% separating gel in a discontinuous buffer system as described by Laemmli (1970). BSA (M_r 68,000), catalase (M_r 60,000), glutamate dehydrogenase (M_r 53,000) and egg albumin (M_r 45,000) were used as molecular weight standards whose molecular weights of polypeptide chains were taken from Weber and Osborn (1969). The gels were fixed and stained for protein with 0.25% Coomassie blue in 50% methanol and 7% acetic acid and destained by the diffusion of unbound dye from gels by extensive washing with 30% methanol.

2.3.10 Immunoblotting

Fish liver microsomes were electrophoresed in 3% stacking and 7.5% separating gels under denaturing conditions in a discontinuous buffer system as described by Laemmli (1970). After electrophoresis the gels were incubated for 1 h in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) and proteins were transferred to nitrocellulose sheets by electrophoresis in the same buffer at a constant voltage of 70 V at 4°C for 1.5 h in Bio-Rad Trans-Blot apparatus.

After transfer the blots were washed with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% (v/v) Tween-20 (TBST). All subsequent incubations and washes were carried out at room temperature with gentle shaking. The blots were first incubated with blocking solution (3% BSA in TBST) for 1 h to block the nonspecific binding. The primary antibody (rabbit anti-cod cytochrome P450c polyclonal antibody) was added at 1:300 dilution to the blocking solution and incubation continued for an additional 1 h. The unbound antibodies were removed by washing three times for 5 min with TBST. Then the blots were incubated with second antibody (Alkaline phosphatase conjugated goat anti-rabbit IgG) in 1% BSA in TBST for 1 h. Finally, blots were washed as previous and incubated with substrate solution (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate system) as described by Ey and Ashman (1986) to visualize the specifically bound antibodies.

3. RESULTS

Cytochrome P450 dependent mixed-function oxidases are regulated by numerous environmental chemicals and physiological agents. In this work, effects of B(a)P pretreatment on gilthead seabream microsomal cytochrome P450 dependent MFO activities were studied using ethylmorphine, aniline and 7-ethoxyresorufin as substrates. Since the results obtained in the previous studies (Nebert and Gonzalez, 1987; Stegeman and Kloepper-Sams, 1987; Stegeman et al., 1990; Goksøyr and Förlin, 1992) have established that oxidative metabolism of 7-ER is associated with PAH inducible cytochrome P4501A1 in fish and in mammals, in this work, first, gilthead seabream liver 7-EROD activity was characterized with respect to substrate concentration, amount of microsomal protein, type of buffer used, incubation period and incubation temperature. The results are given in the following sections.

3.1 Effect of incubation period on 7-EROD activity

Figure 1 shows the effect of incubation period on the rate of 7-ethoxyresorufin O-deethylase activity. The reaction was linear with time up to 12 min under the conditions described in Materials and Methods. The standard assay period of 10 min was adopted for the routine determination of 7-EROD activity.

3.2 Effect of microsomal protein and added BSA

Figure 2 shows the influence of microsomal protein amount on the rate of enzyme activity of gilthead seabream liver microsomes. When enzyme activity (nmole/min) was plotted against the microsomal protein amount, a linear curve was obtained. However, this plot (Curve A) did not extrapolate to the concentration axis at the origin. When BSA was added to the enzyme assay mixture, specific activity was increased and activity versus microsomal protein amount plot (Curve B) was extrapolated to the origin. The optimum concentration of BSA was 1.2 mg ml^{-1}. BSA concentrations of 0.6, 0.9 and 1.2 mg ml^{-1} were tested. For this reason, 1.2 mg BSA was added to the incubation mixture and 100-200 μg microsomal protein was used per ml of assay mixture for routine determinations.

3.3 Effect of incubation temperature on 7-EROD activity

O-Deethylation of 7-ethoxyresorufin by fish liver microsomes was measured at different temperatures such as 15, 20, 25 and 30°C. No significant differences were observed between the enzyme activities at different temperatures (data are not shown) and the enzymatic assay was performed at 25°C.

3.4 Effect of buffers on 7-EROD activity

Two concentrations of phosphate and HEPES buffers at the same pH values were studied to characterize the effect of the buffer system on gilthead seabream liver microsomal 7-EROD activity. A comparison of effects of phosphate and HEPES buffers on fish liver microsomal 7-EROD activity is given in Table 1. It was found that

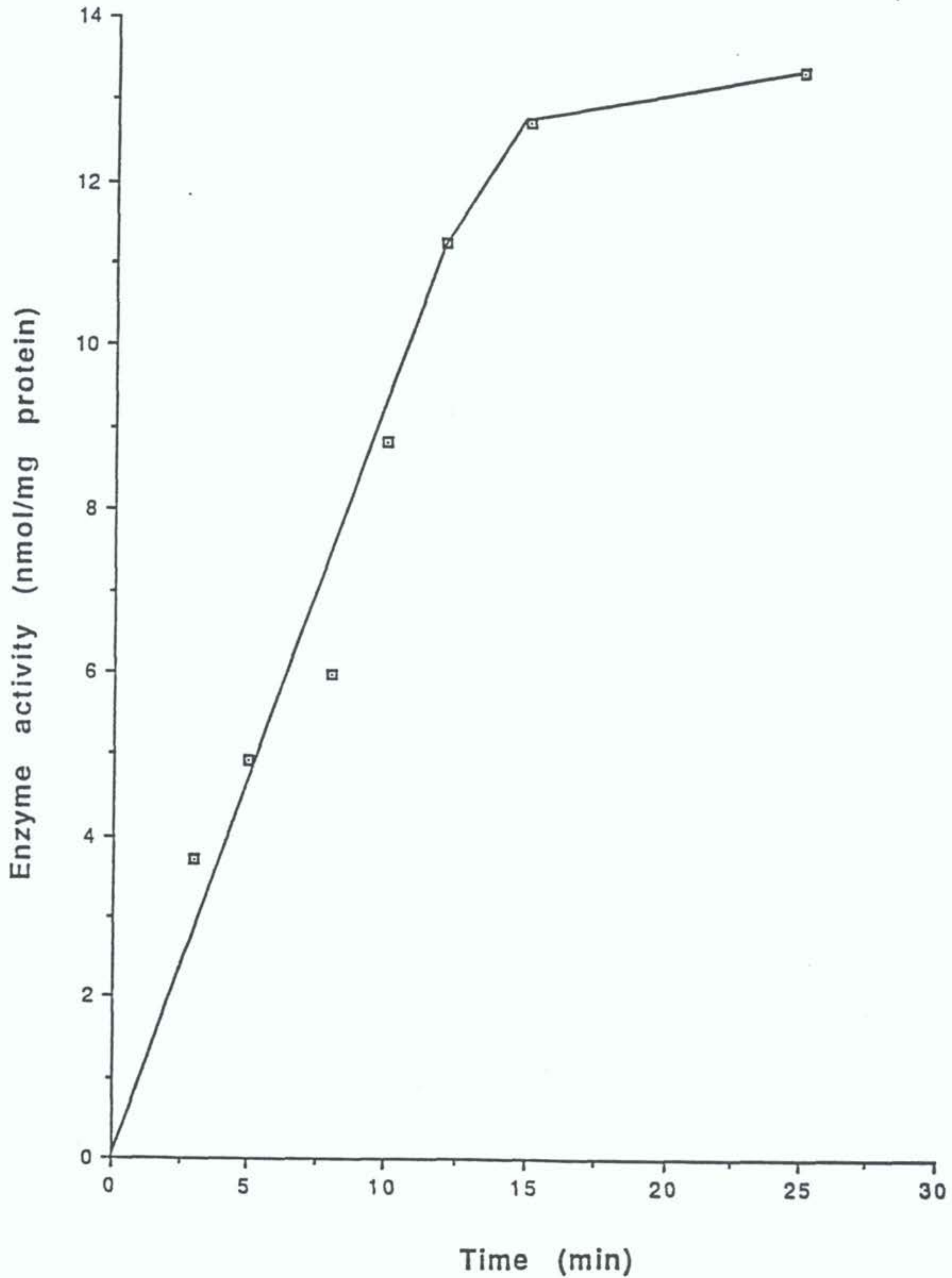

Fig. 1 Effect of incubation time on gilthead seabream liver microsomal 7-EROD activity. The reaction mixture contained 200 μg microsomal protein obtained from B(a)P treated fish liver. The reaction was carried out 25°C as described in Materials and Methods. The points are the means of two sets of duplicate determinations

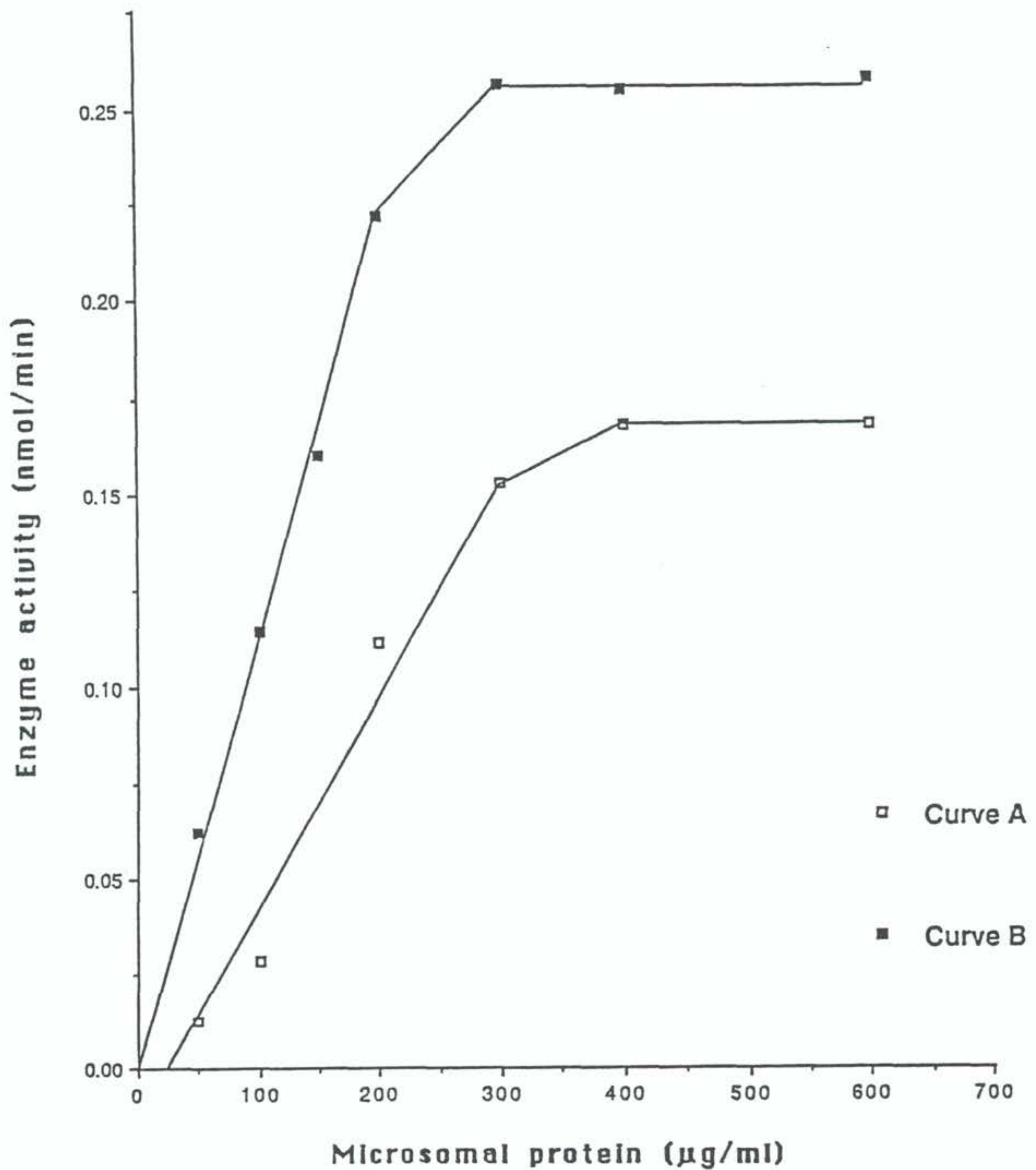

Fig. 2 Effect of microsomal protein and added BSA on hepatic microsomal 7-EROD activity of gilthead seabream. Incubation mixture contained 0.5 mM NADPH, 100 mM phosphate buffer, pH 7.8 containing 100 mM NaCl, 1.5 μM 7-ER, and indicated amount of microsomal protein obtained from control fish liver in a final volume of 1.0 ml. The reaction was carried out at 25°C for 10 min. Curve A shows the 7-EROD activity when the incubation mixture did not contain BSA. Curve B shows the effect of addition of 1.2 mg BSA on 7-EROD activity.

in the presence of phosphate buffers (Buffers A and B), higher 7-EROD activity was obtained. Moreover, 0.1 M phosphate buffer in 0.1 M NaCl also yielded higher activity of the enzyme, and for this reason, buffer B was used in other routine determinations.

Table 1

Effect of different buffers on 7-EROD activity of gilthead seabream liver microsomes

BUFFER*		7-EROD ‡ nmole/min/mg protein
A	(0.05 M phosphate buffer and 0.05 M NaCl)	1.05
B	(0.10 M phosphate buffer and 0.10 M NaCl)	1.27
C	(0.05 M HEPES buffer and 0.05 M NaCl)	0.88
D	(0.10 M HEPES buffer and 0.10 M NaCl)	1.08

* Final concentration and composition of buffers are given
‡ The values are averages of two sets of duplicate determinations

3.5 Effect of substrate concentration on 7-EROD activity

The effect of the substrate, 7-ethoxyresorufin, concentration on fish liver microsomal 7-EROD activity was studied. Substrate, 7-ER, concentration was varied from 0.1 μM to 2 μM. As seen in Fig. 3, fish liver 7-EROD seemed to be saturated by its substrate, 7-ER, at 1.5 μM 7-ER concentration. Thus, throughout this study, enzymatic assays were performed in the presence of 1.5 μM 7-ER.

3.6 Effect of in vivo benzo(a)pyrene treatment on MFO activities and cytochrome P450 components

Table 2 gives cytochrome P450, cytochrome b5 content and NADPH-P450 reductase, NADH-b5 reductase and aniline 4-hydroxylase, ethylmorphine N-demethylase and 7-EROD activities of liver microsomes obtained from control and B(a)P treated gilthead seabream. B(a)P treatment was carried out during the fall of 1990 and spring of 1991. Apparently, NADPH-cytochrome P450 reductase and NADH- cytochrome b5 activities and cytochrome b5 content of liver microsomes were not affected significantly by treatment of gilthead seabream with B(a)P. In addition, p-hydroxylation of aniline in liver microsomes remained unaltered by B(a)P treatment of gilthead seabreams. Seasonal variations in the MFO activities of gilthead seabream liver microsomes were reported before (Arınç and Şen, 1993).

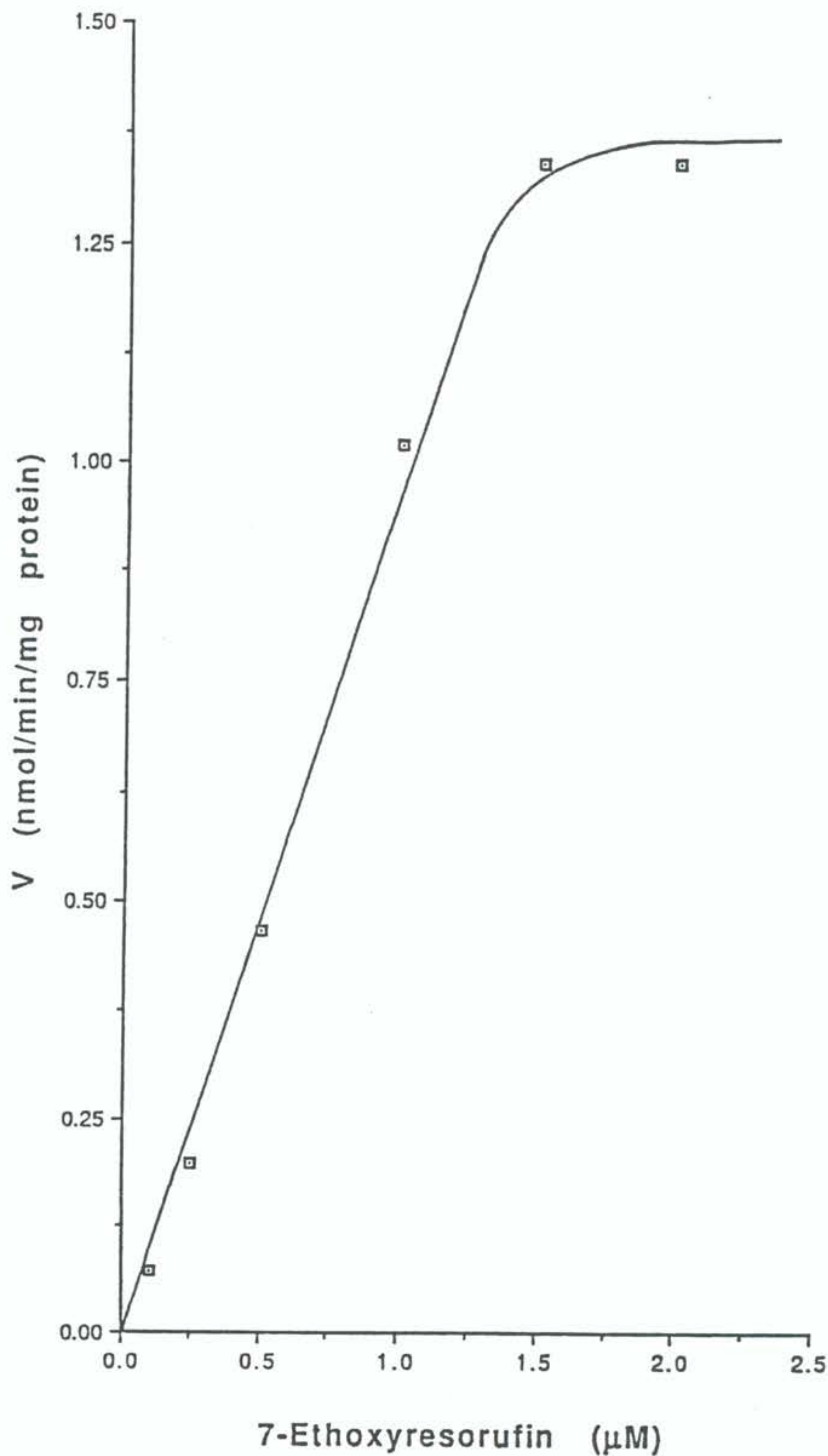

Fig. 3 Substrate saturation curve for 7-EROD of gilthead seabream liver microsomes. Incubation mixture contained 7-ethoxyresorufin concentrations ranging from 0.1 µM to 2.0 µM, 0.5 mM NADPH, 100 mM phosphate buffer, pH 7.8 containing 100 mM NaCl, 200 µg liver microsomal protein obtained from B(a)P-treated fish and 1.2 mg BSA in a final volume of 1.0 ml. The values are averages of two different sets of data each of which was a duplicate determination

Table 2

Cytochromes P450 and b5 content, reductases and some MFO activities of liver microsomes from control and B(a)P treated gilthead seabream

Cytochromes, reductases MFO enzymes	Fall 1990		Spring 1991	
	Control	Treated	Control	Treated
Cytochrome P450 (nmol P450/mg prot.)	0.074 ± 0.010	0.140 ± 0.010	0.083 ± 0.009	0.086 ±0.010
Cytochrome b5 (nmol b5/mg prot.)	0.300 ± 0.038	0.310 ± 0.010	0.323 ± 0.060	0.292 ± 0.028
NADPH-P450 Reductase (Units/mg prot.)	23.3 ± 2.34	26.0 ± 3.12	ND	ND
NADH-b5 Reductase (Units/mg prot.)	1.04 ± 0.20	1.26 ± 0.27	ND	ND
Aniline 4-hydroxylase (nmol pAP/min/mg prot.)	0.019 ± 0.003	0.020 ± 0.003	0.003 ± 0.0006	0.003 ± 0.0006
Ethylmorphine N-demethylase (nmol HCOH/min/mg prot.)	0.263 ± 0.017	0.223 ± 0.020	0.371 ± 0.017	0.300 ± 0.040
7-EROD (nmol resorufin/min/mg prot.)	0.407 ± 0.046	1.27 ± 0.14	0.272 ± 0.026	0.579 ± 0.093

* Microsomes were prepared from three to nine fish livers
ND: Not Determined

Figure 4 illustrates the effect of B(a)P treatment on total cytochrome P450 content. It was found that B(a)P treatment performed in fall caused a 1.7-fold increase in total cytochrome P450 content of fish liver microsomes. However, B(a)P treatment carried out in the Spring caused no significant difference between liver microsomal cytochrome P450 content of control and treated gilthead seabream.

Figure 5 shows the influence of B(a)P treatment on hepatic microsomal 7-EROD activity of gilthead seabream. 7-EROD activity of gilthead seabream liver microsomes was increased by B(a)P treatment. It was observed that in vivo B(a)P treatment selectively increased the 7-EROD activity about 2 to 3-fold.

Figure 6 shows the SDS-PAGE patterns of hepatic microsomes of gilthead seabream. It was observed that there were differences between the protein patterns of liver microsomes of control and B(a)P treated fish. It was found that there was an extra band in the SDS-PAGE pattern of 5-day B(a)P treated gilthead seabream liver microsomes which was less obvious in liver microsomes of 3-day B(a)P treated gilthead seabream. This extra band corresponded to a protein having monomer

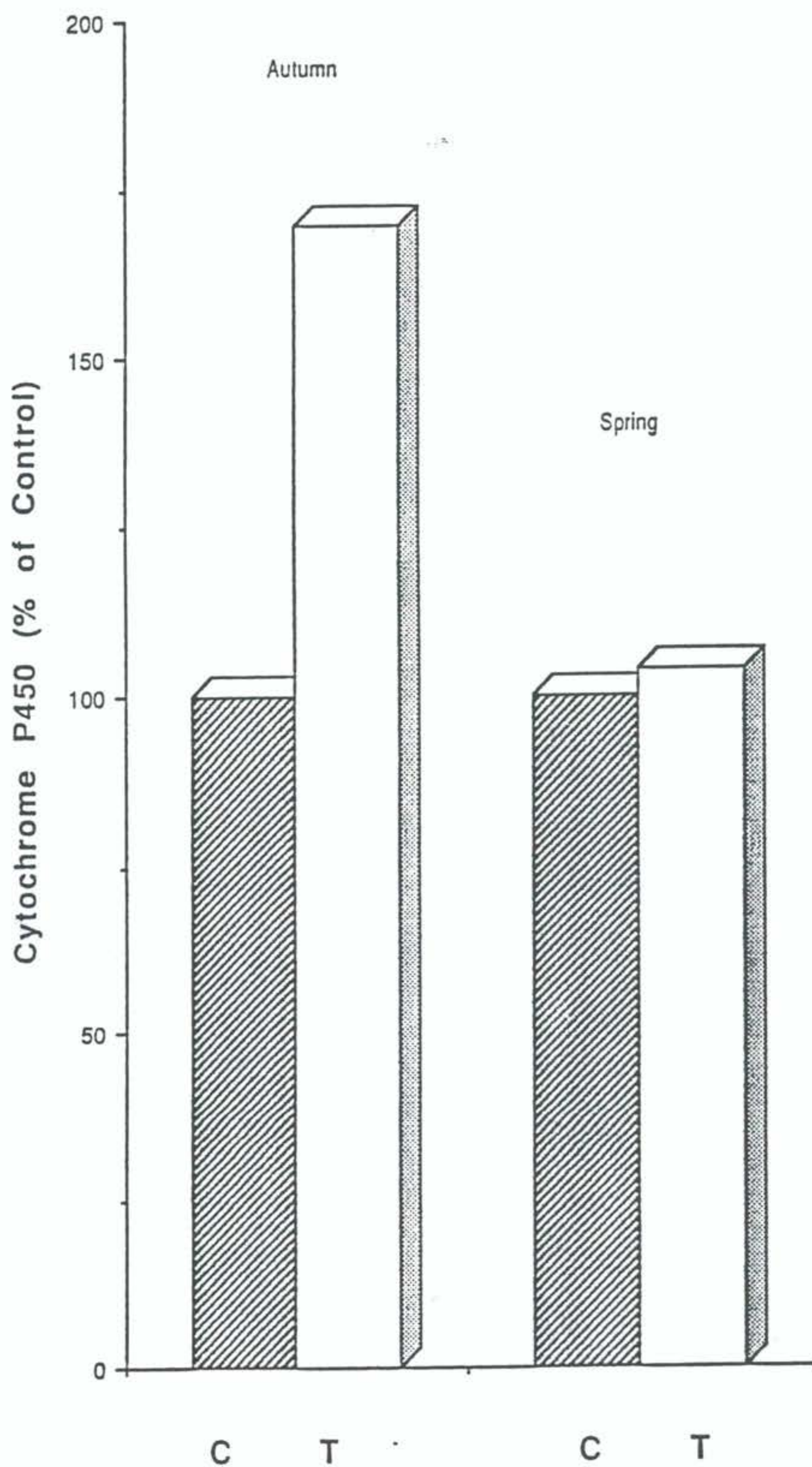

Fig. 4 Influence of B(a)P treatment on total cytochrome P450 content of gilthead seabream liver microsomes. C and T represent the control and treated values, respectively. Control values are taken as 100%

molecular weight of about 58,000 Da and this most probably represents a newly formed cytochrome P450, since the M_r of cytochrome P450 isozymes on SDS-PAGE ranges from 48,000 to 60,000 (Black and Coon, 1986). Moreover, another protein having monomer molecular weight of about 50,000 Da decreased in amount in the microsomes of 5-day B(a)P treated fish (Fig. 6). In conclusion, SDS-PAGE showed that B(a)P treatment caused an overall change in SDS-PAGE protein patterns of fish liver microsomes.

4. DISCUSSION

Several studies have shown that fish respond to 3-MC type induction in cytochrome P4501A1 synthesis (Goksøyr, 1985, 1991; Stegeman and Kloepper-Sams, 1987; Stegeman et al., 1990; Goksøyr and Förlin, 1992). In this study, in vivo treatment of gilthead seabream with B(a)P has also resulted in induction of liver 7-EROD activity which is more likely associated with cytochrome P4501A1.

B(a)P treatment of gilthead seabream selectively caused about a 2 to 3-fold increase in liver microsomal 7-EROD activity of gilthead seabream. As shown in Table 2 and in Figs 4 and 5, increase in 7-EROD activity was accompanied by a small or insignificant increase of total cytochrome P450 content of liver microsomes. This was not an unexpected result since it has been shown that total cytochrome P450 content is not a measurement for demonstration of induction of specific MFO activities (Payne et al., 1987; Kleinow et al., 1987; Arınç et al., 1991; Philpot and Overby, 1993).

Numerous studies have examined the effects of treatment with PCBs and PAHs on cytochrome P450 activities in teleosts (Stegeman et al., 1981; Spies et al., 1982; Klotz et al., 1983; Stegeman et al., 1986; Hahn et al., 1989; Kloepper-Sams and Stegeman, 1989) demonstrating that B(a)P hydroxylase and 7-EROD activities are induced in teleosts as they are in mammals. Recent studies have identified the major forms of cytochrome P450 induced by such compounds in several fish species, that is, cytochrome P450E in scup (Klotz et al., 1983), cytochrome P450c in cod (Goksøyr, 1985) and cytochrome P450LM4 in rainbow trout (Williams and Buhler, 1984). These forms appear to be orthologous to rat cytochrome P450c and rabbit cytochrome P450LM6 and other representatives of cytochrome P4501A1 which are known to be induced by 3-MC, β-NF, and TCDD (according to the nomenclature suggested by Nebert et al., 1987).

Those cytochrome P4501A1 representatives are the major teleost cytochrome P450s induced by 3-MC-type compounds and are the catalysts for B(a)P hydroxylase and 7-EROD activities. It is suggested that 7-EROD is a very useful indicator because of its high sensitivity, specificity and practicability of the reaction (Burke et al., 1985). It has also been shown to be more sensitive and much more specific than the widely used B(a)P hydroxylase assay, which is unreliable because of the multiplicity of products and their further metabolism (Parke, 1985).

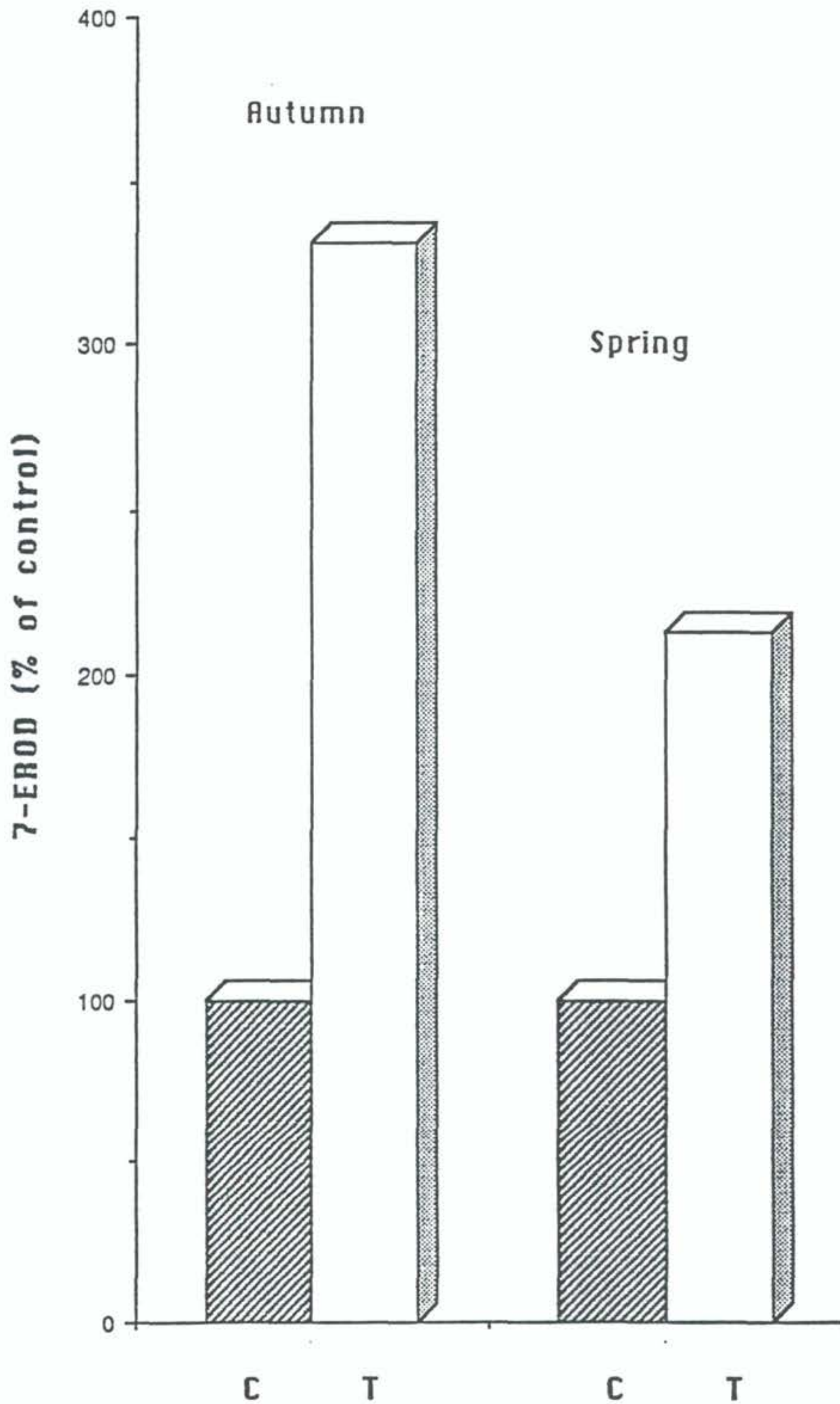

Fig. 5 Effect of B(a)P treatment on 7-EROD activity of gilthead seabream liver microsomes and seasonal variation between the effect of in vivo B(a)P treatment on 7-EROD activity. 7-EROD activity of microsomes was determined by measuring the amount of resorufin formed. C and T represent of control and treated values, respectively. Control values are given as 100%

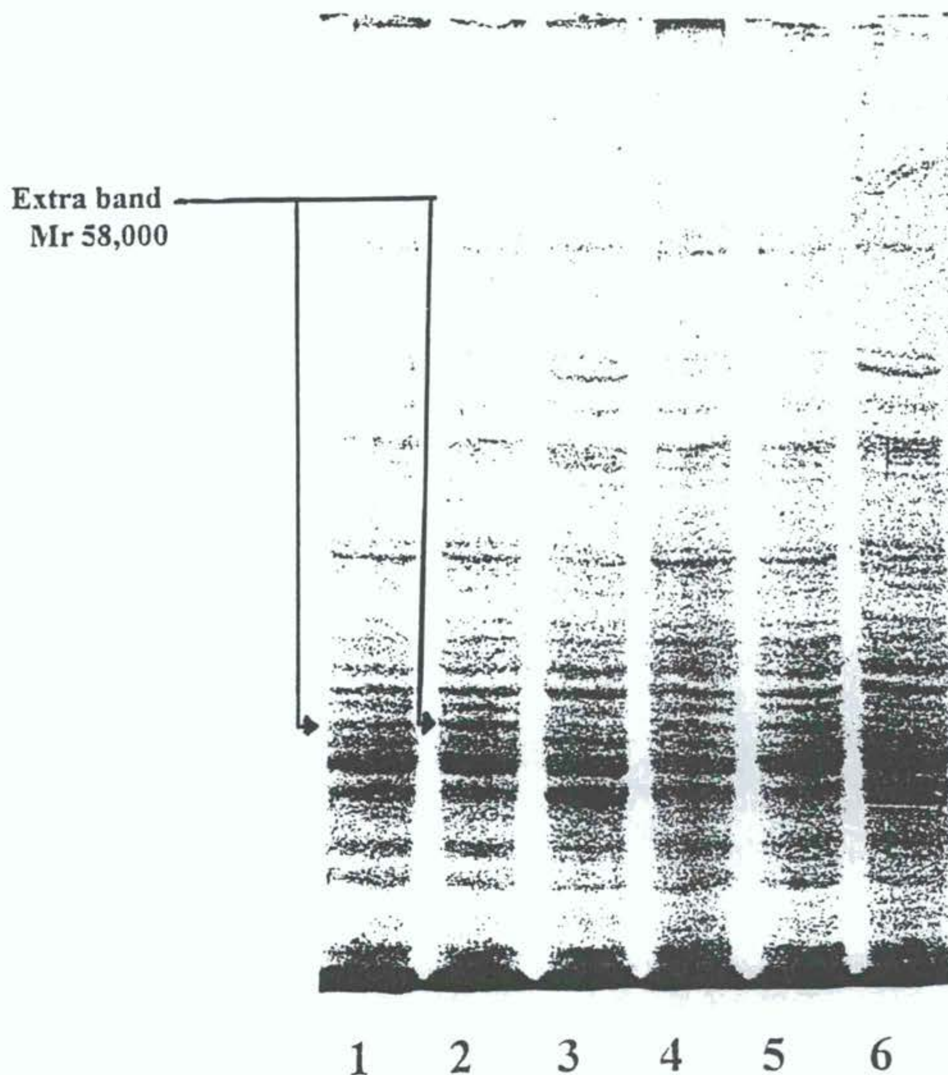

Fig. 6 SDS-PAGE of liver microsomes from untreated and B(a)P-treated gilthead seabream on 7.5% slab gels. Samples were prepared as described by Laemmli (1970), and SDS-PAGE analysis was performed as described in Materials and Methods. Lanes 1 and 4 contained 50 μg of microsomal protein from 3-day B(a)P- treated gilthead seabream; lanes 2 and 5 contained 50 μg of microsomal protein from 5-day B(a)P- treated gilthead seabream; and 50 μg of microsomal protein from untreated fish were in lanes 3 and 6. Arrows show the extra bands observed in liver microsomes of B(a)P-treated gilthead seabream

In this study, it was found that hepatic microsomal 7-EROD activity of gilthead seabream was induced by in vivo B(a)P treatment. Moreover, in vitro effects of several MFO substrates such as 3-MC, B(a)P, ethylmorphine and aniline on hepatic microsomal 7-EROD activity of B(a)P-treated gilthead seabream was also determined (Fig. 7). It was found that acetone (136 and 272 mM concentration), ethylmorphine (0.2 and 2 mM), aniline (0.2 mM), cimetidine (0.2 mM) and p-nitrophenol (0.1 and 0.2 mM) did not alter the 7-EROD activity. However, PAHs, 3-MC and B(a)P inhibited the enzyme activity 70 to 93% even in micromolar concentrations (Fig. 7).

In vitro inhibition of 7-EROD activity significantly by PAH substrates at micromolar concentrations (Fig. 7), and formation of a new cytochrome P450 with an apparent M_r of 58,000 on SDS-PAGE (Fig. 6) and significant in vivo induction of 7-EROD suggested that B(a)P induces a representative of cytochrome P4501A1 in gilthead seabream liver microsomes.

This conclusion is further supported by the fact that β-naphthoflavone (BNF) treatments of gilthead seabream significantly increased the 7-EROD activities of fish liver microsomes and Western blot analysis using the polyclonal antibodies (obtained from Dr. A. Goksøyr) against marine fish cod (Godus morhua) cytochrome P450c (CYP1A1) have demonstrated the induction of cytochrome P4501A1 representative in BNF-treated gilthead seabream liver microsomes (Fig. 8) (Arınç and Şen, 1994).

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

5. ACKNOWLEDGEMENTS

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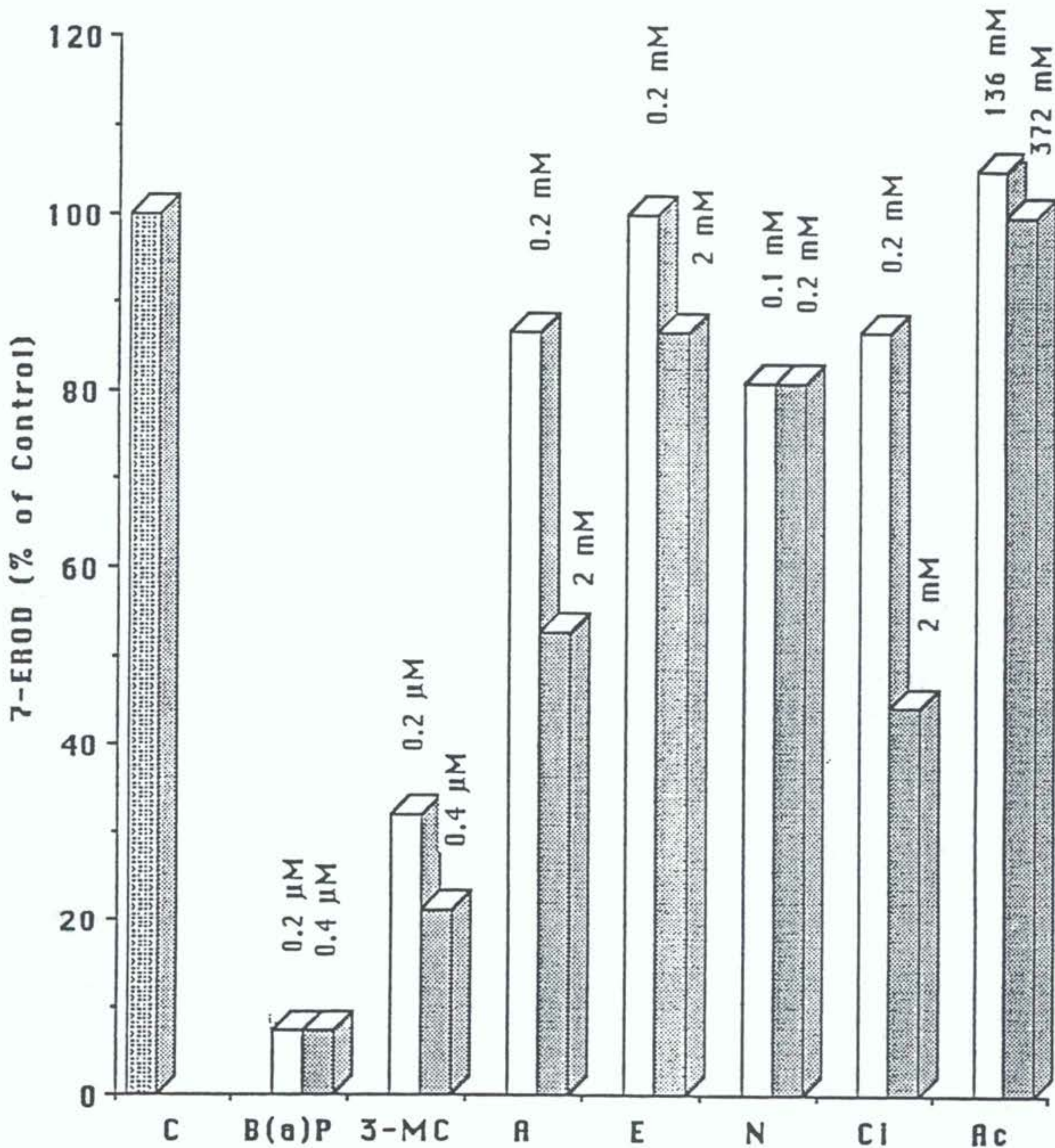

Fig. 7 In vitro effect of several MFO substrates on 7-EROD activity of gilthead seabream liver microsomes. Incubation mixture contained 0.5 mM NADPH, 100 mM phosphate buffer, pH 7.8 containing 100 mM NaCl, 1.5 μM 7-ER, 200 μg microsomal protein and indicated concentrations of tested compounds in a final volume of 1.0 ml. C: control, B(a)P: benzo(a)pyrene, 3-MC: 3-methylcholanthrene, A: aniline, E: ethylmorphine, N: p-nitrophenol, Ci: cimetidine and Ac: acetone

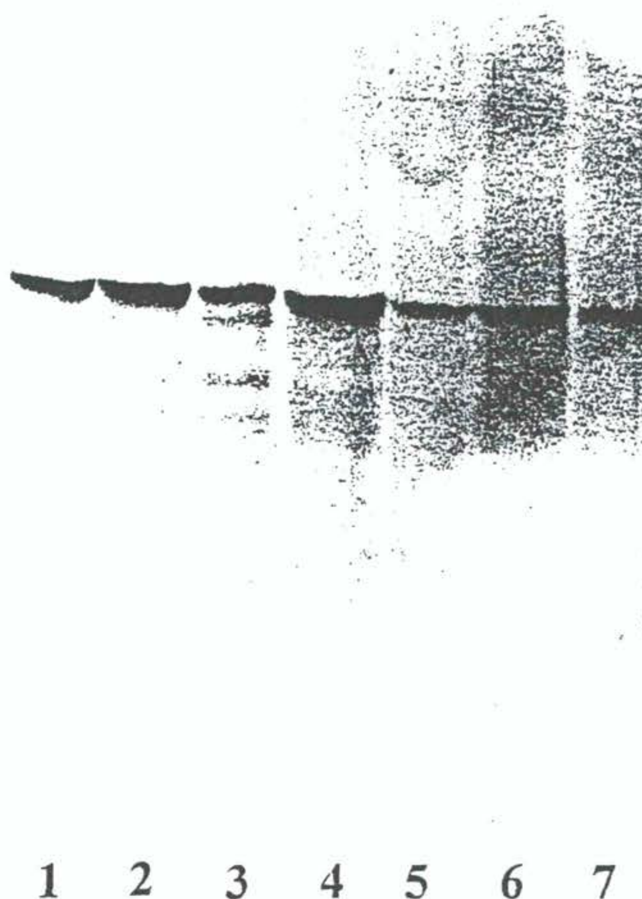

Fig. 8 Immunoblot of liver microsomes obtained from control and BNF-treated gilthead seabreams. Following electrophoretic transfer, the nitrocellulose sheet was incubated with 3% BSA for 75 minutes to reduce the nonspecific binding. The blot was then allowed to react with polyclonal antibodies (produced against purified CYP1A1 from BNF-treated cod) for 1.5 h at room temperature with constant shaking. Then the blot were incubated with second antibody (Anti-Rabbit IgG-Alkaline Phosphatase Conjugate) in 1% BSA in TBST for 1 h. Finally, blots were incubated with substrate solution as described by Ey and Ashman (1986) to visualize the specifically bound antibodies. Lanes 1-4 are liver microsomes from BNF-treated gilthead seabreams and Lanes 5-7 are control fish liver microsomes

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FUNCTIONAL AND PROTEIN EXPRESSION OF MULTIXENOBIOTIC RESISTANCE MECHANISM IN A MARINE MUSSEL AS A BIOMARKER OF EXPOSURE TO POLLUTED ENVIRONMENTS

by

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ABSTRACT

The expression of a multixenobiotic resistance mechanism (MXRM) in a marine mussel Mytilus galloprovincialis living along the pollution gradient in the Rovinj sea area was measured. The MXRM was induced in mussels at polluted sites: gills from mussels living at polluted sites accumulated less vincristine; the vincristine accumulation was less sensitive to verapamil, and in most cases they expressed higher levels of P-glycoprotein. The state of induction of functional activity of MXRM is proportional to the level of pollution, as demonstrated at sites along the well defined pollution gradient in the Rovinj sea area, Northern Adriatic. Since the immunological expression of P-glycoprotein may be compromised by high interindividual differences, season, and some as yet unknown factors, it could not be used as a single marker of exposure. However, the rate of vincristine accumulation and its sensitivity to verapamil offer a new, informatively powerful, molecular biomarker of exposure.

1. INTRODUCTION

Many marine organisms possess an P170-glycoprotein-like driven multixenobiotic resistance mechanism (MXRM) similar to the multidrug resistance (MDR) P-glycoprotein extrusion pump found in tumor cell lines resistant to cytotoxic drugs (Kurelec, 1992; Kurelec et al., 1995a,b). The binding of vincristine (VCR) onto membrane vesicles isolated from their gills was found to be verapamil (VER)-sensitive, and in specimens exposed to water supplemented with VCR, the accumulation of this xenobiotic was VER-sensitive. The activity of MXRM in these organisms is regulated by protein kinase C (PKC), as demonstrated recently by a high modulating activity of PKC-inhibitors, like staurosporine (STA) and bisindolylmaleimide (BIM) (Kurelec, 1995a).

In MDR-positive tumor cells a major determinant of reduced drug accumulation and a dominant feature in a model of classical multidrug resistance is the 170 kD membrane glycoprotein (P170) (Morrow and Cowan, 1988). P170 binds a cytotoxic drug and facilitates its efflux in an energy-dependent manner (Horio et al., 1988). Consequently, P170 mediates a reduction of drug accumulation and causes

drug resistance. The gene coding for glycoprotein P170, mdr1, has been cloned (Ueda et al., 1986) and its amplification and overexpression was found to be proportional to the degree of resistance in resistant cell-lines (Shen et al., 1986; Endicott and Ling, 1989). Some drugs, like verapamil (VER), bind to the active site of glycoprotein P170, causing thereby an inhibition of efflux of cytotoxic drugs and hence restore the previous sensitivity to the cytotoxic agent (Yusa and Tsuruo, 1989). In addition, P170-transporting function can be modulated by phosphorylation (Center, 1985). This post-translational modification is catalyzed by protein kinase C: its activators, like phorbol 12-myristate 13-acetate (Chambers et al., 1990), or its inhibitors, like staurosporine (Ma et al., 1991), stimulate or inhibit the efflux of drugs out of the cell.

Most of these characteristics found in sponges, snail and mussel, as well as in several other aquatic species (Kurelec, 1992; Moore, 1992; Chan et al., 1992; Holland-Toomey and Epel, 1993; Minier et al., 1993), were expressed in specimens living at pristine areas, suggesting that expression of P170-like glycoprotein in these species was inherent. The activity of MXRM however was enhanced in snail and mussel living at polluted sites: they accumulated less of vincristine (VCR) from the water supplemented with this xenobiotic and this accumulation was less sensitive to P170-inhibitors, STA and VER, suggesting that MXRM in mussels living at polluted site was induced (Kurelec, 1995a; Kurelec et al., 1995a). Earlier, Minier et al. (1993) reported that immunochemical expression of P170 was higher in mussels living at polluted site.

In this work we attempted to capitalize the experience from previous experiments and to explore the potential of induction of MXRM in a marine mussel, for example Mytilus galloprovincialis, as a biomarker of exposure to xenobiotics. The results of the screening of mussels along a well defined scale of pollution in the Rovinj sea area, Northern Adriatic, Croatia, by measurement of (1) the rate of accumulation of xenobiotic VCR in gills of exposed mussel, (2) the sensitivity of this VCR-accumulation to model P170-inhibitor, verapamil, and (3) the immunochemical analysis of P170 expression in gills are presented in this work.

2. MATERIAL AND METHODS

2.1 Chemicals

The sources of chemicals and materials used were as follows: (G-^{3}H)vincristine sulfate (VCR) (8 Ci/mmol) from Amersham, Buckinghamshire, England; vincristine sulfate, verapamil, protein molecular weight standards, and alkaline phosphatase conjugated anti-rabbit IgG from Sigma, St. Louis, USA. Rabbit antiserum raised against synthetic 1206-1226 peptide from P-glycoprotein (anti-C) (Yoshimura et al., 1989) was provided by Dr. Akihito Yoshimura from the Institute of Cancer Research, Kagoshima, Japan. Bovine adrenal glands were collected at a local slaughter house from male individuals.

2.2 Animals

Specimens of young (+1 y) marine mussels Mytilus galloprovincialis, 10-12 mm long, were collected during March 1994, at several sites along a well defined pollution gradient in the Rovinj sea area, Northern Adriatic, Croatia (Fig. 1). These were: a polluted Cannery site (a "mixing zone" of a waste from a local cannery); a polluted Market site (close to the town-market, receiving mostly household wastes from several outlets); a polluted Factory site (receiving waste waters from the Tobacco Factory Rovinj); a polluted Hospital site (receiving part of the waste waters from a large hospital); the unpolluted site Škarabe (3 km away from the town of Rovinj); and the unpolluted Lim site (a protected area at Limski Kanal, 10 km North-West from Rovinj). The state of pollution at these sites has been defined by the activity of benzo(a)pyrene monooxygenase in the livers of local fish: Cannery site = 31.2 ± 6.4; Hospital site = 14.1 ± 4.2; Škarabe site = 6.1 ± 1.3 pmoles of benzo(a)pyrene hydroxide/mg protein/min (Kurelec et al., 1977). The state of pollution defined at the Cannery site was taken as a representative also for the Market and Factory sites, whereas the state of pollution at the Lim site was assumed to be the same as the pollution at the Škarabe site. Specimens of mussels for accumulation experiments were used immediately, and their gills were collected and kept at -80°C before use for preparation of membrane vesicles for Western assays.

Fig. 1 Location of sampling sites in the Rovinj sea area, Northern Adriatic. Š: Škarabe-site; F: Factory-site; M: Market-site; C: Cannery-site; H: Hospital-site; and L: Lim-site.

2.3 Bioaccumulation experiments

In bioaccumulation experiments, 4 specimens of mussel were exposed to 20 ml of seawater for a period of 4 h in light-protected, 150 ml glass beakers held on a Rockomat (Tecnomara, Zürich, Swiss) in order to provide the circulation of water. The assays were supplemented with substrates and inhibitors as follows. Typically, 10 μl of stock solution of ^{3}H-VCR (5 μl [1.25 μCi] of original solution dissolved in 95 μl of ethanol) were added under stirring to 100 ml of water together with an ethanol solution of cold VCR so that the final concentration of VCR was 0.5 μM. This volume was divided into five 20 ml-portions (= exposure medium) which were used for exposure of mussels for the determination of the rate of VCR-accumulation, or, after supplementation with different concentrations of verapamil, for the determination of the sensitivity of VCR-accumulation to verapamil. After the exposure period, the mussels were rapidly washed 3-times in 50 ml portions of sea-water and the gills from individual specimens were removed, weighed, transferred to liquid scintillation miniature vials (6-ml), and homogenized in 0.5 ml of sea-water with an Ultra-Turrax homogenizer (IKA Labortechnik, Germany). Five ml of Aquasol were added and the radioactivity of the homogenate was determined in a liquid scintillation counter. The amount of accumulated radioactivity was expressed in cpm/mg of wet weight of gills-tissue.

2.4 Membrane vesicle preparation

Membrane vesicles were prepared from gill-tissue homogenates according to the method of Cornwell et al. (1986). Gills (250 mg) were removed and homogenized in 1 ml of vesicle buffer (0.01 M Tris-HCl, pH 7.5; 250 mM sucrose, 0.2 mM $CaCl_2$) using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 1,000 x g for 10 min, the supernatant was layered onto a 35% sucrose cushion and centrifuged for 30 min at 16,000 x g. The material left on the cushion was collected, diluted in 0.01 M Tris-HCl buffer (pH 7.5; 250 mM sucrose) and centrifuged (45 min; 100,000 x g). The pellet containing the vesicles was resuspended in 10 mM Tris-HCl (pH 7.5; 250 mM sucrose) using a 25-gauge needle. The same method was used for the preparation of membrane vesicles from the cortex of male bovine adrenal glands collected at a local slaughter-house and used as a reference source of P170-glycoprotein. Protein content in resuspended membrane vesicle fractions was determined by the method of Lowry et al. (1951).

2.5 Western blot analysis

Membrane vesicle proteins (100 μg/lane) from gills of mussels, or vesicle proteins from male bovine adrenal cortex (10 - 100 μg/lane), a known rich source of P-glycoprotein (Bradley et al., 1990), were separated by electrophoresis in a 7.5 % sodium dodecyl sulfate polyacrylamide gel using the method of Laemmli (1970). The heating step was omitted in order to avoid formation of aggregates which could not enter the gel (Yoshimura et al., 1989). The transfer to a nylon membrane was performed by a semi-dry method in a LKB apparatus according to the manufacturer's protocol. The blots were blocked with 3% BSA in Tris-saline buffer containing 0.05% Tween 20 overnight at +4°C and incubated with polyclonal antibodies against P-

glycoprotein (anti-C) (diluted 1:500) for 1.5 h at room temperature. The membranes were washed 3 x with Tris-saline buffer, incubated for 1 h with a 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG, dissolved in Tris-saline buffer with 0.05% Tween 20 (containing 1% BSA). After washing 4 times with Tris-saline buffer the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride as described by Harlow and Lane (1988).

Semiquantitative estimation of P-glycoprotein(s) expression was performed following the method of Huet et al. (1992) by visual comparison of different quantities of male bovine adrenal cortex vesicles at the position of 130 kDa (Ishikawa et al., 1991) with the density of spots obtained with vesicle-proteins from the gills of mussel at the position of 140 kDa. The detection limit was the cross-reactivity obtained with 10 μg of adrenal cortex vesicle protein.

3. RESULTS

Specimens of mussel collected at the pristine areas of Lim or Škarabe (sites of collection are shown in Figure 1), exposed to the exposure-medium supplemented with (G-^{3}H)vincristine for a period of 4 hours, accumulated significantly more VCR than specimens collected at the polluted Factory, Hospital, Cannery, and, to a lesser extent, Market sites (Fig. 2). The difference between these populations of mussels becomes more prominent when the exposure-media were supplemented with a competitive inhibitor of the P170-pump, verapamil. Verapamil at 20 μM inhibited much stronger the P-glycoprotein extrusion-pump in specimens living at the pristine sites Škarabe (enhancement in accumulation: 94%) or Lim (82%) than at the pollution-impacted sites Market (23%), Cannery (33%), Factory (42%), and Hospital (50%) (Fig. 2). Thus, both the decrease in the rate of VCR-accumulation and the decrease in the sensitivity of VCR-accumulation to verapamil were correlated with the degree of pollution caused by a planar xenobiotics, e.g. polycyclic aromatic hydrocarbons, at the corresponding sites.

In order to substantiate the presumption that these differences are based on the induced activity of P-glycoprotein in the gills of mussels from the polluted sites, we measured immunochemically the concentration of P-glycoprotein in the membrane vesicles isolated from gills using the semiquantitative Western analysis (Huet et al., 1992). These analyses revealed a general concomitance with the results from VCR-accumulation experiments: the anti-C polyclonal antibody strongly cross-reacted with a protein of about 140 kDa in membrane vesicles isolated from gills of mussel living at the polluted Factory and Market sites (Fig. 3, Table 1). Proteins isolated from gills of mussel living at the pristine Škarabe and Lim sites cross-reacted only at a detection-limit level (Fig. 3, Table 1). Surprisingly, the expression of P-glycoprotein at another polluted site, Cannery site, was unexpectedly low (Fig. 3, Table 1). However, mussels collected at the same site on another occasion (June 17, 1994), revealed the same general picture of P-glycoprotein expression characteristic for polluted sites (not shown). All these results were observed with pooled (gills from 4 specimens) samples. However, differences between individuals within the same group in the expression of

P-glycoprotein may be considerable, i.e. in a ratio of 1:3 (Table 1). Thus, the state of P-glycoprotein expression determined by Western analysis of P-glycoprotein in membrane vesicles isolated from mussel gills was neither a reliable biomarker of exposure of mussel to xenobiotics, nor an indicator of the actual state of pollution in a particular environment.

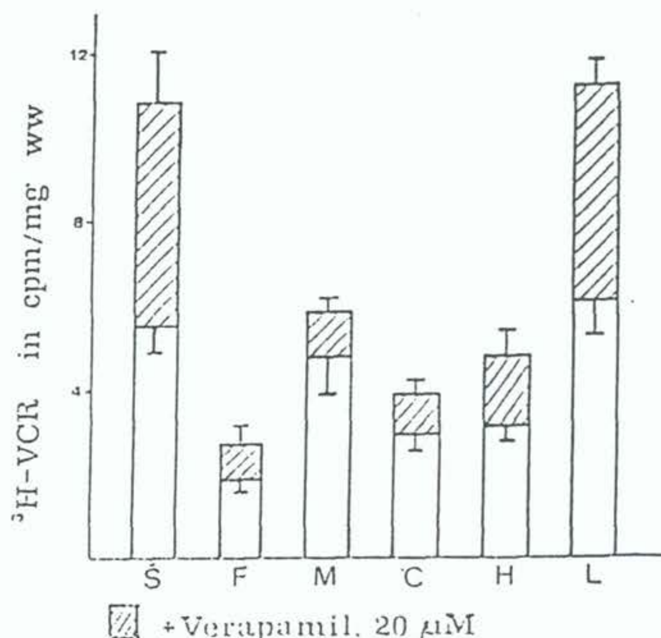

Fig. 2 The accumulation of (G-^{3}H)vincristine and its sensitivity to verapamil (shaded area) in gills of Mytilus galloprovincialis from stations along the pollution gradient (for stations see Fig. 1). The accumulation is expressed in cpm of VCR accumulated in 4 h per mg (ww) of gills tissue. Bars indicate standard deviation.

4. DISCUSSION

Mussels from unpolluted sites contain an inherent P170-pump whose activity allows the accumulation of a certain amount of xenobiotics (Kurelec and Pivčević, 1991). A competitive inhibitor of the pump, verapamil, strongly affects the pumping-out activity of P170, causing thereby the increase in the VCR-accumulation. This sensitivity of VCR-accumulation to verapamil was taken as an argument for the presence of a P-glycoprotein driven pumping-out activity in mussels (Kurelec, 1995a) because all drug-sensitive cells investigated so far were not sensitive to verapamil (Cornwell et al., 1986; Roninson, 1992). Therefore, the level of MXRM-inhibition by verapamil in mussels collected at the unpolluted site indicates, approximately, their natural "fold resistance" (F.res.= IC_{50} cytotoxic in resistant line/IC_{50} cytotoxic in sensitive line; Pierre et al., 1992). Since in nature there are no natural "sensitive"

control specimens of mussel, the maximal sensitization of mussel by verapamil, which inhibits on average about 80% of P170 activity (Ford and Hait, 1990), was the only parameter which indicates the level of the indigenous activity of resistance (defence) mechanism in this species (Kurelec, 1995a). Recently we have estimated the natural state of resistance (F.res.) in a marine snail Monodonta turbinata, using the level of VCR-accumulation in the presence of 20 μM verapamil as a surrogate for the level of accumulation in otherwise nonexisting sensitive species, to be 2.04 (Kurelec et al., 1995a). By applying the same procedure, the F.res. for mussels from pristine site was found to be 2.18 (Kurelec, 1995a).

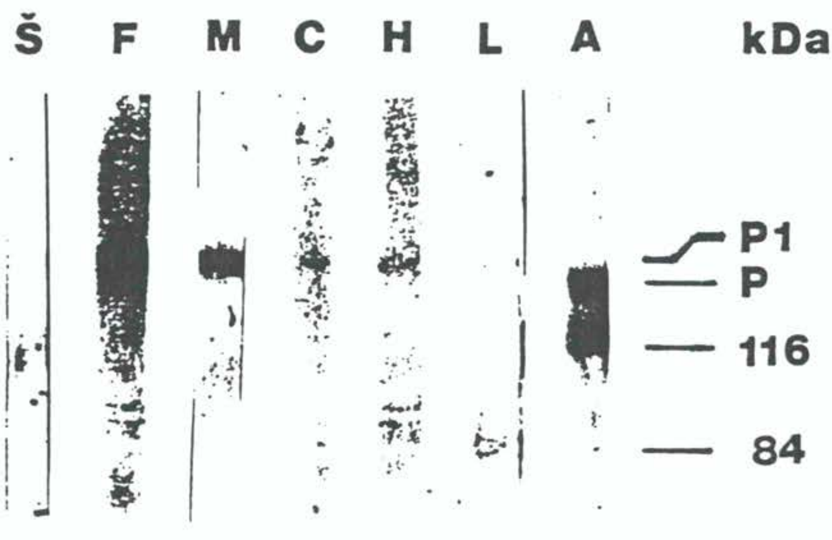

Fig. 3 Western analysis of P-glycoprotein expression in membrane vesicles isolated from pooled samples of gills from mussels living along the pollution gradient in the Rovinj sea area, Northern Adriatic (for stations see Fig. 1). A: membrane vesicles isolated from a male bovine adrenal cortex (100 μg/lane) samples as a reference source of P-glycoprotein. P: the 130 kDa P-glycoprotein from adrenal cortex. P1: P-glycoprotein from Mytilus galloprovincialis. The positions of molecular mass markers (84 and 116 kDa) are indicated.

In mussels living at the polluted sites, the expression of P170 is induced, and consequently, the higher activity of P170 pumps out more of VCR, which, like all other substrates of MDR, enters the cell by passive diffusion across the lipid bilayer (Gros et al., 1992). The same happens with another substrate of the pump, verapamil. The result is lower accumulation of VCR, as well as lower inhibitory effect of verapamil. The results in the present study demonstrate that these two characteristics,

the rate of VCR-accumulation and the sensitivity of VCR-accumulation to P170-inhibitors, are correlated with the degree of pollution along the scale of pollution existing in the sea area. This indicates that the state of MXRM-induction, as well as its (in)sensitivity to MXRM-inhibitors could be used as a biomarker of exposure to at least certain types of environmental pollution.

Table 1

Western analysis of immunochemical expression of P-glycoprotein in pooled and individual gills of the mussel Mytilus galloprovincialis living along a pollution gradient in the Rovinj sea area, Northern Adriatic. Numbers express the intensities of cross-reacting bands as equivalents of intensities of bands from corresponding quantities (in μg) of reference adrenal proteins

Site	Pooled	Individual	(Mean ± SD)
Škarabe	10	10, 20, 15, 10	(13 ± 5)
Factory	200	120, 160, 220, 150	(166 ± 50)
Market	100	60, 100, 120, 130	(102 ± 31)
Cannery	15	15, 30, 10, 40	(24 ± 14)
Hospital	20	15, 50, 25, 40	(32 ± 15)
Lim	10	10, 15, 10, 10	(11 ± 2)

A similar phenomenon of MXRM-induction was described recently in a marine snail Monodonta turbinata (Kurelec et al., 1995a). There, the exposure of the snail for 3 days to a polluted site, or to water containing Diesel-2 oil, resulted in the induction of the P170-activity, expressed as a decrease in the accumulation of VCR with a simultaneous loss of the sensitivity to P170-inhibitors. This promptness in the reactivity of MXRM to inducers demonstrates that MXRM-induction may be used as a biomarker of the current exposure to pollutants.

The direct immunochemical measurement of P-glycoprotein- concentration using Western analysis of membrane vesicles isolated from gills pooled from 4 specimens offers a less reliable indicator of the exposure of mussels to pollutants. For example, the cross-reaction of P170 in mussels from the polluted Cannery site was unexpectedly low. The reason for this discrepancy both with the state of pollution at this site, as well as with the high expression of P-glycoproteins' functional state, as evidenced by a low accumulation of VCR and the relative insensitivity to VER, found in mussels inhabiting this site, remains unclear. It may be explained by randomness amongst individuals in relation to expression of P170: the high interindividual differences in expression of P170 in mussels from the sampling-sites, shown in Table

1, suggest this possibility. Another plausible explanation of this discrepancy might be that certain types of pollutants (substrates), present only seasonally at one particular site (for example on March at a Cannery site), decrease the expression of P170 by a negative regulation of P170, as was shown to be the case with verapamil in a cell line K562/ADR resistant to adriamycin, but not in the parental, sensitive K562 line (Muller et al., 1994). On the contrary, Duensing and Slate (1994) have shown that high levels of P170 in the human colon tumor cell line, LS 174T, were accompanied by the sensitivity to doxorubicin and vincristine. Using anti-P-glycoprotein antibodies in the analysis of membrane and cytoplasmic proteins, they found that P170 in these cells was not associated with the plasma membrane, but was, instead, found intracellularly.

Finally, the use of Western analysis as a single biomarker may be compromised since the amount of P-glycoprotein is not necessarily related with its functional activity, as was demonstrated recently by Chieli et al. (1994). They found that 3-methylcholanthrene added to a rat hepatocyte primary culture increased the amount of immunoblottable protein without producing any concomitant increase in the efficiency to extrude Rhodamine-123. Thus, even a high expression of P170 may not reflect the functional state of multixenobiotic resistance. Thus, the routine application of immunodetection of P170-expression, at the present state of understanding of the mechanism involved, is not a reliable biomarker of exposure to pollutants.

The use of functional analysis of MXRM-activity as a biomarker of exposure has some features important for environmental risk-assessment. We argued recently that P-glycoprotein in aquatic animals, in addition to its physiological role to excrete toxic natural products present in the diet, or endogenous metabolites and steroids, pumps out also "new", man-made toxic chemicals present in polluted waters (Kurelec, 1995b; Kurelec et al., 1995a). The fact that the same xenobiotics induce the expression of MDR-gene simultaneously with the expression of genes encoding a series of mechanisms belonging to a general biological defence system (drug-metabolizing enzymes, glutathione S-transferase, heat-shock proteins) (Thorgeirsson et al., 1987; Burt and Thorgeirsson, 1988; Moscow et al., 1989; Gant et al., 1991; Kioka et al., 1992), support the conclusion that P-glycoprotein has a physiological function in the protection of cells from environmental stress. Based on this, we named this phenotype in aquatic organisms as a multixenobiotic defence mechanism (MXDM). Resistance, or MXRM, might develop as the consequence of the induction of MXDM.

The discovery of such a defence mechanism in aquatic organisms may have profound implications on ecotoxicology. One of the most striking is the effect of xenobiotics, which can inhibit the MXDM, which we call "chemosensitizers". They deserve a top rank among environmentally hazardous compounds because even though not acutely toxic themselves they can block the basic biological defence mechanism, the MXDM, and revert natural resistance to a pathobiological sensitivity. This was demonstrated by a ten-fold increase in the single-strand brakes in DNA from gills of mussel exposed to 2-aminofluorene in the presence of verapamil (Waldmann et al., 1995), by the increase of 2-aminofluorene-adducts in the DNA from sponges exposed to 2-aminofluorene in the presence of verapamil (Kurelec, 1992), by the

increase in the level of induction of benzo(a)pyrene monooxygenase in fish exposed to Diesel-2 oil in the presence of verapamil (Kurelec, 1995b), and by the increased accumulation of xenobiotics in the presence of verapamil observed in all aquatic organisms studied so far. We demonstrated also that such MXDM-chemosensitizers are present not only in concentrates of sediments and waters from polluted sites, but also (albeit to a less extent) from unpolluted environments (Kurelec et al., 1995b). The results of this study indicate that without the protective function of the indigenous activity of their P-glycoprotein pump, the population of mussels from an unpolluted site would be, theoretically, exposed to higher intracellular concentrations of environmental xenobiotics. Furthermore, these results offer an explanation why a pollution accident can cause much more severe effects at a pristine than at an already polluted areas the population of mussels living at a polluted site, thanks to its induced MXDM, will considerably diminish the bioavailability of accidental xenobiotics. All these examples illustrate the potential power of functional analysis of MXDM as a biomarker.

Therefore, the expression of the functional state of multixenobiotic resistance mechanism in mussels, measured by the rate of vincristine accumulation, and by the sensitivity of the VCR-accumulation rate to the MXDM-inhibitor, verapamil, offer a new, informatively powerful, molecular biomarker of exposure.

5. ACKNOWLEDGEMENTS

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PHYSIOLOGICAL RESPONSES OF MARINE INDICATOR ORGANISMS TO GLOBAL POLLUTION

by

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SUMMARY

Physiological response criteria such as Scope for Growth and Growth efficiency were applied for the first time in 4 regions of the Greek coastal zones (2 in Saronikos Gulf and 2 In Larymna site, Central Greece) to establish appropriate clean/uncontaminated reference sites and to initiate a preliminary basis of results for further establishment of environmental management programmes.

1. INTRODUCTION

A number of strategies have been developed to measure biological effects and alterations in the characteristics of ecosystems resulting from exposure to pollutants either of natural or anthropogenic origin. While there is no single biological effect measurement that can satisfy all environmental situations, the role of physiological responses measurements and their integration (bioenergetics) have been outlined as ideal in environmental monitoring programmes (Widdows et al., 1981; Donkin and Widdows, 1986; Widdows and Johnson, 1988; Widdows et al. 1995). Measurements through the use of physiological responses can provide a rapid assessment of the energy status of the animal as well as an insight into the individual components which affect changes in the growth rate (Widdows, 1985).

Physiological changes can be quantified by estimating the value of biological parameters called "stress indices" whose variations may be utilized to evaluate the physiological status of the organisms (Bayne et al., 1979; Bayne et al., 1988). Among the stress indices are the "Scope for Growth" and the "Growth Efficiency". These indices describe a general stress syndrome of the whole organism in response to a total environmental stimulus, which may include both natural and anthropogenic stressors with all the possible interactions between them.

The Scope for Growth refers to the energy available for growth and reproduction, and provides both an index of the condition of the organism and a measure of potential ecological damage. It is not a direct measurement but it is derived from the substraction of energy respired and excreted from the energy absorbed from the food (Bayne and Newell, 1983; Widdows, 1985).

Growth efficiency (gross and net) are measures of the efficiency with which food is converted into body tissues. Such an index not only indicates the areas or conditions under which the growth of the animal is most efficient but also provides a measure of the most economical use of food. In addition, relationships between growth efficiency and ration (i.e ingestion and respiration rates) can provide a useful means of comparing data from individual animals in relation to other factors (e.g. size, ration, temperature etc.) (Thompson and Bayne, 1974).

Up to date, the Scope for Growth has been successfully applied to the marine water environments as a tool for the examination of sublethal, toxic effects and environmental monitoring programmes (Widdows and Donkin, 1992; Widdows et al., 1995).

Indicator organisms such as mussels have been extensively used in detecting environmental changes by measuring and integrating their physiological responses since they are sessile, and filter-feeding organisms able to accumulate within their tissues many of the pollutants present in the sea water. Moreover, they show a wide geographical distribution and permit the survey of extensive coastal areas.

Although many monitoring programmes have been based on physiological response criteria for pollution assessment, in monitoring programmes of the Mediterranean region and more particularly of Greek coastal waters, pollution assessment has been based on other response criteria (UNEP/FAO, 1986; UNEP/WHO, 1986).

The purpose of the present study was:

* to apply physiological response criteria such as "Scope for Growth" and Growth Efficiency for the first time in Greek coastal regions.
* to locate mussel population and establish appropriate clean/uncontaminated reference sites.
* to initiate a preliminary basis of results (based on physiological response criteria) for further establishment of environmental monitoring and management programmes of the Greek coastal zones.

2. MATERIALS AND METHODS

2.1 Source and treatment of organisms

Mussels of Mytilus galloprovincialis were collected from native populations of three sites in Greece : the Western basin of the Saronikos Gulf (W. Saronikos native), Larymna 1 (west of the main harbour of Larymna) and Larymna 2 (near an aquaculture unit in the Lagonisi area). Mussels of all sites have had the same treatment of transportation and storage (packed with ice bags and remained at 4°C for 24 hours prior to any physiological response measurement). The mussels from the

W. Saronikos population were divided in two groups (group 1 and group 2). The physiological responses of mussels of group 1 were assessed immediately after transportation and storage of the mussels at 4°C for 24 hours. The mussels of group 2 were transplanted at the site of Agios Kosmas (Ag. Kosmas caged) where they remained for a period of 15 days prior to physiological responses measurements.

2.2 Physiological responses measurement

a) Scope for Growth

This technique involves monitoring the energy budget of individuals for growth and reproduction and testing how the various components of bioenergetics (e.g. feeding, respiration etc.) are affected by stress. The amount of energy available for growth and reproduction is calculated from the balanced energy equation of Winberg (1960) based from data on feeding and oxygen consumption rates and on absorption efficiency as follows :

$$C - F = A = R + U + P$$

where:
C= energy consumed as food
F= energy lost as faeces
A= energy absorbed
R= energy metabolized (measured as respiration)
U= energy lost as excretion and
P= energy available for growth and production.

Therefore, the definition of Scope for Growth (SfG) is:

$$SfG\ (P) = A - (R + U)$$

The SfG can be either positive indicating that energy is available for growth and reproduction; zero, when energy input balances energy expenditure; or negative, when animals must use their body reserves for essential metabolism.

The physiological measurements necessary for the calculation of SfG include : i) clearance rate, ii) food absorption efficiency and iii) respiration rate. They were carried out in the laboratory under controlled conditions of temperature (18°C) and were based on procedures given by Widdows and Salkend (1992) with minor modifications.

i) Clearance rate

Clearance rate, defined as the volume of water cleared of particles per hour, was estimated by measuring the removal of particles (> 3 μm diameter) by the mussels, as synthetic sea water of known rate passed through an experimental chamber containing a mussel (Fig. 1a). The particles were laboratory cultured algal cells of Dunaliella tertiolecta which were added to a mixing chamber with 1L pre-filtered synthetic seawater. Pre-filtered water was achieved in a continuous closed circulation system equipped with two types of cartridge filters, one with pores of 3μ

and the other with pores of 1μ plus a filter of activated carbon. Individuals of M. galloprovincialis were placed in sixteen experimental chambers with the inflow at the bottom and the outflow from an overflow tube at the top (Fig. 1a). Two additional chambers without animals acted as "controls".

The synthetic seawater (with approx. 38‰ salinity at 18°C), was pumped from a reservoir to the mixing chamber and then was passed through plastic tubes in each chamber. At a flow rate of 160 - 180 mL min^{-1}, the water was collected either in cylindrical tubes (when measurement was to be taken) or in a tank from which it entered again into the circulation system. The mixing chamber was used to ensure that the algae concentrations entering each of the experimental chambers were not significantly different. At the beginning of the experiment the mussels were left undisturbed for at least 12 hours to allow the organisms to recover from transportation, for ventilation to be resumed and the system to establish an equilibrium. Water samples were collected simultaneously from the outflow of all chambers four times at intervals of 45 minutes. The flow rate through each chamber was recorded at every sampling time (mL of sample min^{-1}) and the particle concentrations were measured by means of a Coulter counter (Model Z1). The sensitivity settings of the Coulter counter was adjusted that only particles of > 3 μm were counted and there was no coincidence counting. For each sample the mean of three counts was recorded.

The water samples from the control chambers represented the inflowing particle concentration (C_1) and the water samples from each experimental chamber contained a mussel represented the outflow particle concentration (C_o). Clearance rate was then calculated as follows :

$$\text{Clearance rate (L h}^{-1}) = (C_1 - C_o)/C_1 X \text{ Flow rate (L h}^{-1}).$$

ii) Food Absorption Efficiency.

Absorption efficiency was measured by the ratio method of Conover (1966). The procedures for estimating food concentration and faeces were according to Widdows and Salkend (1992). Then, the Conover Ratio for absorption efficiency was calculated as follows :

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

where : F=ash-free dry weight : dry weight ratio of food (algal culture)
E=ash-free dry weight : dry weight ratio of faeces.

iii) Respiration rate

Rates of oxygen consumption by individual mussels were measured in "closed" transparent plastic respirometers (modified Quickfit chambers of 500 mL). An individual mussel was introduced into the respirometer chamber and allowed to attach on a perforated glass plate over a stirrer bar (Fig. 1b). Rates of oxygen were measured for sixteen mussels placed six at a time in six respirometers chambers. At

30 minutes intervals, small volumes were sampled from each respirometer by means of a plastic syringe coupled to a stainless steel needle passing through a rubber stopper in the top of each respirometer. Each sample was then slowly injected into the thermostated microcell (MC 100) and an oxygen reading was obtained in mL L^{-1} at a Strathkelvin Model 781 oxygen meter after 2 min. The decline in oxygen concentration was measured by sampling from each respirometer on at least two occasions. One at the beginning, when each mussel was placed in the respirometer and allowed 5 minutes to open its valve, and the other after 30 minutes, exceptionally after 45 to 60 minutes in case where no decline was observed during the period of 30 minutes.

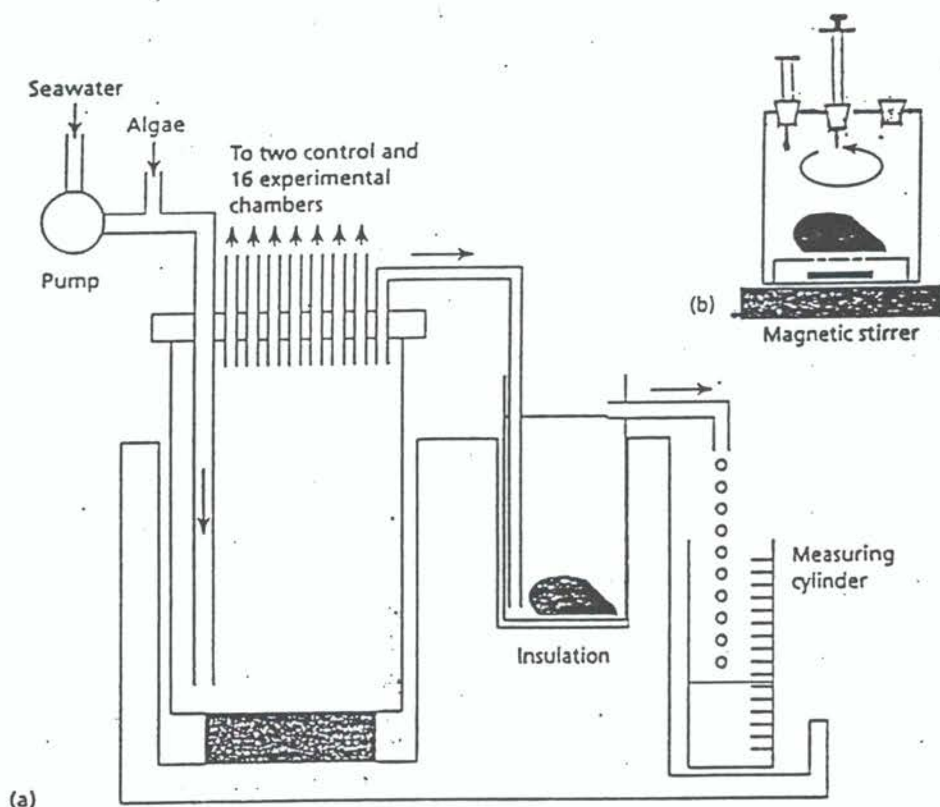

Fig. 1 (a) Flow-through apparatus for measurement of clearance rate by mussels.
(b) Chamber for measurement of oxygen consumption

Before obtaining the animal's dry tissue mass, the displacement volume of each individual was determined with a measuring cylinder. Finally, the rate of oxygen consumption was calculated as follows:

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

where : t_o, t_1= start and finish times (min) of the measurement period;
C(t) = concentration of oxygen in the water (mL O_2 L^{-1}) at time t;
Vr = volume of respirometer minus the volume of the mussel.

At the final result the values of oxygen consumption were expressed as μL O_2 g^{-1} h^{-1}.

2.3 Calculation of "Scope for Growth"

After all physiological measurements were completed, the shell length and the dry tissue weight of each mussel were recorded. Body tissues were dissected and dried at 110°C for 24 hours. Physiological rates (i.e. clearance and respiration) were corrected to a "standard body size" (i.e. 1 g dry weight) using appropriate weight exponents (e.g. b= 0.67, Widdows and Salkeld, 1992). The physiological components of the energy equation were converted to energy equivalents (J/g/h) and used in the balance energy equation to calculate the energy available for growth and reproduction (SfG) as follows:

i. Energy consumed ($J\ g^{-1}\ h^{-1}$)=C= clearance rate ($L\ g^{-1}\ h^{-1}$) x algal cells (mg L^{-1}) x 23 J mg^{-1} ash-free dry weight of algal food.
ii. Energy absorbed ($J\ g^{-1}\ h^{-1}$)=A= C x absorption efficiency.
iii. Energy respired ($J\ g^{-1}\ h^{-1}$)=R= ($\mu mol\ O_2\ g^{-1}\ h^{-1}$) x 0.456 J μmol^{-1} O_2.
iv. SfG ($J\ h^{-1}\ g^{-1}$) = A - R.

b) Growth efficiency

Growth efficiency was calculated from the energy equation, in order to provide further information on animal's condition. The scope for growth as a proportion of the ingestion ration represents the gross efficiency (K_1) and as a proportion of the absorbed ration represents the net efficiency (K_2). Gross (K_1) and net (K_2) growth efficiency were calculated according to:

$$K_1 = A\text{-}R/C \text{ and } K_2 = A\text{-}R/A$$

3. RESULTS

M. galloprovincialis shell length and tissues dry weight from all collection sites are indicated in Table 1. Similar shell lengths are presented since the selection was based on comparable results of the biological parameters. The physiological responses measurements (i.e clearance and respiration rates) are shown in Table 2. Clearance rates values of the mussels of Larymna 1 population were significantly different from the mussels collected from all the other sites ($p < 0.1$). Furthermore, coefficient of variation values of all sites were low and ranged from 3% to 8% and from 8% to 15% for the clearance and respiration rates, respectively.

The components of the energy budget (i.e. energy consumed, energy respired and energy absorbed) are indicated in Table 3. All components of the energy budget of mussels of Larymna 1 population except that of energy respired for W. Saronikos population showed the lowest values, which were significantly different from the respective values of the mussels of the other populations and the transplanted mussels at site of Ag. Kosmas (group 2).

Table 1

Shell length and tissue weight of Mytilus galloprovincialis collected from different coastal areas of Greece

Population	No of individuals	Shell length (mm)	Tissue weight (dry weight, g)
W. Saronikos (native)	10	55.9 ± 0.6	0.40 ± 0.03
Ag. Kosmas (caged)	13	59.0 ± 0.9	0.36 ± 0.02
Larymna 1 (native)	9	43.9 ± 0.8	0.29 ± 0.02
Larymna 2 (native)	6	58.6 ± 0.9	0.36 ± 0.04

Table 2

Physiological responses of Mytilus galloprovincialis collected from coastal areas of Greece

Population	Clearance rate ($L\ g^{-1}\ h^{-1}$)		Respiration rate ($\mu L\ O_2\ g^{-1}\ h^{-1}$)	
	Mean ± SE	CV%	Mean ± SE	CV%
W. Saronikos (native)	5.16 ± 0.43	8.3	10.08 ± 1.49	14.8
Ag. Kosmas (caged)	6.35 ± 0.25	3.9	27.61 ± 3.91	14.2
Larymna 1 (native)	2.21 ± 0.06*	2.7	20.71 ± 1.65	8.0
Larymna 2 (native)	5.71 ± 0.24	4.2	26.44 ± 3.44	13.0

SE = standard error
CV = coefficient of variation
* = significantly different from the others

Table 3

Components of the energy budget of Mytilus galloprovincialis from coastal areas of Greece (C= energy consumed; A= energy absorbed; R= energy respired)

Source	C ($J\ g^{-1}\ h^{-1}$)	A ($J\ g^{-1}\ h^{-1}$)	R ($J\ g^{-1}\ h^{-1}$)
W. Saronikos (native)	45.92 ± 3.80	11.16 ± 0.22	4.59 ± 0.68
Ag. Kosmas (caged)	42.52 ± 1.68	33.21 ± 1.31	12.59 ± 1.78
Larymna 1 (native)	19.15 ± 0.51	5.34 ± 0.14	9.44 ± 0.75
Larymna 2 (native)	50.83 ± 2.09	22.57 ± 0.93	10.41 ± 1.57

The calculated Scope for Growth measurements are presented in Figure 2, while gross and net growth efficiencies in Figure 3. The SfG and Growth efficiency of mussels of Larymna 1 population indicated negative values while Growth efficiencies were positive and much higher for mussels of Ag. Kosmas site.

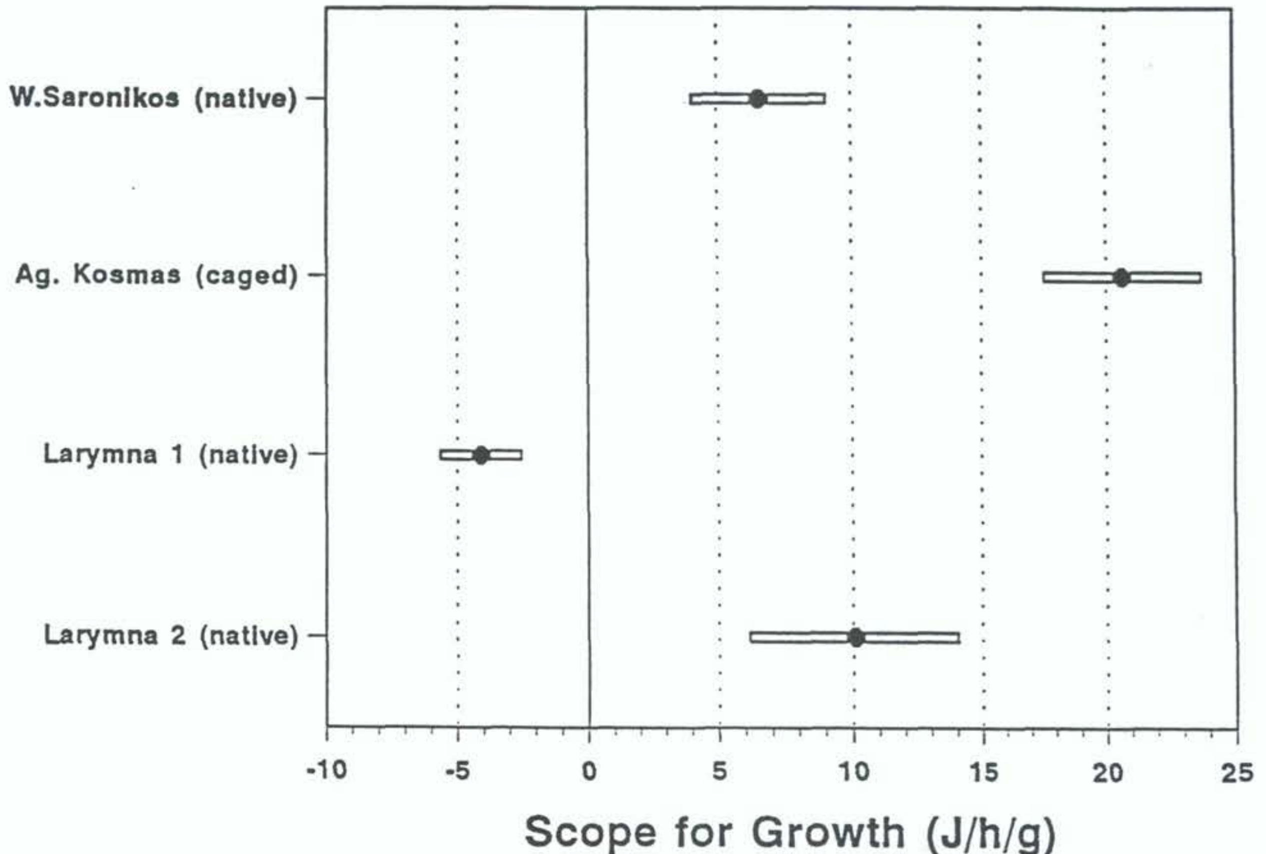

Fig. 2 Scope for Growth of Mytilus galloprovincialis from coastal areas of Greece (mean ± 95% CL)

4. DISCUSSION

In general, an important aspect of any pollution monitoring programme is the selection of an appropriate "clean/uncontaminated" reference site to be compaired with other sites (e.g. contaminated). If the reference site is not far from contaminant inputs, then the degree of contamination and pollution impact will be underestimated (Widdows and Donkin, 1992). In the majority of environmental studies, however, it is difficult to establish a cause/effect relationship because of the number of stressors that can influence the biological response. In the present study, those natural environmental stressors that are most likely to affect the physiological condition of the

animals, such as temperature, salinity, dissolved oxygen and mainly the total seston concentration are not the same at all sites (NCMR, 1985; 1991). Besides, anthropogenic environmental impacts, mainly of industrial activities, have been recognized in some of these sites (Voutsinou-Taliadouri, 1981).

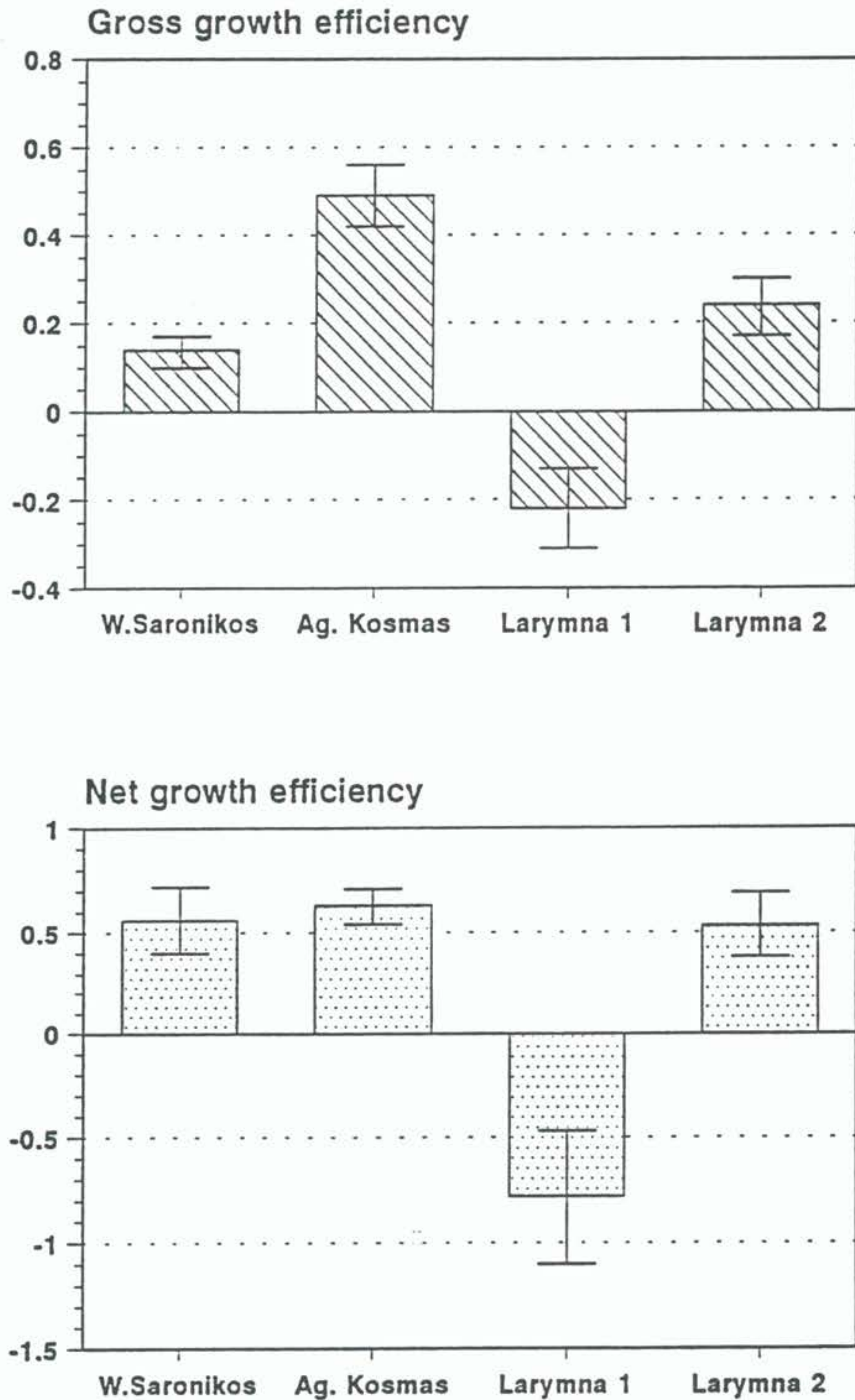

Fig. 3 Gross and net growth efficiency of native and transplanted populations of Mytilus galloprovincialis from coastal areas of Greece (mean ± 95% CL)

The physiological response values at the W. Saronikos site were lower thatn those at Ag. Kosmas site although both sites are parts of a bigger region (Saronikos Gulf). This is probably a synergistic effect of the environmental conditions of the Elefsis Bay and the Western Saronikos since a mixture of water masses from those areas occurs in the W. Saronikos site (Friligos, 1987; Friligos and Barbetseas, 1990). According to the literature, the Elefsis Bay is an eutrophic region with periodical anoxic conditions resulting from the influence of natural and anthropogenic (local industry) activities, while the Western Saronikos region and the Ag. Kosmas site at the outer region of Saronikos Gulf is an oligotrophic, clean/uncontaminated environment far away from any pollution source (Psyllidou-Giouranovits et al., 1990; Siokou-Frangou et al., 1990; Pagou, 1995).

Nevertheless, based on the physiological response criteria the degree of the environmental impact at Larymna sites is well documented from the dumping of metalliferous residue of the process of laterite to extract nickel and one could distinguish the two sites that had substantial differences in the degree of impacts. Previous investigations indicated higher concentrations of the heavy metals Ni and Fe in water (Nacopoulou and Hadjigeorgiou, 1987) and in animal tissue analyses (NCMR, 1995) as compared to other sites in Greece. These, however, were no harmful concentrations for the human health (NCMR, 1985) but as it appears from the present study probably are harmful to the environment.

Consequently, regarding the status of the environmental quality in the examined regions, the conclusions based on physiological response criteria, enhance the work that has been done previously by other researchers (UNEP/FAO, 1986) although the latest have been based on other types of response criteria. Furthermore, the findings of the present study imply that physiological response criteria are sensitive enough and they can give measurable values for regions that are affected by nearby disturbed (contaminated) environments. Furthermore, they can distinguish the degree of the impact between sites when they consist parts of larger regions.

On the other hand, eventhough it appears that based on physiological response criteria one is able to assess environmental impacts, the type of the impacts (i.e. anthropogenic from natural) cannot be distinguished. Therefore, it is suggested that although physiological stress indices alone can provide a measure of the health and/or the condition of individuals exposed to different environments, they can not provide information if the causes are of anthropogenic stressors or of natural environmental variables (e.g. seston concentration). Thus, the importance of a combination of physiological stress indices (e.g. SfG) together with other types of biological stress analyses appear to be important for the identification of anthropogenic and natural stressors.

Based on the results derived from the mussels transplanted from W. Saronikos to Ag. Kosmas site, it can be assumed that in the future and in a first phase of a monitoring programme this site could be operated as a recovery environment and be able to play the role of a reference site in the area of the Saronikos Gulf. Further research, however, must be performed using Scope for Growth measurements as a tool to distinguish eutrophic/oligotrophic environments.

Additionally, Larymna 1 site can be characterized as a impure environment compared to Larymna 2 site; the latter can be used as a territorial reference site in the area of Larymna.

5. ACKNOWLEDGEMENTS

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ECOLOGY, ENZYMOLOGY AND POPULATION DYNAMICS OF SOME SELECTED LITTORAL MOLLUSCS

by

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ABSTRACT

The shallow sandy subtidal of Haifa Bay is populated by the infaunal molluscs Donax trunculus, Mactra corallina (Bivalvia), Arcularia gibbosula and Natica millepunctata (Gastropoda), that form a large biomass at 5-25 m from the shoreline and at a depth of 20-100 cm. Donax trunculus is dominant, numbering up to 2000 per m^2. Analysis of metal residues was performed on samples of all four species collected in 1988, 1989 and 1990. Two of the sites studied were: a) in the vicinity of "Frutarom" - a polyvinyl chloride (PVC) factory, and b) near the city of Akko on the Haifa Bay, Mediterranean coast of Israel. The third site for comparison, Qiryat Yam, was situated at a distance of 6 km along the coast from the first site. Higher levels of metals were found in soft tissue and shells of the molluscs sampled near the Frutarom discharge sites, as compared with those from the clean site. The level of mercury was periodically found to reach particularly high levels (up to 20 ppm/dry weight) at the sampling site near the PVC factory, indicating the factory as the direct cause for pollution. In some instances, a correlation was found between age (dimensions of the shell) and levels of metal. Although bioaccumulation of metals was also found in the shells of the studied animals, the main site of deposition was the soft tissues. Higher levels of cytochrome P-450 at the Frutarom site compared to other sites, indicate that this site is also polluted by hydrocarbon pollutants.

1. INTRODUCTION

The Mediterranean coast of Israel is approximately 180 km long and ecologically can be clearly divided into two types of ecosystems: a) shallow water sandy habitats and b) intertidal and shallow sub-tidal rocky habitats, composed mainly of the special sand beachrock termed "kurkar" (Fishelson, 1966; Lipkin and Safriel, 1971). The soft-bottom habitat was primarily formed through the accumulation of sand

continuously introduced into the sea by the Nile floods. From the Nile delta of Egypt the south-north shore currents carry the sand across the wide-based shallow water flats up to the Israeli littoral zone.

These intertidal and shallow subtidal sandy bottoms are populated by the typical east Mediterranean infaunal community of species that form the Donax trunculus - Mactra corallina zone. Apart from these two dominant species, Natica millepunctata and Arcularia gibbosula are also very common. Less frequent are Angulus planatus, Solen marginatus (Mollusca), Gastrosaccus sanctus (Mysidacea), Carcinus mediterraneus and Portunus pelagicus (Crustacea, Brachyura), and several species of polychaetes. Of these sand-dwelling Israeli species, the ecology of Donax was partly studied by Neuberger-Cywiak et al. (1990), and data on some trace elements in this species are given by Hornung et al. (1981, 1984) and Mizrahi and Achituv (1989).

During the last few decades, immense urban and industrial development has occurred along these shores, especially in the Haifa Bay area of northern Israel. Consequently, high levels of xenobiotics, including heavy metals and organochlorides, have been introduced into the environment (Fishelson et al., 1994), through effluents discharged directly into both the sea and into the air. The impact of these releases on the Israeli marine fauna has recently been studied by Manelis and Fishelson (1989), Dotan et al. (1989), Bresel and Fishelson (1994).

The use of sand-bottom dwelling molluscs as bioindicator organisms for metal pollution has been documented in several publications (Roméo and Gnassia-Barelli, 1988; Szefer and Szefer, 1990). Most of the recommendations to use mytilid molluscs as key organisms for pollution studies along rocky shores are also valid for sand-dwelling species. As all these are sessile organisms that filter microorganisms and debris from the surrounding water, they are excellent traps for various natural and anthropogenic material and residues.

This study focuses on the intertidal and shallow subtidal population of molluscs along Haifa Bay, especially those dwelling in the vicinity of Akko and the Frutarom industrial plant, a complex of PVC production that discharges part of its waste waters into the sea-shore sands. For comparison, sampling was also performed at Qiryat Yam, 6 km further south (Fig. 1). Previous studies of these waters (Hornung et al., 1981, 1984) revealed high levels of mercury. The aim of this study, carried out within the framework of a comprehensive investigation on pollution in this region was to establish to what extent the Frutarom output can be considered a source of the metal ions observed in marine organisms found in its vicinity.

2. METHODS

Four species of molluscs common to this site were selected for analysis: Donax trunculus and Mactra corallina (Bivalvia); Arcularia gibbosula and Natica millepunctata (Gastropoda). For this study the Frutarom sea shore area was divided

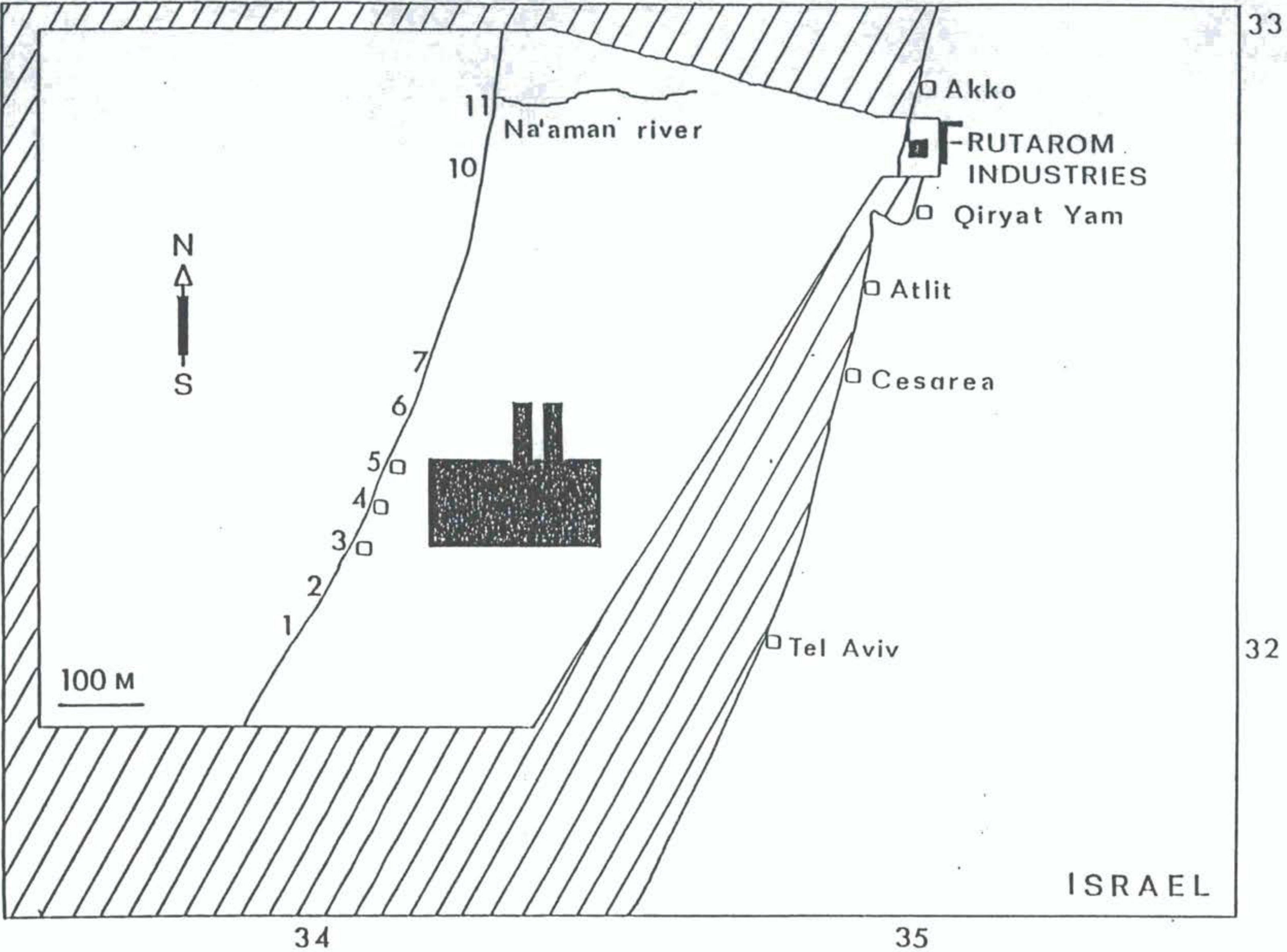

Fig. 1 The study site near the Frutarom chemical complex. Numbers along the seashore mark the sites of sampling

into three regions (Table 1): the waste-water discharge region located in front of the factory, at sampling stations 4-6; the site north of the discharge (Akko), stations 7-11; and the site south of the discharge, stations 1-3.

Table 1

Frutarom beach - position and distances between sites of sampling

	South site			Frutarom Factory Sewage discharge			Akko (cleaner)				
Station no.	1	2	3	4	5	6	7	8	9	10	11
Distance (m)	0	50	100	140	180	230	280	380	480	580	780

All the stations will be referred to henceforth by their numbers only. Animals were collected from these sites during the period 1988 to 1991. During 1988-1989 three to five grab samples of 15 x 15 cm were taken bi-monthly at each station at a water depth of 20-100 cm, from the range of the wash zone close to the beach. In addition, sampling along selected transects perpendicular to the shore was carried out to study the ecology, population density and age composition (dimensions) of the studied molluscs. This was primarily performed from 1990 with a specially designed sand dredge comprising a metal frame 30 cm wide and 15 cm high, with an obliquely-directed 10 cm wide blade attached to the lower front. At the rear, a collecting net was attached with an obliquely-directed handle in front. The dredge was pulled forward with this handle so that the blade sank into the sand within a 30 x 100 cm frame sunken on the bottom. In such a way samples (1 m x 30 cm x 20 cm) were taken along transects at distances of 5,10,15,20 and 25 m from the shoreline. As the overwhelmingly dominant biomass of infaunal organisms was formed by the Donax population, the analysis was focused primarily on this species. Donax were also studied for demography and population dynamics. The data on these bivalves were pooled into 6 size groups of 3-4 mm intervals (Table 2).

All samples were passed through a 5 mm mesh sieve, transported to the laboratory and divided according to maximum shell length. The analysis of heavy metal residues was performed by atomic absorption spectrophotometry (AAS), according to the following methodology (see also Hornung et al., 1981; Hornung and Rumelow, 1987): Aliquots of wet homogenized tissue (for mercury) and dry lyophilized samples were digested for 3 h at 140°C with concentrated nitric acid in decomposition vessels. The cooled digests were diluted in volumetric flasks with double distilled water to appropriate volume. With every batch run, blank and standard reference materials were also analyzed. The concentration of mercury was measured by cold vapor absorption spectrophotometry on a Coleman Mercury Analyzer MAS-50A. The standards used were provided by NBS-NIST from U.S.A. The

concentrations of Cd, Pb, Cu and Zn were measured in an IL-951 atomic absorption spectrophotometer at the following wavelength: 228.8nm for Cd, 283.3nm for Pb, 324.7nm for Cu, and 213.9nm for Zn. Background corrections were made for Cd and Zn. In the composite samples for each year, groups of specimens from the same point of collection and the same time were analyzed, as follows: Donax - 5 to 13 specimens/sample; Mactra - 6 to 9; Natica - 4 to 20; Arcularia - 3 to 6. Such pooling was intended to acquire sufficient tissue for 2 to 4 parallel runs for AAS. To correlate the observed levels of metals with physiological functions, we also studied the content of P-450 in microsomal fractions from the molluscs' digestive gland. The method used was that reported previously (Yawetz et al., 1984; Manelis et al., 1993).

Statistical evaluation: Data were analyzed using: single classification ANOVA, Student-Newman-Keuls test for multiple comparisons among means (Sokal and Rohlf, 1969).

3. RESULTS

3.1 Ecology

The sand-dwelling populations of molluscs along the Haifa Bay inhabit the bottom substrate in shallow water. Of these, Donax trunculus is the most dominant from the wash-zone to a depth of 2 m. This dominance continues along the entire 10 km length of the Bay. As shown in the study of Neuberger-Cywiak et al. (1990) Donax reproduces at the end of summer, and during those months (September - November) the fraction of juvenile (smaller) individuals in the population greatly increases. Overlapping this population of Donax is that of the gastropod Arcularia gibbosula and dispersed sparsely between is Natica millepunctata. Mactra corallina starts to appear on the more seaward fringe of this belt, forming larger populations in deeper water and mingling there with other deeper digging sand-dwelling molluscs, such as the bivalves Angulus spp., Ensis spp. and Solen spp. At the study site, which extends along + 1500 m of the subtidal zone, Donax trunculus was found to bury itself in the bottom at a depth of 3-18 cm during the daytime or in rough weather. Arcularia occurred in the top 5 cm of sand, while Natica and Mactra were found below 10-12 cm sand. In the evening or early morning the siphons of Donax were clearly seen above the sand and Arcularia was seen gliding on the bottom, often aggregating in groups of 10-15. The other species were seldom observed to be active. The analysis of age-distribution and biomass was performed on Donax.

The smallest measured individuals were of 10-11 mm shell length, 0.205 g total weight and 0.075 g wet soft body weight. With growth, the relationship between the weight of shells and tissue, as well as length of shell, drastically changed (Table 2). The largest Donax trunculus found on this shore was 34 mm long, had a total weight of 3.741 g and a wet flesh weight of 1.269 g. Table 2 provides data on these parameters in groups of Donax pooled together for our analyses. As seen from Table 3, the biomass of flesh per total weight remains constant in all groups. However, there is a constant increase in the ratio of flesh per mm increase in length. The increase in total weight per mm length also changed with growth.

Table 2

The relationship between total shell length range, total weight and net weight of flesh in studied groups of Donax trunculus (n = 960)

	Length (mm)		Total weight (g)		Weight of flesh (g)	
Group	Range	Mean	Range	Mean	Range	Mean
1	12-16	14	0.205-0.464	0.337	0.075-0.149	0.116
2	17-20	18.5	0.582-0.833	0.710	0.192-0.299	0.243
3	21-24	22.5	1.022-1.518	1.243	0.383-0.497	0.422
4	25-27	26	1.688-2.131	1.880	0.582-0.733	0.635
5	28-30	29	2.340-2.772	2.602	0.787-0.937	0.882
6	31-34	32.5	2.937-3.741	3.460	1.038-1.269	1.215

Table 3

The relationship between means of length (mm) and wet weight (mg) in the groups of Donax trunculus (n = 960)

Group	flesh wt / total wt	flesh wt / shell length	total wt / shell length
1	0.344	8.29	24.07
2	0.342	13.50	39.40
3	0.340	19.18	56.50
4	0.337	24.42	73.30
5	0.338	30.41	89.72
6	0.351	37.97	108.12

This study on demography and biomass distribution of Donax and other sand-dwellers provides a clearer picture of the relative importance of Donax in this habitat. Table 4 shows an example of a typical transect extending 25 m seaward on the clean site south of Akko (Fig. 1). This transect, one of twelve, shows that the calculated number of Donax per 1000 m^2 can range from 417,000 at 5 m from shore

to 1,130,000 at 20 m distance. The same applies for the biomass: from 273.6 - 796.5 kg/1000 m^2 to a maximal 745.0 - 2,237 kg at 20 m from the shoreline. As this habitat extends along 10 km and is very uniform, and the bottom occupied by Donax is more than 25 m wide, the enormous biomass of flesh and shells that this species produces in the ecosystem can be clearly seen. This also enables a quantification of metal or other xenobiotics trapped in the food-web of Donax.

Table 4

The sampled and calculated numbers and biomass of Donax trunculus (Akko, Transect June 1990)

Distance from shore	Average per sample			Average per m^2		
	No.	Biomass (g)		No.	Biomass (kg)	
		flesh wt.	total wt.		flesh wt.	total wt.
5m	125	82.2	237.4	417	0.274	0.791
10m	255	182.0	531.7	850	0.606	1.770
15m	254	199.5	576.4	847	0.664	1.920
20m	339	223.8	671.7	1130	0.745	2.237
25m	242	192.6	569.3	806	0.642	1.895

3.2 Residues of metals in soft tissues

The residual content of iron, zinc, cadmium, lead, copper and mercury were exposed in the soft tissues of Donax trunculus of different body sizes, and sampled during the years 1988, 1989 and 1990 from the three sites located in Haifa Bay, namely, Akko, Frutarom and Qiryat Yam (Fig. 1).

The concentrations of both iron and zinc in the soft tissues of Donax collected from Akko and Frutarom in the years 1988 and 1989 was practically identical (Fig. 2). In 1990 there was a significant ($P<0.01$) decrease in the content of iron in Donax from both Akko and Frutarom, and the levels of iron and zinc did not differ significantly from the low concentrations of these metals in Donax collected from Qiryat Yam in the same year.

The overall accumulation of cadmium, copper and mercury in Donax of different body size is presented in Fig. 3. For each body size, data from three years, and three sampling sites were combined. The accumulation of each of the three cations did not differ significantly with respect to the Donax body size.

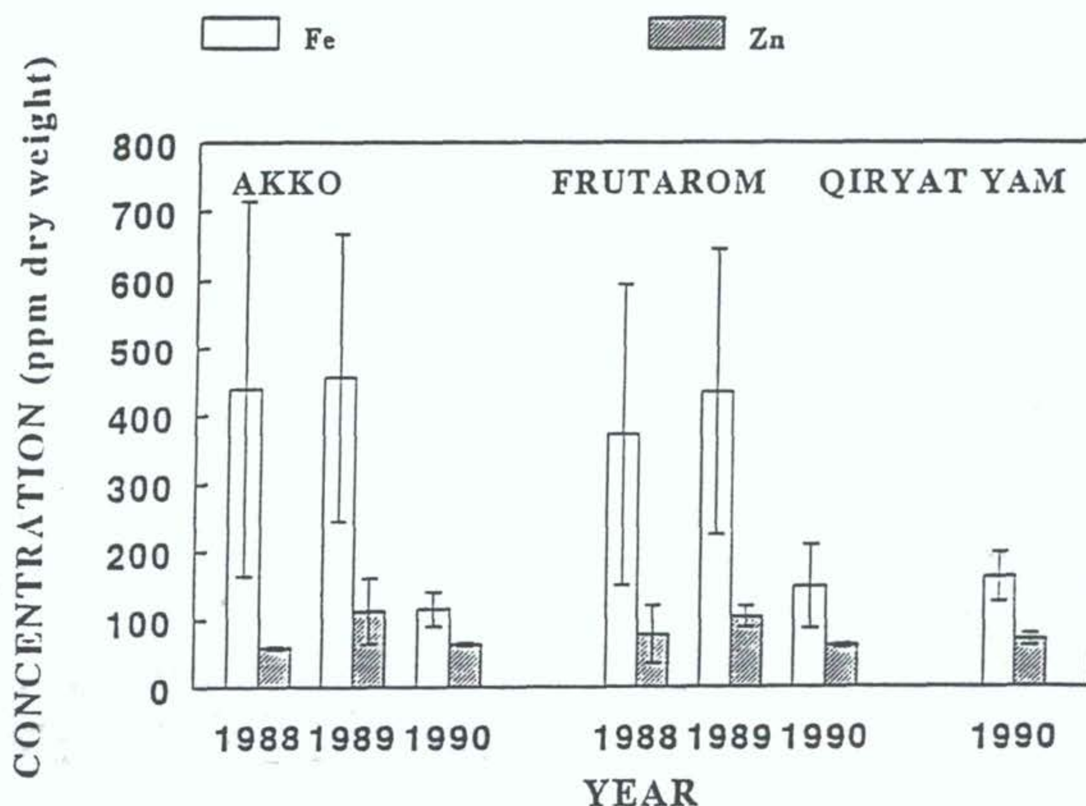

Fig. 2 Annual changes in the concentration of iron and zinc in the soft tissues of Donax trunculus from different sampling sites in Haifa Bay (results are means + SD)

The concentration of copper in Donax from Akko, Frutarom and Qiryat Yam did not differ significantly (Fig. 4). Mercury concentration in Donax from Frutarom was significantly higher ($P<0.001$) than the value recorded in Donax from Akko (Fig. 4). The most outstanding measurement was 20 ppm mercury in Donax from Frutarom, a particularly alarming observation, being higher than any previous observation along the Israeli Mediterranean coast (Table 5).

The concentration of cadmium (Fig. 5) in Donax from Frutarom was significantly ($P<0.01$) lower compared to that found in Donax from Akko and Qiryat Yam. Lead was found only in Donax from Akko. Annual changes in the concentrations of copper (Fig. 6) and cadmium (Fig. 7) were insignificant both in Akko and Frutarom. The decrease of lead levels in Donax from Akko in 1989 compared to 1988 (Fig. 7)

was insignificant. In contrast, the concentrations of mercury in Donax from Frutarom and also in those from Akko do show a significant ($P<0.01$) trend of reduction, with time. The content of this highly toxic metal in Donax (Fig. 6) was reduced dramatically in 1990, both in Akko and Frutarom.

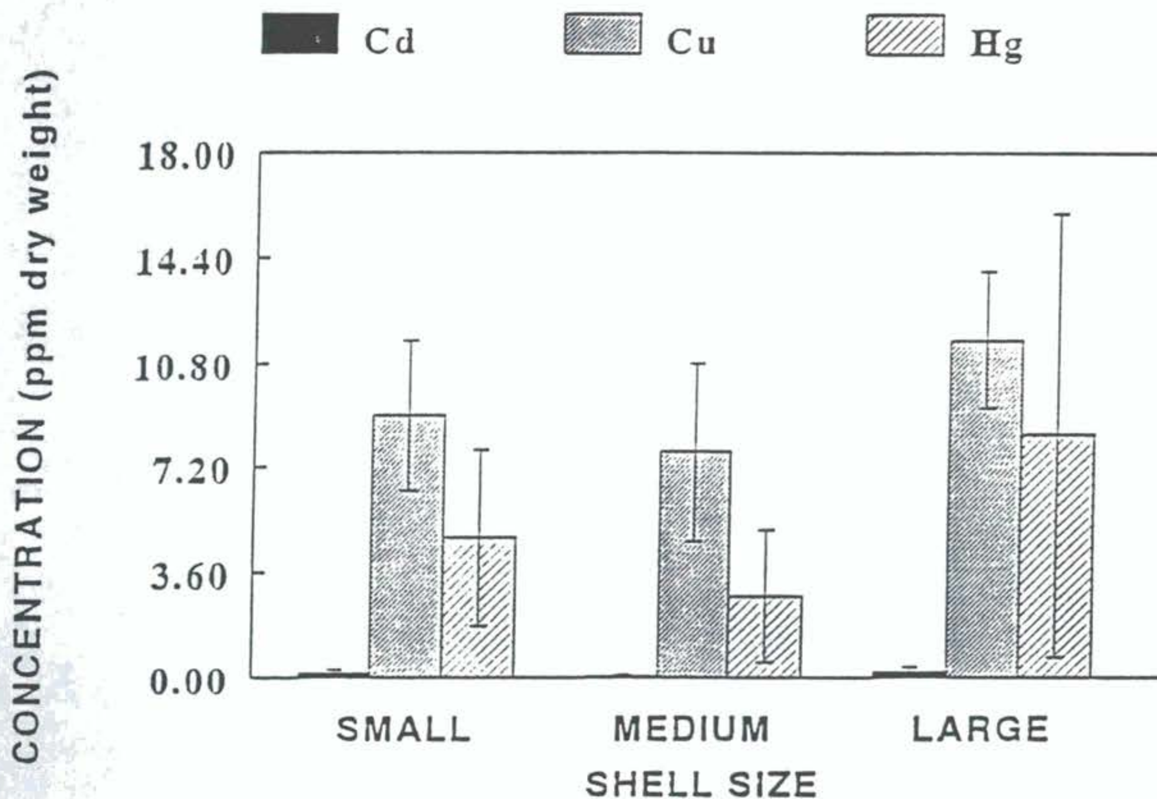

Fig. 3 The effect of size on the accumulation of cadmium, copper and mercury in Donax trunculus. (results are means + SD)

3.3 Residues of metals in shells

As with the soft tissues, the shells of D. trunculus can also serve as evidence for the occurrence of pollutants in the environment (Table 6). Evidently, when the metal content of soft tissue is compared with that of the shells, the main location for the deposition of the xenobiotics is in the body and not in the shell. However, nickel was found only in the shells and not in the soft parts; conversely, aluminium was found in soft parts but not in shells. The levels of magnesium, although found in soft parts and shells, were significantly different, between 170-192 ppm d.w. in shells (Table 6) and much lower in the soft tissue, with no specific correlation to centres of pollution. It is also interesting to note that although the level of aluminium in the soft, sandy bottom was 1947 ppm dry sand at the polluted site, the highest level observed in the soft tissue of Donax from this site was 869 ppm, while in Natica millepunctata

it was only 501 ppm d.w. Recent studies of Bresler and Fishelson (1994) showed that the mantle of Donax constitutes a depot for nickel accumulation and transportation to the shell.

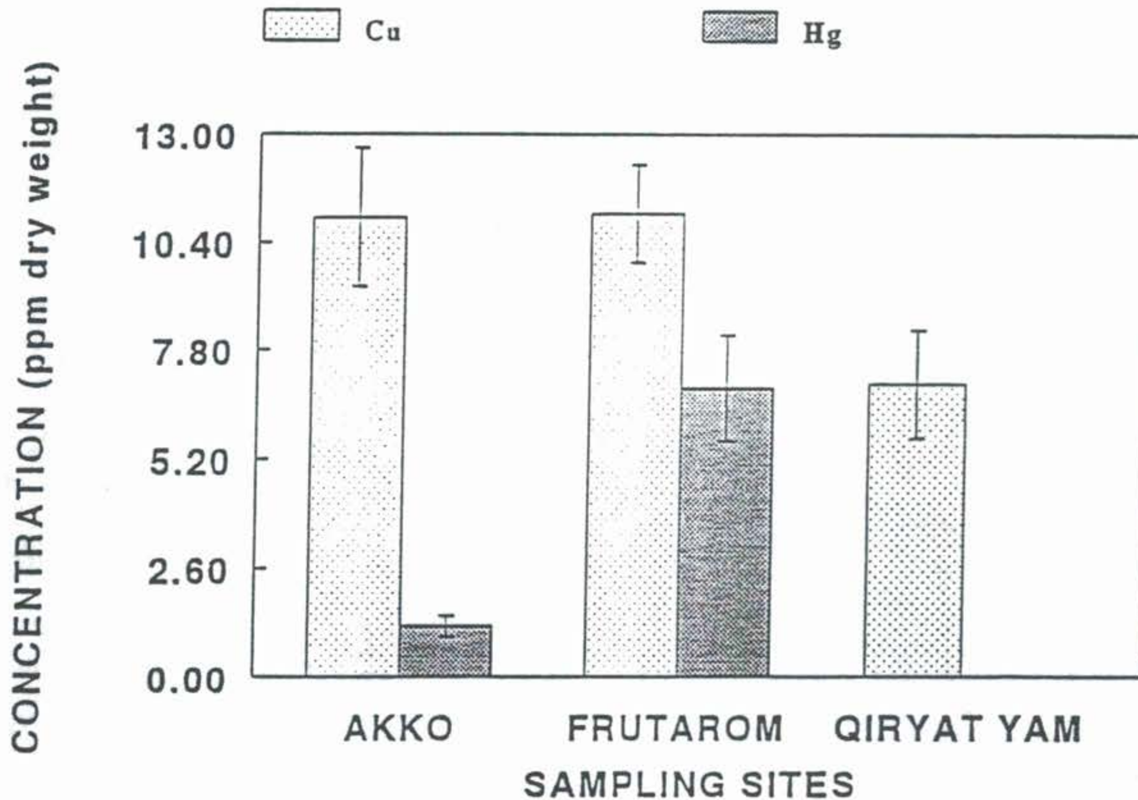

Fig. 4 Copper and mercury content in Donax trunculus from different sampling sites in Haifa Bay. (results are means + SD)

In 1991, following a relatively small crude oil spill at Qiryat Yam, a site south of the sampling region, a sample of molluscs from this site was collected and analysed, as well as from a much cleaner site adjacent to it. Sediment from the oil polluted site was also analysed. During this sampling it was observed that the oil spill caused mass mortality of Mactra corallina, the shells of which covered the shore, together with Angulus planatus, some Venus spp. and even Ensis spp., all of which are species that dwell in deeper water and dig deeper into the sand than Donax. In contrast, the shallow-water Donax population remained on the site and only a few were killed. Following the oil-spill, the soft tissues of the studied molluscs showed a radical increase in some metals, especially chromium (32. ppm d.w.) as compared to zero on the clean site; lead 60 ppm compared to 2.48, and copper 191 ppm compared to 15.8. On the oil-polluted site nickel was also found accumulating in the soft body parts (32 ppm d.w.), but was not found in the same species at other sites

of collection, except in shells. It is possible that the high load of the other metals has suppressed the defence system and "paved the way" for nickel bioaccumulation in Donax from the oil polluted site.

Table 5

Maximal metal content in the studied molluscs. Comparison of sites 4-5 (discharge) and 10-11 (Akko) in 1989 (ppm/dry weight) (N = samples of 6-8 specimens)

Species	Near the factory (4-5)				500 m away (10-11)			
	Cd	Zn	Pb	Hg	Cd	Zn	Pb	Hg
Donax trunculus	1.08	222.9	12.70	20.00	0.72	151.9	8.25	1.72
Mactra corallina	1.45	74.6	4.02	1.87	1.00	78.6	9.60	0.64
Natica millepunctata	0.56	56.2	2.19	6.04	0.40	67.0	ND	ND
Arcularia gibbosula	5.47	622.3	9.43	1.45	3.00	606.0	ND	ND

ND = not detectable

Table 6

Maximal metal content in shells of D. trunculus from clean and polluted sites in Haifa Bay (ppm/dry weight)

Station	n	Pb	Mg	Zn	Al	Ni	Cu	Fe
Clean (st. 10-11)	10	43.1	192.0	ND	ND	11.2	8.4	48.0
Polluted (st. 3-5)	12	60.5	170.0	12.2	ND	12.6	9.2	52.0

n = number of samples

3.3 Enzymology

Cytochrome P450 was determined in the microsomal fraction of Donax hepatopancreas in order to compare the specific content of the hemoprotein in Donax collected from Frutarom to that collected from Akko. Cytochrome P450 content is a good biomarker for monitoring exposure of molluscs to aliphatic and aromatic

hydrocarbons including polychlorinated biphenyls (PCB) and dibenzodioxins that are chemical pollutants from industrial origin (Yawetz et al., 1992). The carbon-monoxide (CO) difference spectra for microsomes of Donax collected from Frutarom was always higher (Fig. 8A) than that measured in Donax from Akko (Fig. 8B). The constant higher state of induction of cytochrome P450 in Donax from Frutarom indicates exposure of the molluscs to industrial waste, of the nature mentioned above. Frutarom sampling site is therefore polluted with hydrocarbonic waste as well as mercury ions.

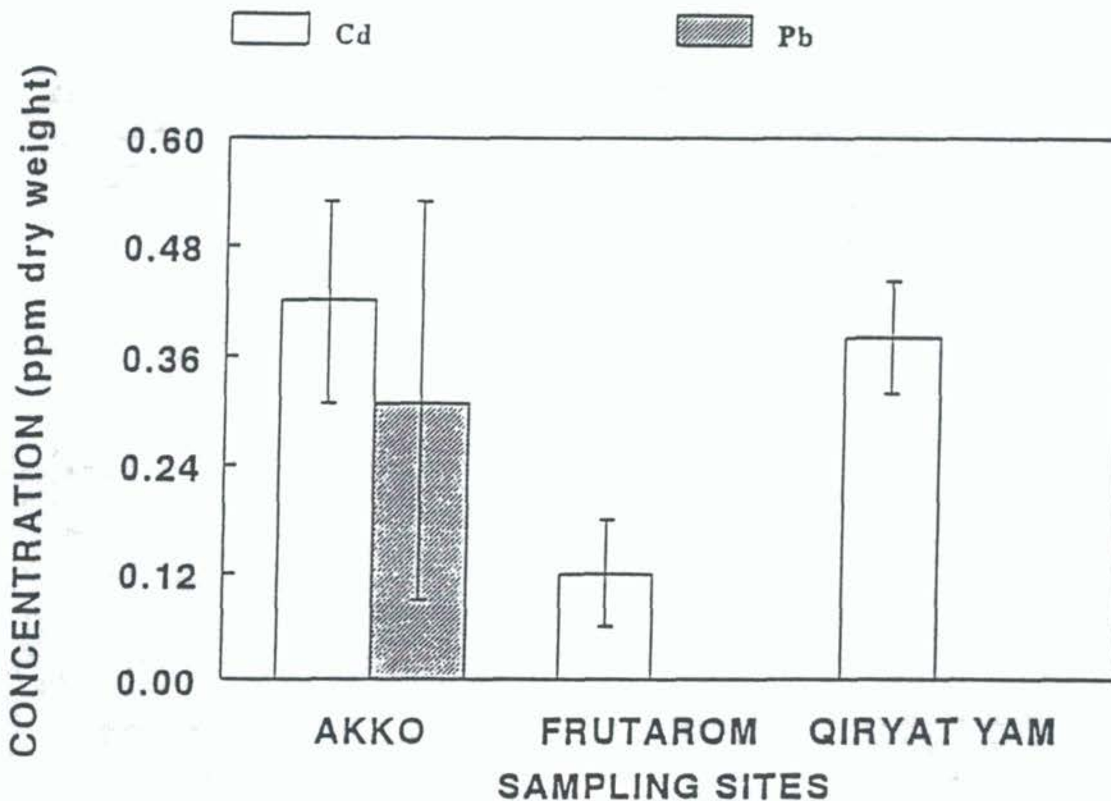

Fig. 5 Cadmium and lead content in Donax trunculus from different sampling sites in Haifa Bay. (results are means + SD)

4. DISCUSSION

The occurrence of high levels of heavy metals in shallow-water invertebrates of the Mediterranean Sea has been extensively documented during the last two decades. Particularly alarming is the high presence of mercury, copper, lead, zinc and cadmium, recognized as the most toxic and detrimental to the various species (Connell and Miller, 1984). As shown by Yawetz et al. (1984) nickel can induce anomalies in the function of hepatic tissue. Concentrations of iron, copper and zinc in our Donax trunculus are much higher than in the same species from Mauritania

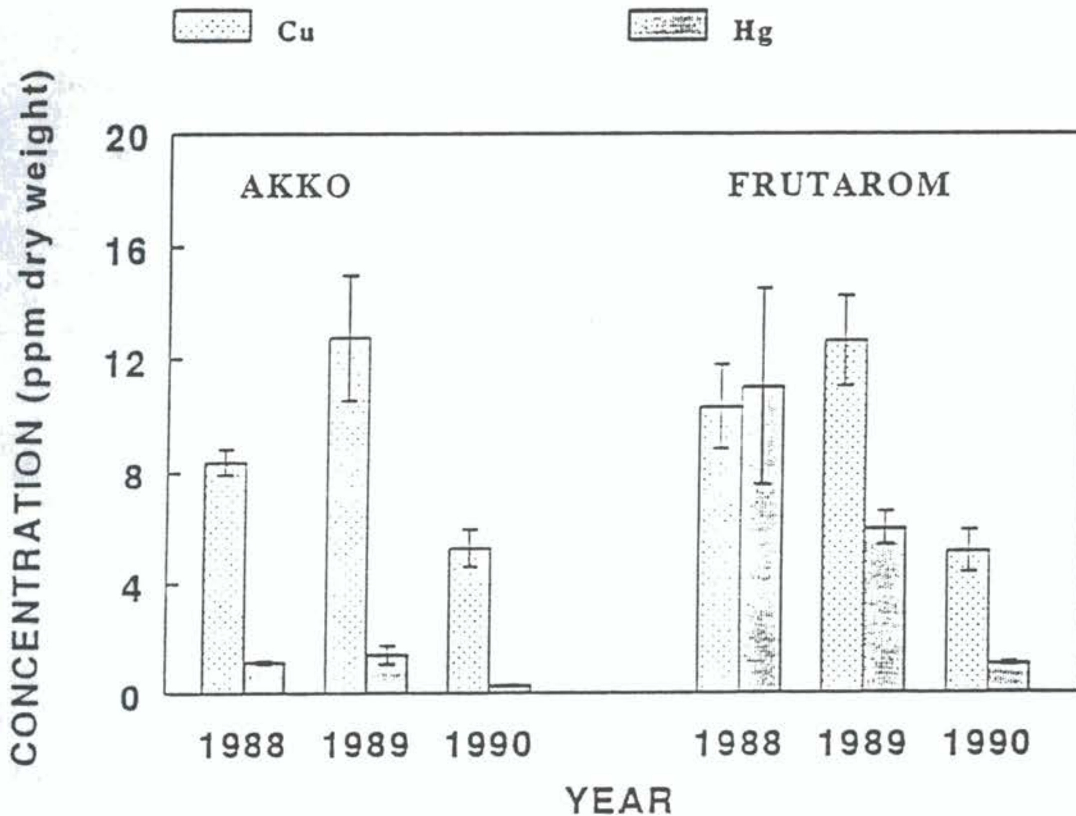

Fig. 6 Annual changes in copper and mercury content in Donax trunculus from different sampling sites in Haifa Bay. (results are means + SD)

(Roméo and Gnassia-Barelli, 1988). The high levels of these metals in the shallow-water infaunal sand-dwelling species of Haifa Bay, especially on the doorstep of the Frutarom PVC factory, makes this factory the suspected source of pollution. This is especially true for mercury, since it is used as a catalyst in their production processes. It is possible that once stressed by mercury, the molluscs also become more vulnerable to other metals. The data thus far collected, especially on Donax trunculus, show that this point-pollution is very localised, and several hundred meters laterally and 2-3 meters deep, the load of mercury and other metals is not much higher than in populations of cleaner stations along the Israeli Mediterranean sea-shore. This explains the lower figures in Hornung et al. (1984), who collected at depths outside this belt. However, metals are retained in the bottom or detritus for many years at their localities, and this accumulation may at any time, under optimal conditions, become absorbed, bioaccumulate, and so *ab novo* penetrate the food-web of the ecosystem. Such pollution, involving the animal food-webs can induce teratogenic changes such as sarcomas (Farley et al., 1986) or induce phenomena of genotoxicity and in this way destroy vital parts of the ecosystem (Bresler and

Fishelson, 1994). The observed higher concentrations of Donax, such as mercury, in smaller mussels indicates the possibility that a) they metabolize more food in correlation with their growth, thereby accumulating more, as was also postulated by Roméo and Gnassia-Barelli (1988), and b) the mortality is specifically higher in older individuals. Regarding the high levels of the so-called less toxic metals, such as aluminium and magnesium, the question remains open as to how they will behave in synergism with the more toxic ones (Connell and Miller, 1984), especially in the high ambient water temperatures of the eastern Mediterranean. As biomagnification leads toward higher concentration of the refined material, it should be expected that the pollutant levels at any trophic level will be a fraction of those finally detected.

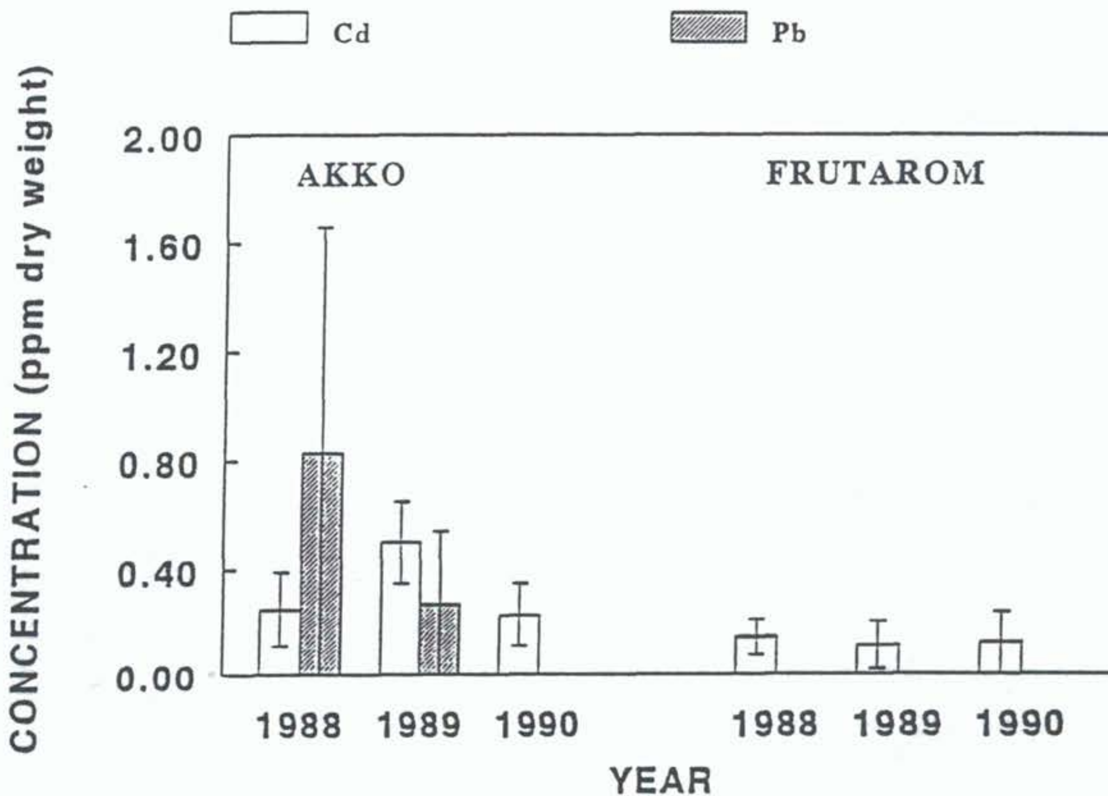

Fig. 7 Annual changes in cadmium and lead content in Donax trunculus from different sampling sites in Haifa Bay. (results are means + SD)

The population of the shallow water molluscs, especially Donax trunculus, seems to be extremely well adapted to this harsh environment, frequently disturbed by strong water currents and sediment transport during winter, and high water temperatures during summer. Additionally, there are the man-induced stresses, especially metal and organic pollution. In spite of these obstacles, we are witnessing

a biomass of Donax that reaches 2400 kg/1000 m^2, the highest registered along the Mediterranean littorals. This possibly indicates an excellent resistance to the various stresses, probably based on specific defense systems, one of which is the transport of metal residues from the soft body into the shells. The existence of such transport is evident from the concentration of nickel and lead in shells of Donax. This transport was recently studied by Bresler and Fishelson (1994) by means of marker cations, anions and fluorescence.

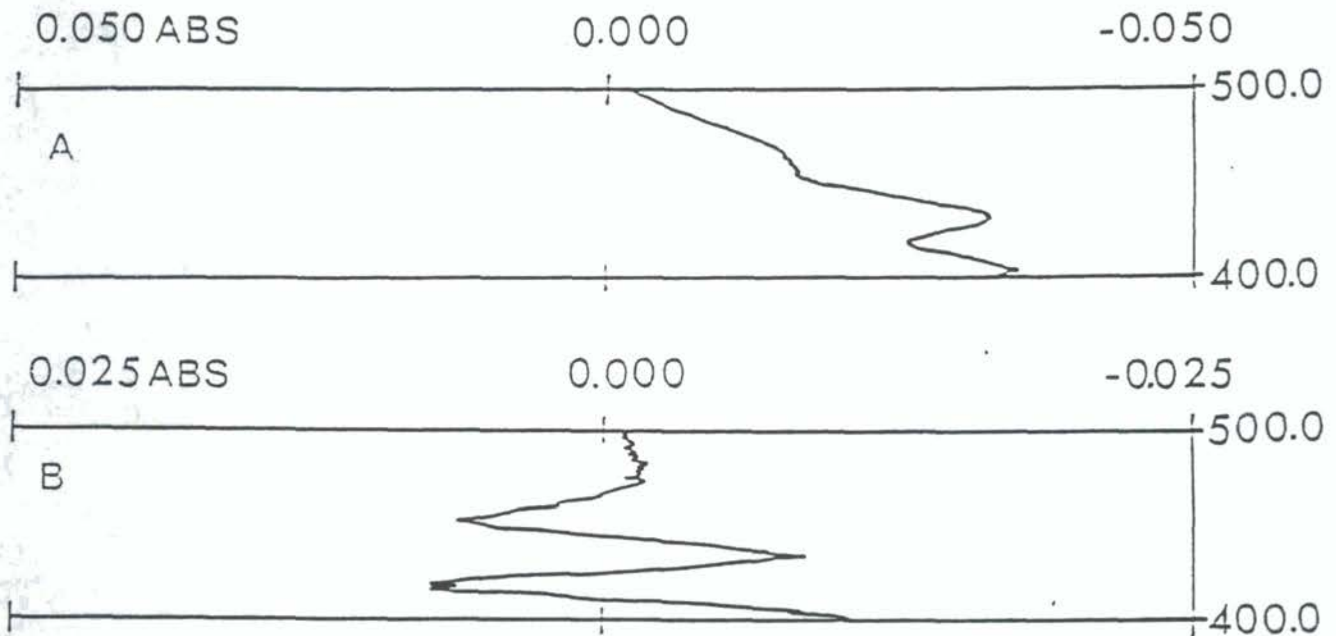

Fig. 8 The carbon-monoxide reduced versus reduced microsomes prepared from the hepatopancreas of Donax trunculus collected from the sampling sites near Frutarom (A) and Akko (B). The high absorption band at 450 nm present in the specimens collected near Frutarom as compared to Akko represents induction of cytochrome P450 as a result of exposure to various hydrocarbons present in the environment

5. ACKNOWLEDGMENT

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ACCUMULATION OF SEVERAL HEAVY METALS AND THEIR RELATION TO INDUCED METAL-BINDING PROTEINS IN Artemia

by

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ABSTRACT

The brine shrimp Artemia is highly tolerant to polluted environments and it shows a high capacity to accumulate heavy metals.

A Mediterranean strain of Artemia (La Mata strain) was used in order to study the accumulation of Cd and Hg under sublethal concentrations, and their relation to induced metallothionein (MT).

Cadmium accumulation after different exposure times for pre-exposed and non pre-exposed animals was studied. There was no significant effect of cadmium pre-exposure on cadmium accumulation, however, cadmium pre-exposure modified cadmium accumulation rate temporarily but that it had no effect on the cadmium saturation level.

The silver-saturation method was used to determine MT content. This method is a rapid quantitative assay for MT quantification that may be used for crustacean tissues when polarography is not available. Silver determination by atomic absorption spectrophotometer has proved to be sensitive enough for our purposes.

MT content in Artemia increases in a time-dependent fashion. There is a remarkable increase in MT content between 12 and 24 h of cadmium exposure. However, prolonging exposure to cadmium for another 24 h did not cause a further increase in MT.

After 48 h of exposure, the MT content in Artemia increased in a dose responsive manner in relation to the amount of cadmium present in the water. The high responsiveness that Artemia shows in the MT induction by cadmium can explain the high resistance that it exhibits to this particular metal.

The clear relationship that we have found between cadmium concentration in water and MT levels in tissues support the idea that MT levels in crustaceans can be a good biomarker for cadmium exposure. Nevertheless, we are aware that field studies are needed before we can apply our findings to pollutant monitoring.

The effects of a short-term exposure to Cd on Zn stored in the brine shrimp Artemia and the possible involvement of MT in this process, was also studied. The natural zinc content of shrimps is not altered by exposure to 10 mg Cd l^{-1}. However, when extra zinc (5 mg Zn l^{-1}) is added to the seawater after cadmium exposure, significant differences are observed. Zinc accumulation was lower in cadmium pre-exposed shrimps as compared with their respective control.

Cadmium-exposed groups showed higher content of MT than control groups for all the times studied. The difference occurred as a consequence of Cd exposure. Zn exposure did not affect Cd elimination. However, some interaction between Cd and Zn can be seen from our results. The Cd pre-exposed group accumulated less Zn from the water than non pre-exposed.

Mercury accumulation and metallothionein synthesis in Artemia, was also studied. In treated groups, mercury content in supernatant increased with exposure time. The mercury uptake rate during the first 48 h was much higher for 20 μg Hg l^{-1} exposed groups than for 5 and 10 μg Hg l^{-1}. MT content was slightly increased after 48 h of exposure to 20 μg Hg l^{-1}, but there is not increment in MT content until 72 h in the groups treated with lower concentrations.

1. INTRODUCTION

Metal ions are serious pollutants, especially in the aquatic environment, since they can be incorporated into food chains and concentrate in aquatic organisms to a level which affects their physiological state. There are studies on the toxicity and deleterious effects of heavy metals and/or their accumulation on marine invertebrates. However, there are only few studies dealing with physiological disturbances produced by them.

Metallothionein (MT) is a small cysteine-rich protein that binds to heavy metals, and its synthesis is induced by some of the metal to which it binds. Elevated levels of such proteins have been suggested as indicating involvement in uptake, storage, transport, and elimination of toxic metals and in the routine metabolism of metals (Engel and Brouwer, 1989). On the other hand, MTs have been proposed as specific biochemical probe for metal exposure to aquatic organisms (Brown et al., 1977). Because of that, we consider that the study of MTs in crustaceans is very interesting for understanding the mechanisms associated with the toxicology of metals, and for its potential use for monitoring metal-contaminated environments. Both approaches require the determination of MT levels in the tissue.

The brine shrimp Artemia is highly tolerant to polluted environments and it has been used extensively on environmental toxicity studies. The purpose of our project is to study the capacity of Artemia to accumulate heavy metals (cadmium and mercury), and to isolate and to characterise metal binding proteins. Special attention has been payed to the toxicologically-relevant parameters associated with MT as its level of induction and the enhanced binding of metal associated with this induction.

2. ACUTE TOXICITY OF CADMIUM IN Artemia

Approaches to measuring lethal toxicity vary in their complexity, in terms of the procedures employed, the apparatus required and the methods of collecting and processing the data produced. There are corresponding differences in the amounts of information yielded, the degree of confidence which may be placed in the results, and the purposes for which those results may validly be used. The results of toxicity tests may be significant in connection with pollution control legislation. Some of the simpler methods (like LC_{50}) are widely used but provide limited information, and their results need to be interpreted with caution. A summary of the acute toxicity results for cadmium for various concentrations (LT_{50}) and various exposure periods (LC_{50}) is given in Tables 1 and 2.

Table 1

The LT_{50} values and 95% confidence limits for different concentrations of cadmium. Each LT_{50} value represents the average of 3 replicates

Concentration (mg Cd l^{-1})	LT_{50} (hours)	Slope
50	85 (122-59)	3.17
60	84 (144-49)	2.15
70	41 (60-28)	3.04
80	43 (64-29)	2.96
90	32 (49-21)	2.77
100	22 (54-9)	1.28
120	12 (24-6)	1.69

Table 2

The LC_{50} values and 95% confidence limits for cadmium at different exposure periods. Each LC_{50} value represents the average of 3 replicates

Time (hours)	LC_{50} (mg Cd l^{-1})	Slope
	(124.19-78.23)	
36	80.01 (93.61-63.38)	4.10
48	60.48 (67.01-54.58)	6.55
72	47.07 (51.63-42.90)	7.26
96	42.89 (47.39-38.81)	8.23

The percentages of mortality were calculated in each concentration and exposure time and converted to probits. The metal concentrations were converted to Logs. The LT_{50} or LC_{50} and their 95% confidence limits and the slope of the probit line were calculated using the method of Litchfield and Wilcoxon (1949).

3. CADMIUM ACCUMULATION AND METALLOTHIONEIN SYNTHESIS

Cadmium is a ubiquitous non essential element which possesses high toxicity and is easily accumulated from the environment by aquatic organisms. Cadmium uptake from water can take place by either passive diffusion through the gills or through the body. Among all macromolecules that interact with metals, there is a class of low molecular weight, cytoplasmic, metal-binding proteins, which have similar characteristics to mammalian metallothioneins, that have been observed in a variety of marine invertebrates. These proteins have a high affinity for various toxic heavy metals, particularly cadmium. Elevated levels of such proteins have been suggested as indicating involvement in uptake, storage, transport and elimination of toxic metals and in the routine metabolism of metals (Engel and Brouwer, 1989).

Artemia shows a high capacity for accumulation of heavy metals, including cadmium and displays a notable resistance to this particular metal (Jennings and Rainbow, 1979). The presence of cadmium binding proteins in this crustacean has also been shown (Thall and Acey, 1985; Jayasekara et al., 1986; Acey et al., 1989). In this work we have studied the effect of pre-exposure to cadmium on the accumulation of this metal and the involvement of cadmium binding ligands.

3.1 Materials and methods

Stock cultures of Artemia ("La Mata" strain) were maintained in aerated natural sea water under continuous fluorescent illumination at 22°C, within a salinity range of 36 to 38‰. Both nauplii and adults were fed on cultured Tetraselmis algae. For all the experiments, 20-day old adults were used. Two groups of about one thousand adult Artemia were kept in sea water. Brine shrimp of group A were kept during 24 h (pre-exposure period) in 15 l experimental aquaria containing 20 mg Cd l^{-1}. Group B was maintained in clean sea water during this period. After the pre-exposure period animals of group A were transferred to clean sea water aquaria for 48 hours. Afterwards, groups A and B were exposed to 10 mg Cd l^{-1}. Samples from both groups were obtained after 1, 3, 6, 10, and 14 days for cadmium determination and for the analysis of cadmium binding proteins.

For separation of Cd-binding proteins, samples were homogenized in Tris-HCl buffer (0.06 M Tris, 0.01 M NaCl, pH 8.6) with 0.1 mM PMSF to prevent protease activity and 1 mM DTT to maintain reducing conditions. The homogenate was centrifuged at 30,000 g for 30 min. at 4°C. The supernatant was heat treated at 60°C for 10 min and centrifuged again at 30,000 g for 30 min. at 4°C. Supernatant was then applied to a column of Sephadex G-75 (1.3 x 90 cm) and was eluted at 4°C with Tris-HCl buffer (0.06 M Tris, 0.01 M NaCl, pH 8.6) and 0.5 mM DTT at a constant rate of 15 ml h^{-1}. Three milliliter fractions were collected. Selected fractions were applied to an ion exchange DEAE-Sephadex A-25 chromatography, and eluted with Tris-acetate buffer (pH 8.6) gradient of increasing ionic strength (10 mM-300 mM Tris-acetate). In some cases further Sephadex G-75 chromatography in the presence of either 10 mM 2-mercaptoethanol or 0.5 mM DTT was performed.

Cadmium concentrations were determined with a Perkin-Elmer model 5000 flame atomic absorption spectrophotometer equipped with a model 561 recorder and a deuterium background corrector. UV absorbance at 254 and 280 nm was measured on a HP 8452A Diode Array Spectrophotometer.

Two-way Analysis of Variance was used to determine cadmium pre-exposure and time effects on cadmium accumulation. Mean separation was accomplished with Fisher PLSD test. The significance level was probability of ≤ 0.05.

3.2 Results and discussion

Cadmium accumulation after different exposure times for pre-exposed and non pre-exposed animals is shown in Fig. 1. The two-way ANOVA showed the effect of exposure time on cadmium accumulation (p=0.0001). There was no significant effect of cadmium pre-exposure on cadmium accumulation. However, the significant interaction between cadmium pre-exposure and time effects (p=0.0001) showed that cadmium pre-exposure modified cadmium accumulation pattern. The accumulation rate of cadmium in non pre-exposed animals was very high (25 μg Cd g^{-1} wet weight) during the first day. Subsequently, the uptake slowed down and became linear (1.7 μg Cd g^{-1} wet weight day^{-1}) until day 10 when it levelled off. In pre-exposed brine shrimp

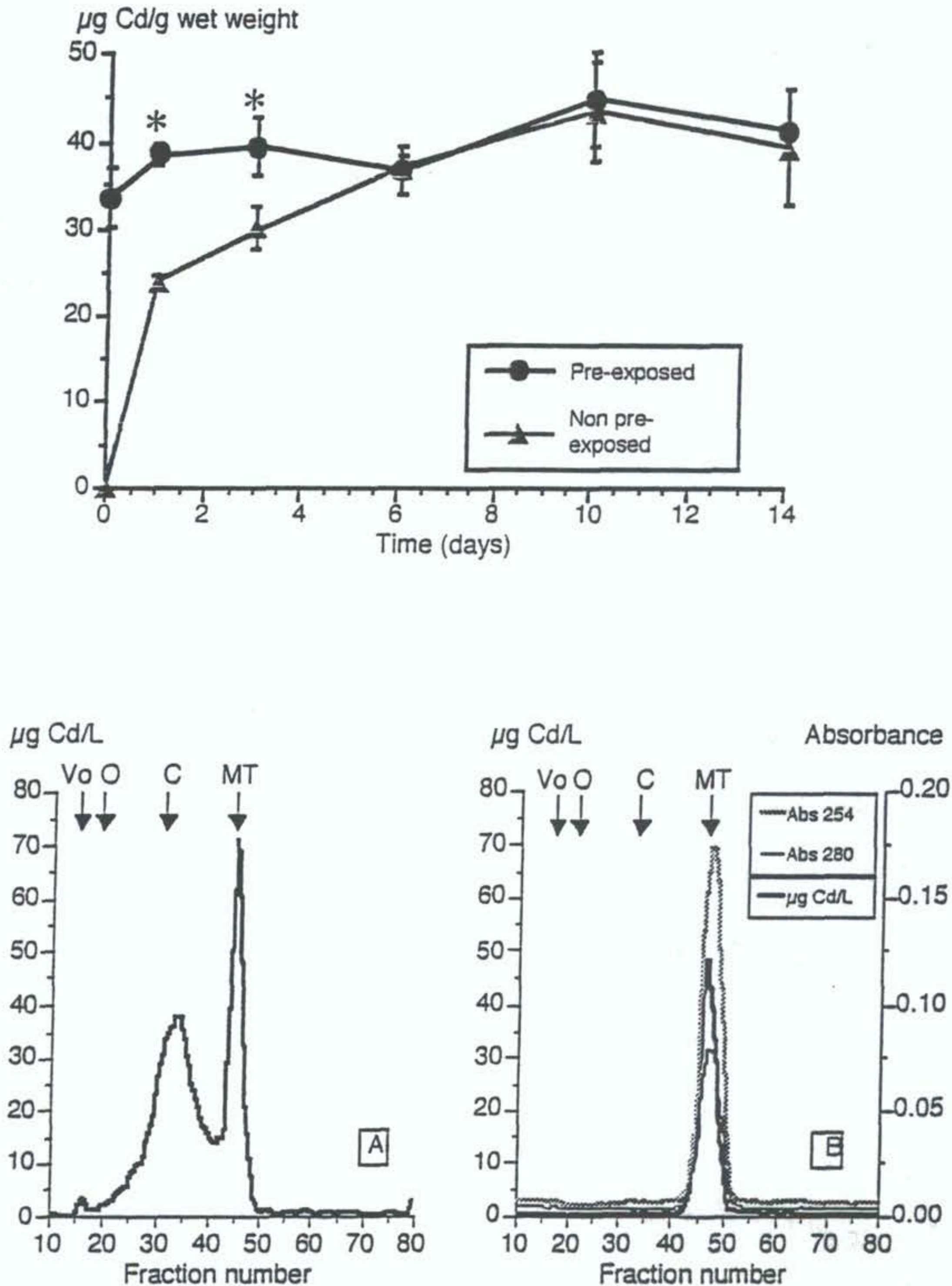

Fig. 1 **(Above)** Accumulation of cadmium after 1, 3, 6, 10, and 14 days of cadmium exposure for pre-exposed and non pre-exposed animals. Error bars indicate data range. (*) Significantly different from non pre-exposed organisms (Fisher PLSD test, $p \leq 0.05$).
(Below, A) Sephadex G-75 elution profile from non pre-exposed animals after 24 hours exposure to 10 mg Cd l^{-1}.
(Below, B) Sephadex G-75 profile of 6000 daltons peak after purification by anion exchange chromatography. (0=ovoalbumin, C=cytochrome C, MT=metallothionein)

the accumulation rate was much lower than in non pre-exposed animals (5 μg Cd g^{-1} wet weight) during the first 24 hours and there was no significant increase in cadmium concentration between day 1 and 6. Both experimental groups reached the same cadmium concentration at 6 days, after which their cadmium accumulation pattern was identical until the end of the exposure period. Our results show that cadmium pre-exposure modifies cadmium accumulation rate temporarily but that it has no effect on the cadmium saturation level.

In cadmium pre-exposed animals cytosolic cadmium was associated with a peak with an approximate molecular weight of 12,000 daltons at all the exposure times considered. This was also true for non pre-exposed animals except for 24 hours exposure when a second peak with an approximate molecular weight of 6,000 was also observed (Fig. 1A). When fractions corresponding to the 6,000 daltons peak were further processed by DEAE-Sephadex anion exchange chromatography only one peak was observed. This peak eluted at the same ionic strength (65 mM Tris) as the standard MT-1 from rabbit liver. Fig. 1B shows the fractionation pattern after Sephadex G-75 gel chromatography of pooled fractions from the anion exchange column. A single, symmetrical peak containing cadmium can be discerned, its absorbance being more pronounced at 254 nm than at 280 nm. The elution volume of this peak coincides with that of standard mammalian metallothionein.

In order to investigate whether the higher molecular weight component is a dimer of the lower molecular weight component, fractions from the 12,000 peak were reduced with 10 mM of 2-mercaptoethanol and rechromatographed on Sephadex G-75. The profile obtained was similar to Fig. 1B. Our results support the hypothesis that the 12,000 cadmium peak is due to the dimeric forms of metallothionein through intermolecular sulphide bond formation. We detected metallothionein in monomeric form only after a short cadmium exposure time (24 hours) in non pre-exposed animals when the cadmium accumulation rate is very high and metallothionein induction is expected to be very active. The presence of only dimeric forms in all the other experimental conditions when the cadmium accumulation rate is lower may indicate that the dimeric form is a physiological reality and it is not just an oxidation by-product of the isolation procedure.

4. METALLOTHIONEIN QUANTIFICATION IN Artemia

Metallothionein (MT) has been seen as a potential biomarker for heavy metal pollution. However, it is recognised that its usefulness as a monitoring tool depends on the full understanding of its function and on the availability of a standard measurement method (Roesijadi, 1992). Immunochemical and electrochemical procedures have been shown very useful for basic research but expensive equipment and trained staff are not always available. Usually biomonitoring programs involve laboratories from several countries that may have different budgets and the choice of an MT quantification assay should take this fact into account.

4.1 Materials and methods

Samples of whole Artemia (50 individuals pooled) and crayfish hepatopancreas were obtained after 12, 24, and 48 hours of cadmium exposure. Cadmium exposure was carried out in a semistatic way. Cadmium solution was removed every day to reduce the build-up of metabolic wastes and to keep the concentration of cadmium near the nominal level.

For MT estimation (see Fig. 2), samples were weighed and placed in a homogenizing tube with a solution of 0.25 M sucrose and the mixture was homogenized with a motor-driven Teflon pestle at 4°C. The homogenate was centrifuged at 20,000 g for 20 min. at 4°C. Aliquots of 750 μl supernatant were analyzed for MT content by the silver-saturation method (Scheuhammer and Cherian, 1986) with small modifications. Samples were incubated with 1mL of 20 mg l^{-1} silver solution for 15 min. at 20°C to saturate the metal binding sites of MT. Excess metal was removed by the addition of 200 μl human red blood cell hemolysate to the assay tubes followed by heat treatment in a water bath (100°C for 2 min.). The heat treatment caused precipitation of Ag^+ bound haemoglobin and other proteins, except for MT which is heat stable. The denatured proteins were removed by centrifugation at 1000 g for 5 min. The hemolysate addition, heat treatment and centrifugation were repeated three times in each sample. The amount of Ag^+ in the final supernatant fraction is proportional to the amount of MT present. Silver concentrations were estimated by flame in a Perkin-Elmer model 5000 atomic absorption spectrophotometer equipped with a model 561 recorder, and a deuterium background corrector. In a similar way, cadmium from supernatant fraction obtained from homogenate was estimated by atomic absorption spectrophotometer. The detection limit was 0.03 μg ml^{-1} for silver and 0.015 μg ml^{-1} for cadmium. Assay tubes containing purified MT from horse kidney (Sigma) in a range of concentrations from 1 to 40 μg ml^{-1} underwent the same process.

Some of the samples where MT had been estimated were applied to a column of Sephadex G-75 and eluted at 4°C with 10 mM Tris-HCl buffer, pH 8.6. Silver concentrations of fractions were determined. A single peak of silver was measured. Equally, a purified MT solution from horse kidney was applied to column and UV absorbance at 254 nm was measured. An absorbance peak from purified MT coincided with the silver peak from the sample exposed to cadmium.

Two-way analysis of variance was used to determine treatment and time effects on the various parameters studied. Mean separation was accomplished with test Fisher PLSD. The significance level in all instances was probability of $p \leq 0.05$.

4.3 Results and discussion

The cadmium-saturation assay (Eaton and Toal, 1982) has been successfully used for the routine measurement of MT content in tissues and cells from mammals and fishes. However, MT from crustaceans has a high copper content (Scheuhammer and Templeton, 1990). Since cadmium displaces Cu from MT poorly, this method underestimates the total MT content. The silver-saturation method was

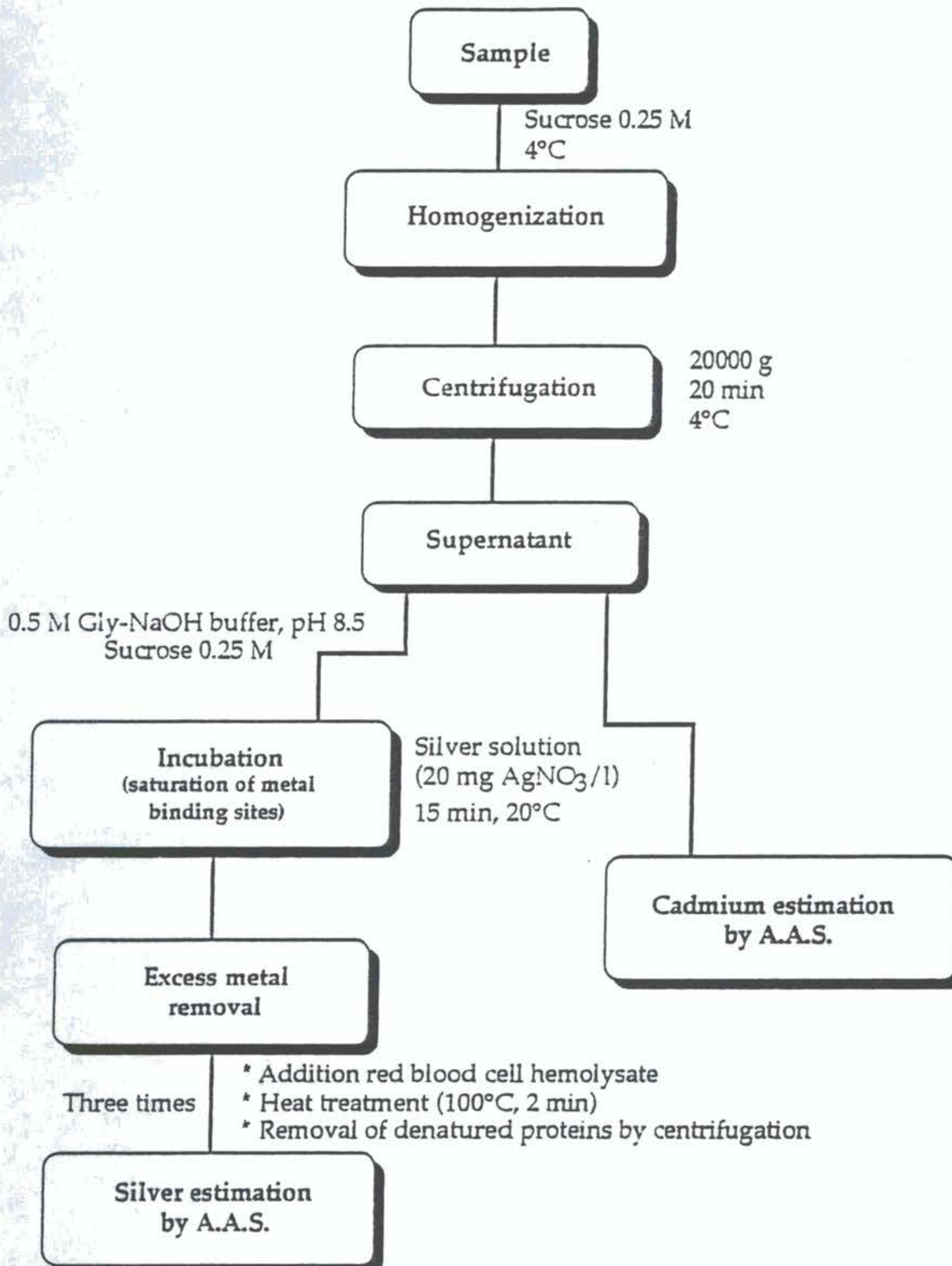

Fig. 2 Protocol for MT determination by silver saturation method

shown to be superior to the Cd-hem assay when measuring MT with a high content of Cu (Scheuhammer and Cherian, 1986). Furthermore, although the original method is based on the use of radioactive isotopes to increase sensitivity, silver determination by atomic absorption spectrophotometer has proved to be sensitive enough for our purposes. The regression coefficient for the MT standard curve was r^2= 0.999.

Gel filtration chromatography showed that all silver measured in the supernatant fraction from Artemia appeared in a peak similar in molecular weight and absorbance properties to that found when purified mammalian MT was applied to the same column.

As can be seen in Fig. 3A, MT content in Artemia increases in a time-dependent fashion. There is a remarkable increase in MT content between 12 and 24 h of cadmium exposure. However, prolonging exposure to cadmium for another 24 h did not cause a further increase in MT. The Fisher PLSD test showed that MT contents at 24 and 48 h were significantly different (p= 0.0005) from those at shorter times of cadmium exposure. Similar results were found for P. clarkii hepatopancreas (Del Ramo et al., 1995). However, the control values for MT in Artemia were higher than in crayfish hepatopancreas. When cadmium contents were measured in the similar supernatant fractions where MT was estimated (Fig. 3B), cadmium levels were much higher in crayfish hepatopancreas than in Artemia. Although these results can be partially explained by the remarkable role of hepatopancreas in the metal storage and metabolism of decapod crustaceans. The high dissimilarities of Cd/MT ratio in both systems deserve further investigation.

After 48 h of exposure, MT content in Artemia increased in a dose responsive manner in relation to the amount of cadmium present in the water (Fig. 4A). At 20 mg Cd l^{-1}, the MT contents in the two systems studied are very similar, despite the higher MT content in Artemia at the beginning of cadmium exposure. A linear relationship (r^2= 0.95) between MT levels and cadmium concentration in water was found, but this relationship was different for Artemia and crayfish hepatopancreas.

The effectiveness of cadmium in MT induction, as defined by Scheuhammer and Templeton is at least 300 μg MT g^{-1} for Artemia. The high responsiveness that these crustaceans show in the MT induction by cadmium can explain the high resistance that they exhibit to this particular metal. It was found that MT protects from Cd-induced toxicity by reducing the amount of Cd that was bound to ligands other than MT. A comparison of effectiveness of cadmium on MT induction among crustaceans cannot be made until similar studies are carried out in different species. It will allow correlation of effectiveness and potency in MT induction with the heavy metal resistance that these species exhibit.

The silver saturation method is a rapid quantitative assay for MT quantification that may be used for crustacean tissues when polarography is not available. This taken together with the clear relationship that we have found between cadmium concentration in water and MT levels in tissues support the idea that MT levels in crustaceans can be a good biomarker for cadmium exposure. Nevertheless, we are aware that field studies are needed before we can apply our findings to pollutant monitoring.

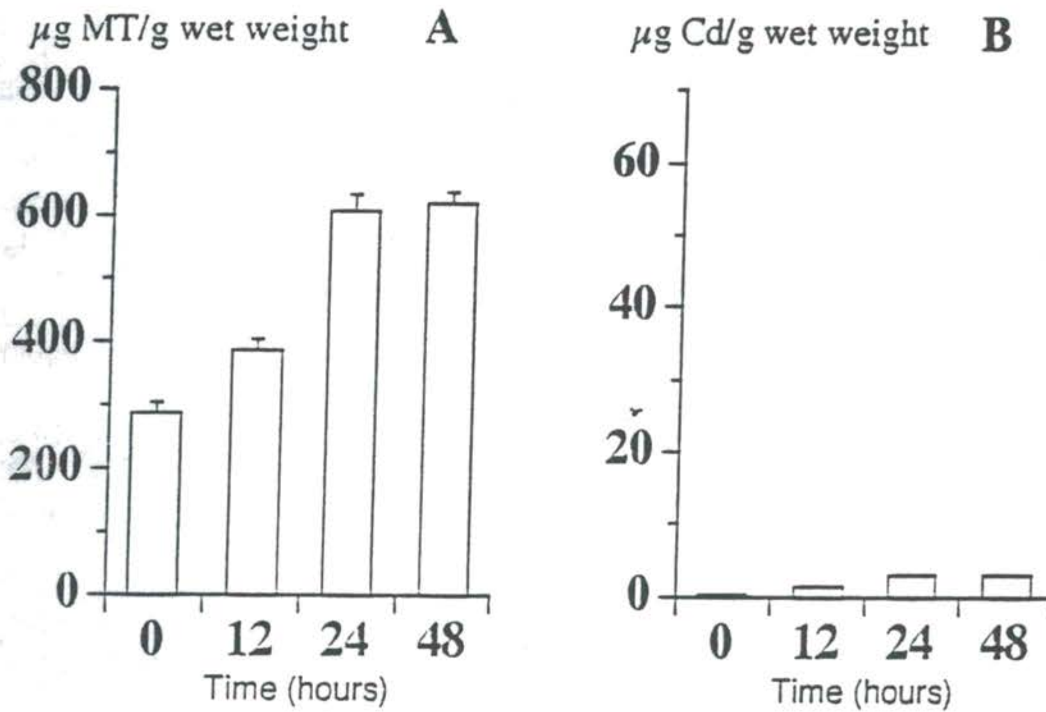

Fig. 3 MT concentrations (A) and cadmium content (B) in Artemia after 0, 12, 24, and 48 hours of exposure to 10 mg Cd l^{-1}

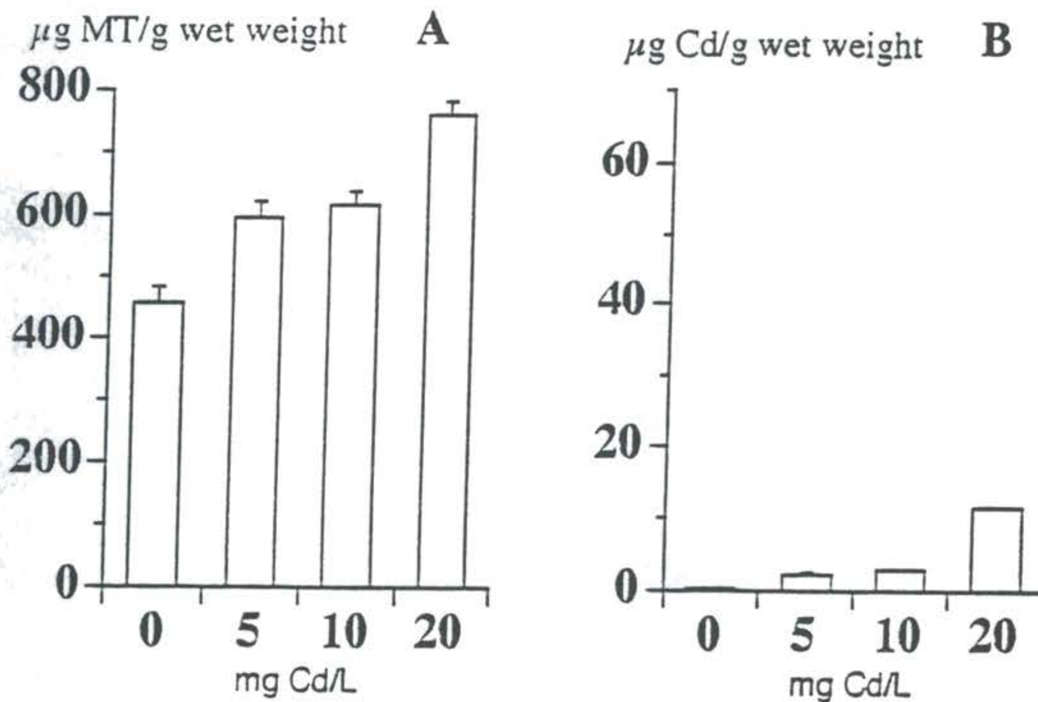

Fig. 4 MT concentrations (A) and cadmium content (B) in Artemia after 48 hours of exposure to 0, 5, 10, and 20 mg Cd l^{-1}

5. CADMIUM EFFECT ON THE ACCUMULATION OF ZINC. INVOLVEMENT OF METALLOTHIONEIN

Cadmium is among the most toxic metals in the aquatic environment, has no known biological role and exhibits high toxicity if allowed to accumulate at metabolically-active sites. On the contrary, zinc is one of the most important essential heavy metals because it is an integral part of a number of metalloenzymes and a cofactor for regulating the activity of specific zinc-dependent enzymes, including carbonic anhydrase involved in ecdysis. Zinc and Cd have been reported as metabolic antagonists, so that high zinc intakes in animals afford some protection against the potentially toxic effects of cadmium exposure. In the same way, Cd has been shown to interfere with Zn transport in vitro (Torreblanca et al., 1992). Induction of metallothionein (MT) synthesis occurs in aquatic invertebrates following the exposure to metals such as cadmium (Del Ramo et al., 1995) and zinc (Roesijadi, 1992). We considered it interesting to study the mechanisms of interaction between essential and non-essential heavy metals.

The objective of the present study was to investigate the effects of a short-term exposure to Cd on Zn stored in the brine shrimp Artemia and the possible involvement of MT in this process.

5.1 Materials and methods

Dried Artemia cysts from "La Mata" strain (parthenogenetic diploid, 1988) were hatched at 27°C under conditions of continuous illumination and aeration. Nauplii were transferred to clean sea water and stock cultures were maintained in aerated sea water under continuous fluorescent illumination at 22°C. Artificial sea water (Instant Ocean) of 36‰ was used in all experiments. Nauplii and adults were fed on cultured Tetraselmis.

Adult animals were pre-exposed to cadmium (as cadmium chloride) and exposed to zinc (as zinc sulphate) during the periods described in Fig. 5. They were divided into four groups (A, B, C, and D) and were kept in 25 l experimental aquaria containing sea water. Groups A and B were pre-exposed to 10 mg Cd l^{-1} for 24 hr. After the pre-exposure period, animals of group A were exposed to 5 mg Zn l^{-1}, and animals of group B were transferred to clean water aquaria. Animals of group C were maintained in clean water during 24 hr and then exposed to 5 mg Zn l^{-1}. Animals of group D were maintained in clean water during all the experiment (control). During the experiment, animals were fed only 1 hour after the Zn exposure.

During the test, the mean water temperature was 20°C (±0.2), pH= 7.9 (±0.2), hardness 250 (±30) mg l^{-1} as $CaCO_3$.

Samples from each experimental group were obtained after 0, 24, 48 and 72 hr from the start of the experiment to determine the metal accumulation (three replicates of 25 brine shrimps) and metallothionein concentration (three replicates of 50 brine shrimps).

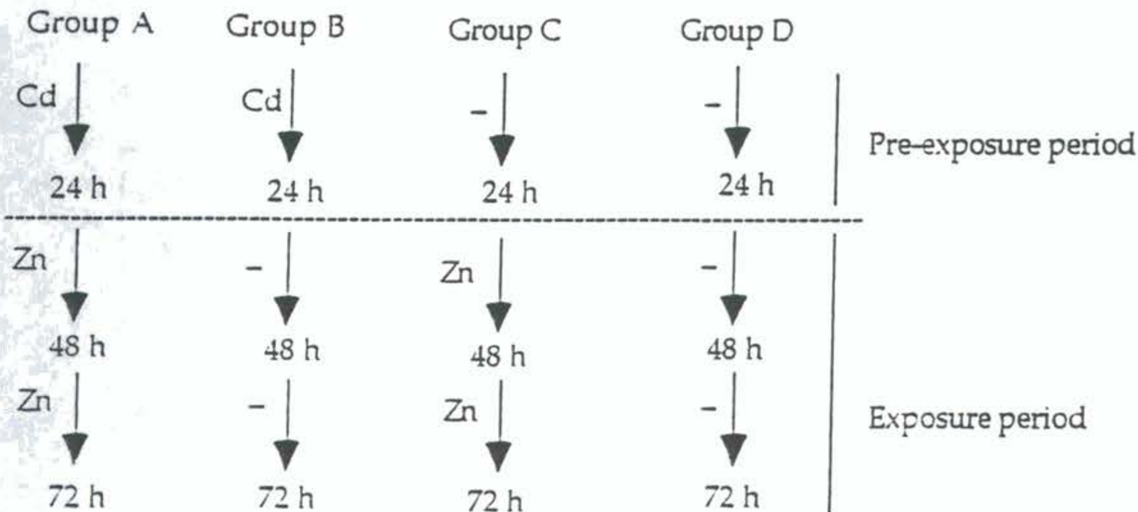

Fig. 5 Treatment regimen for Artemia used in the study. Animals were exposed to Cd (10 mg l^{-1}) and to Zn (5 mg l^{-1}) at the indicated times

Sublethal concentration of cadmium and zinc was used (Trieff, 1980). No mortality was observed during the experiment.

Concentrations of Cd and Zn were estimated by atomic absorption spectrophotometry using an air-acetylene flame. Previously, samples ranging in weight from 0.07-0.15 g were digested in 1 ml of concentrated nitric acid (Baker's 65%) at room temperature for 48 h. Solutions were diluted with ultrapure water until 10 ml. In all experiments several blanks were processed to ensure that contamination was not occurring. Concentrations of MT in tissues were estimated by the silver saturation method (Scheuhammer and Cherian, 1986) using non-radioactive Ag (Martínez et al., 1993). Silver was measured by air-acetylene flame atomic absorption spectrophotometry using an Ag cathode lamp.

Two-way analysis of variance was used to determine treatment and time effects on the various parameters studied. Mean separation was accomplished with Fisher PLSD test. P values of $\leq$ 0.05 were considered to indicate statistically significant differences.

5.2 Results and discussion

Cadmium content in brine shrimp is shown in Fig. 6. Groups A and B exhibit similar cadmium concentrations throughout the experiment. During the pre-exposure period they accumulated cadmium until about 35 μg Cd g^{-1}. From 24 to 72

h after the start of the experiment (cadmium elimination period) there was a decrease in cadmium content. As was expected, no cadmium was detected in samples corresponding to animals from groups C and D for any of the exposure times considered.

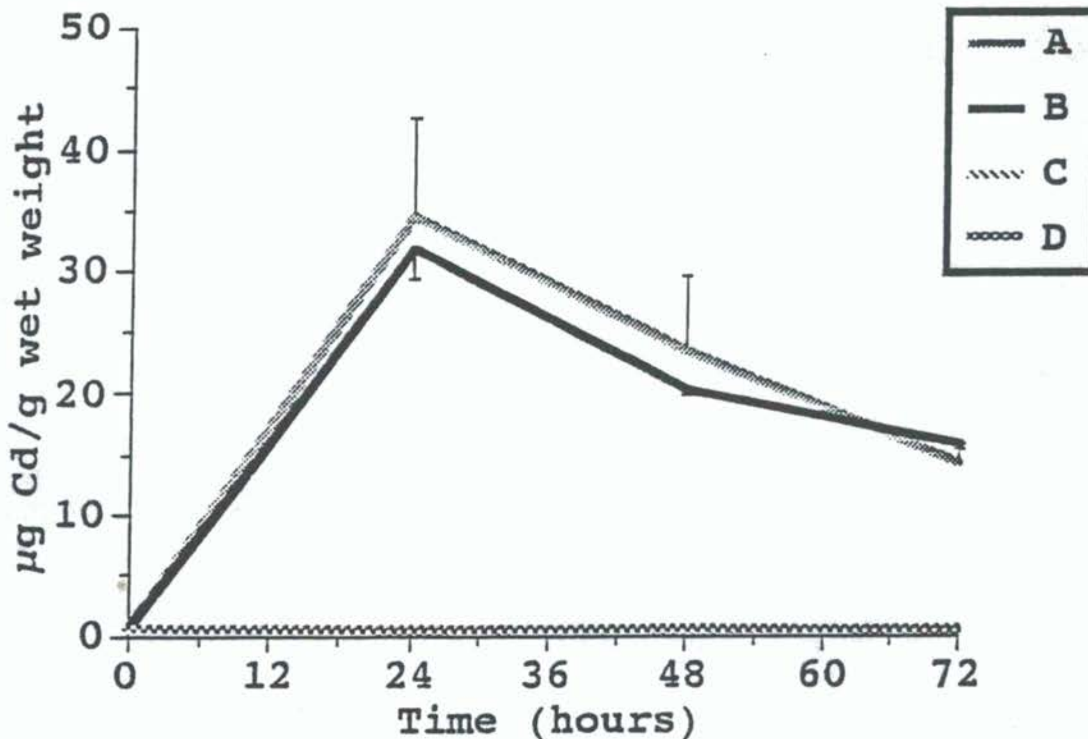

Fig. 6 Accumulation of Cd at different times from the start of the experiment. Values represent the means ± S.D. (n= 3). Error bars indicate S.D.

Zinc concentration in Artemia is shown in Fig. 7. Zinc levels were similar for all the experimental groups after the first 24 h. Therefore, the natural zinc content of shrimps is not altered by exposure to 10 mg Cd l^{-1}; and this is true as long as the animals are kept in clean seawater (groups B and D). However, when extra zinc (5 mg Zn l^{-1}) is added to the seawater after the cadmium exposure time (groups A and C) significant differences due to cadmium exposure show up. Zinc accumulation was lower in cadmium pre-exposed shrimps (A) as compared with their respective control (C).

Cadmium-exposed groups (A and B) showed higher content of MT than groups C and D for all the times studied (Fig. 8). The difference occurred as a consequence of Cd exposure. No statistical differences were found between the two groups exposed to Cd (A and B) or the groups non Cd exposed (C and D) due to the exposure to Zn.

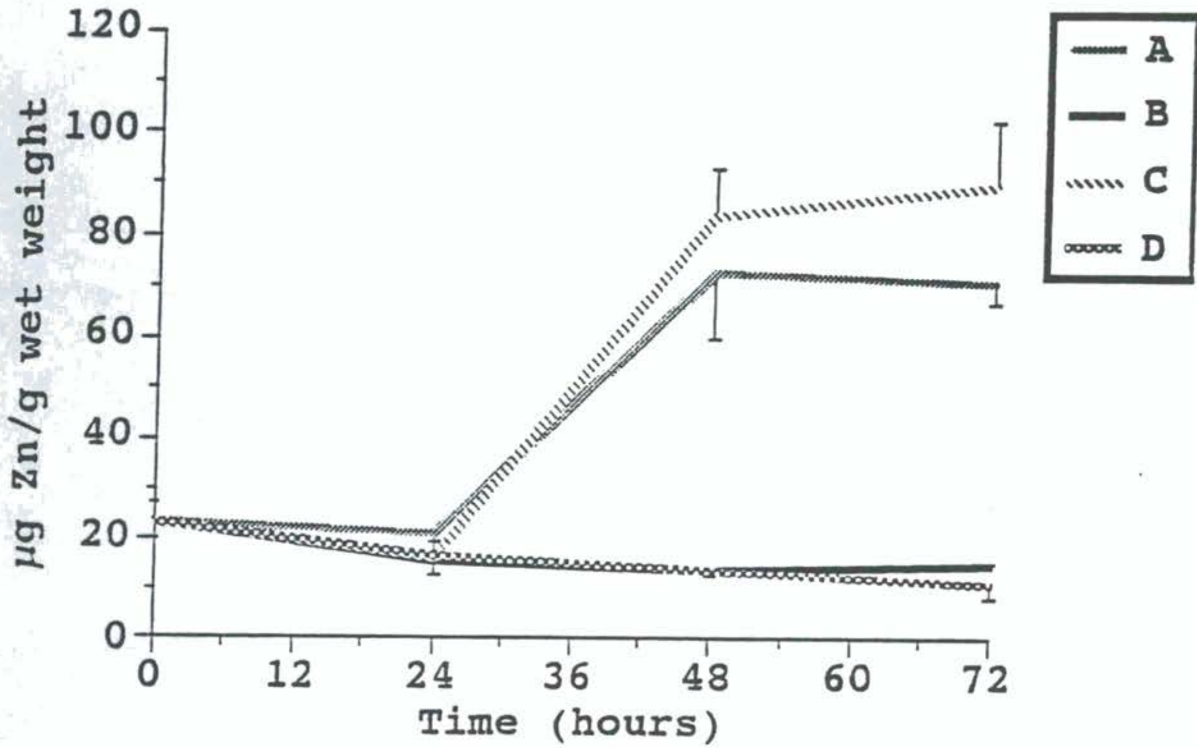

Fig. 7 Accumulation of Zn at different times from the start of the experiment. Values represent the means ± S.D. (n= 3). Error bars indicate S.D.

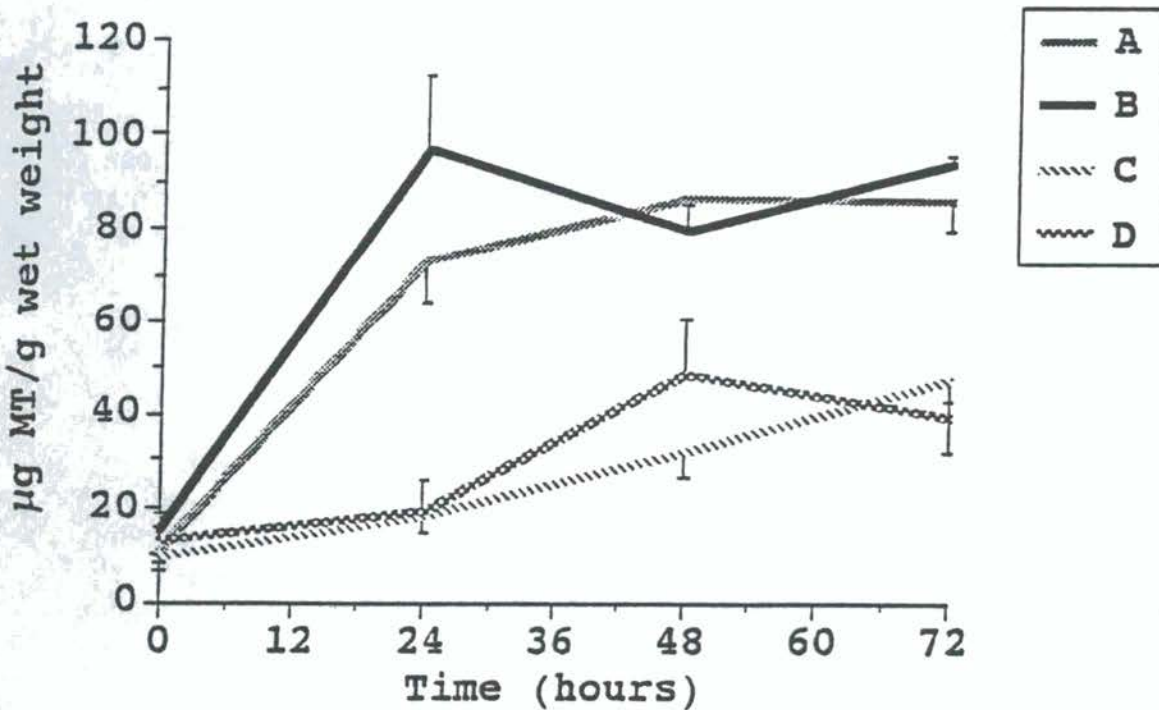

Fig. 8 MT concentrations at different times from the start of the experiment. Values represent the means ± S.D. (n= 3). Error bars indicate S.D.

As can be seen in Fig. 6, Zn exposure did not affect Cd elimination since no differences between groups A and B arose. On the contrary, some interaction between Cd and Zn can be seen from our results. The Cd pre-exposed group (A) accumulated less Zn from the water than non pre-exposed (C) (Fig. 7). However, it seems that this interaction is not very important in natural conditions since we did not find differences between groups B and D. Therefore, the physiological significance of the interaction would become important only when shrimps are accidentally exposed to high concentrations of Zn. Our results support the idea that Zn is highly regulated in crustaceans as has been reported by other authors (Engel and Brouwer, 1987; Depledge and Rainbow, 1990).

As in other systems studied (Hamer, 1986; Suzuki et al., 1990), Cd is a more potent inducer of MT synthesis than Zn. After the increase of MT levels by Cd exposure (groups A and B) no further increase was produced by the following exposure to Zn (Fig. 8) since MT content remains the same for both groups.

The site for interaction between Cd and Zn in biological systems has not yet been precisely identified. Whereas some works demonstrated the involvement of MT (Torreblanca et al., 1992), other authors support that high molecular weight cytoplasmatic proteins other than MTs are the interaction sites.

6. MERCURY ACCUMULATION AND METALLOTHIONEIN SYNTHESIS IN Artemia

Marine aquatic organisms at all levels accumulate mercury into tissues. This mercury is retained for long periods if it is in an organic form.

Factors which affect the toxicity of mercury to aquatic invertebrates include the concentration and species of mercury, the developmental stage of the organisms, and the temperature, salinity, water hardness, and flow rate. Methylmercury is more toxic than aryl and inorganic mercury. The larval stage is apparently the most sensitive of the organism's life cycle. Mercury toxicity increases with temperature and decreases with water hardness.

The objective of the present study was to investigate the effects of a short-term exposure to Hg on metallothionein synthesis.

6.2 Materials and Methods

Dried Artemia cysts from "La Mata" strain (parthenogenetic diploid, 1988) were hatched at 27°C under conditions of continuous illumination and aeration. Nauplii were transferred to clean sea water and stock cultures were maintained in aerated sea water under continuous fluorescent illumination at 22°C. Artificial sea water (Instant Ocean) of 36‰ was used in all experiments. Nauplii and adults were fed on cultured Tetraselmis.

Adult animals were divided into three groups of 600 animals each. These were kept in 4 litre experimental aquaria containing sea water. Aliquots of mercury (as $HgCl_2$) stock were added to each test aquaria to bring the mercury concentrations to the desired levels of 5, 10, and 20 μg Hg l^{-1}. All aquaria were kept at a constant temperature (22°C) and on a 12 h light-dark photoperiod for the 3-day duration of the experiment. The water was changed every day to reduce the build up of metabolic wastes and to keep the concentration of mercury near the nominal level. A fourth group served as a control and was kept in 4 litre of clean water.

Samples (ranging in weight from 0.074 to 0.43 g) from each experimental group were obtained after 0, 24, 48 and 72 hours from the start of the experiment to determine metallothionein concentration (three replicates of 50 brine shrimps).

Concentrations of MT in tissues were estimated by the silver saturation method (Scheuhammer and Cherian, 1986) using non-radioactive Ag (Martínez et al., 1993). Silver was measured by air-acetylene flame atomic absorption spectrophotometry using an Ag cathode lamp.

Mercury in supernatant fractions was determined using cold vapour flameless atomic absorption spectroscopy after addition of 2 ml H_2SO_4 and 3.25 ml of potassium permanganate and heating for 2 h at 70°C. Determination of mercury was carried out at 253.7 nm with $NaBH_4$ as reductor agent and argon as purging gas, with 0.5 ml of sample. The detection limit was 12 ng for mercury. Several blanks were processed to ensure that contamination was not occurring.

Two-way analysis of variance was used to determine treatment and time effects on the various parameters studied. Mean separation was accomplished by Fisher PLSD test. P values of ≤ 0.05 were considered to indicate statistically significant differences. Sublethal concentration of mercury was used (Trieff, 1980).

6.2 Results and discussion

The MT content corresponding to each time and treatment is shown in Fig. 9. MT content was slightly increased after 48 h of exposure to 20 μg Hg l^{-1}, but there is no increment in MT content until 72 h in the groups treated with lower concentrations. A longer period of exposition to mercury might be necessary to detect higher levels of induction. The induced MT/metal accumulated ratio is higher for cadmium than for mercury (Del Ramo et al., 1995).

As can be seen in Fig. 10, the concentrations of mercury in the control group did not change as a function of time during the 72-h exposure. In treated groups, mercury content in supernatant increased with exposure time. We observed a similar behaviour over time in animals exposed to 5 and 10 μg Hg l^{-1}, although mercury content was higher after 48 hr of exposure in the second group. On the other hand, in the group exposed to 20 μg Hg l^{-1}, there was a constant increase in mercury content throughout the whole experiment. So, mercury content in supernatant at 48

hr was double that at 24 h. We did not observe any plateau of saturation during the 72 h period. Therefore, a longer period would be necessary to achieve mercury content saturation. The difference in accumulation rate between the highest concentration (20 μg Hg l^{-1}) and the low concentrations (5 and 10 μg Hg l^{-1}) can be explained by the overloading of the detoxification systems when the animals are exposed to concentrations higher than 10 μg Hg l^{-1}.

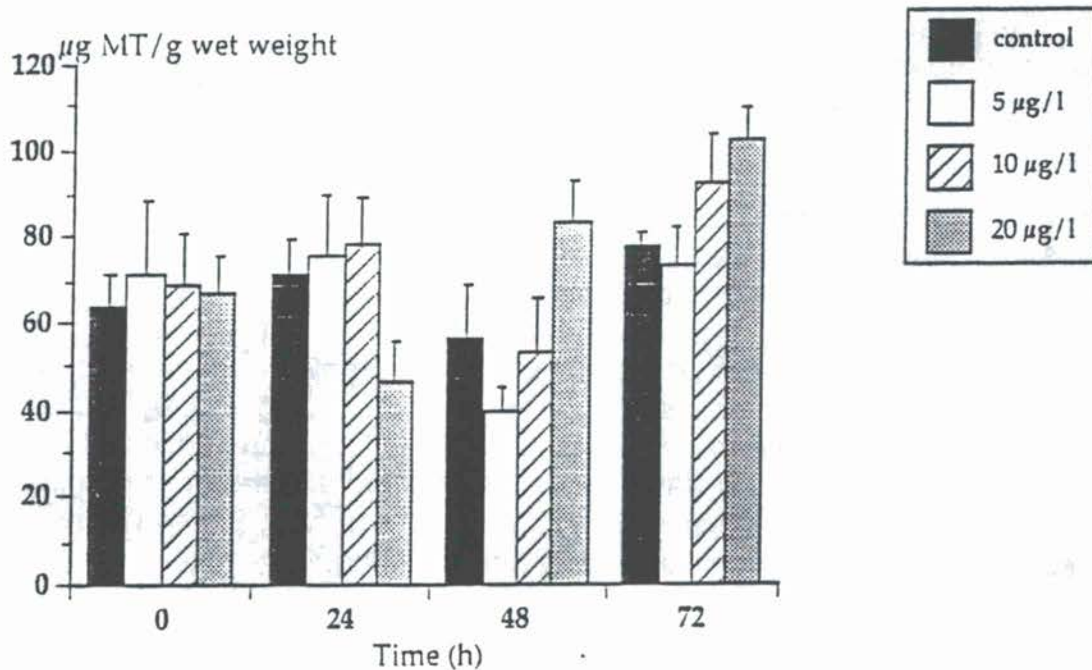

Fig. 9 MT concentration after 0, 24, 48 and 72 hours of exposure to 5, 10 and 20 μg Hg l^{-1}. Values represent the means ± S.D. (n= 3). Error bars indicate S.D.

7. ACKNOWLEDGEMENTS

The project was executed in the framework of the MED POL programme and an MTF contribution was received through FAO.

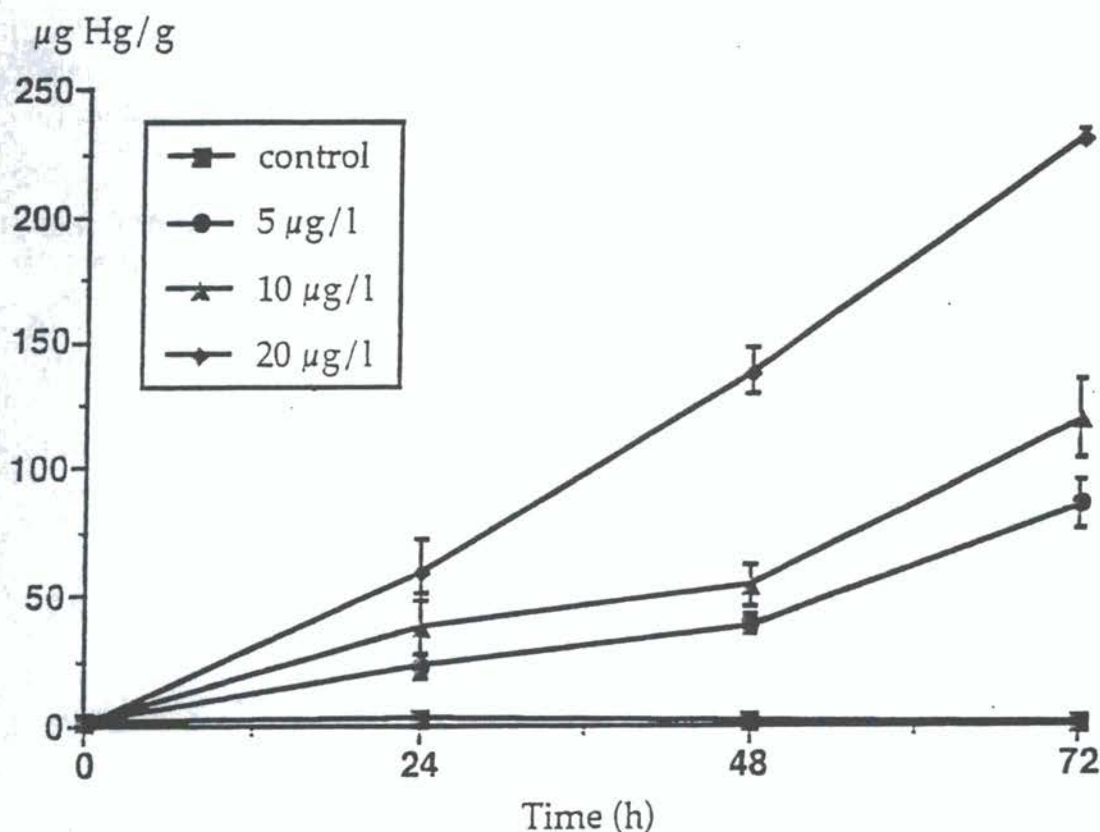

Fig. 10 Hg concentration after 0, 24, 48 and 72 hours of exposure to 5, 10 and 20 μg Hgl $^{-1}$. Values represent the means ± S.D. (n= 3). Error bars indicate S.D.

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LES HYDROCARBURES AROMATIQUES POLYCYCLIQUES DANS L'ENVIRONNEMENT MARIN MEDITERRANEEN: SEDIMENTS ET MOULES

par

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RESUME

Les hydrocarbures aromatiques polycycliques prioritaires définies par l'USEPA ont été recherchées dans des échantillons de sédiments et de moules de la façade méditerranéenne française.

Un gradient de pollution est très nettement observé. Ces données ont été corrélées aux activités enzymatiques déterminées dans les moules. L'origine des composés aromatiques (pyrolytique, pétrogénique) est aussi discutée.

1. INTRODUCTION

Les résultats présentés dans cette étude ont trait à la détection des composés aromatiques polycycliques dans les sédiments et les moules de plusieurs sites de Méditerranée occidentale. Il s'agit de la mission océanographique GICBEM IX qui s'est déroulée en juillet 1991.

Les composés aromatiques polycycliques (CAP) représentent une famille de composés cancérigènes ou mutagènes dans l'environnement. L'agence américaine de protection de l'environnement (US EPA) a ainsi définie 16 composés polluants prioritaires à rechercher en prioritaire dans les matrices environnementales (fig. 1).

Les origines des CAP sont classées en deux grandes familles (Soclo, 1986; Sicre, 1987):

- les CAP d'origine pyrolytique formés par la combustion incomplète à haute température de la matière organique récente et fossile (fumées industrielles, gaz d'échappement, etc: origine pyrolytique),
- Les CAP d'origine pétrogénique et provenant du pétrole, qui est formé lors de l'enfouissement de la matière organique dans les bassins sédimentaires.

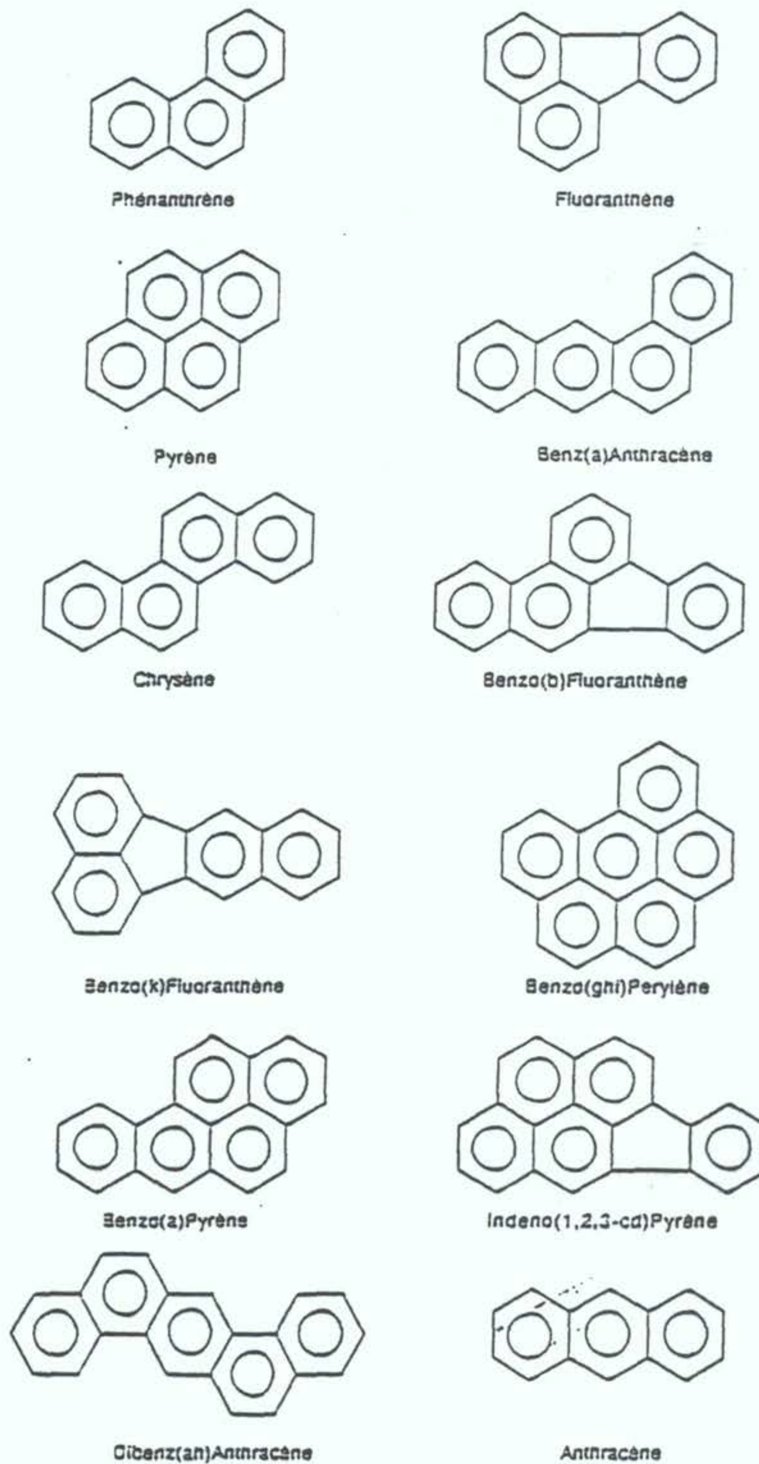


Fig. 1 Les composés aromatiques polluants prioritaires recherchés dans cette étude

Parallèlement à l'origine des CAP, il existe d'autres critères de différenciation basés sur les modes de transfert à l'environnement marin, qui sont principalement les voies éoliennes ou aqueuses, reflétant ou non une activité humaine.

2. CHOIX DES ECHANTILLONS REPRESENTATIFS

Nous avons choisi les sédiments et les moules comme échantillons représentatifs de la contamination d'un endroit donné dans le milieu marin.

Les composés aromatiques subissent dans la matrice sédimentaire des processus de dégradation quantitativement peu importants. Ce sont aussi des composés très stables qui sont présents dans toutes les couches du sédiment, qu'elles soit superficielle (contamination récente) ou plus ancienne (couches plus profondes du sédiment).

Les moules sont souvent utilisées comme organismes bioindicateurs des programmes de surveillance des niveaux de contamination des environnements côtiers (rapport Réseau National d'observation, 1988, programme "Mussel watch" au USA). Ainsi les moules (Mytilus galloprovincialis) possèdent des mécanismes de filtration et d'aspiration capables de concentrer les contaminants organiques et plus particulièrement les hydrocarbures. Ceux-ci peuvent alors être soit stockés dans les lipides, soit métabolisés par des systèmes enzymatiques liés au cytochrome P-450 (Ribera, 1990).

3. LOCALISATION GEOGRAPHIQUE DES SITES DE PRELEVEMENT

Les sites de prélèvement se répartissent essentiellement sur le littoral méditerranéen français (fig. 2): plusieurs échantillons de sédiments ont été prélevés dans la baie du Lazaret (près de Toulon), avec comme objectifs d'étudier la représentativité de la collecte des sédiments. Des échantillons de moules ont aussi été collectés sur des tables à moules. Des échantillons de moules et de sédiments ont été prélevés à La Fourmigue (Phare en face de Cannes) à la fois sur une balise (noté B) et sur les rochers (noté rock), au Cap Martin (frontière entre l'Italie et la France), à Scandola (dans la réserve marine corse), à Spotorno (en Italie) non loin du lieu où avait eu lieu le naufrage du pétrolier "Le Haven" en avril 1991.

4. PROTOCOLE EXPERIMENTAL D'ANALYSE

Le protocole expérimental d'analyse comprend la collecte des échantillons, la préparation des échantillons (tamisage, lyophilisation), l'étape d'extraction et l'étape d'analyse.

Les échantillons de sédiment ont été collectés en scaphandre autonome dans des eaux de 2 à 25 m de profondeur (tableau 1). Les sédiments sont stockés à bord et congelés à -18°C, jusqu'à traitement au laboratoire. Les échantillons de moules

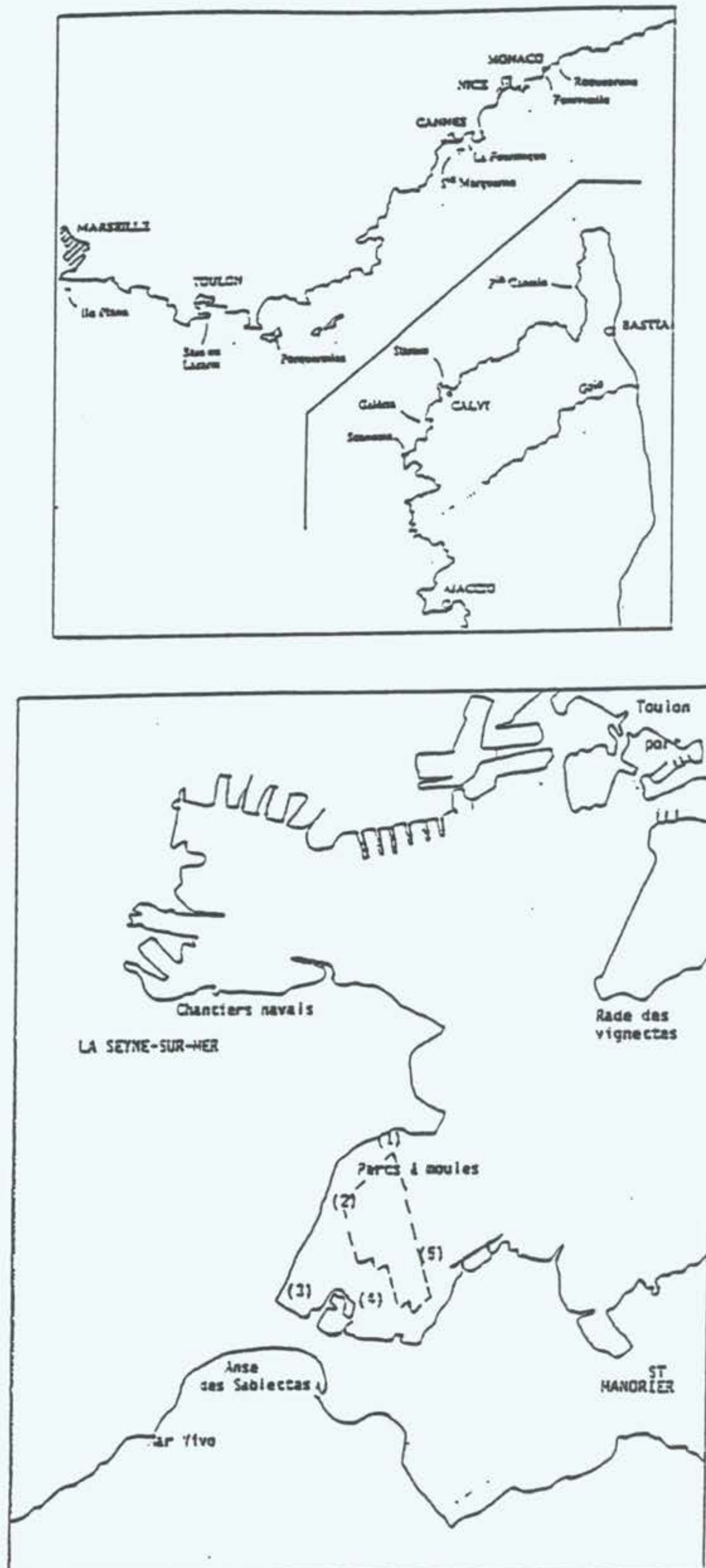


Fig. 2 Sites de prélèvement des échantillons de moules et de sédiments. Les échantillons notés Lazaret 1, 2, 3, 4 et 5 correspondent à différentes localisations dans la baie du Lazaret. L'échantillon Lazaret 1 correspond à l'échantillon Lazaret M dans le texte

sont prélevés par plongeurs en apnée. La chair est extraite de la coquille et stockée à -18°C pour les analyses chimiques et à la température de l'azote liquide (-190°C) pour les analyses biochimiques.

Les échantillons de sédiments et de moules sont lyophilisés, broyés, puis tamisés à 2mm pour éliminer cailloux, coquilles et autres fragments végétaux grossiers qui ne sont pas réellement constitutifs de la matrice analysée.

Tableau 1

Caractéristiques (aspect, profondeur) des sédiments

Stations de prélèvement	Profondeur (m)	Faciès sédimentaires
Lazaret.M	2,5	Vase avec débris végétaux
Lazaret.3	1,5	Sable fin
Lazaret.2	1,7	Vase avec débris végétaux
Lazaret.4	2,5	Vase avec débris végétaux
Lazaret.5	1,5	Vase avec débris coquillers
Fourmigue	15	Sables grossier avec débris coquillers
Cap Martin	12	Sable fin
Spotorno	9	Sable fin
Scandoia	15	Sable grossier avec débris coquillers

L'extraction est ensuite réalisée à l'aide d'extracteur Soxhlet en utilisant le dichlorométhane comme solvant d'extraction. La durée de l'extraction est comprise entre 24h et 72h suivant le degré de contamination de l'échantillon.

L'extrait organique obtenu est reconcentré à l'aide d'un évaporateur rotatif, jusqu'à un volume de 2 mL. L'évaporation est ensuite poursuivie sous jet d'azote jusqu'à mise à sec de l'extrait qui est repris rapidement par du pentane pour être ensuite purifié sur une microcolonne de florisil. L'extrait est déposé en tête de la microcolonne, nettoyée par 40 mL de méthanol, puis éluée avec 40 mL de pentane.

L'analyse est ensuite effectuée par chromatographie en phase liquide avec détection spectrofluromértrique (colonne VYDAC 201 TP, L:25cm, 1:4,6mm). La fluorescence est observée pour différents couples de longueurs d'onde d'excitation et d'émission, choisis en fonction des composés étudiés, présents successivement dans la cellule de mesure du spectrofluorimètre en fonction de l'ordre d'élution (tableau 2); la sélectivité de la fluorescence permet de détecter préférentiellement un composé qui n'a pas été séparé d'un autre par l'étape chromatographique. L'élution est réalisée en mode gradient en utilisant l'eau et l'acétonitrile comme éluants. L'analyse quantitative est réalisée en utilisant des étalons internes deutérés (pyrène deutéré) qui ne sont pas présents dans les échantillons naturels (fig. 3).

Tableau 2

Conditions d'analyse (longueur d'onde d'émission et d'excitation) des extraits de sédiments et de moules

PAH	EX(nm)	EM(nm)
Phe	252	378
Ant	252	378
Flt	288	462
Pyr-d10	317	406
Pyr	317	406
BaA	254	409
Chry	266	363
BdNT	273	351
BeP	287	411
BbF	287	411
Per	405	440
BKF	281	429
BaP	281	429
dBahA	298	419
BghiP	298	419
InPyr	300	502

5. RESULTATS ET DISCUSSIONS

5.1 Distribution des CAP dans les sédiments

Les teneurs en CAP dans les échantillons de sédiment prélevés lors de la mission GICBEM IX sont présentés dans le tableau 3 et sont exprimées en ng g^{-1} de sédiment sec. Rappellons qu'il n'existe pas de normes internationales concernant des niveaux de contamination à ne pas dépasser.

Les sédiments les plus contaminés en CAP totaux proviennent des zones de la Baie du Lazaret (rade de Toulon) et surtout du sédiment sous les tables à moules. Le sédiment noté Lazaret 4 se situe à l'entrée du port (fig. 2). Sa forte contamination peut s'expliquer par l'importance des gaz d'échappement des moteurs de bateaux.

La source pyrolytique est la source essentielle de contamination des sédiments (fig. 4). En effet la distribution des CAP est dominée par à la fois les composés de faible poids moléculaire (inférieur au chrysène, 4 cycles aromatiques et moins) et des composés de haut poids moléculaire (supérieur au chrysène, 4 à 6 cycles aromatiques). Les CAP sont essentiellement introduits dans le milieu marin associés aux aérosols atmosphériques et aux particules.

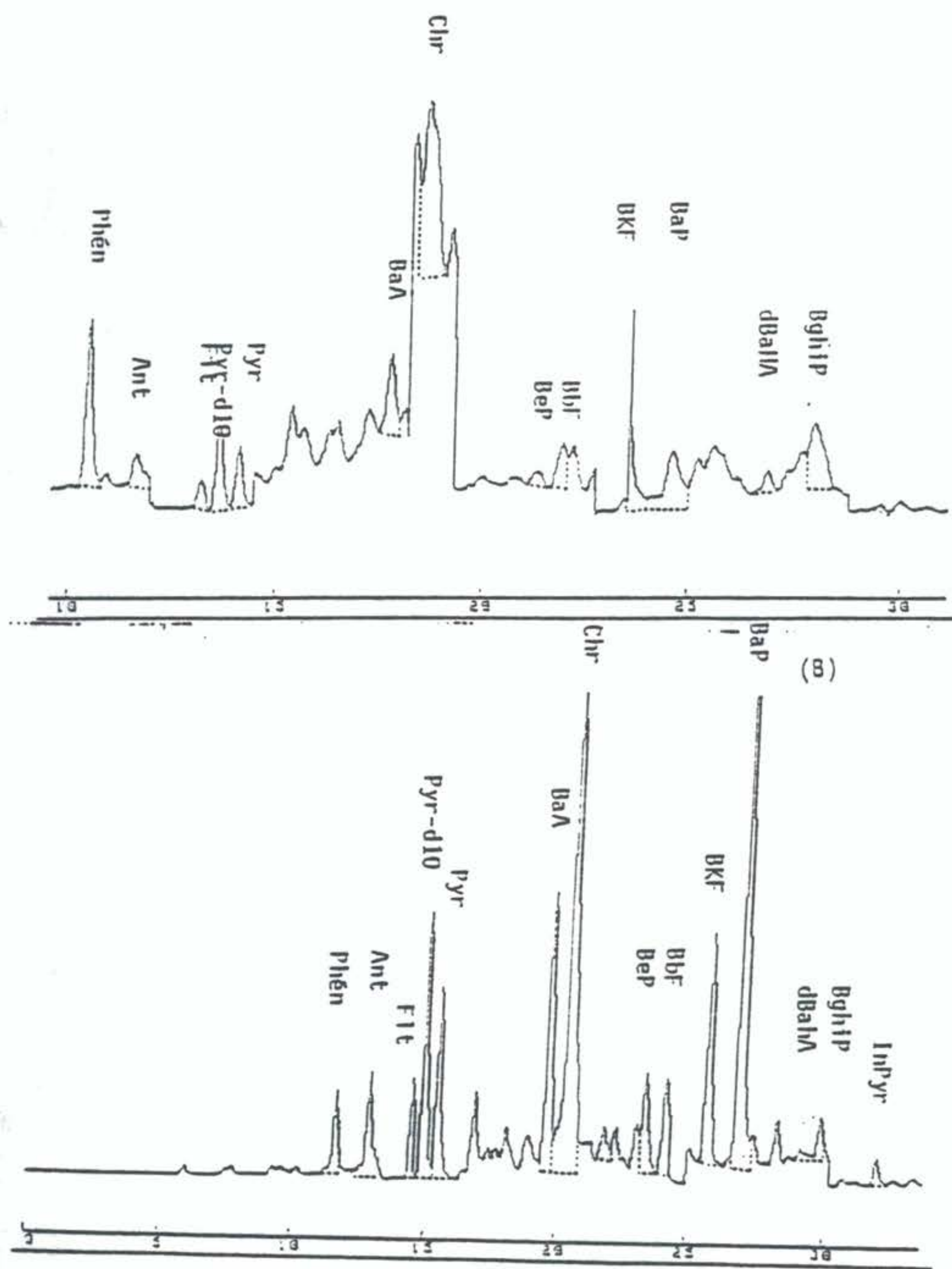


Fig. 3 Chromatogramme d'un échantillon de moule (en haut) et d'un échantillon de sédiment (en bas)

Tableau 3

Concentrations (ng g^{-1} de poids sec) des composés aromatiques dans les sédiments (en bas) et les tissus de moules (en haut)

Stations de prélèvement	Phe	Ant	Flt	Pyr	BaA	Chry	BeP	BbF	Per	BKF	BaP	dBah	BghiP	InPyr	Total[a]
Lazaret 7	7	1	0,5	4	7	1	10	90	Nd	6	4	1	6	6	143,5
Fourmigue.B	5	2	6	5	7	4	4	12	Nd	8	4	4	3	6	70
Fourmigue rock	5	0,5	4	1	3	1	3	4	Nd	4	1	4	6	Nd	36,5
Cap Martin	30	1	30	20	30	20	20	40	Nd	40	10	6	30	20	297
Spotorno	10	1	10	1	10	1	10	20	Nd	10	2	Nd	Nd	Nd	75

a: ng g^{-1} moule entier (poids sec)
Nd: non détécté

Stations de prélèvement	Phe	Ant	Flt	Pyr	BaA	Chry	BeP	BbF	Per	BKF	BaP	dBah	BghiP	InPyr	Total[b]
Lazaret 2	70	10	230	180	210	10	50	170	Nd	120	190	60	180	150	1630
Lazaret 3	90	14	280	190	210	30	300	260	40	100	140	40	250	220	2164
Lazaret 4	1110	690	6850	4060	5250	1960	4690	3780	Nd	2400	3690	830	3380	1290	39980
Lazaret 5	50	10	130	90	100	90	180	160	Nd	60	120	70	180	130	1440
Fourmigue	3190	1230	8230	2790	1990	4670	5590	4690	2230	2830	3900	720	3240	2790	48090
Cap Martin	4	0,5	1	10	12	10	13	12	Nd	6	8	1	3	6	86,5
Spotorno	40	10	170	140	200	110	260	190	Nd	100	180	12	30	150	1592
Scandola	90	20	350	190	210	110	180	120	Nd	75	130	30	120	95	1720
	8	5	13	8	12	8	20	12	Nd	6	10	1	3	13	119

b: ng g^{-1} sédiment lyophilisé
Nd: non détécté

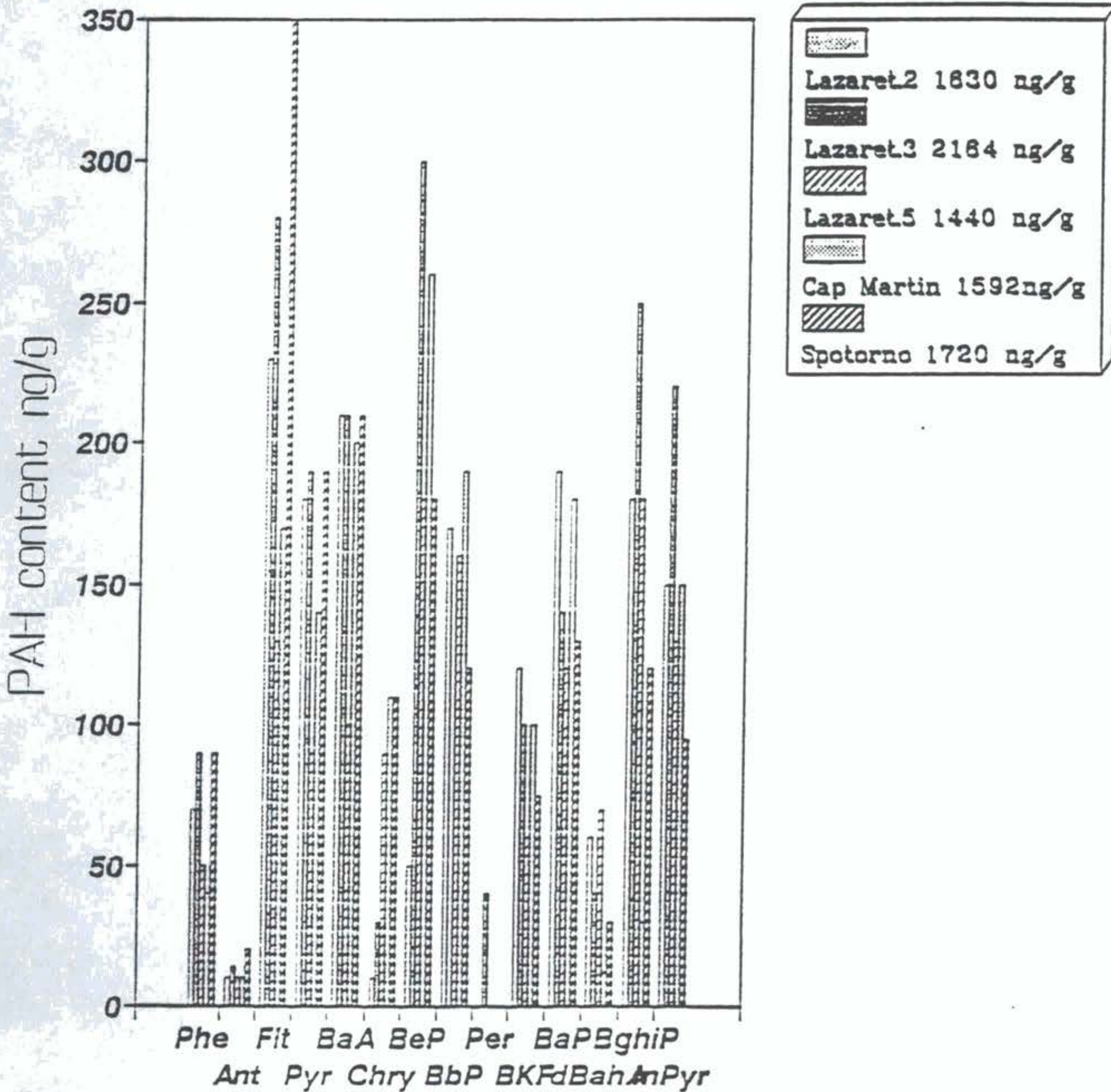


Fig. 4 Distribution des composés aromatiques dans les sédiments

Des outils ont été développés par Garrigues et al. (1991), basés sur les rapports des concentrations de composés aromatiques isomères comme le phénanthrène et l'anthracène, le pyrène et le fluoranthène (fig. 5). Ainsi des rapports supérieurs à 10 pour Phe/An indique une contamination pétrolière des sédiments, tandis qu'un rapport de concentration nettement inférieur à 10 indique une contamination pyrolytique. De même des valeurs inférieures à 1 pour le rapport Pyr/Fluo est classiquement attribué à une origine pyrolytique des CAP.

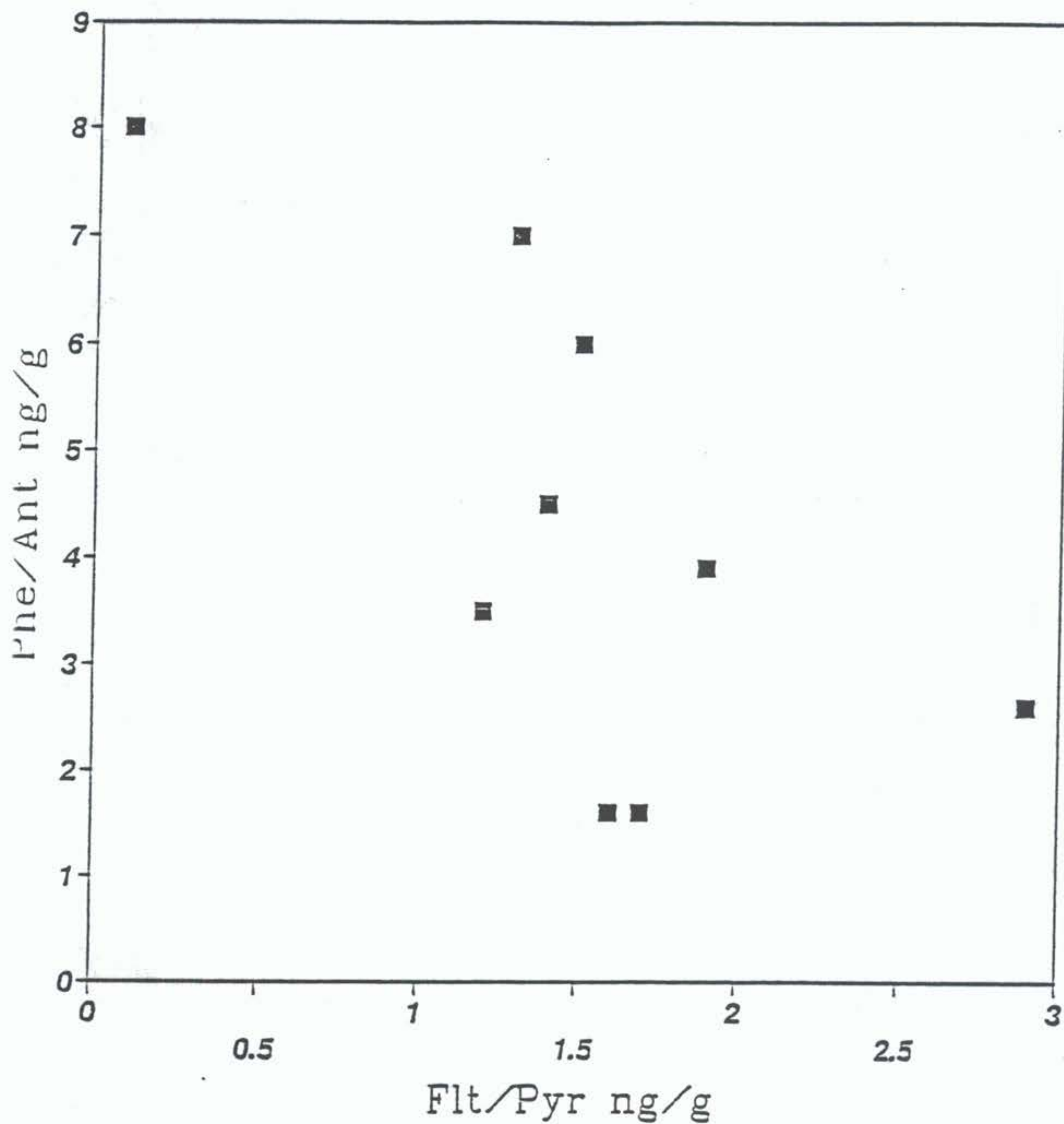


Fig. 5 Répartition des points de prélèvement en fonction des rapports de concentration Phénanthrène/Anthracène et Fluoranthène/Pyrène

Ainsi pour l'échantillon de sédiment noté Lazaret 4, les valeurs de ces deux rapports (respectivement Phe/An = 1,61 et Pyr/Fluo = 1,69) confirment l'origine pyrolytique de la contamination. La forte teneur des CAP dans l'échantillon Lazaret M (prélevé sous les tables à moules), peut s'expliquer par la capacité des moules à concentrer les CAP particulaires présents dans l'eau. Aux CAP particulaires incorporés dans les sédiments par sédimentation directe se rajoutent le cas des CAP particulaires incorporés aux biodépôts organiques (Prahl et al., 1979; Burns et al., 1983; Readman et al., 1987; Raoux, 1991). Par ailleurs les végétaux jouent le rôle de pièges à CAP (Raoux et Garrigues, 1993).

Les sédiments prélevés à Scandola (Corse) et à La Fourmigue (Cannes) présentent des contenus totaux en CAP très faibles (de l'ordre de 100 ng g^{-1}). Ceci peut s'expliquer d'une part par la qualité du sédiment qui est grossier et riche en débris coquilliers et d'autre part par la profondeur de la collecte qui est supérieure à celle des autres échantillons.

Le rapport Phe/Ant dans ces sédiments est très élevé (8) par rapport à celui des autres échantillons, alors que le rapport Fluo/Pyr est nettement supérieur à 1. On peut donc attribuer à ces échantillons une faible contamination d'origine pétrogénique. Les CAP d'origine pétrogénique sont communément considérés comme plus biodisponibles que ceux d'origine pyrolytique. Les CAP pétrogéniques sont introduits directement sous forme dissoute, colloïdale ou associés à des particules en suspension dans l'eau, alors que les composés pyrolytiques sont probablement plus fortement associés aux particules émises par les sources pyrolytiques.

Quant aux autres sites de prélèvement, le contenu total en CAP indique une contamination moyenne des sédiments (fig. 6). Il est compris aux environs de 2000 ng g^{-1} de sédiment. Cette contamination peut être soit d'origine pyrolytique atmosphérique (Phe/An > 10 et Fluo/Pyr > 1), soit d'origine anthropique (rejets des eaux usées urbaines et eaux de ruissellement provenant de lessivage du sol) au vu de la localisation des sites de prélèvement (Lazaret près de Toulon, Cap Martin près de Menton et Monaco).

5.2 Distribution des CAP dans les moules

Dans le cas de l'analyse des tissus de moules, on observe de nombreux pics chromatographiques qui créent des interférences (fig. 3).

Les moules du Cap Martin sont celles qui présentent une forte contamination (figs. 7 et 8). On n'observe pas de corrélation entre la concentration des CAP dans le sédiment et le niveau de contamination des sites de prélèvement. En fait ces teneurs calculées par rapport aux masses correspondantes de chair lyophilisée ne tiennent pas compte de la taille des moules qui peut varier d'un facteur 10 entre les moules cultivées de la Baie du Lazaret et les moules sauvages qui croissent dans des infractuosités des rochers battus par les vagues.

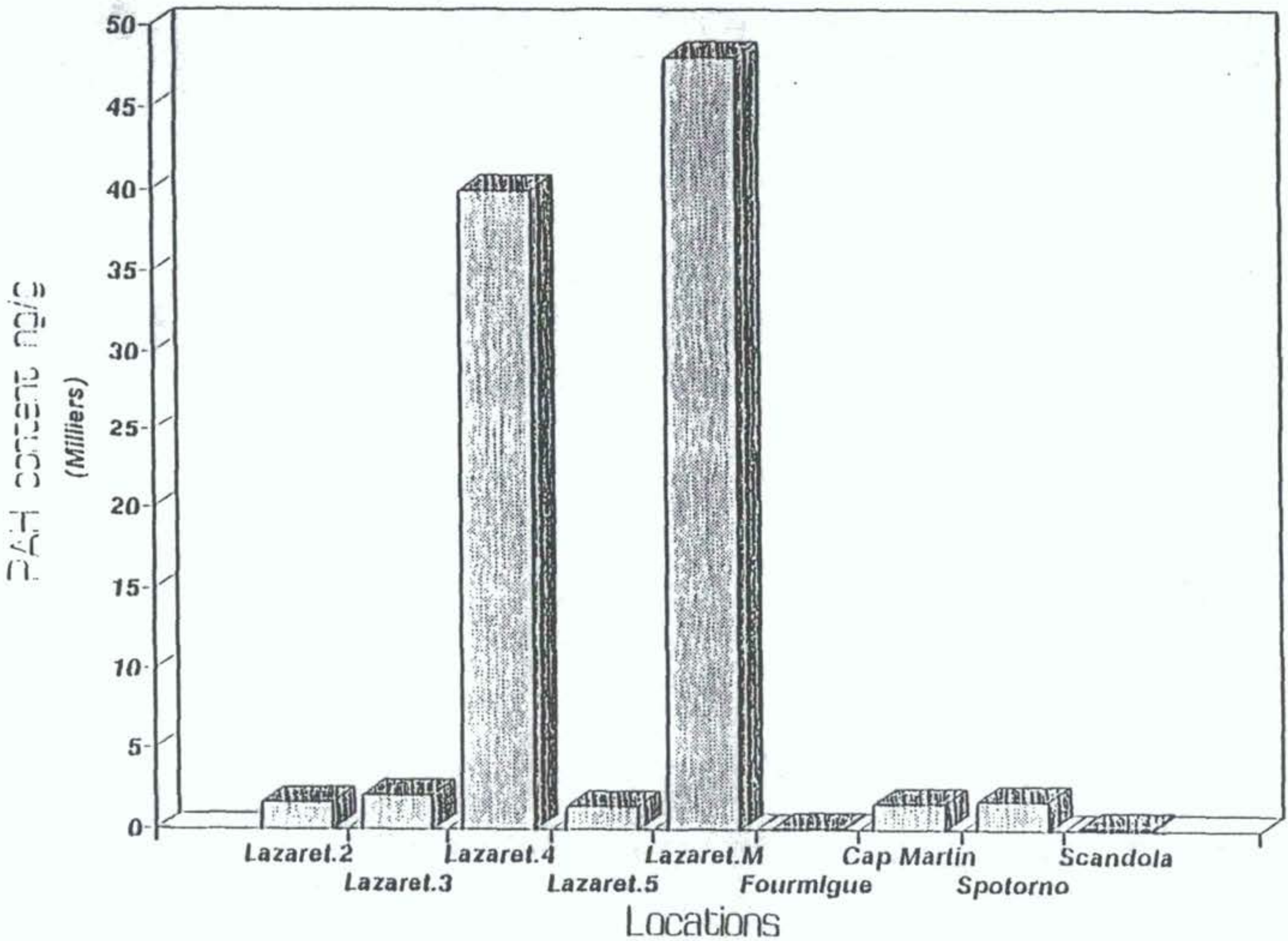


Fig. 6 Concentration totale des CAP dans les sédiments

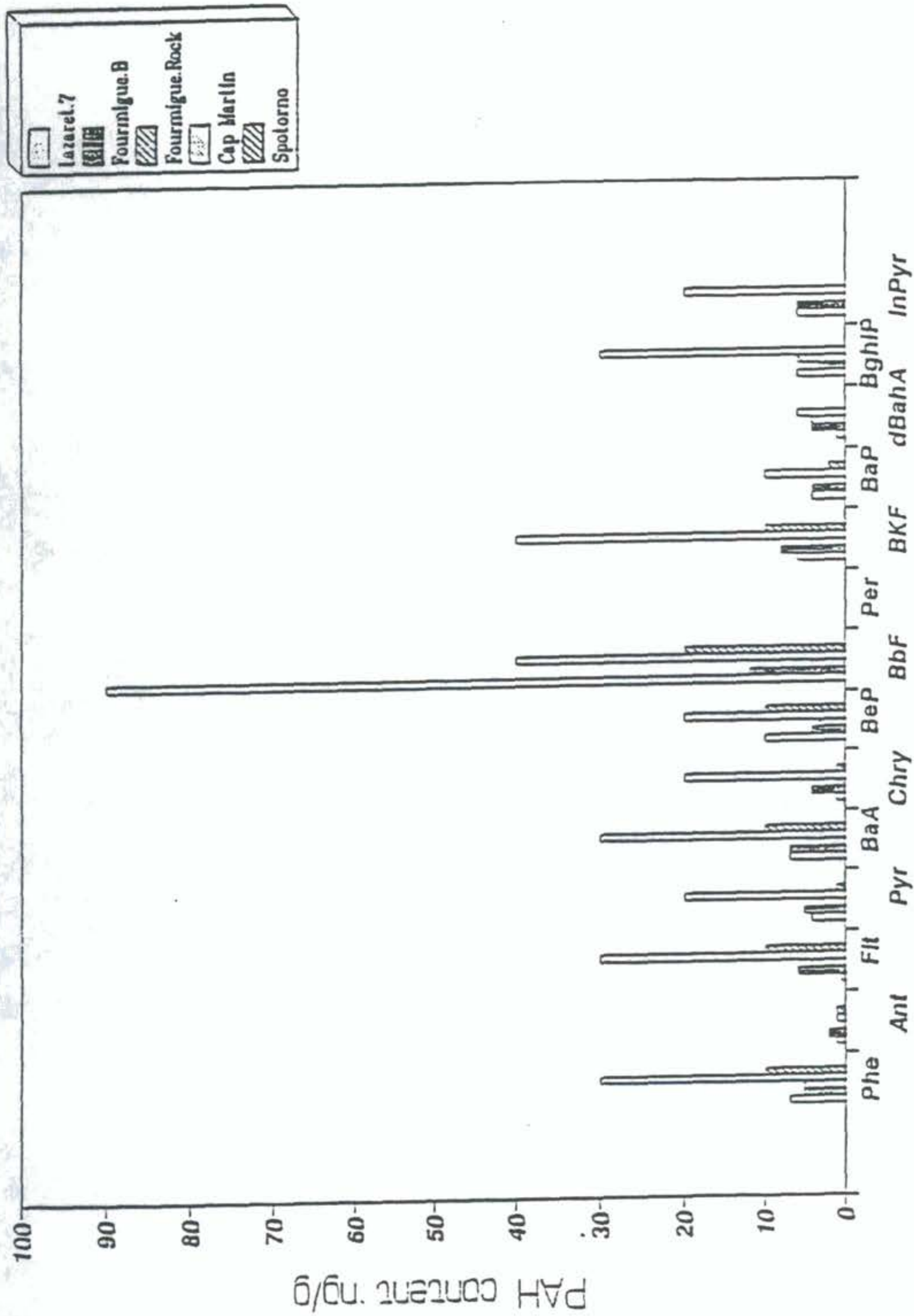


Fig. 7 Distribution des composés aromatiques dans les tissus de moules

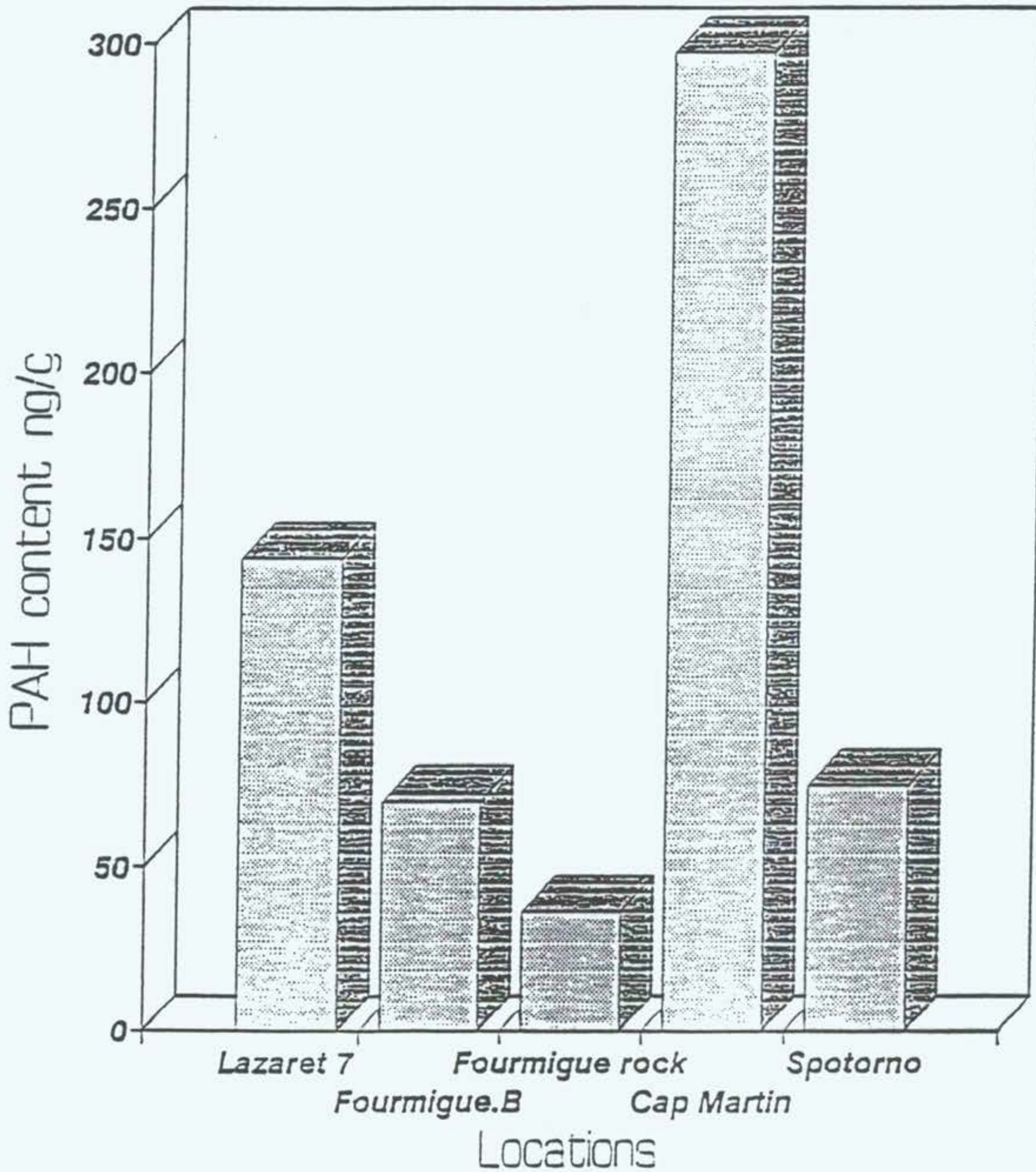


Fig. 8 Concentration totale des CAP dans les moules

L'hétérogénéité des prélèvements (moules sauvages ou d'élevage, tailles et ages différents), ne permet pas de considérer les moules prélevées comme possédant la même capacité intrinsèque de bioaccumulation des CAP. La mode de contamination de ces organismes marins semble être liée à leur capacité de filtration. Ainsi une contamination par voie indirecte (alimentation) devient prépondérante lorsque le milieu est riche en polluants sous forme particulaire (Raoux, 1991).

Par ailleurs, dans le cas d'une pollution pétrogénique, la contamination des organismes marins par les voies dissoutes des xénobiotiques (voie directe) semble être le mode principal de contamination (Farrington et al., 1983).

Le contenu des moules en CAP n'a donc qu'une signification biologique limitée: il ne permet pas d'apprécier l'ampleur des perturbations d'ordre biologiques induites par la présence des contaminants dans les organismes (induction des systèmes enzymatiques, atteintes physiologiques). Le stress subi par les organismes peut cependant être évalué grâce à l'étude des systèmes de biotransformation des xénobiotiques.

5.3 Corrélation de la contamination par les CAP avec les activités enzymatiques

L'activité de la benzo(a)Pyrène hydroxylase a été mesurée dans les moules aux différents points de prélèvement. Cette activité est mesurée par une méthode radiométrique en déterminant la fluorescence par mg de protéine ou par g de tissu (fig. 9).

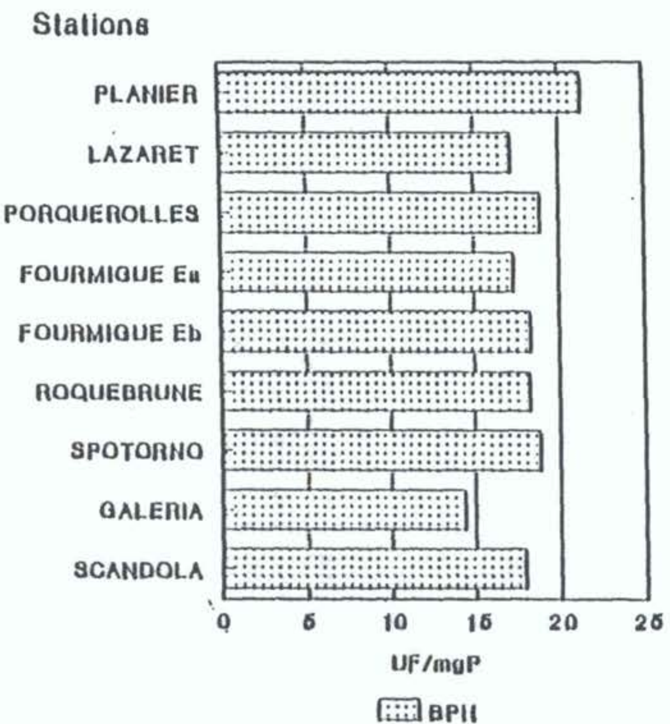
On observe une forte réponse à Spotorno (19 UF mg^{-1}; (CAP) = 1700 ng g^{-1}), à Scandola (18 UF mg^{-1}; (CAP) = 115 ng g^{-1}) et à La Fourmigue (18 UF mg^{-1}; (CAP) = 83 ng g^{-1}).

Le site de la Baie du Lazaret, qui est le plus contaminé au point de vue sédiment, présente une activité enzymatique plus faible que les autres sites moyennement ou faiblement contaminés. Certains auteurs ont avancé une inhibition possible des activités enzymatiques par des métaux présents en grande concentration. Cela pourrait être le cas de ce site, situé près du port de Toulon qui abrite la Marine Française, très consommatrice de peintures antisalissures à base de métaux lourds.

Si l'on exclue le site de la Baie du Lazaret, on observe une corrélation évidente entre les activités enzymatiques et teneurs en CAP des sédiments des stations correspondantes. Ces résultats confirment une nouvelle fois que l'activité Benzo(a)pyrène hydroxylase chez la moule constitue un excellent indicateur spécifique de la contamination des moules par les CAP (Narbonne et al., 1991).

6. REMERCIEMENTS

Le projet a été exécuté dans le cadre du programme MED POL et grâce à un concours financier du Fonds d'affectation spéciale pour la Méditerranée octroyé par l'entremise du Bureau FAO du Plan d'Action pour la Méditerranée.



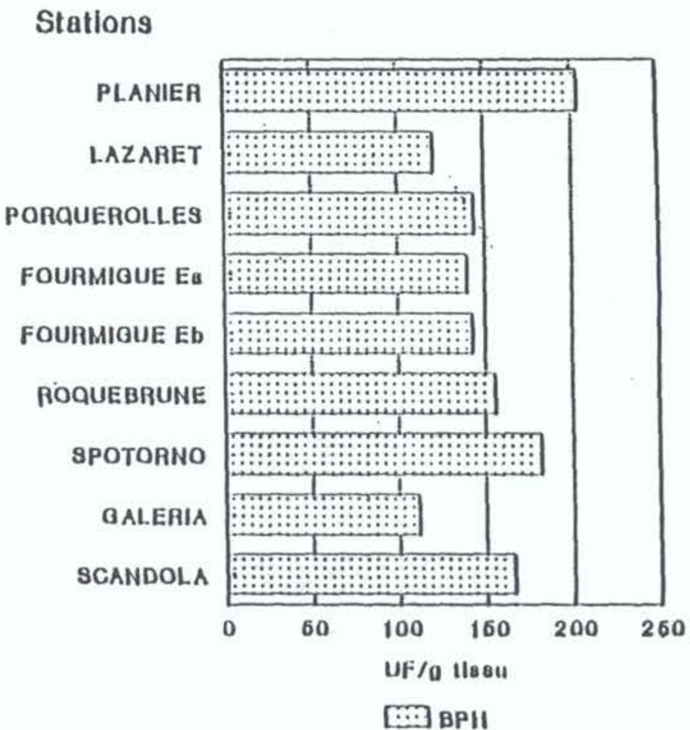


Fig. 9 Activités enzymatiques (benzo(a)Pyrène hydroxylase) au niveau des microsomes hépatopancréatiques des moules dans les différentes stations

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