MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

FINAL REPORTS ON RESEARCH PROJECTS DEALING WITH MERCURY, TOXICITY AND ANALYTICAL TECHNIQUES

RAPPORTS FINAUX SUR LES PROJETS DE RECHERCHE TRAITANT DU MERCURE, DE LA TOXICITE ET DES TECHNIQUES ANALYTIQUES

MAP Technical Reports Series No. 51

UNEP
Athens, 1991

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For bibliographic purposes this volume may be cited as:

UNEP/FAO: Final Reports on research projects dealing with mercury, toxicity and analytical techniques. MAP Technical Reports Series No. 51. UNEP, Athens, 1991.

Pour des fins bibliographiques, citer le présent volume comme suit:

PNUE/FAO: Rapports finaux sur les projets de recherche traitant du mercure, de la toxicité et des techniques analytiques. MAP Technical Reports Series No. 51. UNEP, Athens, 1991.

This volume is the fifty-first 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 cinquante-et-unième numéro de la série des Rapports techniques du Plan d'action pour la Méditerranée.

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

INTRODUCTION

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

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

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

MED POL - Phase I (1976-1980)

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

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

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

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

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

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

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

MED POL - Phase II (1981-1990)

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

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

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

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

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

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

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

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

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

For this purpose, monitoring was organized on several levels:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Research area I - Characterization and measurement

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

Research area II - Transport and dispersion

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

Research area III - Effects

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

Research area IV - Fates/Environmental transformation

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

Research area V - Prevention and control

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

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

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

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

The present volume includes final reports on research projects implemented under Research Activities A, D, G and K and deals with some aspects of the biogeochemical cycle of mercury, the analytical methodology for arsenic and organophosphorus pesticides as well as the toxicity of some heavy metals. Final editing and compilation of this volume was done by Mr. G.P. Gabrielides, FAO Senior Fishery Officer (Marine Pollution) while Ms V. Papapanagiotou, FAO Secretary, was responsible for the typing.

INTRODUCTION

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

- Planification intégrée du 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.

MED POL - Phase I (1976 - 1980)

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

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

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

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

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

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

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

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

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

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

MED POL - Phase II (1981 - 1990)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Les Parties contractantes au cours de leur sixième réunion ordinaire (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 exécutés au titre des activités A, D, G et K et traitant des divers aspects du cycle biogeochimique du mercure, des méthodologies analytiques pour l'arsenic et les pesticides organophosphorés ainsi que de la toxicité de certains métaux lourds. 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, Sécretaire FAO était chargée de la dactylographie.

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EVALUATION DES TENEURS EN MERCURE, METHYLMERCURE ET SELENIUM DANS LES POISSONS ET COQUILLAGES DES COTES FRANCAISES DE LA MEDITERRANEE

par

Y. THIBAUD et J. NOEL

IFREMER, Centre de Nantes
France

RESUME

Une interprétation originale des teneurs en mercure et en méthylmercure rencontrées dans les coquillages et les poissons de la Méditerranée, est présentée. Les relations entre les niveaux de présence dans les organismes et dans l'eau de mer, sont expliquées à l'aide d'une modélisation, aux conditions d'équilibre, du cheminement du mercure en milieu marin.

1. INTRODUCTION

L'objectif de l'étude était initialement orienté vers la connaissance des niveaux de présence en mercure et en méthylmercure dans les espèces de poissons et de coquillages couramment consommés afin d'évaluer les risques encourus par les populations qui s'en nourrissent. Mais depuis lors, le problème de la pollution par le mercure en Méditerranée a été abordé globalement en introduisant la notion de cycle biogéochimique; il a été particulièrement bien posé au meeting FAO/UNEP/WHO/IOC/IAEA de Sienne par Aston et Fowler (1985).

Nous avons préparé en décembre 1985 un rapport et 3 annexes qui regroupaient les données brutes obtenues (IFREMER, 1985). Nous présentons maintenant le rapport définitif du projet de recherche référencé FRA/9-D.

L'interprétation originale de nos résultats donnée ici, fait apparaître des éléments de réponse aux questions posées concernant la pollution mercurielle dans la région méditerranéenne.

2. MATERIELS ET METHODS

2.1 Collecte des échantillons, préparation et prétraitement

Le Tableau I regroupe un ensemble d'informations déjà présentées dans un autre rapport (IFREMER, 1985) et qui concernent le mode d'échantillonnage, les espèces prélevées, les lieux des prélèvements et les caractéristiques des échantillons. La figure 1 indique de façon approximative les zones de pêche concernées.

Après leur collecte, les prélèvements de coquillages et de poissons ont été congelés. Ils ont été ensuite acheminés jusqu'au laboratoire (Centre de Nantes) par transport rapide en utilisant des caisses isothermes réfrigérées.

Tableau I

Organismes marins collectés en Méditerranée
et leurs caractéristiques.

Espèce (°)	Date de pêche	Région de pêche (*)	Nombre échantillons	Poids moyen des individus en g
Moule (23) *Mytilus galloprovincialis*	4 à 11/83 9 à 11/84 1 á 7/83 11/84 4 á 12/84 10/84	 Leuca Ia Etang de Thau IIa Golfe de Fos IIa Toulon IIIa	 5 9 11 1 26	1,6
Sardine (17) *Sardina pilchardus*	6/83	Sète II	3	38
Anchois (06) *Engraulis encrasicholus*	11/84	Port Vendre I	1	22
Rouget barbet (16) *Mullus barbatus*	11/84	Sète II	3	71
Sole (18) *Solea solea*	11/83 11/84	Sète II	5	277
Maquereau (12) *Scomber scombrus*	6/83 11/84	Sète II	7	152
Daurade royale (10) *Chrysophrys aurata*	11/84	Port Vendres Port la Nouvelle I	3	301
Bar (07) *Morone labrax*	11/83 11/84	Sète II	5	411
Lingue (14) *Molva molva*	4/83	Marseille II	1	76
Rascasse (15) *Scorpena scorfa*	4/83 11/84	Marseille Sète II	5	51

Tableau I (suite)

Espèce (°)	Date de pêche	Région de pêche (*)	Nombre échantillons	Poids moyen des individus en g
Capelan (08)				
Trisopterus	6/83			
minutus	11/84	Sète II	8	69
Grondin (11 et 19)				
Trigla sp.	4/83	Marseille		
	11/84	Sète II	4	71
Thon rouge (20)				
Thunnus	11, 12/76			
thynnus	10/77	Port Vendres I	14	
1) moins de 35 kg	7 à 12/76			
	8 à 12/77	Sète et Marseille II	38	
	6 à 9/76			
	6 à 9/77	Nice III	16	
	6/76			
	8/77	Golfe de Gênes IV	9 77	20600
2) plus de 35 kg	10/77	Port Vendres I	1	
	7 et 8/76			
	10/77	Sète et Marseille II	13	
	6 à 9/76			
	à 9/77	Nice III	9	
	6/76	Golfe de Gênes IV	1 24	67560
Merlu (13)				
Merluccius	11/84	Port Vendres I	2	
merluccius		Marseille II	3 5	648
Raie bouclée (21)				
Raja	5 et 7/76	Sète II	30	731
clavata				
Congre (09)				
Conger	4/83			
conger	11/84	Sète et Marseille II	10	411
Roussette (22)				
Scyliorhinus	12/76	Port Vendres I	4	
canicula	5 à 7/76	Sète II	12 16	904

(°) Le chiffre entre parenthèses est le numéro du "code des espèces" utilisé dans le rapport provisoire (IFREMER, 1985).

(*) Le chiffre romain correspond aux zones qui ont été délimitées sur la carte de la figure 1; l'indice indique que le prélèvement a été effectué sur le littoral.

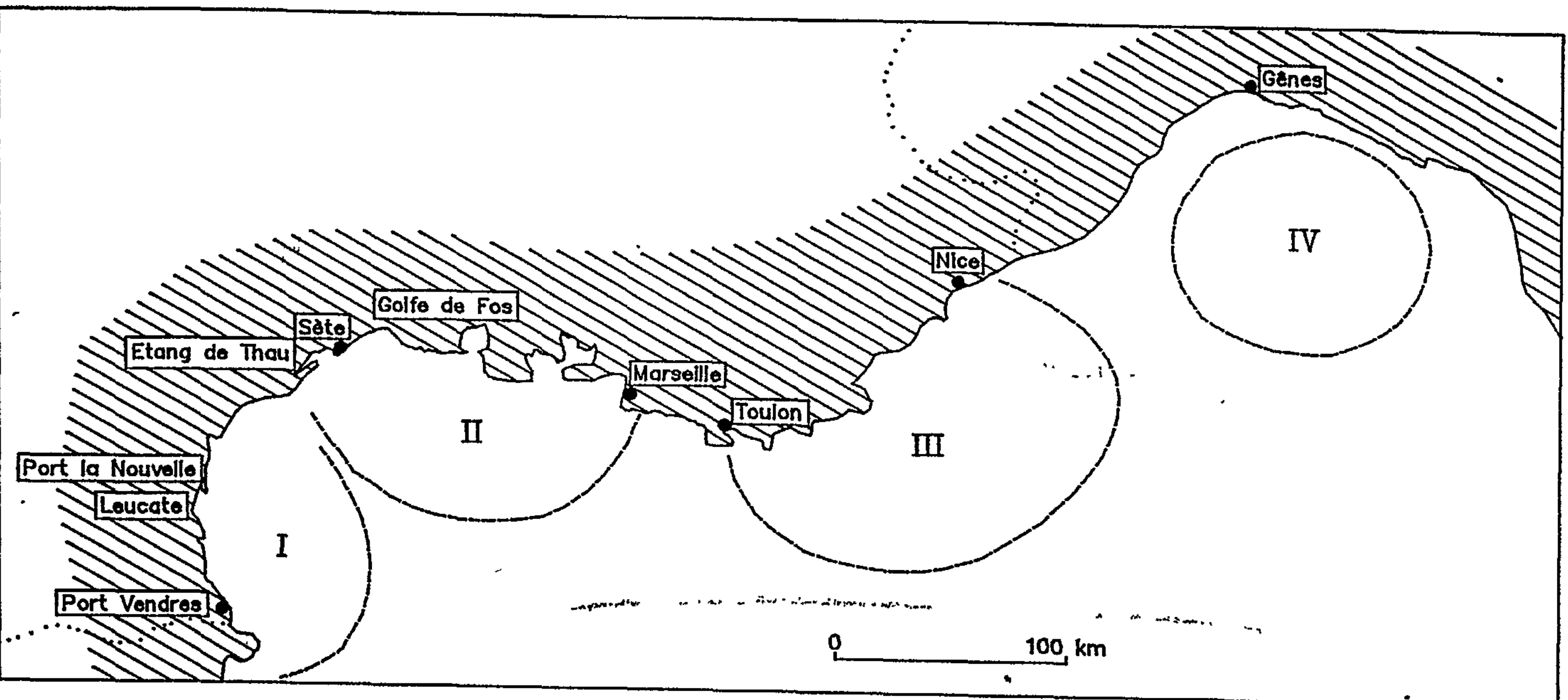

Fig. 1 Lieux de pêche approximatifs des organismes marins collectés en Méditerranée

Pour ce qui est des échantillons de moules, ils ont été décoquillés et leurs parties molles séparées. Les petits poissons, sardine, anchois, ont été étêtés et éviscérés, tandis que les autres poissons ont été découpés et les muscles séparés puis gardés.

Les chairs des différents types de prélèvements ont été ensuite broyées en utilisant un homogénéiseur VIRTIS type 23, puis lyophilisées en utilisant un appareil DURA DRY de FTS équipé d'une pompe à vide 2012 de ALCATEL.

Les lyophilisats correspondants ont été réduits en poudre avec un broyeur à couteau IKA A 10 puis conservés dans des piluliers en verre bouchés par une cape en polyéthylène, en attendant les analyses.

2.2 Techniques analytiques

Sur tous les échantillons lyophilisés, il a été recherché trois types différents de contaminants: le mercure total, le mercure méthylé et le sélénium.

Les déterminations du mercure total ont été réalisées par spectrophotométrie d'absorption atomique sans flamme selon une technique précédemment décrite (Thibaud, 1983). La minéralisation a été effectuée sur 0,2 à 0,5 g de matière sèche en chauffant à 60°C avec un mélange 1/1 d'acide nitrique et d'acide sulfurique puis en ajoutant à froid une solution de permanganate de potassium. Après dilution et filtration, Hg^{2+} a été réduit par du chlorure stanneux à l'état de Hg° et son absorption simultanément mesurée dans l'ultraviolet à 254 nm.

Les déterminations du mercure méthylé ont été effectuées par deux méthodes différentes: la chromatographie en phase gazeuse en utilisant une technique qui a été tout récemment décrite (Averty, 1989) et la spectrophotométrie d'absorption atomique en opérant suivant un procédé antérieurement décrit par Capelli et al. (1979).

Les déterminations du sélénium ont été réalisées par spectrophotométrie d'absorption atomique avec four électrothermique.

Il a été minéralisé 2 à 3 g de chair lyophilisée dans des réacteurs en téflon en utilisant 50 ml d'acide nitrique et 75 mg de pentoxyde de vanadium (V_2O_5) et en chauffant sous pression à une température voisine de 170°C pendant 3 heures. L'acide nitrique a été ensuite évaporé en chauffant lentement, puis le résidu obtenu a été mis en solution en utilisant du chlorure d'ammonium. Du nickel a été finalement ajouté, de manière à obtenir 0,5% de Ni dans les solutions; ceci permet la formation de composés stables entre Ni et Se avant l'atomisation dans le four électrothermique.

Les mesures proprement dites ont été effectuées à 196 nm sur un appareil IL 157 couplé avec un four IL 555, équipé d'une lampe à cathode creuse et de la correction de l'absorption non spécifique au deutérium. Il a été utilisé un tube en graphite non pyrolytique et de l'azote comme gaz de purge; le programme de montée en température du four était le suivant: séchage 100-400°C (70s), calcination 850°C (45s), atomisation 1800°C (1s).

Les techniques analytiques qui ont été employées pour cette étude sont utilisées couramment dans les laboratoires qui effectuent des dosages de contaminants métalliques; elles permettent en particulier d'atteindre des limites de détection et des reproductibilités tout à fait satisfaisantes.

Nous nous assurons par ailleurs de la validité de nos résultats en participant périodiquement à des exercices d'intercalibration des méthodes d'analyse de ces contaminants dans les tissus biologiques. Ainsi, en 1983, dans le cadre du programme MED POL - Phase II, nous avons déterminé le mercure sur des échantillons de chair de moule et de poisson (IAEA, 1984) et tout récemment, sous l'égide du CIEM, nous avons coordonnée un exercice interlaboratoire concernant la détermination du méthylmercure dans les tissus des organismes marins (Thibaud et Cossa, 1989).

3. RESULTATS

L'ensemble des résultats des analyses est regroupé sur le Tableau II. Les teneurs en mercure total, mercure méthylé et sélénium, sont données pour chaque espèce en mg kg^{-1} de poids sec.

La première constatation qui se dégage et qui a été dans le passé souvent soulignée est celle d'une concentration en mercure total énormément plus élevée dans les organismes que dans l'eau de mer elle-même dont la concentration est voisine des 2-5 ng Hg l^{-1} (Copin-Montegut _et al._, 1986); ce qui correspond à un facteur de concentration voisin de 60000 (concentration en mg kg^{-1} de chair humide).

La deuxième constatation est celle de l'augmentation des teneurs en Hg total et en Hg méthylé dans les espèces avec leur niveau trophique et le poids des organismes.

Ces deux observations montrent la forte rétention par la biomasse des formes chimiques du mercure stables en solution: Hg^{2+} et surtout CH_3Hg^+, qui sont ingérées à la fois par l'eau et par la nourriture.

En examinant les valeurs du Tableau II, il est en particulier remarquable que les petits poissons pélagiques, sardine et anchois, dont la nourriture est constituée essentiellement de zooplancton, possèdent des teneurs en mercure plus faibles que le maquereau, un poisson pélagique se nourrissant d'organismes planctoniques et accessoirement de petits poissons. Il est de même remarquable que le marquereau lui-même possède des teneurs en mercure beaucoup plus faibles que la raie et la roussette dont le mode de vie est plus sédentaire, qui sont des carnassiers se nourrissant de petits crustacés, de mollusques et de petits poissons.

Une telle rétention est de toute évidence gouvernée par les interactions du mercure avec les systèmes biologiques. La forte affinité des formes chimiques du mercure les plus stables (Hg^{2+} et CH_3Hg^+) pour le soufre des tissus biologiques, présent dans les enzymes, dans certaines protéines comme la métallothionéine ou dans des composés biochimiques plus simples comme la cystéine et le glutathion, favorise la formation et le transport de bio-complexes dans les fluides et les cellules en permettant la pénétration des membranes biologiques.

Tableau II

Teneurs en mercure total, en mercure méthylé et en sélénium dans les organismes marins en mg kg^{-1} (poids sec).
n = nombre d'échantillons analysés; m = concentration moyenne; sd = déviation standard ou écart-type.

Espèces	Mercure total (HgT)			Mercure méthylé			Sélénium (Se)			Observation
	n	m	sd	n	sd	(% de HgT)	n	m	sd	
Moule	26	0,132	± 0,06	0,051	± 0,02	38%	26	2,05	± 0,73	Dans la totalité de la chair
Sardine	3	0,320	± 0,06	0,180	± 0,02	56%	3	1,36	± 0,59	Dans le poisson étêté
Anchois	1	0,270		0,260		96%	1	0,43		et éviscéré
Rouget barbet	3	0,410	± 0,09	0,340	± 0,13	83%	3	0,20	± 0,11	Dans le muscle
Sole	5	0,472	± 0,17	0,386	± 0,12	82%	5	0,46	± 0,34	"
Maquereau	7	0,585	± 0,27	0,389	± 0,18	66%	6	0,84	± 0,24	"
Daurade	3	0,563	± 0,33	0,410	± 0,25	73%	3	0,41	± 0,26	"
Bar	5	0,704	± 0,20	0,540	± 0,13	77%	5	0,62	± 0,35	"
Lingue	1	1,230		0,790		64%	1	1,02		"
Rascasse	5	1,612	± 1,05	0,962	± 0,56	60%	4	1,29	± 0,50	"
Capelan	8	1,654	± 1,21	1,126	± 0,74	68%	8	1,08	± 0,34	"
Grondin	4	1,729	± 0,44	1,407	± 0,31	81%	4	0,69	± 0,38	"
Merlu	5	3,148	± 0,95	2,134	± 0,53	68%	5	0,99	± 0,29	
Thon rouge										
1) moins de 35000g	77	3,317	± 1,08	2,360	± 0,85	71%	19	1,80	± 0,36	"
2) plus de 35000g	24	6,746	± 3,25	4,496	± 1,98	67%	5	2,00	± 0,47	"
Raie	30	3,869	± 3,51	2,384	± 2,11	62%				"
Congre	10	4,491	± 2,79	2,684	± 1,38	60%	9	1,40	± 0,55	"
Roussette	16	9,431	± 5,16	5,144	± 2,91	55%				"
Bonite <u>Sarda sarda</u> (*)										
1) 1920 à 3400g										
m = 2710g	11	3,912	± 1,89	3,538	± 1,99	89%	9	1,08	± 0,34	"
2) 390 à 676g										
m = 566g	5	0,739	± 0,32	0,50	± 0,36	68%	1	1,19		"

(*) Résultats de Capelli et al. (1987).

Les processus biochimiques concernés et le cheminement des métaux, notamment du mercure, au niveau cellulaire ont été décrits par plusieurs auteurs (Moore, 1981; Kägi et Hapke, 1984; Viarengo, 1985).

S'agissant du sélénium, l'examen du Tableau II reflète un comportement de cet élément tout à fait différent vis-à-vis des systèmes biologiques. La teneur moyenne, proche de 1 mg kg^{-1} (poids sec) correspond approximativement à 0,25 mg kg^{-1} (poids humide) une valeur voisine de celles qui ont été trouvées récemment par d'autres auteurs; Capelli et al. (1987) pour de la bonite de Méditerranée et Chvojka (1988) pour des poissons d'Australie; elle correspond à un facteur de concentration approximatif de 1100 beaucoup plus faible que celui du mercure, en se basant sur une teneur dans l'eau de mer de 240 ng l^{-1} en Se total (Measures et Wrench, 1983).

Les concentrations en sélénium reportées sur le tableau II ne présentent pas par ailleurs autant de variabilité d'une espèce à l'autre que les concentrations du mercure total ou du mercure méthylé.

Cela s'accorde avec le fait que le sélénium est un élément essentiel qui doit assurer des fonctions biologiques. Il parcourt chez les organismes, ses propres voies metaboliques, étant en particulier un précurseur pour la construction d'enzymes comme par exemple la peroxydase glutathion (Diplock, 1976).

Son rôle particulier dans les systèmes biologiques explique assez bien que les teneurs en Se du tableau II ne soient reliées ni aux teneurs en mercure total, ni aux teneurs en mercure méthylé, ni aux mêmes paramètres biologiques comme l'espèce ou le poids des individus.

Une représentation particulièrement commode et adéquate de l'ensemble de nos résultats concernant le mercure est obtenue en portant sur un même graphique les concentrations en Hg total et en Hg méthylé toutes espèces confondues, en fonction du poids des individus. Il est obtenu alors après transformation logarithmique une droite de régression à la fois pour le mercure total et pour le mercure méthylé (Fig. 2).

4. DISCUSSION

4.1 Considérations théoriques

Une théorie du phénomène de bioaccumulation des métaux trace par les organismes, a été présentée par Fagerstrom (1977) afin d'expliquer les relations entre les concentrations rencontrées et le poids des individus. Une théorie comparable a été proposée simultanément par Norstrom et al. (1976); elle a été vérifiée par ces derniers auteurs dans le cas particulier du mercure méthylé.

Ces deux théories reposent sur la conception du "poisson adsorbant" suivant laquelle les tissus des organismes se comportent comme des supports chimiques qui peuvent admettre des fixations et des éliminations successives du mercure inorganique (Hg^{2+}) et du mercure organique (CH_3Hg^+) présents à la fois dans l'eau environnante et dans la nourriture.

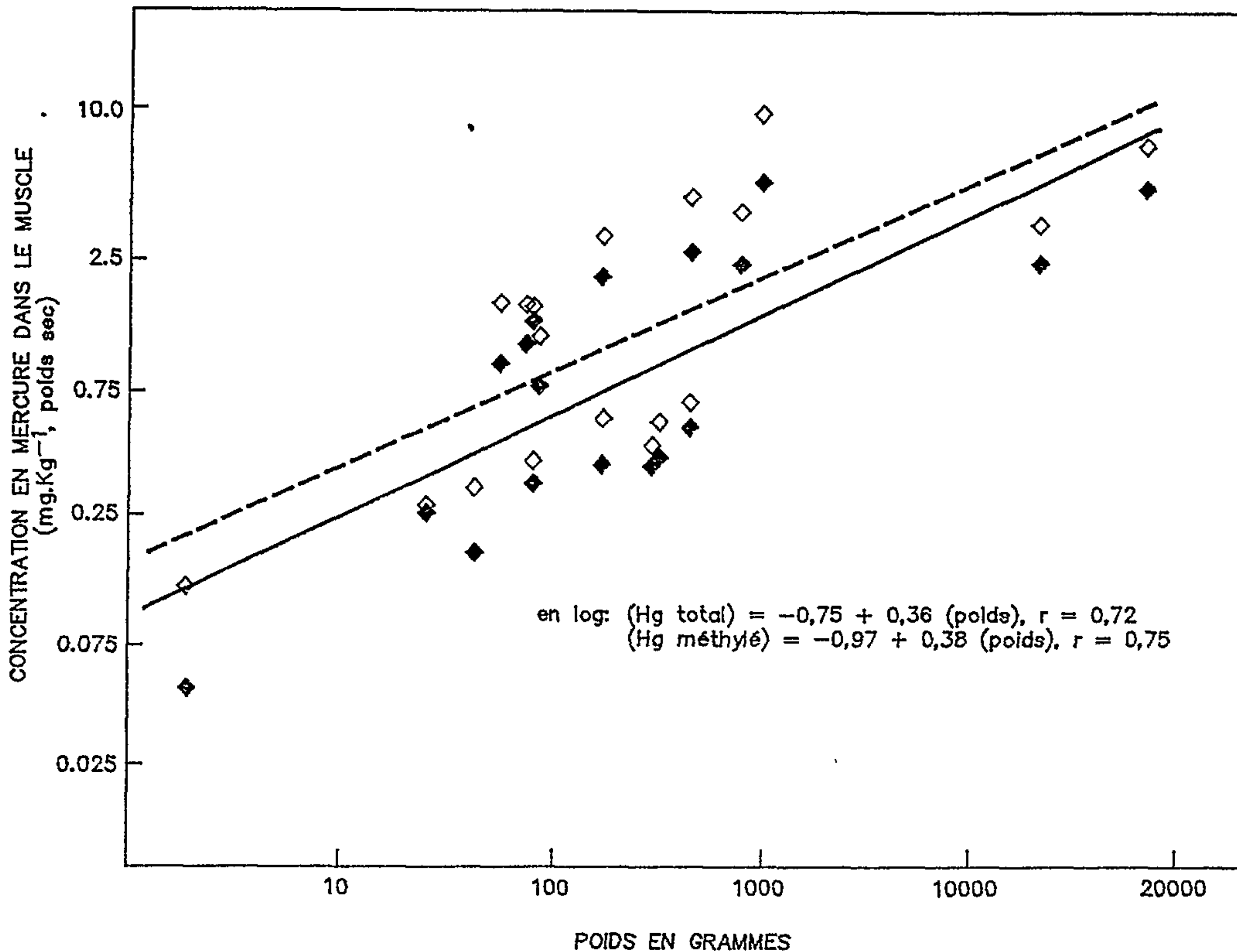

Fig. 2 Concentration en mercure méthylé (◆) et en mercure total (◇) dans les organismes en fonction du poids des individus

Elles s'appliquent facilement au milieu naturel en supposant l'existence des conditions de "l'état permanent". Il y a alors équilibre dynamique; les flux d'entrée et de sortie qui traversent les tissus des organismes sont égaux.

Plus récemment et en utilisant les mêmes conceptions, Thomann (1981) a proposé une modélisation globale concernant toute la biomasse marine.

Les mécanismes de sorption, de désorption du contaminant et de la consommation des aliments sont associés à une représentation schématique d'une chaîne alimentaire à 4 niveaux comprenant phytoplancton, zooplancton, petits poissons et gros poissons (Fig. 3).

Les équations "balance de masse" écrites pour chacun des composants de la chaîne alimentaire dans les conditions de "l'état permanent" correspondent alors au flux du contaminant à travers des compartiments successifs définis en termes biologiques spécifiques.

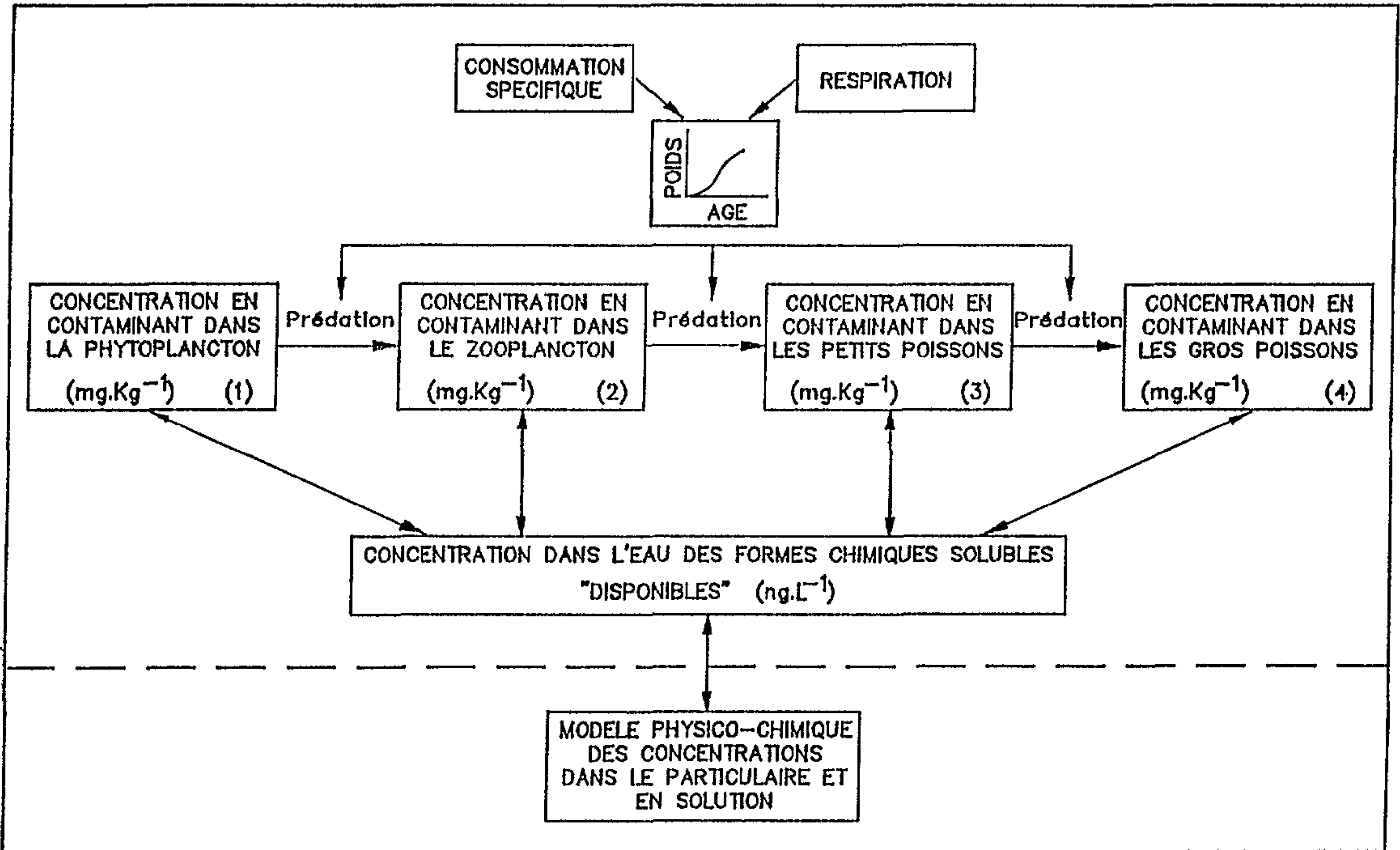

Fig.3 Représentation schématique d'une chaîne alimentaire aquatique de quatre compartiments. D'après Thomann (1981)

Avec la définition des symboles donnés sur le Tableau III, nous avons dans le cas du phytoplancton

$$\frac{d\nu_1}{dt} = k_{u1}c - K_1 \nu_1$$

La quantité du contaminant pour un organisme de niveau i est donnée par

$$\frac{d(\nu W)_i}{dt} = k_{ui}cW_i + \alpha_{i,i-1}C_{i,i-1}\nu_{i-1}W_i - K_i\nu_i W_i$$

La concentration du contaminant pour un organisme de niveau i, avec les symboles du Tableau III est donnée par

$$\frac{d\nu_i}{dt} = k_{ui}c + \alpha_{i,i-1}C_{i,i-1}\nu_{i-1} - K'_i\nu_i$$

sachant que $K'_i = K_i + G_i$ et que $G_i = \frac{dW_i}{W_i}/dt = a_{i,i-1}C_{i,i-1} - r_i$.

G_i, la croissance, est la différence entre la nourriture consommée $a_{i,i-1}C_{i,i-1}$ et la perte r_i due à l'excrétion et au métabolisme.

Beaucoup de termes de cette dernière équation sont dépendants du poids W_i des organisms. Les relations entre G_i, r_i, les taux k et K du mercure inorganique (Hg^{2+}) et du mercure méthylé (CH_3Hg^+) et le poids des individus sont données sur le Tableau IV.

Dans les conditions de "l'état permanent", avec la modélisation de Thomann (1981), la concentration d'un organisme s'écrit finalement:

$$\nu_i = \frac{k_{ui}c}{K'_i} + \frac{\alpha_{i,i-1}C_{i,i-1}\nu_{i-1}}{K'_i} \qquad (°)$$

Une forme plus générale de l'équation (°) a été donnée aussi par Thomann en introduisant le facteur de concentration:

$$\frac{\nu_i}{c} = N_n \quad \text{(n nombre de compartiments);}$$

elle devient: $$N_n = N_{nw} + \sum_{j=1}^{n-1} \left(\frac{\alpha_i C_i}{K'_i}\right)^{n-j} N_{jw}$$

Le terme $\alpha_i C_i/K'_i$ est le facteur de transfert par chaîne alimentaire.

L'indice n correspond au nombre de compartiments, une notion introduite par la modélisation; tandis que l'indice i correspond aux niveaux trophiques des organismes.

Adaptation de la modélisation de Thomann aux résultats obtenus

L'expression (°) qui découle de la modélisation de Thomann n'est autre qu'une relation entre la concentration en contaminant dans les organismes de la biomasse, les taux de fixation et d'élimination, les paramètres bioénergétiques et de croissance présentés sur le Tableau IV, qui sont reliés eux-mêmes au poids des individus. Cette relation apparaît directement en observant la représentation graphique de nos résultats en Hg total et en Hg méthylé (figure 2).

L'expression (°) peut être ajustée dans le cas du mercure méthylé pour se confondre avec la droite (échelle logarithmique) de la figure 2 en utilisant les paramètres du tableau IV dépendants du poids des individus et en supposant une teneur en eau de 74% dans les tissus des organismes.

Tableau III

Symboles utilisés dans le modèle de Thomann (1981)
et leur description.

Symboles	Signification
c	concentration dans l'eau
ν_1	concentration dans le phytoplancton
ν_i	concentration dans l'organisme de niveau i
k_{u1}, k_{ui}	taux de sorption du phytoplancton, de l'organisme de niveau i
K, K_1, K_i	taux de désorption, du phytoplancton, de l'organisme de niveau i
$\alpha_i, \alpha_{i,i-1}$	efficacité d'assimilation du contaminant dans l'organisme prédateur de niveau i, en consommant la proie de niveau i-1
$C, C_{i,i-1}$	consommation, de l'organisme de niveau i en organisme proie de niveau i-1
$a_{i,i-1}$	efficacité d'assimilation de l'organisme prédateur de niveau i en organisme proie de niveau i-1
W, W_i	poids de l'organisme, de niveau i
r_i	perte en poids de l'organisme de niveau i due à l'excrétion et au métabolisme
N_n	facteur de concentration au compartiment n
N_{nw}	facteur de concentration au compartiment n dû uniquement à l'entrée de l'eau
$G_i = \frac{dW_i}{W_i}/dt$	croissance de l'organisme de niveau i
$K'_i = G_i + K_i$	

La progression des concentrations (ν_i) ou celle des facteurs de concentration (N_n) est alors fonction à la fois du poids des organismes et du nombre n de compartiments traversés par le contaminant; après le 1er compartiment correspondant au phytoplancton, il y a un 2ème, un 3ème et un 4ème compartiment comprenant les individus dont le poids est compris entre 50 et 10000 g, puis un 5ème compartiment pour les individus dont le poids est supérieur à 10000 g. La bonne correspondance obtenue dans le cas du mercure méthylé est montrée graphiquement sur la figure 4.

Ce bon ajustement permet de calculer, et cela est un résultat important, une concentration en mercure méthylé dans l'eau de mer approximative de 0,4 ng Hg l^{-1}.

Notre simulation a été étendue au mercure total en s'efforçant d'obtenir également un bon recouvrement des droites en échelle logarithmique; celle qui correspond à nos résultats et celle qui est issue de la modélisation de Thomann. Pour cela il a été supposé que le mercure total est représenté par les deux formes chimiques Hg^{2+} et CH_3Hg^+ et il a été utilisé les différents paramètres indiqués sur le tableau IV.

L'ajustement obtenu est montré graphiquement sur la figure 4; il correspond à une concentration en mercure total dans l'eau de mer approximative de 2,5 ng Hg l^{-1}; une valeur assez proche de celles qui ont été effectivement mesurées en Méditerranée (Copin-Montegut et al., 1986; Ferrara et Maserti, 1988).

Une aussi bonne correspondance avec les résultats des analyses effectuées sur l'eau de mer confirme la validité de la modélisation de Thomann (1981); elle s'accorde aussi parfaitement avec les teneurs en mercure rencontrées habituellement dans les organismes marins originaires de la Méditerranée.

Au cours de ces dernières années, il a été effectué beaucoup de déterminations de mercure total mais très peu de déterminations par contre de mercure méthylé, sur des poissons de Méditerranée. Récemment, Capelli et al. (1987) ont trouvé des teneurs en Hg total et en Hg méthylé dans de la bonite *Sarda sarda* pêchée dans le Golfe de Gênes qui s'accordent très bien avec nos propres résultats et qui sont reportées sur le tableau II.

5. CONCLUSIONS

Les résultats présentés ici et interprétés à l'aide de la modélisation de Thomann qui est basée sur le cheminement du mercure à travers la biomasse marine, apportent un éclairage complètement nouveau à la question qui reste toujours posée de la contamination mercurielle en Méditerranée, plus importante que dans d'autres régions marines et dont Aston et Fowler (1985) ont présenté les différents aspects.

Il est montré ici que des concentrations en mercure total dans l'eau de mer proches de 2,5 ng Hg l^{-1} peuvent bien correspondre à des concentrations dans le thon rouge aussi élevées que 6,8 mg kg^{-1} (poids sec) lorsque une proportion de 16% environ de ces 2,5 ng Hg (0,4 ng) est du mercure méthylé.

<u>Tableau IV</u>

Valeurs des taux d'entrée et d'élimination du mercure, du taux de croissance et d'autres paramètres utilisés pour appliquer la modélisation de Thomann (1981) à des organismes marins.

Paramètres			Valeur	Référence
. Taux d'entrée pour un organisme de poids W	k	(Hg^{2+}) (Hg méthylé)	14.6 $W^{-0,19}$ j^{-1}, g^{-1} 49.6 $W^{-0,43}$ j^{-1}, g^{-1}	Pentreath (1976a) Pentreath (1976b)
. Taux d'élimination pour un organisme de poids W	K	(Hg^{2+}) (Hg méthylé)	0.014 $W^{-0,22}$ j^{-1}, g^{-1} 0.03 $W^{-0,58}$ j^{-1}, g^{-1}.	Pentreath (1976a) Norstrom <u>et al.</u> (1976)
. Facteur de concentration pour du phytoplancton	N_{1w}	(Hg^{2+}) (Hg méthylé)	$10^{3,3}$ $10^{4,08}$	Fowler (1985) Fowler (1985)
. Efficacité d'assimilation du contaminant	α_i		0,9	Thomann (1981)
. Croissance de l'organisme	G_i		0,01 $W^{-0,22}$ (25°C) j^{-1} W en g	Thomann (1981)
. Perte en poids de l'organisme due à l'excrétion et au métabolisme	r_i		0,036 $W^{-0,2}$ (25°C) j^{-1} W en g	Thomann (1981)
. Efficacité d'assimilation de la nourriture	a		0,8	Thomann (1981)

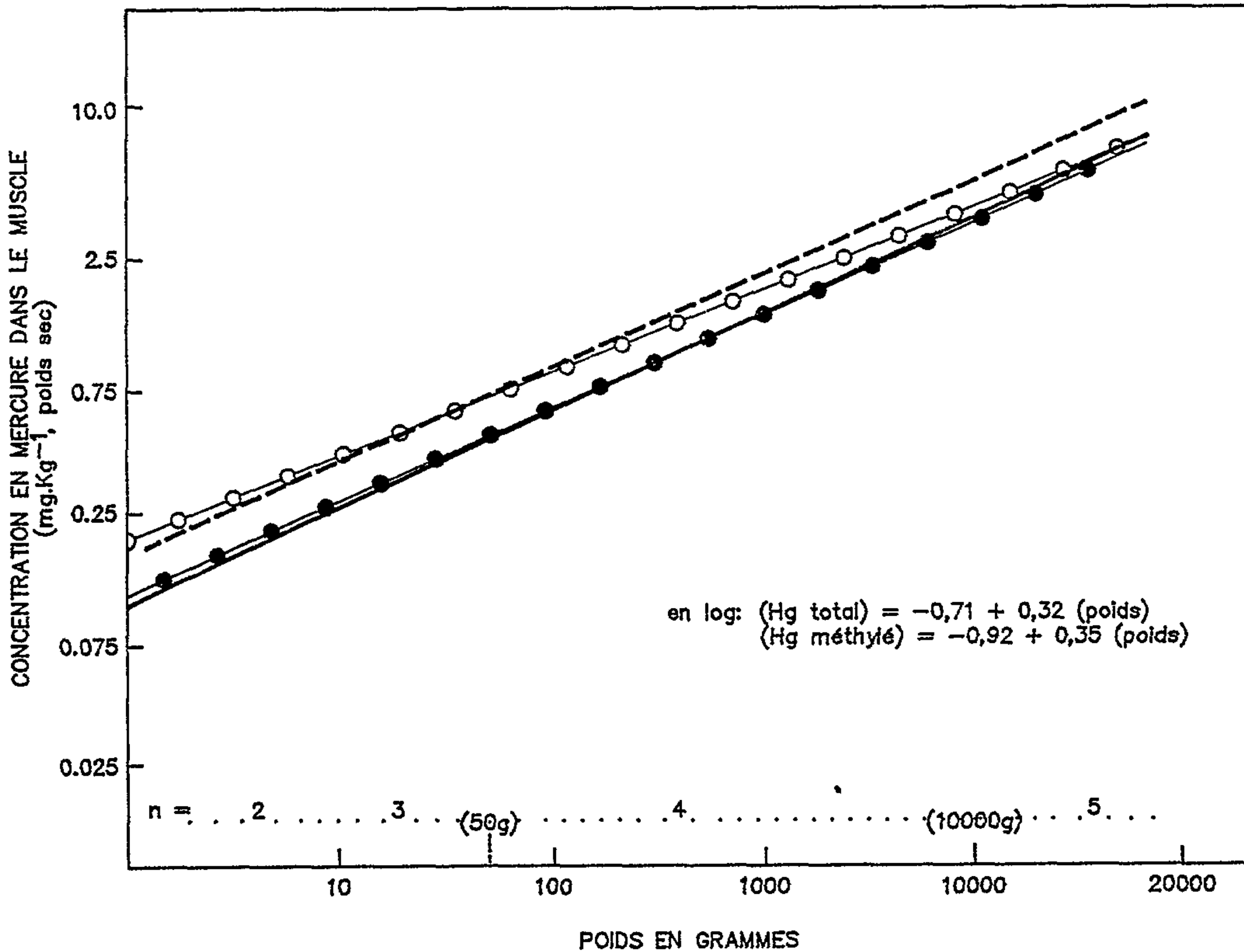

Fig. 4 Adaptation de la modélisation de Thomann (1981); simulation de la relation entre la concentration en mercure méthylé (-●-●-) et entre la concentration en mercure total (-o-o-) et le poids des individus

Les critiques de Aston et Fowler (1985) portant sur la reliabilité des mesures analytiques et le manque de données sur le mercure inorganique et sur le mercure méthylé dans la région méditerranéenne ont moins de raisons d'être présentées aujourd'hui. Des améliorations ont été apportées à la fois dans la qualité et dans la quantité des analyses.

S'agissant de la remarque particulière et déjà ancienne suivant laquelle les teneurs en mercure des poissons de Méditerranée sont plus élevées que dans les poissons originaires d'autres régions marines, notamment de l'Atlantique, elle ne doit pas s'expliquer par un mécanisme biologique propre aux poissons eux-mêmes. Ce travail montre qu'une variation du niveau en mercure total dans l'eau de mer ou une

faible variation de sa proportion en Hg méthylé, entraîne une variation des teneurs chez les organismes marins, qui est d'autant plus importante qu'ils appartiennent comme les thonidés à des niveaux trophiques élevés.

Une diminution par exemple de la concentration en Hg total de l'eau de mer de 2,5 ng Hg l^{-1} dont 16% est méthylé à 1,5 ng Hg l^{-1} dont 16% est méthylé, entraîne une diminution de concentration en Hg total chez le thon rouge de 8,63 à 5,18 mg kg^{-1} (poids sec). De même une diminution de la proportion du mercure total qui est méthylé de 16% (0,4 ng Hg l^{-1} de 2,5 ng l^{-1} en Hg total) à 10% (0,25 ng Hg l^{-1} de 2,5 ng l^{-1} en Hg total), entraîne une diminution de la concentration en Hg total chez le thon rouge de 8,63 à 5,70 mg kg^{-1} (poids sec).

En considérant la proportion dans l'eau de mer du mercure méthylé par rapport au mercure total calculée à partir de nos résultats (16%), il ne nous est pas possible de dire que cette proportion est plus élevée en Méditerranée que dans d'autre mers ou océans. Nous ne disposons pas de mesures de mercure méthylé de fiabilité suffisante en eau de mer concernant une quelconque région marine.

Il est toutefois permis de penser que cette proposition est relativement importante en Méditerranée en se référant au pourcentage de mercure méthylé par rapport au mercure total, proche de 1%, qui est rapporté pour l'eau de mer en général dans la littérature (Craig, 1986).

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ISOLATION AND ASSESSMENT OF BACTERIA WHICH TRANSFORM MERCURY IN EFFLUENTS NEAR CINNABAR AND OTHER SULFIDE ORE DEPOSITS: METHODS TO EVALUATE THE BIOTRANSFORMING ACTIVITY

by

F. BALDI

Dipartimento di Biologia Ambientale
Università di Siena
Siena, Italy

ABSTRACT

Strains of bacteria has been isolated from Hg polluted areas by using enrichment media. The bacteria, which grow on iron peptone agar amended with 10 $\mu g\ ml^{-1}$ of $HgCl_2$, were checked for the mechanism of Hg-resistance. All these strains were able to reduce Hg(II) to Hg°. Few of them (8 strains) could degrade also organomercurials. In this study methylation of Hg(II) is not a mechanism of Hg resistance in aerobic heterotrophic bacteria. Moreover, an extractable-toluene form of Hg was commonly determined in cultures of many isolates, even though with specific analyses, this Hg species was not methylmercury. Methods for detecting routinely bacterial transformation of Hg species were based on headspace analysis of Hg°, and/or volatile hydrocarbon determination.

1. INTRODUCTION

Hg pollution in the Mediterranean basin is due mostly to natural sources rather than anthropogenic activities (Baldi and D'Amato, 1986). This is especially true for the Tyrrhenian sea, where Hg input comes from the weathering of cinnabar (HgS) deposits (Baldi and Bargagli, 1982), natural degassing of volcanoes (Buat-Menard and Arnold, 1978; Legittimo et al., 1986) and hot springs, and from geothermal boreholes (Breder and Flucht, 1984), but also in part from industries such as chlor-alkali plants (Baldi and Bargagli, 1984). Many reports have shown that air, water, sediment, and biota from this area are contaminated by Hg.

Certain marine organisms from the Mediterranean, especially those at the top of the food chain such as tuna (Buffoni et al., 1982) seabirds (Renzoni et al., 1982) and sea mammals (Thibaud and Dugny, 1973), have high methylmercury levels in muscle tissue. However the levels of Hg in Mediterranean waters are similar to those in the Atlantic Ocean (Seritti et al., 1982). Despite the amount of studies on the distribution and occurrence of the Hg in the Mediterranean sea, the role of microorganisms in biogeochemical cycling of Hg in this environment has not been investigated.

Bacteria resistant to various forms of Hg have been isolated from areas containing elevated concentrations of anthropogenic Hg inputs; these include aquatic (Sprangler et al., 1973; Olson et al., 1979a), soil and sediment (Isaki, 1981; Timoney et al., 1978) and clinical (Porter et al., 1982) environments. The most frequently documented

bacterial mechanism of resistance, which is termed "narrow spectrum" Hg resistance, consists in production of volatile Hg° by enzymatic reduction of Hg(II) (Summer, 1984; Robinson and Tuovinen, 1984). Less common biotransformation is the degradation of organomercurials (Baldi et al., 1988b). Strains with a "broad spectrum" mercury resistance can cleavage the C-Hg bond and producing Hg° and the respective hydrocarbons. Methylation of mercury by microorganisms has been described since the 1968 (Imura et al., 1971), but the real mechanism is not yet understood, even though it has been demonstrated that methylcombalamine coenzyme is a methyl donor for Hg(II) methylation (Wood et al., 1968).

The aim of this project, partially supported by FAO/UNEP programme, is to pin point the role of microorganisms in mercury polluted environments especially in transforming and controlling the Hg species.

2. MATERIALS AND METHODS

2.1 Sampling areas

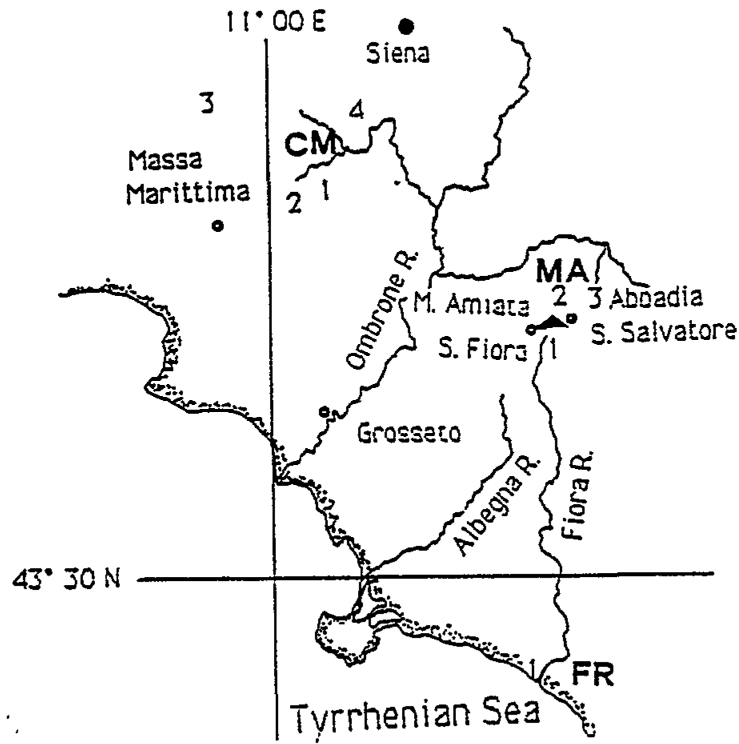
The samples were collected in different areas of southern Tuscany (Fig. 1). Comprehensive studies have shown elevated levels of Hg in alluvional sediments (Dall'Aglio et al., 1966) and soils (Bargagli and Baldi, 1984), over this area.

The first sampling area was a pyrite and mixed sulfide (Cu, Pb, and Zn), deposit in the Colline Metallifere (CM on the map). Four sites were sampled: 1) a collection pond draining the pyrite mines of Campiano (pH 5.5); 2) a run-off stream 100 m downstream from the collection pond (pH 5); 3) water from waste heaps of the pyrite mines of Niccioleta (pH 3); 4) Feccia Creek (pH 7), about 15 km from the pyrite mines in a non-mining drainage.

The second sampling area surrounded the cinnabar mines at Monte Amiata (MA on the map). The site 1 was a collection pond at the mine of Abbadia San Salvatore. Site 2 was a small creek at Bagni San Filippo. Site 3 was a Formone Creek, 7 km downstream from Abbadia San Salvatore.

The third sampling area was at the mouth of the Fiora River (FR on map), which drains the Monte Amiata area.

The forth sampling area was along the Fiora River and sampling was carried out 2 years later. The sites were located at the Fiora mouth and proximities (A-D), at about 10 km from the river source (F-I), where the Morone cinnabar mine effluent flows into the Fiora River, and at the river source (K and L). The Fiora is a rapidly flowing river with highly variable flow whose waters bear small crystals of cinnabar which are deposited in the river sediments and in sediments of the inner continental shelf in the sea near the mouth of the river (Anselmi et al., 1976; Baldi and D'Amato, 1986).



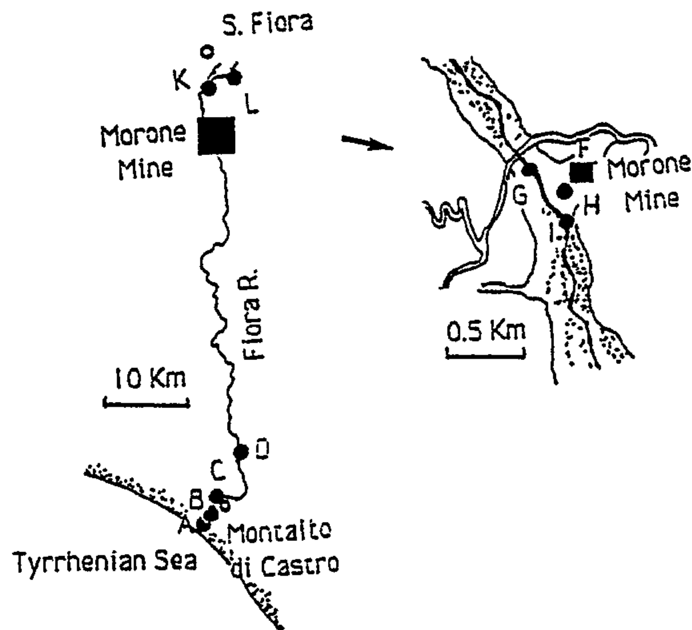


Fig. 1 Sampling sites for isolation of strains. CM (Campiano mine areas), MA (Monte Amiata area), FR (Fiora River mouth). In the two insets, stations are respectively located in the Fiora River and near the Morone cinnabar mine

2.2 Bacteria isolation

Surface river water and sediments were collected in whirl-pak bags (Nasco). The samples were stored at - 80°C for few days before bacterial analysis (Boeye et al., 1975). No significant changes in numbers of viable bacteria (as determined by plate counts) were detected in a sample (Site G in February) even after 3 weeks of frozen storage.

Aerobic, heterotrophic bacteria were enumerated using modified iron peptone medium (FeP) (Ferrer et al., 1963) containing (per liter) 5.0 g polypeptone (Merck), 0.5 g glucose, 0.1 g $(NH_4)_2SO_4$, and $FeSO_4.7H_2O$. The medium was solidified with agar (Difco) at 1.5% final concentration. Hg resistant bacteria were enumerated on FeP agar amended with 2 and 10 $\mu g\ ml^{-1}$ mercury as $HgCl_2$ (Merck); these media are hereafter referred to as 2FePA and 10FePA, respectively. Dilutions of river water were added to duplicate tubes of molten agar (47°C) FeP agar, 2FePA, and 10FePA, and plates were poured from these tubes. Colonies were counted after 2 and 7 days incubation at 28°C. Colonies of differing shape, color, and morphology were noted and restreaked twice on fresh plates on fresh plates for isolation of strains. Isolates were stored in cryogenic vials at -80°C in FeP containing 20% glycerol (final concentration).

2.3 Identification of strains

Mercury-resistant strains were tested for gram stain reaction and motility. Gram-negative strains were identified by using API 20 E and 20 NE kits (API System).

2.4 Plate assay of Hg-resistance

Isolated growing in FeP broth were induced for Hg resistance with 0.2 $\mu g\ ml^{-1}$ Hg as $HgCl_2$. After incubation overnight in broth, the cultures were streaked onto 10FePA. After 24 hours aerobic incubation at 28°C, the 10FePA plates were observed for growth.

2.5 Mercury reduction assay

Strains that grew on 10FePA were transferred in tests tubes containing 10 ml of FeP broth. Pseudomonas putida strain FB-1, shown previously (Baldi et al., 1988a) to possess plasmid-coded mercury reductase, was used as a positive control. After 24 hours, the cells were centrifuged at 3,600 x g for 20 min, washed twice in potassium phosphate buffer (pH 7.4), and resuspended in Nelson medium (NeM) (Nelson et al., 1973), which contains (per liter) 5 g casamino acids (Difco), 2 g glucose, 1 g yeast extract (Difco), 10 g NaCl, and 0.1 g $MgSO_4.7H_2O$. This medium was designated to reduce the precipitation of Hg by sulphur-containing compounds. 1μg of Hg as $HgCl_2$ was added to 1.0 ml of NeM in a test tube which was immediately sealed with a "minert" cap (Supelco) (Fig. 2). After 10 min, 1.0 ml of headspace gas was removed with a gas sampling syringe and the gas was injected into a quartz cell of an atomic absorption spectrometer (AAS) (Perkin Elmer 300S) equipped with a Hg hollow cathode lamp (Perkin Elmer). Quantitation of the gaseous Hg° produced by the bacteria was determined by comparison a peak hights with those from various amounts of vapor equilibrated with liquid Hg° at a given temperature.

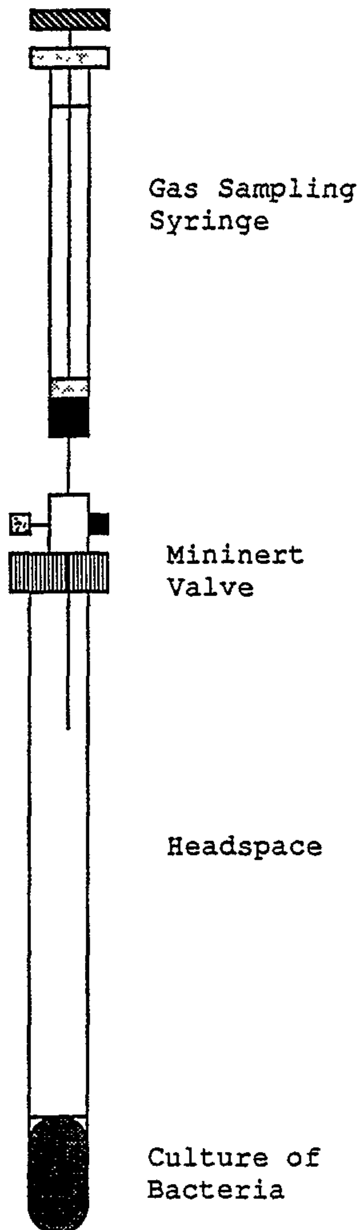


Fig. 2 Method to determine Hg° in the headspace produced enzymatically by narrow and broad mercury resistant bacteria

2.6 Methylmercury degradation assay

All isolates were streaked onto FeP agar containing 1.0 μg Hg ml^{-1} as methylmercury chloride (MeFePA) (Merck), and those that were able to grow were tested for Hg° production, indicative of methylmercury degradation. The chosen strains were grown in FeP broth overnight, washed, and resuspended in NeM as above. One μg of methylmercury chloride was added to 1.0 ml of culture, and gaseous Hg° was determined after 30 min as described above.

2.7 Mercury methylation assay

All strains of Hg-resistant and non-resistant bacteria were tested for methylation of inorganic mercury. The isolates were inoculated into 50 ml test tubes containing 40 ml of FeP broth containing 1.0 μg ml^{-1} and incubated at 28°C for 15 days. The tubes were loosely capped so that a gradient of oxygen was established in the medium. After incubation, 4.0 ml 37% HCl was added to each sample. A 9.0 ml aliquot was extracted three times each with 1.0 ml of benzene to extract methylmercury. Then 1.0 ml of 0.01 M aqueous $Na_2S_2O_3.5H_2O$ was added to the combined benzene extracts and spun for 30 s on a vortex mixer. The aqueous layer was analysed for total Hg content by AAS (Perkin Elmer 372, equipped with HGA 5000 graphite furnace) (Filippelli, 1987). Conversion of 1% of inorganic Hg to methylmercury would have been detected, based on addition of spikes of methylmercury carried through the analysis sequence. Methylmercury partitions into the organic phase whereas inorganic mercury remains in the aqueous phase.

To determine if the benzene extractable Hg was methylmercury, 0.5 ml of 0.5 M aqueous $CuCl_2$ solution plus 0.5 ml of benzene was added to the aqueous thiosulfate extracts. The layers were mixed on a vortex mixer for 30 s. A 5.0 μl aliquot of the benzene layer was injected into a gas chromatograph (Carlo Erba Fractovap, model 2350) equipped with a ^{63}Ni electron capture detector. The column was glass 4 mm i.d. x2m packed with 2% carbowax 20M on Chromosorb W(HP) 100-200 mesh operated at 195°C (Filippelli, 1987).

3. RESULTS

3.1 Bacteria distribution

The distribution of Hg-resistant bacteria was determined only in sites along the Fiora River from the source to the mouth in two different period of the year: February and May. The percentage of Hg-resistant bacteria depended on the time and site of the samples and on the concentration of Hg in the medium (Table I). The mean percentage of bacteria from all sites growing on 2FePA was 10.2% in February and 3.1% in May and on 10FePA was 1.8% and 0.04% respectively. In May the only two samples containing bacteria growing on 10 FePA were from two stations at the mouth of the river. The highest percentages of bacteria growing on the Hg-amended agar were found in samples from the upper sites, especially in February in the areas around the mine effluent (sites G, H, I) and at the mouth of the river. In February, waters were turbid due to the rainfall, and particulate matter levels were high. In this sampling 106 strains of aerobic, heterotrophic bacteria were isolated from samples of the river water from all stations during the two sampling periods.

3.2 Hg transformations

144 bacterial cultures were obtained from the sampling sites by selecting morphologically different colonies from agar plates for isolation and purification. 36 of the isolates grew in FeP broth containing 10 μg ml^{-1} of Hg (narrow spectrum resistance), whose 8 strains grew well in FeP broth containing Hg as methylmercury chloride 1 μg ml^{-1} (broad spectrum resistance).

Table I

Percentage (%) of isolates that formed colonies on Hg-amended agar plates*. (From Baldi *et al.*, 1989)*

Site	February 2FePA	February 10FePA	May 2FePA	May 10FePA
A (mouth)	No sample collected		5.6	0.1
B	5.4	0.50	2.1	0.3
C	No sample collected		0.6	0
D	0.9	0.06	2.0	0
G	6.9	0.40	2.6	0
H	32.2	9.00	1.9	0
I	10.4	0.02	4.2	0
L (source)	5.6	0.50	5.8	0
K (source)	No sample collected			0

* Compared to FePA counts (non-Hg amended)

Mercury ion resistance in bacteria has been shown to be associated with mercury reductase enzyme, which converts mercury ion to volatile elemental mercury.

In a separate experiment we measured the levels of Hg(II) in FeP broth after addition of 2 $\mu g\ ml^{-1}$ $HgCl_2$ and inoculation with Hg(II)-resistant isolates. A set of 16 cultures that grew in the presence of 2 $\mu g\ ml^{-1}$ $HgCl_2$ fell into two groups, based on the amount of mercury remaining in the growth medium. One group of six isolated showed only 20 to 35% Hg remaining, and the other group of 9 isolates showed >85% of Hg remaining (Fig. 3). All of the group of six were resistant to 10 $\mu g\ ml^{-1}$ $HgCl_2$. In separate experiments, Hg°, but not other volatile species, was found in the headspace gas above all these six cultures and above all the other 36 strains which were able to grow on FeP agar amended with 10 $\mu g\ ml^{-1}$ $HgCl_2$. This suggests that bacteria are resistant to Hg(II) by reduction and volatilization of Hg° due to mercury reductase (Silver and Misra, 1984). No Hg° was found in the headspace above any of strains sensitive to 10 $\mu g\ ml^{-1}$ $HgCl_2$.

The Hg° volatilizing activity was inducible in all strains of mercury-reducing bacteria. A lag phase, ranging from 5 to 30 min occurred before the uninduced cultures began to produce detectable Hg° (Fig. 4). The rate of transformation of Hg(II) to Hg° depends on number of bacteria ml^{-1} (Fig. 5). With the closed test tubes, the production of elemental mercury for induced and uninduced strains began to level off in less than 1 h, with induced cell and after 2-3 h with uninduced cells. The constant concentration of Hg° is correlated to the temperature and pressure and represents a chemical equilibrium between Hg° in liquid phase and the Hg° in a gaseous phase. In fact the same value of gaseous Hg° is determined above a drop of metallic mercury.

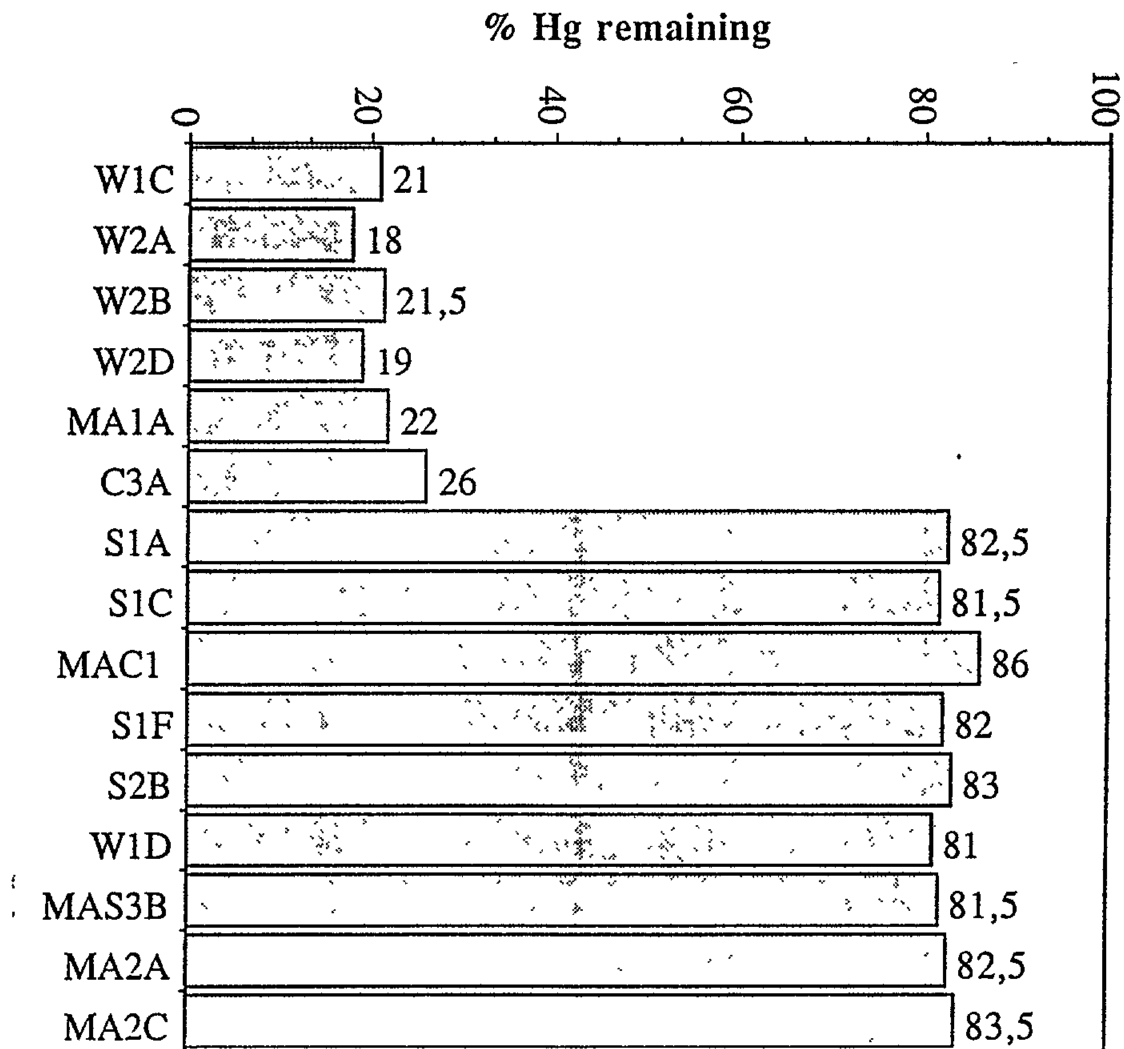


Fig. 3 Percentage of total mercury which remains after 24 hours incubation at 28°C with sensitive and mercury resistant strains

The isolates that grew well in presence of 10 $\mu g\ ml^{-1}$ $HgCl_2$ were rather sensitive to 1.0 $\mu g\ ml^{-1}$ MeHgCl, and only 8 strains were resistant to that concentration. These strains were gram-positive and gram-negative bacteria, and only one of them was identified as a Pseudomonas putida, strain FB1. This strain produced Hg° when incubated with methylmercury chloride and also with ethyl- and phenylmercury species (Fig. 6), attributable to the production of an organomercurial lyase enzyme (Begley et al., 1986). This enzyme cleaves the R-Hg bond to R-H (hydrocarbon) and Hg(II) which is subsequently reduced by mercuric reductase to Hg°. Always the

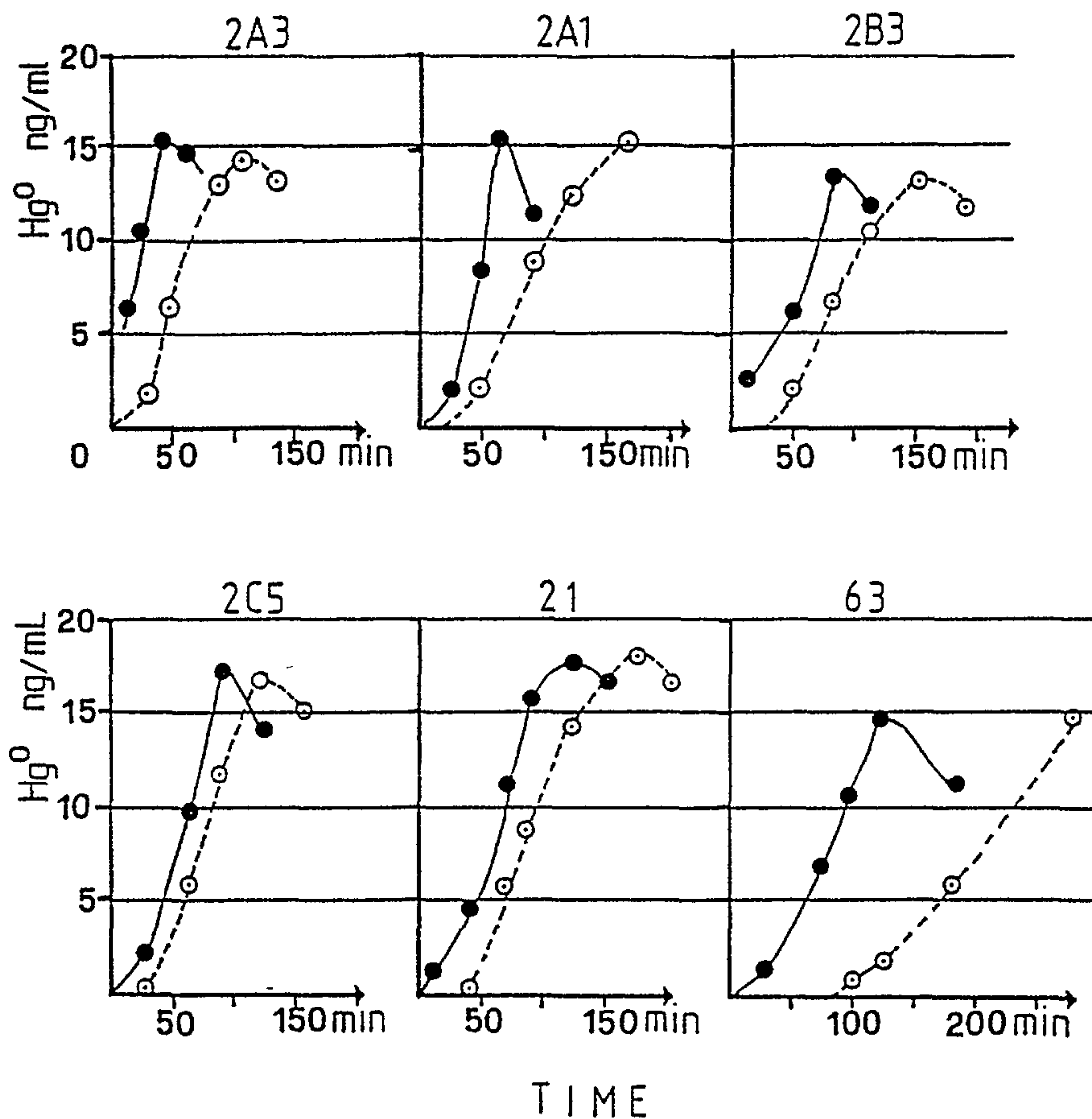


Fig. 4 Production of elemental mercury by induced and uninduced representative cultures. Uninduced cultures were grown for five transfers in Hg-free FeP broth. Induced cells were incubated overnight in FeP plus 0.2 $\mu g\ ml^{-1}\ HgCl_2$ (From Baldi et al., 1989)

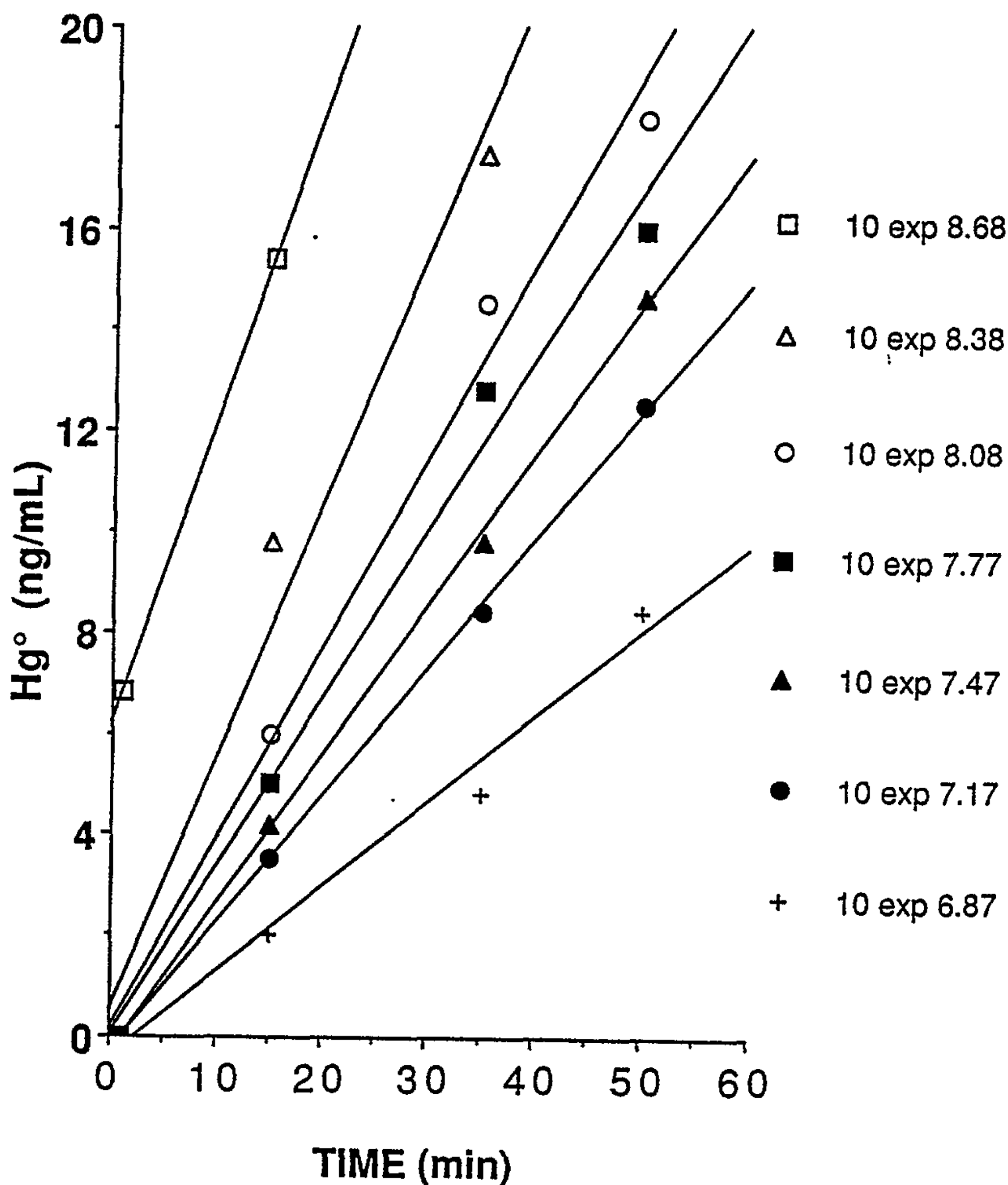


Fig. 5 The enzymatic volatilization rate of Hg°, at a constant temperature and pressure, is strictly related to number of cells ml^{-1} of a mercury resistant strain

organomercurial lyase is coupled with the mercuric reductase (Robinson and Tuovinen, 1984), and never it has been found alone. The production of methane in the headspace depends of the degradation rate of methylmercury: in 15 min, 50% of 1 μg of methylmercury is degradated, whereas 98% of methylmercury is degradated in 2 h (Fig. 7). Methane was specifically detected with gas-chromatograph in line with a Fourier transform infrared spectroscopy (GC-FTIR) and the bacterial origin was pin pointed by the use of a "cured" strain FB4 (Baldi et al., 1988b) (Fig. 8).

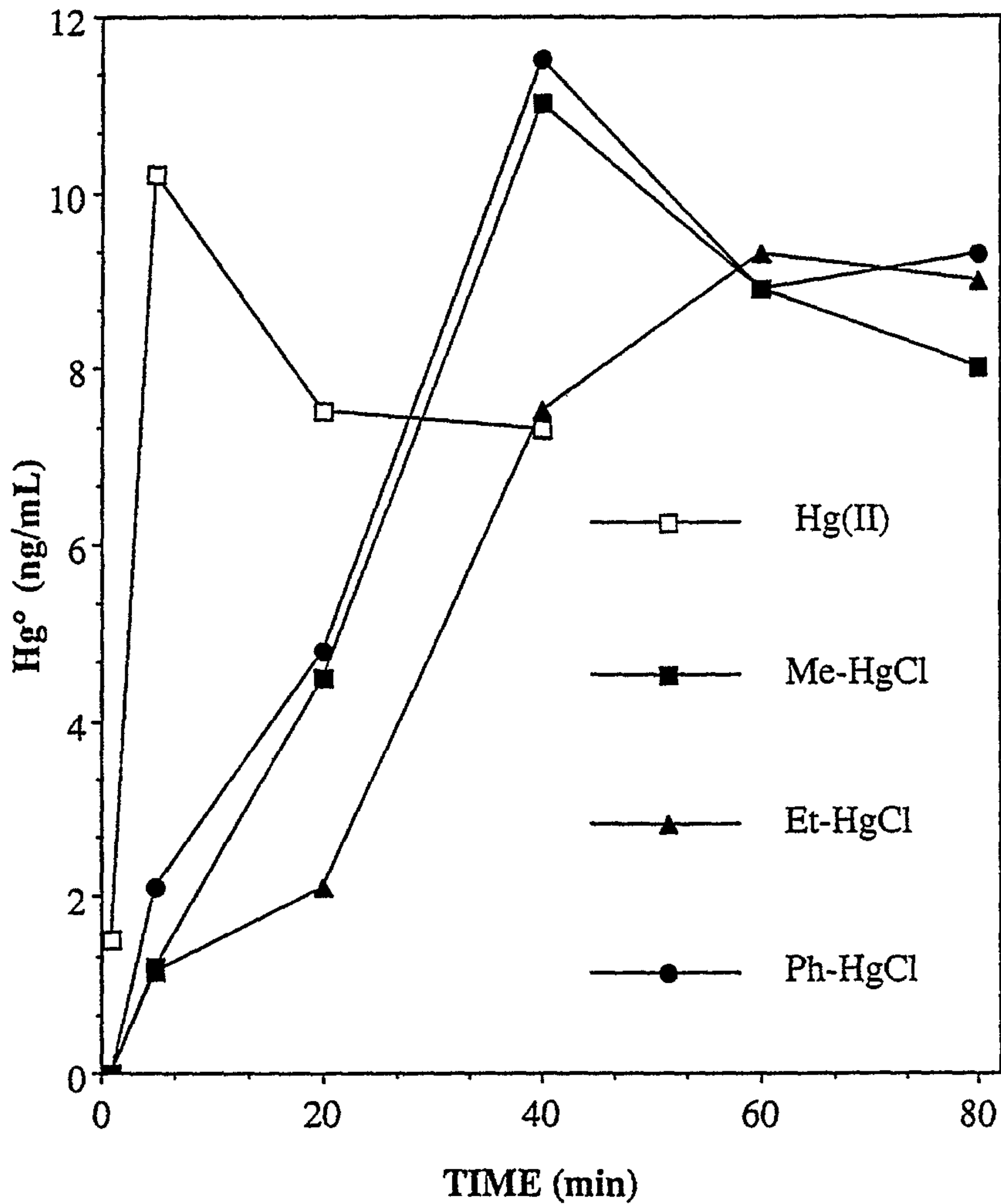


Fig. 6 Transformation of $HgCl_2$, methylmercury (Me-Hg), methylmercury (Et-Hg) and phenylmercury (pH-Hg) to elemental mercury (Hg°) by a Pseudomonas putida strain FB1 (From Baldi et al., 1988a)

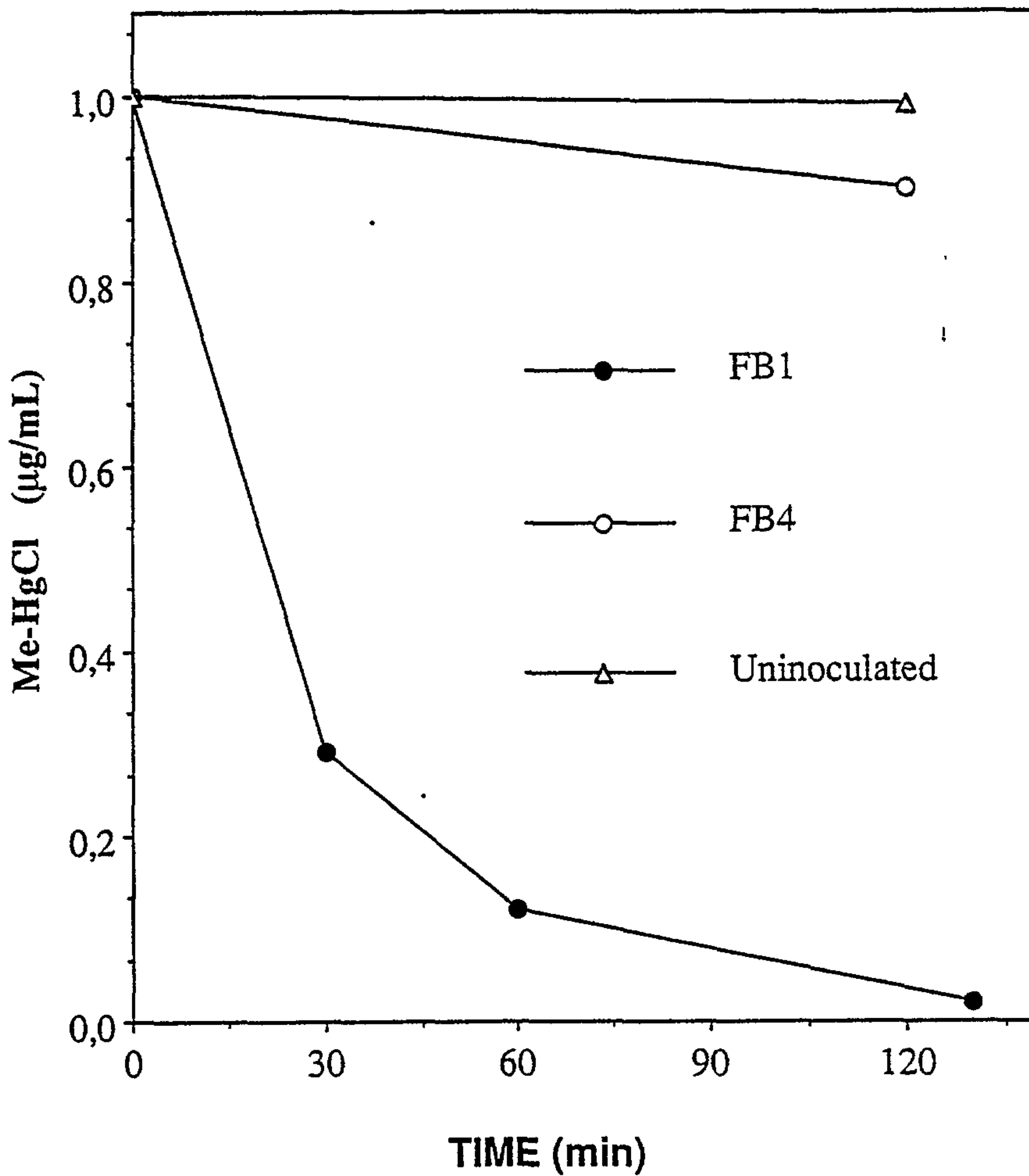


Fig. 7 Determination of the disappearance of Me-Hg in culture medium inoculated with FB1, FB4 strain and in the uninoculated samples. FB4 strain is a "cured" strain, which lost by chemical treatment the plasmid harbouring mercury resistance (From Baldi et al., 1988b)

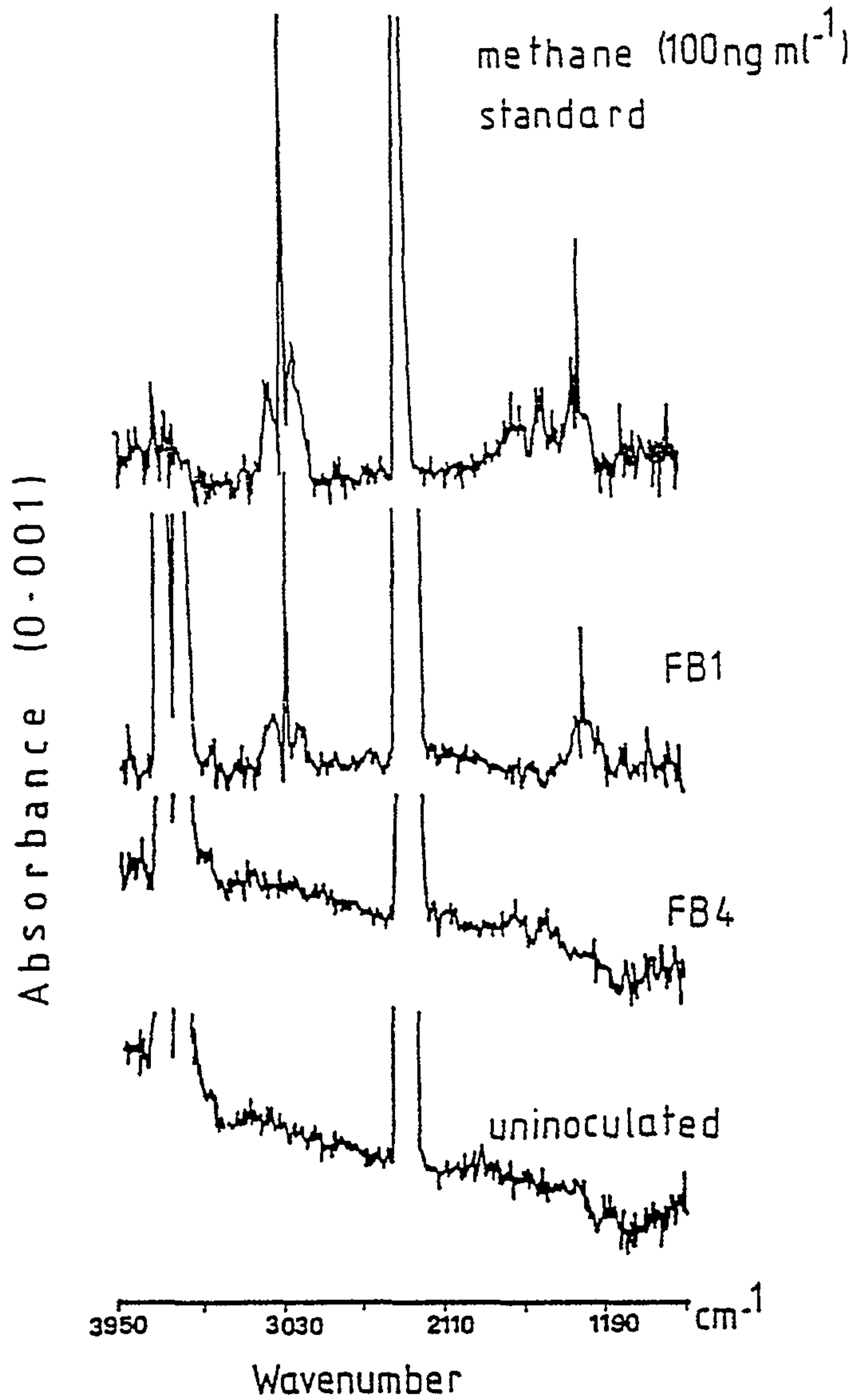


Fig. 8 GC/FTIR spectra of methane produced by the strain FB1 is compared with spectra of an uninoculated sample and the cured strain FB4. A spectrum of methane standard (0.1 μg ml^{-1}) is also shown (From Baldi et al., 1988b)

The methane detected for instance in the headspace arises only from the degradation of methylmercury and not from other methyl-metals, so the enzyme involved is a specific one (Baldi et al., 1989).

Methylation of Hg(II) was checked in all strains, the detection limit of the methylmercury moiety was 5 ng ml^{-1} in broth, representing 0.25% of the spike (2 μg ml^{-1}) of $HgCl_2$. After 15 days incubation, traces of benzene-extractable Hg were detected in 15 cultures comprising both sensitive and resistant strains. Strain 2A4, identified as a Flavobacterium species, produced a benzene extractable Hg compound (0.25 μg ml^{-1}) representing about the 2.8% conversion (Fig. 9). However no methylmercury was detected when this sample was analysed by GC for the confirmation of methylmercury. This form of Hg was soluble in benzene and was not volatile at 200°C in the graphite furnace with thiosulfate, whereas Hg(II) is volatile at 60°C. In addition, Hg was not covalently bound to carbon in this compound. This may be an unstable metallorganic complex.

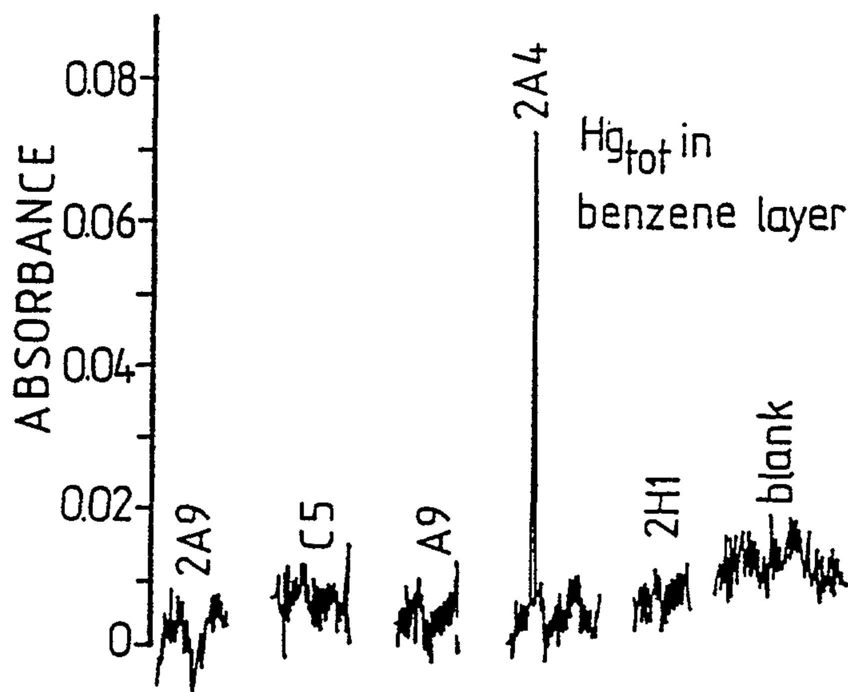


Fig. 9 Detection by AAS of a benze soluble mercury species from strain 2A4. No Me-Hg species were detected when this sample was analysed by gas chromatography (From Baldi et al., 1989)

4. DISCUSSION

This paper characterizes mercury transformations by Hg-resistant microorganisms obtained from natural cinnabar and metal sulfide deposits. Other investigators working with organisms obtained from diverse environments have reported that reduction is the main mechanism of microbial resistance to Hg(II) (reviewed by Robinson and Tuovinen, 1984). However methylation of Hg has been suggested as a

means of Hg detoxification (Ladner, 1971; Pan-Hou and Imura, 1982). Other microorganisms are tolerant to Hg(II) by virtue of less specific mechanisms of resistance. Such organisms may grow at Hg(II) concentrations above those tolerated by the majority of the microbial population, but would be inhibited at levels at which Hg(II)-reducing cells can function. Such nonspecific resistances as cell envelope complexation or H_2S production would also alleviate toxicity of other heavy metal cations. These kinds of resistances may have accounted for the behavior of the freshwater environment in Barkay's study (Barkay, 1987). Thus, there is a difficulty in defining microbial Hg resistance and concentrations indicative of resistance. In this study, we define resistance as the ability to produce Hg° from $HgCl_2$ or organomercurials, since we did not detect organisms from the study environment that grew well on 10FePA or MeFPA that did not produce Hg°. In an environmental sense all mechanisms, general or specific, are important in understanding the community response to a metal toxicant. However, in a physiological sense, certain levels of the resistance may help to define specific enzymatic mechanisms of the metal detoxification.

Many papers have documented the aerobic and anaerobic production of methylmercury by bacteria and fungi (Hamdy and Noyes, 1975; Ladner, 1971; Olson et al., 1979b; Pan-Hou and Imura, 1982; Rowland et al., 1975; Vonk and Sijpesteijn, 1973). In these studies, only a small percentage of Hg(II) (usually 0.1-2%) is converted to methylmercury. This suggests that Hg(II) may be methylated indirectly by methyl-donating metabolites excreted by cells (Huey et al., 1974). For example, methylcobalamin is known to nonenzymatically methylate Hg(II) (Ridley et al., 1977). Iodomethane produced by fungi and marine algae, reacts with Hg(I) to produce methylmercury (Craig et al., 1983). Enzymatic methylation of mercury in aerobic heterotrophic bacteria has never been conclusively demonstrated, and the biological function of this project is unclear. Several authors have claimed that biomethylation of Hg(II) is a mechanism of Hg resistance in microorganisms: however, methylmercury is at least as toxic as Hg(II) to microorganisms (Jones et al., 1984; Nakamura et al., 1986). The results from this study show that 144 bacterial strains isolated from Hg mine-impacted environments in southern Tuscany, the 46 that grew on agar media amended with 10 $\mu g\ ml^{-1}$ Hg as $HgCl_2$, without exception were resistant to Hg by virtue of Hg reduction (indicative of mercuric reductase enzyme).

5. CONCLUSION

Hg-resistant bacteria are common in the southern Tuscany, where there is a contamination from cinnabar deposits and other sulfide deposits. Plate methods and AAS are useful for screening strains for Hg resistance. Growth on 10 FePA was always associated with Hg° production. Although several papers have documented the biomethylation of mercury by many strains of bacteria, this process was not detected in aerobic, heterotrophic bacteria isolated from this Hg-impacted environment nor was it involved as a mechanism of Hg resistance. The biodegradation of organomercurials was specific and no activity of the organisms toward other organometallic compounds was detected.

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DETERMINATION OF MERCURY (TOTAL AND ORGANIC) AND SELENIUM IN SEAFOOD FROM THE LIGURIAN SEA FOR THE STUDY OF THE CORRELATION Hg TOTAL/Hg ORGANIC/Se

by

R. CAPELLI, V. MINGANTI and R. DE PELLEGRINI

Istituto di Analisi e Tecnologie
Farmaceutiche ed Alimentari
Università di Genova
Genova, Italy

ABSTRACT

This paper presents the results obtained during a 3 years study on mercury (total and organic) and selenium concentrations in marine organisms of the Ligurian Sea. Species considered have been: European hake (Merluccius merluccius), Norway lobster (Nephrops norvegicus), Bogue (Boops boops), and Atlantic mackerel (Scomber scombrus). Total mercury and selenium were determined by atomic absorption spectrometry on the solution obtained by mineralization of the samples with nitric acid, respectively with the cold vapour and hydride generation techniques. Organic mercury was determined by cold vapour atomic absorption spectrometry after extraction in toluene and back extraction in L-cysteine solution. Results obtained show that both total and organic mercury increase with the size of the specimens, while no correlations between selenium content and size is evident.

1. INTRODUCTION

The FAO/WHO Joint Expert Committee on Food Additives (JECFA) has suggested that a person of 70 kg bodyweight must not be exposed to a quantity of mercury exceeding 0.3 mg per week (Provisional Tolerable Weekly Intake, PTWI). Methylmercury, expressed as mercury, should not exceed 0.2 mg per week.

Data concerning mercury and methylmercury concentrations in seafood and data concerning seafood consumption (12.5 kg year in term of live weight) show that Italian population can be considered not at risk (Nauen et al., 1983).

This may not be true for groups of person with a seafood consumption higher than the average, or which eat seafood with a high mercury content.

The effort to establish any mercury and methylmercury exposure/health effects relationships, needs data concerning mercury and methylmercury concentrations in seafood.

The scope of the present research is: (i) to improve the knowledge about the presence of mercury (total and organic) in marine organisms from the Ligurian Sea; (ii) to study possible relationships between mercury and biological parameters (size, species, etc.) and (iii) to supply data necessary to an epidemiological study.

Selenium has been taken into consideration because it is indicated as antidote of the toxicity of the organic and inorganic mercury.

2. MATERIALS AND METHODS

2.1 Sampling and samples preparation

Sampling has been carried out in the area between La Spezia (East of Genova) and Varazze (West of Genova), both by means of local fishers ("Cooperative Pescatori, Camogli") and by means of fishing cruises organized in collaboration with Prof. G. Relini and Prof. L. Relini-Orsi ("Istituto di Zoologia", Department of Biology, University of Genova).

Samplings have been carried out between December 1984 and June 1986.

In the area studied, the most important possible sources of mercury are two power plants that burn fossil fuel, and agriculture activities in the western part of the area with the possible use of chemical containing mercury compounds. No other activities that can contribute to mercury pollution are present in the area studied.

The preparation and dissection of the specimens was carried out according to UNEP/FAO/IAEA/IOC (1984a) reference method.

Most samples consisted from one individual. Only when the sample amount was too small for analysis, composite samples have been used.

2.2 Reagents and apparatus

Mercury (total and organic) was determined using cold vapour atomic absorption spectrometry (AAS), and selenium was determined using hydride generation AAS.

For each run, two or more "blanks" obtained with the same procedure of the samples were examined to check the purity of the reagents and possible contamination.

Solvents and reagents were of analytical grade. Nitric acid was distilled just before its use. Working standards were daily prepared from stock solutions commercially available. Mercury standards were stabilized by means of nitric acid (5 mL in 100 mL of standard) and potassium bichromate (0.01 % w/v).

Special care was taken in cleaning the glassware, which were washed with 1.5 M nitric acid.

For all analyses a Perkin-Elmer Model 560 Atomic Absorption Spectrophotometer and an IL 951 AA/AE Spectrophotometer have been used. All instrumental conditions have been chosen according to the manufacturer's instructions.

Cold vapour and hydride generation accessories were studied and built in our laboratory.

2.3 Analytical methodologies

2.3.1 Sample decomposition

The determination of total mercury and selenium requires a complete mineralization of organic matter. After homogenization, a portion of the sample (4-6 g) exactly weighed was placed in a Pyrex round-bottomed flask which was supplied with a condenser, with 90% nitric acid added to the sample in the proportion of 3:1 (v/w), and left to pre-digest at room temperature as long as possible (overnight). When freeze-dried samples were analyzed, they were re-hydrated with deionized water before the addition of acid. The samples were then slowly heated to boiling point and left boiling for about 3 hours. Several mls of deionized water were added and the mixture was kept boiling until the disappearance of the red vapours. It was then allowed to cool and the solution was brought to volume in a volumetric flask, after having carefully washed the flask and the condenser.

2.3.2 Total mercury determination

For the determination of the total mercury a portion of the solution was placed in an aeration flask, then 1.0 ml of reducing solution was added. This solution contains 5 ml of 96% sulfuric acid, 3 g hydroxylammonium chloride, 3 g sodium chloride, and 5 g tin(II) chloride-2-hydrate.

A stream of air at 0.3 min^{-1} was used to strip the mercury from the aeration flask and to transfer it into a cell, with quartz windows, placed across the AAS beam. Peak height readings were taken.

2.3.3 Selenium determination

For the selenium determination a portion of the solution was diluted with the addition of 37% hydrochloric acid in order to reduce to Se(IV) the Se(VI) which might have been present. The amount of hydrochloric acid was calculated in order to obtain a 5 N final concentration. The standard solutions were also made to 5 N hydrochloric acid.

A portion of the solution to be analyzed was placed in the aeration flask. After degassing with nitrogen, 2.0 ml of 2.5% (w/v) sodium borohydride in 2% (w/v) sodium hydroxide solution were added. The selenium hydride formed was then transferred by a stream of nitrogen at 3.0 min^{-1} into a quartz cell, electrically heated at 850-900°C, and placed across the AAS beam. Peak height readings were taken.

2.3.4 Organic mercury determination

For the determination of organic mercury a portion of the sample (4-6 g) was placed into a 100 ml polyethylene tube with a pressure cap, and mixed with deionized water (10 ml), 47% hydrobromic acid (7 ml), and toluene (35.0 ml). The sample was shaken manually for 5 min. and centrifuged. A portion of the toluene (25.0 ml) was transferred into another 100 ml polyethylene tube and 7.0 ml of an aqueous solution of L-cysteine (1.00 g of L-cysteine hydrochloride monohydrate, 0.775 g of

sodium acetate trihydrate and 12.5 g of anhydrous sodium sulfate in 100 ml) were added. After shaking (5 min.) and centrifuging (antifoam agent can be added if any foam appears) a portion of the aqueous phase (5.0 ml) was transferred into a 25 ml volumetric flask and diluted with deionized water.

A portion of this solution was placed in the aeration flask and 1 ml of 16 N sulfuric acid, 1.0 ml reducing solution (50% (w/v) tin(II) chloride-2-hydrate, 10% (w/v) cadmium chloride monohydrate solution), and 3.0 mL of 45% (w/v) sodium hydroxide solution were added in order to reduce the mercury to the metallic form.

The operation conditions were the same as reported for the total mercury determination (section 2.3.2).

This analytical procedure allows the determination of organic mercury, but papers in literature (MacCrehan and Durst, 1978; Holak, 1982) reported that in fish the organic mercury is present mainly as methylmercury.

2.3.5 Fresh weight/dry weight ratio

For all samples analyzed the fresh weight/dry weight ratio has been determined by drying a portion of the homogenate (1-2 g) in the oven at 105°C until a constant weight was reached.

2.4 Quality control

On the basis of repeated analyses carried out on Standard Reference Materials (SRM) samples obtained from the U.S. National Bureau of Standards (NBS), and from the International Atomic Energy Agency (IAEA/Monaco), precision and accuracy of the methods were checked (Table I).

Unfortunately the lack of reference samples for the organic mercury makes it difficult to estimate the accuracy of the organic mercury determination. However the method used has been compared with different analytical methods used in other laboratories with good results (personal communications).

Participation to intercalibration exercises organized by IAEA/Monaco assured comparability of analytical procedures used with Reference Methods issued by UNEP/FAO/IAEA/IOC (1984b).

Samples have been analyzed in duplicate and results reported are the mean values of the two determinations.

3. RESULTS

The analyses described have been carried out on 41 specimens of European hake, 36 specimens of Norway lobster, 15 specimens of Bogue, and 18 specimens of Atlantic mackerel. The results obtained are reported respectively in Tables II-V.

For each species the correlations matrix has been calculated and results obtained are reported in Tables VI-IX.

Table I

Comparison of results obtained with those certified by U.S. NBS or with results of IAEA/Monaco intercalibration exercises. Results are reported in $\mu g\ g^{-1}$ dry weight. Number of samples is between brackets.

Monaco MA-A-2 Fish homogenate		
	own result	IAEA results
Hg (12)	0.53 ± 0.04	0.49 ± 0.02
IAEA/Monaco MA-M-2/TM Mussel tissue homogenate		
	own result	consensus values
Hg (3)	0.94 ± 0.04	0.95(0.85-1.06)
Se (3)	2.0 ± 0.2	2.27(1.70-2.56)
NBS SRM 1566 Oyster tissue		
	own result	certified values
Hg (6)	0.052± 0.014	0.057± 0.015
Se (13)	1.8 ± 0.2	2.1 ± 0.5
NBS SRM 1577a BOVINE LIVER		
	own result	certified value
Se (4)	0.70 ± 0.02	0.71 ± 0.07

For each species a scatter plot of most relevant data are shown Figures 1-4. These plots are aligned so that the same variable appears on the X axis in any given column, and the same variable on the Y axis in any given row.

Comparison of distributions for Hg-o, Hg-t, Hg-o%, and Se between different species are given in Figures 5-8. In these graphical displays a box is shown for each species, around the central 50 percent of the data values, a central line at the median, and whiskers out to the extreme. Individual points beyond 1.5 times the box length (interquartile range) are plotted as individual values. A notch is added to each box giving an approximate confidence interval for the median. The notches are scaled so that any pair of boxes with vertically non- overlapping notches would show a significant difference between medians at the 5 percent level of significance. The width of the box is scaled proportionally to the square root of the number of values in each group.

4. DISCUSSION

Tables VI-IX and Figures 1-4 show significant correlations between mercury (both total and organic) and size (both weight and length) of the specimens for all species studied.

No correlation has been observed between size and selenium content, except for Norway lobster. The same is true for correlations between selenium and mercury (both total and organic) contents.

Table II

Concentration (in $\mu g\ g^{-1}$ fresh weight) of mercury (total and organic) and selenium in European hake (Merluccius merluccius). Sample code (SC), standard length in cm (SL), total length in cm (TL), weight in g (W), fresh weight/dry weight ratio (FW/DW), and percentage of organic mercury (HGO) are reported.

SC	SL	TL	W	FW/DW	HGO	HGT	HGO%	SE
N01	14	18	41	5.59	0.040	0.093	43	0.142
N04	20	24	97	5.49	0.036	0.078	46	0.116
N05	28	33	243	4.94	0.100	0.207	48	0.100
N06	23	27	155	4.96	0.058	0.106	55	0.142
N07	21	24	112	5.22	0.037	0.080	46	0.140
N08	38	44	710	4.90	0.143	0.191	75	0.198
N09	33	38	422	5.14	0.314	0.334	94	0.138
N10	25	29	204	5.06	0.053	0.120	44	0.137
N11	21	25	108	4.91	0.083	0.100	83	0.207
N12	15	17	31	5.37	0.154	0.171	90	0.150
N15	42	50	800	5.03	1.033	1.195	86	0.142
N16	38	41	537	4.95	0.230	0.280	82	0.152
N17	35	38	462	4.76	0.100	0.110	91	0.130
N18	12	15	20	5.16	0.020	0.107	19	0.164
N19	42	48	658	5.19	0.434	0.918	47	0.106
N20	46	52	1148	5.43	0.394	0.702	56	0.080
N21	38	45	684	5.25	0.382	0.492	78	0.174
N22	22	27	115	4.78	0.138	0.190	73	0.122
N23	29	35	270	4.87	0.092	0.206	45	0.118
N24	17	21	46	5.40	0.074	0.360	21	0.135
N25	16	19	49	5.36	0.116	0.194	60	0.135
N26	13	16	25	5.18	0.077	0.114	68	0.186
N27	20	23	82	5.24	0.339	0.824	41	0.108
N29	40	45	449	4.70	0.169	0.223	76	0.154
N30	27	32	214	5.27	0.163	0.250	65	0.101
N31	19	23	85	4.98	0.096	0.107	90	0.111
N32	26	31	207	5.01	0.077	0.126	61	0.154
N33	19	23	66	5.43	0.116	0.139	83	0.103
N34	25	29	173	4.71	0.088	0.141	62	0.190
N35	32	40	414	4.73	0.283	0.559	51	0.111
N36	6	8	3	5.39	0.193	0.255	76	0.209
N37	5	6	2	5.06	0.209	0.314	67	0.226
N38	17	21	58	5.32	0.213	0.242	88	0.090
N39	11	13	14	5.14	0.146	0.200	73	0.161
N41	38	44	696	4.90	0.212	0.364	58	0.126
N42	36	42	486	5.12	0.350	0.494	71	0.112
N43	23	27	143	4.87	0.134	0.140	96	0.104
N44	17	21	71	4.84	0.088	0.115	77	0.137
N45	14	17	28	4.77	0.130	0.138	94	0.275
N46	11	14	15	4.91	0.150	0.175	86	0.33

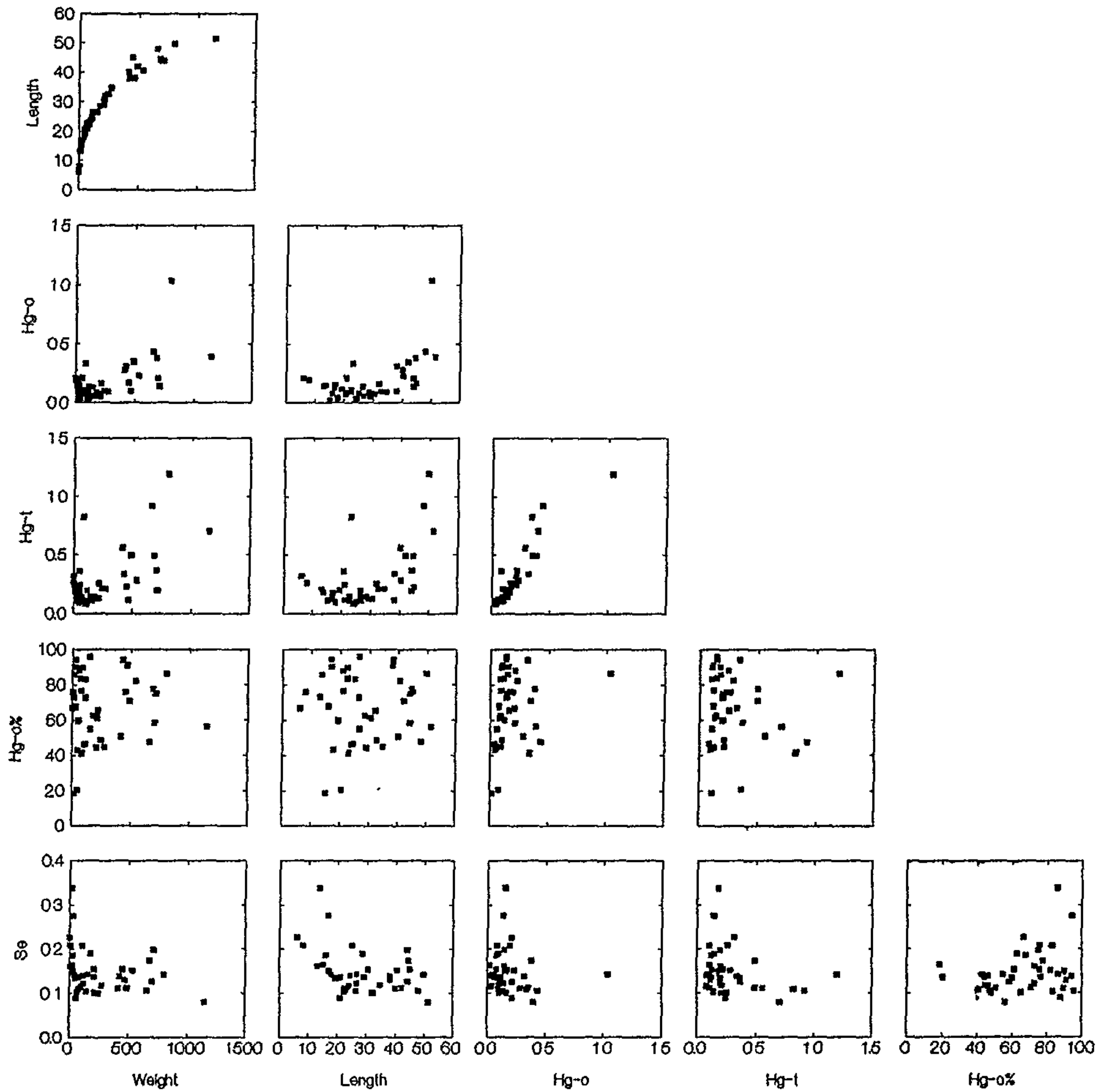

Fig. 1 Scatter plot of data obtained for European hake (<u>Merluccius merluccius</u>). This plot is aligned so that the same variable appears on the X axis in any given column, and the same variable on the Y axis in any given row. Concentrations are in $\mu g\ g^{-1}$ fresh weight, weight in g, and standard length in cm

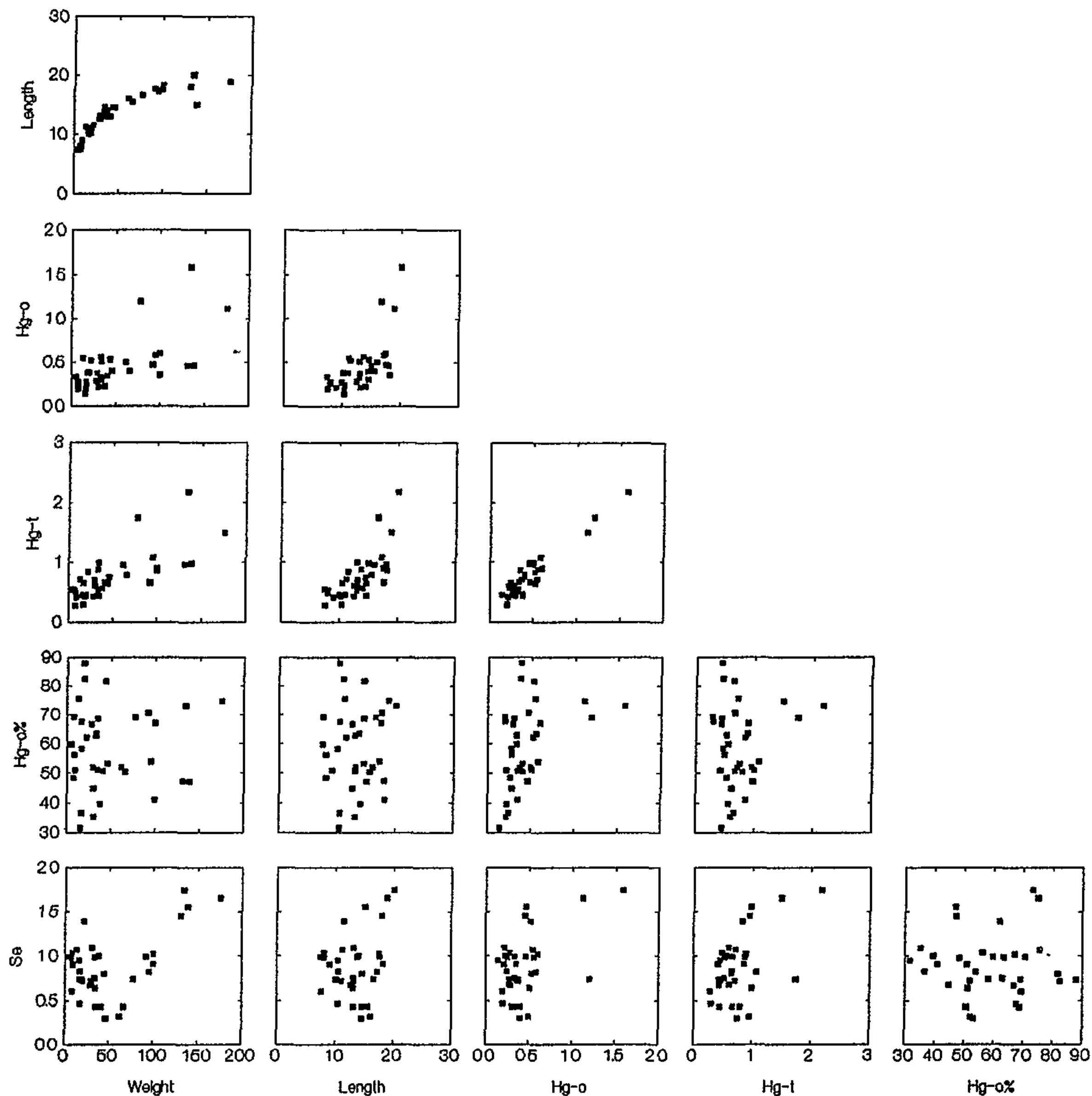

Fig. 2 Scatter plot of data obtained for Norway lobster (Nephrops norvegicus). This plot is aligned so that the same variable appears on the X axis in any given column, and the same variable on the Y axis in any given row. Concentrations are in $\mu g\ g^{-1}$ fresh weight, weight in g, and Rostrum-Uropod length in cm

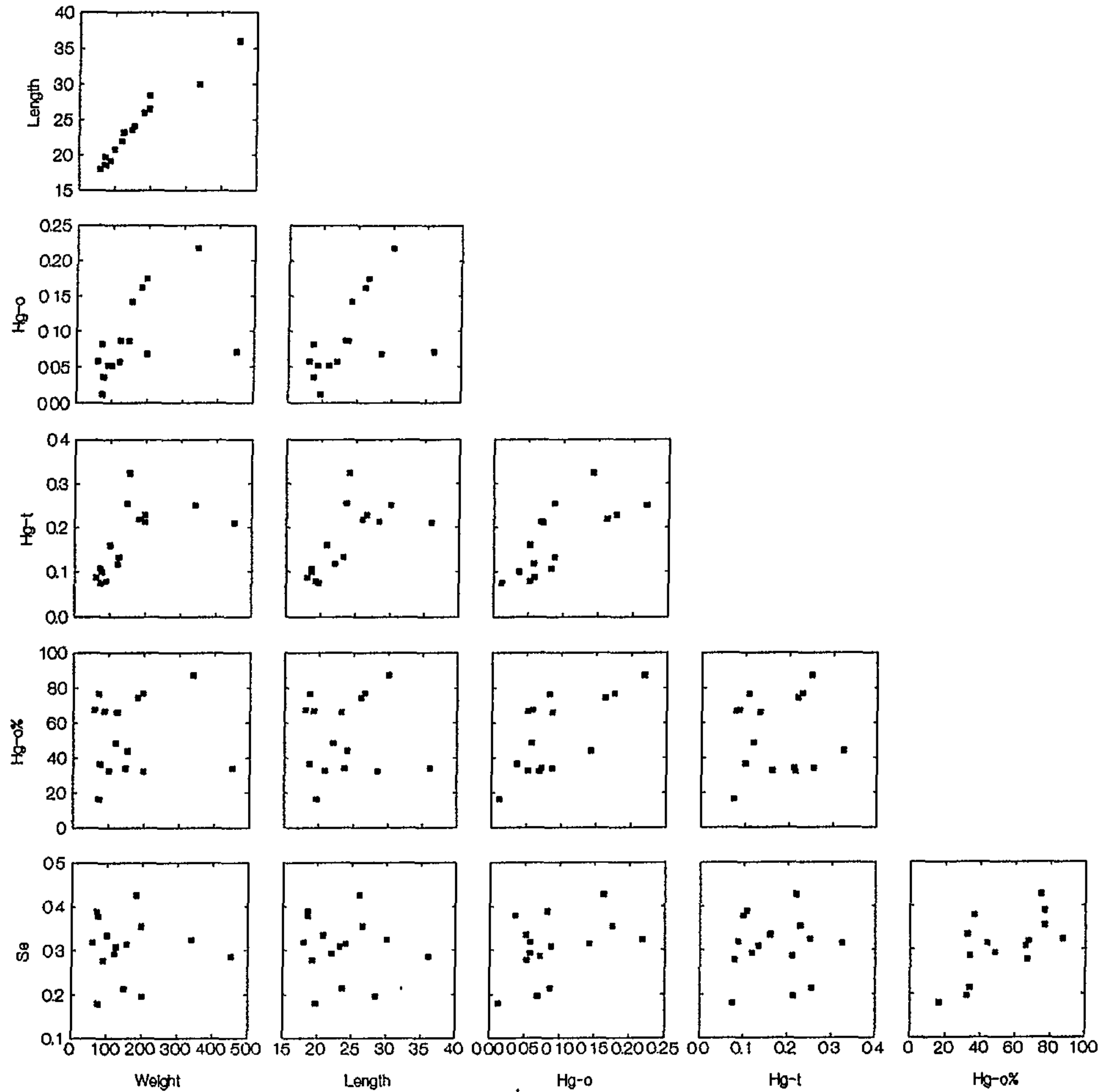

Fig. 3 Scatter plot of data obtained for Bogue (<u>Boops boops</u>). This plot is aligned so that the same variable appears on the X axis in any given column, and the same variable on the Y axis in any given row. Concentrations are in $\mu g\ g^{-1}$ fresh weight, weight in g, and standard length in cm

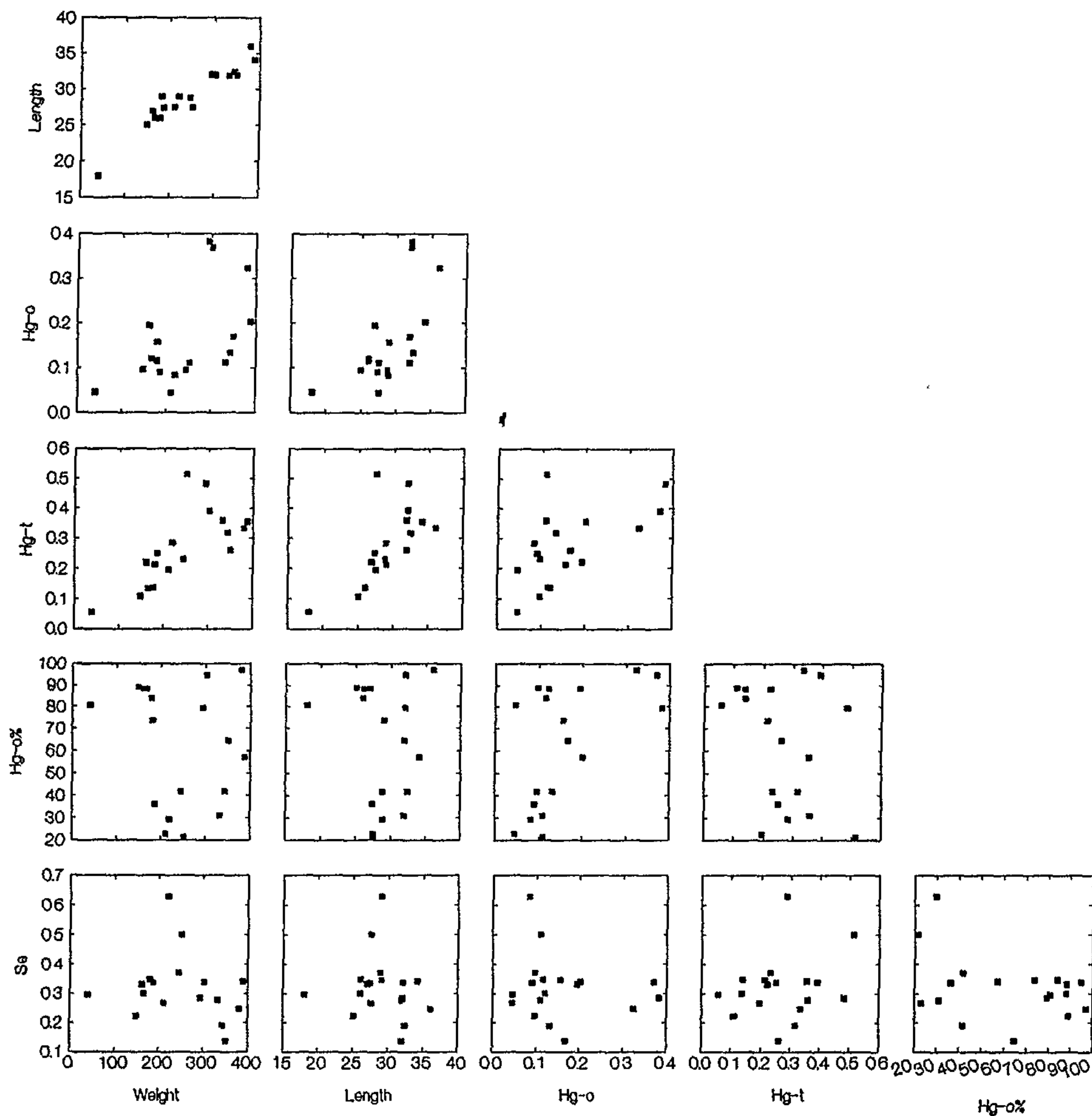

Fig. 4 Scatter plot of data obtained for Atlantic mackerel (Scomber scombrus). This plot is aligned so that the same variable appears on the X axis in any given column, and the same variable on the Y axis in any given row. Concentrations are in $\mu g\ g^{-1}$ fresh weight, weight in g, and standard length in cm

Table III

Concentration (in $\mu g\ g^{-1}$ fresh weight) of mercury (total and organic) and selenium in Norway lobster (*Nephrops norvegicus*). Sample code (SC), Carapace length in cm (CL), Rostrum-Uropod length in cm (RUL), weight in g (W), fresh weight/dry weight ratio (FW/DW), a percentage of organic mercury (HGO) are reported.

SC	CL	RUP	W	FW/DW	HGO	HGT	HGO%	SE
S090	6	20	134	5.10	1.585	2.170	73	1.740
S091	5	17	78	4.16	1.200	1.741	69	0.740
S092	4	15	44	4.78	0.528	0.647	82	0.800
S093	6	19	175	4.54	1.116	1.493	75	1.650
S094	3	10	19	4.93	0.379	0.431	88	0.730
S095	3	11	20	4.63	0.378	0.459	82	0.720
S096	6	15	138	4.78	0.460	0.978	47	1.550
S097	6	18	131	4.30	0.456	0.965	47	1.450
S098	6	18	100	4.21	0.352	0.860	41	0.910
S099	5	16	66	4.97	0.399	0.790	51	0.430
S100	4	15	46	4.79	0.398	0.750	53	0.300
S101	4	13	41	4.34	0.340	0.670	51	0.430
S102	2	7	5	4.77	0.330	0.552	60	0.990
S103	3	10	16	4.66	0.271	0.466	58	0.740
S104	5	16	62	4.28	0.496	0.954	52	0.320
S105	4	13	34	4.44	0.508	0.993	51	0.640
S106	5	17	95	4.06	0.578	1.073	54	0.820
S107	4	13	30	4.66	0.274	0.612	45	0.680
S108	4	14	38	5.15	0.224	0.566	40	1.000
S109	4	13	31	4.81	0.214	0.608	35	1.090
S110	3	10	16	4.90	0.142	0.451	31	0.950
S111	4	15	35	4.66	0.302	0.440	69	0.430
S112	3	10	17	5.43	0.200	0.296	68	0.470
S113	5	18	100	5.01	0.601	0.896	67	1.020
S114	5	18	92	4.56	0.467	0.662	71	0.990
S115	4	13	33	5.12	0.329	0.524	63	0.750
S116	3	9	9	5.22	0.213	0.418	51	0.905
S117	3	8	8	4.56	0.275	0.489	56	1.038
S118	2	8	7	4.85	0.257	0.531	48	0.976
S119	3	11	17	4.24	0.241	0.659	37	0.826
S120	4	14	33	4.66	0.557	0.878	63	0.986
S121	4	12	22	4.61	0.520	0.838	62	1.391
S122	2	8	8	4.60	0.196	0.283	69	0.604
S123	4	11	13	4.73	0.545	0.722	75	1.072
S124	4	13	29	4.76	0.369	0.711	52	0.721
S125	4	13	28	4.81	0.290	0.435	67	0.669

Table IV

Concentration (in $\mu g\ g^{-1}$ fresh weight) of mercury (total and organic) and selenium in Bogue (Boops boops). Sample code (SC), standard length in cm (SL), total length in cm (TL), weight in g (W), fresh weight/dry weight ratio (FW/DW), and percentage of organic mercury (HGO) are reported.

SC	SL	TL	W	FW/DW	HGO	HGT	HGO%	SE
B01	23	28	199	4.57	0.068	0.212	32	0.196
B02	17	20	74	4.13	0.012	0.074	16	0.180
B03	17	19	88	4.17	0.052	0.078	67	0.278
B05	18	21	101	4.52	0.052	0.160	33	0.334
B06	16	19	76	4.47	0.036	0.099	36	0.378
B07	19	23	125	4.44	0.087	0.132	66	0.308
B08	21	24	155	4.38	0.142	0.324	44	0.314
B09	19	24	148	4.24	0.086	0.254	34	0.214
B10	26	30	340	3.85	0.218	0.250	87	0.324
B11	23	27	197	4.04	0.175	0.228	77	0.354
B12	22	26	182	4.12	0.162	0.218	74	0.426
B13	15	18	59	4.09	0.058	0.086	67	0.318
B14	16	19	71	3.95	0.082	0.107	77	0.388
B15	19	22	120	4.61	0.057	0.118	48	0.292
B16	28	36	451	4.19	0.071	0.210	34	0.286

Table V

Concentration (in $\mu g\ g^{-1}$ fresh weight) of mercury (total and organic) and selenium in Atlantic mackerel (Scomber scombrus). Sample code (SC), standard length in cm (SL), total length in cm (TL), weight in g (W), fresh weight/dry weight ration (FW/DW), and percentage of organic mercury (HGO) are reported.

SC	SL	TL	W	FW/DW	HGO	HGT	HGO%	SE
SG01	30	34	389	2.09	0.202	0.354	57	0.341
SG02	28	32	350	2.49	0.167	0.259	64	0.137
SG03	29	32	343	2.73	0.132	0.317	42	0.190
SG04	28	32	332	2.66	0.110	0.358	31	0.276
SG06	24	28	251	3.25	0.110	0.514	21	0.500
SG07	24	28	210	3.07	0.044	0.194	23	0.266
SG08	25	29	244	3.85	0.096	0.231	42	0.370
SG09	24	27	186	4.32	0.090	0.250	36	0.336
SG10	23	27	161	4.24	0.194	0.220	88	0.331
SG11	25	29	219	4.14	0.083	0.284	29	0.628
SG12	28	32	301	3.17	0.370	0.391	95	0.338
SG13	31	36	380	2.97	0.323	0.333	97	0.246
SG14	28	32	293	2.82	0.382	0.482	79	0.284
SG15	25	29	180	4.14	0.156	0.212	74	0.346
SG16	16	18	41	4.15	0.046	0.057	81	0.295
SG17	23	26	166	4.10	0.120	0.136	88	0.299
SG18	22	25	148	4.17	0.096	0.108	89	0.222
SG19	23	26	178	4.05	0.115	0.137	84	0.347

Table VI

Correlation matrix for European hake (Merluccius merluccius). The correlation coefficient is significant (41 samples) at a 95% level of confidence when is greater than 0.308.

	SL	TL	W	FWDW	HGO	HGT	HGO%	SE
SL	1.000							
TL	0.997	1.000						
W	0.924	0.921	1.000					
FWDW	-0.262	-0.255	-0.126	1.000				
HGO	0.523	0.540	0.622	0.030	1.000			
HGT	0.524	0.543	0.616	0.109	0.910	1.000		
HGO%	0.050	0.037	0.060	0.286	0.224	0.085	1.000	
SE	0.393	0.406	0.264	0.256	0.122	0.233	0.243	1.000

Table VII

Correlation matrix for Norway lobster (Nephrops norvegicus). The correlation coefficient is significant (36 samples) at a 95% level of confidence when is greater than 0.329.

	CL	RUL	W	FWDW	HGO	HGT	HGO%	SE
CL	1.000							
RUL	0.959	1.000						
W	0.933	0.875	1.000					
FWDW	-0.235	-0.265	-0.249	1.000				
HGO	0.598	0.643	0.661	-0.158	1.000			
HGT	0.686	0.721	0.736	-0.286	0.943	1.000		
HGO%	0.031	0.054	0.059	0.141	0.416	0.116	1.000	
SE	0.400	0.279	0.539	0.046	0.483	0.497	0.023	1.000

Table VIII

Correlation matrix for Bogue (Boops boops). The correlation coefficient is significant (15 samples) at a 95% level of confidence when is greater than 0.514.

	SL	TL	W	FWDW	HGO	HGT	HGO%	SE
SL	1.000							
TL	0.991	1.000						
W	0.965	0.967	1.000					
FWDW	-0.171	-0.120	-0.242	1.000				
HGO	0.575	0.501	0.489	-0.491	1.000			
HGT	0.674	0.658	0.577	-0.057	0.717	1.000		
HGO%	0.082	0.015	0.071	-0.596	0.675	0.050	1.000	
SE	-0.048	-0.103	-0.040	-0.245	0.427	0.024	0.630	1.000

Table IX

Correlation matrix for Atlantic mackerel (*Scomber scombrus*). The correlation coefficient is significant (18 samples) at a 95% level of confidence when is greater than 0.468.

	SL	TL	W	FWDW	HGO	HGT	HGO%	SE
SL	1.000							
TL	0.999	1.000						
W	0.955	0.951	1.000					
FWDW	-0.733	-0.726	-0.869	1.000				
HGO	0.624	0.630	0.531	-0.408	1.000			
HGT	0.684	0.684	0.707	-0.622	0.577	1.000		
HGO%	-0.075	-0.067	-0.166	0.203	0.543	-0.301	1.000	
SE	-0.165	-0.156	-0.213	0.368	-0.171	0.225	-0.363	1.000

Good correlations exist between total mercury and organic mercury for all species, especially for European hake and Norway lobster.

Mercury content in Norway lobster has been already determined during MED POL in the period 1976-1980, obtaining an average value (89 samples) of 0.68 $\mu g\ g^{-1}$ fresh weight. A comparison of data obtained during this study in specimens weighing 31.0-65.3 g (in order to compare specimens of the same size) show excellent agreement (average value 0.71 $\mu g\ g^{-1}$ fresh weight, 9 samples).

Comparison between species studied (Figs 5-8) show the Norway lobster has mercury (both total and organic) and selenium contents higher then the other species, and European hake a lower selenium content.

As far as the percentage of organic mercury is concerned no statistically relevant differences are detectable (Fig. 7).

5. ACKNOWLEDGEMENTS

This study has been supported by FAO(GFCM), "Ministero della Pubblica Istruzione", and "Consiglio Nazionale delle Ricerche".

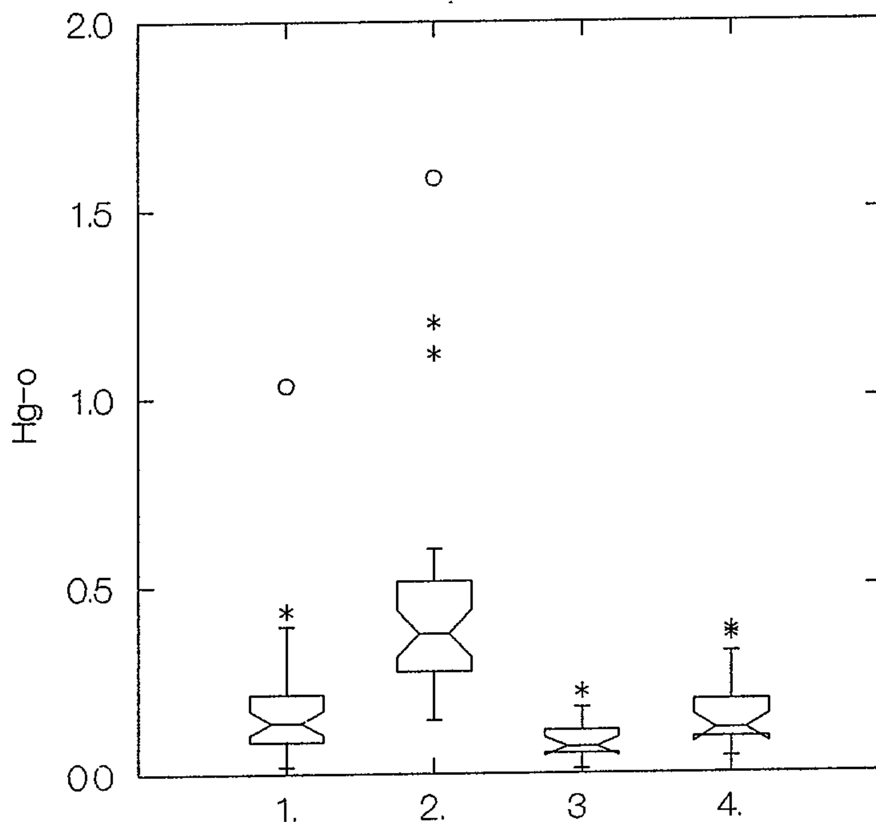

Fig. 5 Comparison of distributions for organic mercury concentration (in $\mu g\ g^{-1}$ FW) between the different species. 1 = European hake (Merluccius merluccius); 2 = Norway lobster (Nephrops norvegicus); 3 = Bogue (Boops boops); 4 = Atlantic mackerel (Scomber scombrus). For explanation of the figure see text (3. RESULTS)

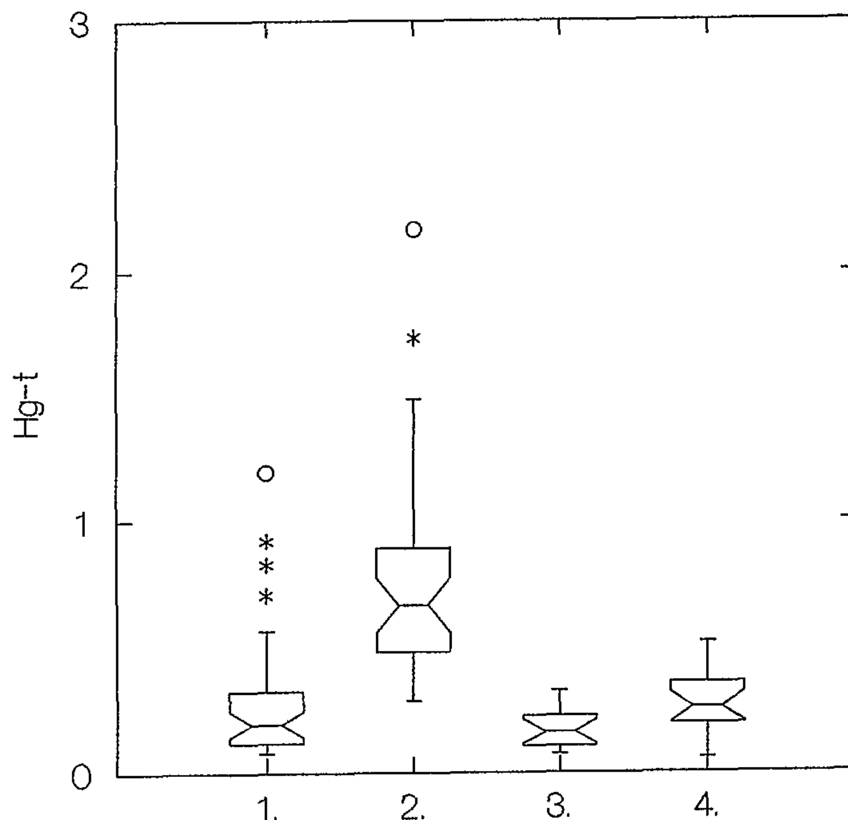

Fig. 6 Comparison of distributions for total mercury concentration (in $\mu g\ g^{-1}$ FW) between the different species. 1 = European hake (Merluccius merluccius); 2 = Norway lobster (Nephrops norvegicus); 3 = Bogue (Boops boops); 4 = Atlantic mackerel (Scomber scombrus). For explanation of the figure see text (3. RESULTS)

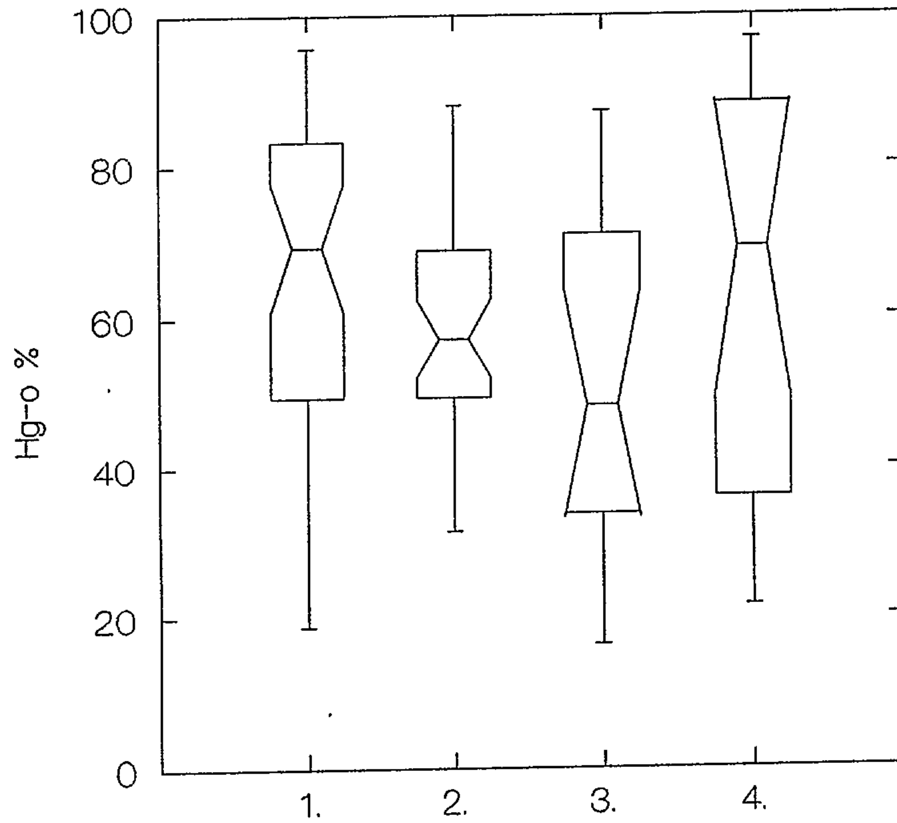

Fig. 7 Comparison of distributions for the percentage of organic mercury (ratios between organic and total mercury) between the different species. 1 = European hake (Merluccius merluccius); 2 = Norway lobster (Nephrops norvegicus); 3 = Bogue (Boops boops); 4 = Atlantic mackerel (Scomber scombrus). For explanation of the figure see text (3. RESULTS)

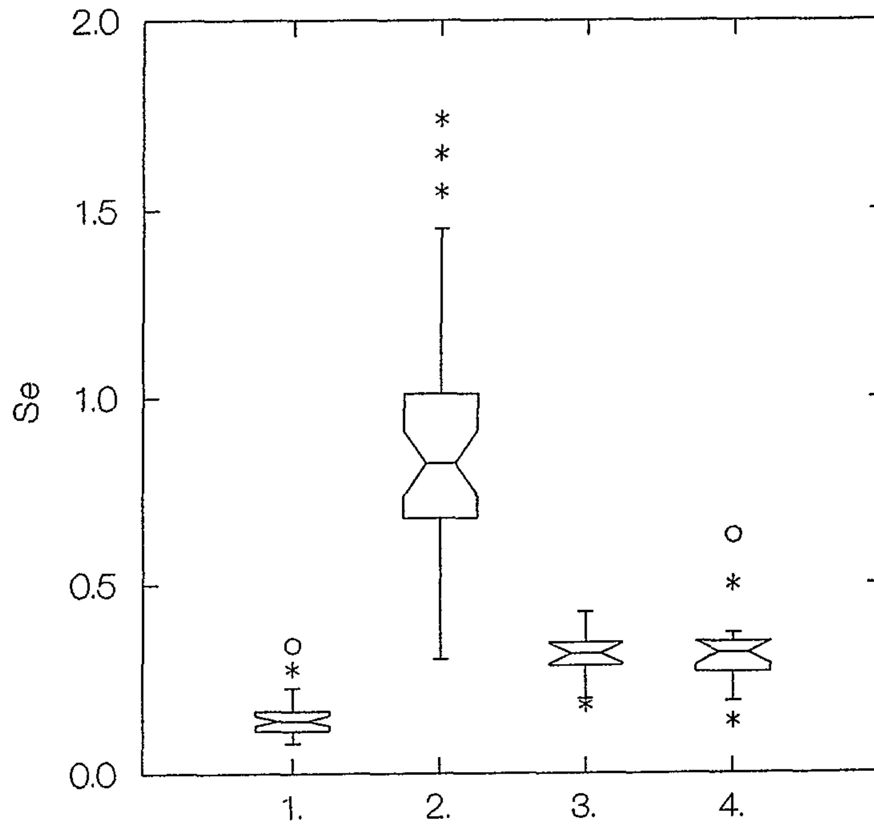

Fig. 8 Comparison of distributions for selenium concentration (in μg g^{-1} FW) between the different species. 1 = European hake (Merluccius merluccius); 2 = Norway lobster (Nephrops norvegicus); 3 = Bogue (Boops boops); 4 = Atlantic mackerel (Scomber scombrus). For explanation of the figure see text (3. RESULTS)

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BIOACCUMULATION OF MERCURY AND ITS DISTRIBUTION IN VARIOUS ORGANS OF SOME SHORE FISHES OFF THE MEDITERRANEAN SEA COAST (ISRAEL)

by

H. HORNUNG

Israel Oceanographic & Limnological Research
National Institute of Oceanography
P.O. Box 8030, Haifa 31080, Israel

ABSTRACT

Total mercury concentrations were determined in tissues and organs of *Epinephelus alexandrinus*, *Epinephelus guaza*, *Pagrus ehrenbergii*, *Oblada melanura* and *Diplodus sargus* obtained from polluted and unpolluted areas along the Mediterranean coastline of Israel, during 1981-1985. These inshore species, which are commercially important, are most abundant at depths of 5-20m.

Out of 10 different tissues and organs of *D. sargus*, mercury concentrations were higher in specimens from the polluted (Haifa Bay) area than in specimens from the unpolluted (Zarqa, Tel-Shikmona) area. Mercury in the muscle tissue ranged from 0.072 to 1.02 $\mu g\ g^{-1}$ wet wt. (mean: 0.531) in the polluted area, and from 0.059 to 0.212 $\mu g\ g^{-1}$ (mean: 0.123) in the unpolluted one. Highest mercury levels were found in the liver: up to 3.53 $\mu g\ g^{-1}$ (mean: 1.05) in specimens from Haifa Bay and Akko, and up to 0.269 $\mu g\ g^{-1}$ (mean: 0.161) in specimens from Zarqa. The mean mercury concentration in food found in the intestines of specimens from Haifa bay and Akko, was about 8 times higher than the corresponding value for specimens from Zarqa.

The results suggest that mercury accumulation by *D. sargus* is associated with its food and feeding habits, as well as the size.

Additional tissues and organs of *Upeneus moluccensis* taken by trawl were analyzed for mercury content. This species was chosen because of its abundance in trawl fishery and its known affinity for mercury accumulation. A similar trend in the distribution was observed in *D. sargus* (inshore) and *U. moluccensis* (offshore).

1. INTRODUCTION

Previous studies of mercury concentrations in inshore and offshore fish species caught by trawlers from the Mediterranean coast of Israel showed that some species caught within a given area accumulate more mercury in their flesh than others (Levitan *et al.*, 1974; Yannai and Sachs, 1978; Hornung *et al.*, 1980). A preliminary study of inshore fish indicated that species taken from polluted areas of Haifa Bay had higher levels of mercury than the same species taken from south of the bay (Hornung *et al.*, 1984, 1985; Hornung and Cohen, 1986).

To obtain a better understanding of the distribution and accumulation of mercury in fish, various tissues and organs of individual fish specimens were analyzed. The selected species included: Epinephelus alexandrinus, Epinephelus guaza, Pagrus ehrenbergii, Oblada melanura and Diplodus sargus. These species are common throughout the Mediterranean and are among the most important comemrcial inshore species. Out of a number of inshore species of fish collected along the shoreline, D. sargus was considered most suitable for the purpose of monitoring trends because it is benthic, usually confined to shallow coastal waters (5 to 20m depth). It is also one of fish that is likely to have spent a significant proportion of its time in the same area (Ben-Tuvia, 1971).

Simultaneously, the distribution of total mercury in the goat fish, Upeneus moluccensis, taken by trawlers, was investigated. This is also a commercially fished species. It resides in trawling grounds from 10 to 100m depth. U. moluccensis has also been shown previously to accumulate mercury in its tissues (Hornung et al., 1980; Aydogdu et al., 1983).

These surveys were designed to provide information related to the route and bioaccumulation of mercury by the organs and tissues of inshore and offshore species of fish.

2. MATERIALS AND METHODS

2.1 Sampling area

The sampling sites are presented in Figure 1. The Akko and Haifa Bay areas were chosen because of elevated levels of mercury found in nearshore sediments and benthic fauna as well as in a few species of fish examined in a preliminary investigation (Hornung et al., 1984). A chlor-alkali plant, which is situated adjacent to the northern part of the Bay, is the major source of mercury pollution in the area. Zarqa and Caesarea, south of Haifa Bay, were selected as control stations since no industrial pollution occurs there. The Tel-Shikmona sampling site is half-way between the Bay and the control areas.

2.2 Sample collection and preparation

The fish were obtained from catch locations in each geographical area. Inshore fishes were caught mostly with entangling nets along the shore between 3.5 and 18.0m depth. Specimens of U. moluccensis were caught by trawling at depths ranging between 30-75m.

Each fish was measured, weighed, washed, and dissected. Muscle tissue was removed from both sides of each specimen and homogenized. Each organ was carefully removed and placed separately into a clean, pre-washed plastic bag and frozen until analysis.

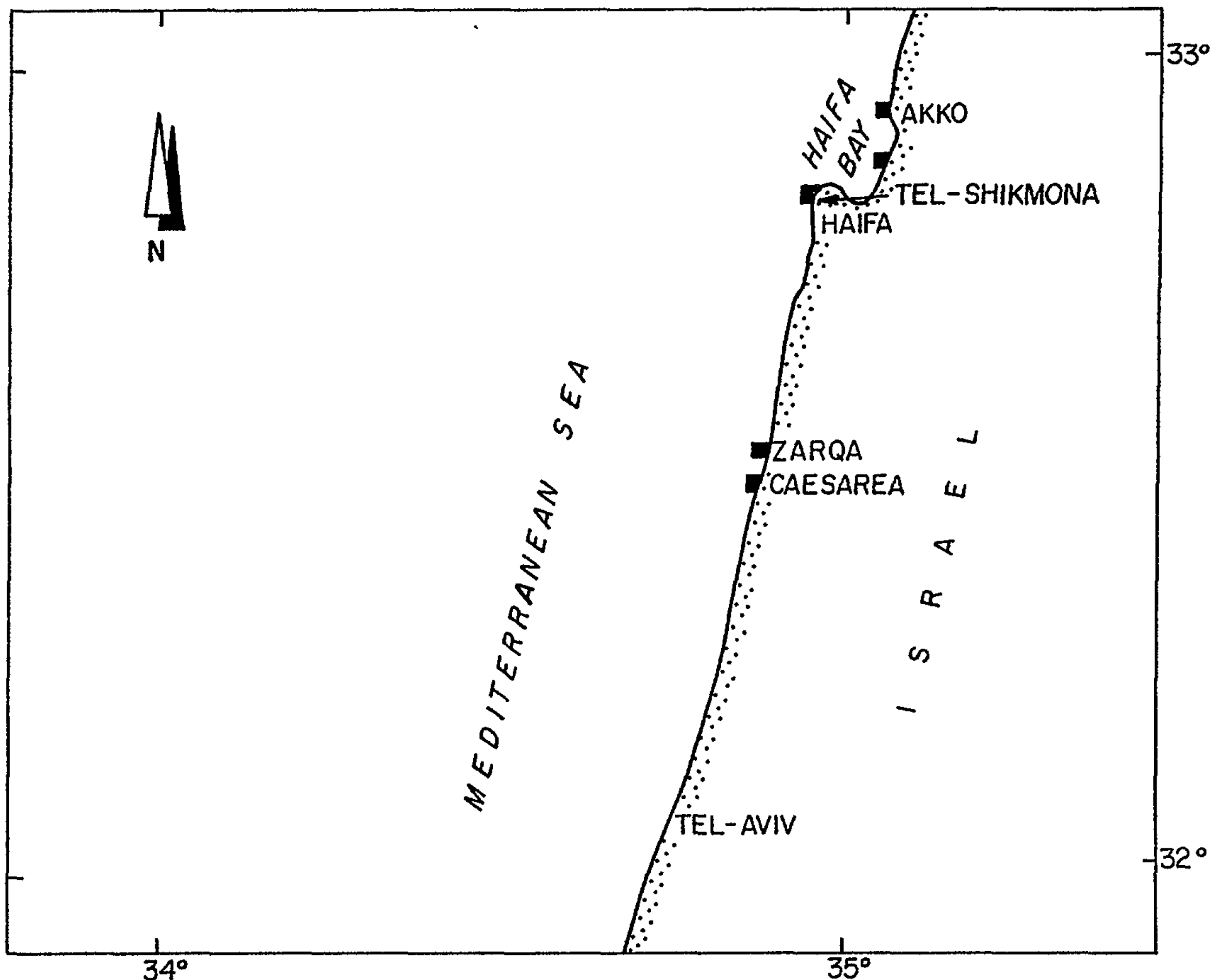

Fig. 1 Map of sampling sites

2.3 Chemical analysis

Aliquots of wet homogenized tissue ranging from 0.3 to 1.0 g were digested with concentrated nitric acid (65%) in Uniseal decomposition vessels for 3 hours at a temperature between 135-140°C, in a preheated oven. The digested samples were cooled, transferred to 50 ml volumetric flasks and diluted to volume with mercury-free distilled water. The ionic mercury was reduced to the elemental form with stannous chloride and the concentration measured on a Coleman Mercury Analyzer, MAS-50A, with a detection limit of 0.005 μg Hg g^{-1} of sample. Estimated precision on replicate samples gave a value of 3.4%. With each batch of samples, a known certified standard (NBS Albacore Tuna) was analyzed. Results of replicate analyses of the reference material were in good agreement with the NBS values (0.932±0.033 μg Hg g^{-1}- 0.95±0.10 NBS value).

All mercury concentrations presented in this study are expressed as μg g^{-1} wet weight of the sample.

3. RESULTS AND DISCUSSION

3.1 Inshore species

Ranges and averages of mercury content in tissues and organs of E. alexandrinus, E. guaza, P. ehrenbergii and O. melanura are given in Table I. The concentrations of mercury in tissues and organs of D. sargus are presented in Tables II-IV, and ranges and averages for the three areas sampled are given in Table V.

3.1.1 Food and feeding habits

The Serranids, Epinephelus alexandrinus and Epinephelus guaza are common in the Mediterranean and inhabit inshore waters. E. alexandrinus forms small shoals in coastal waters over rocky grounds, swimming in mid water or near the bottom. It feeds mainly on fish and cephalopods. E. guaza is a sedentary and solitary species, living among rocks and in caves between 10 and 120m depth, feeding mainly on octopus and other cephalopods but also on fish and crustaceans. Both species are caught occasionally with gill and trammel nets, but mainly taken on lines or spear guns as they frequent areas not easy to net (Smith, 1961).

Pagrus ehrenberghii lives over gravel bottoms in the vicinity of seaweeds and around rocks, down to depths of about 60m. It feeds on fish, crabs and shrimps. The gut contents of the examined species were practically indiscernable but it was possible to find remains of molluscan shells, polychaetes and crustaceans.

Table I

Ranges, averages and standard deviation (based on individual specimens) of total mercury, $\mu g\ g^{-1}$ wet wt. in five species of inshore fishes (Mediterranean, Israel).

Species	No. of speci-mens	Range of total length cm	Range of weights g	Hg concentration		
				Range	Av.	S.D.
Epinephelus alexandrinus	5	27.5-29.1	246.5- 334.3	0.389-0.544	0.449*	0.066
	3	24.6-29.0	174.2- 268.9	0.184-0.205	0.195**	0.011
Epinephelus guaza	4	15.5-30.1	55.8- 501.2	0.640-0.981	0.790*	0.167
	3	34.9-40.7	599.0-1090.0	0.291-0.480	0.366**	0.101
Pagrus ehrenbergii	14	10.3-39.0	18.3- 848.2	0.063-0.585	0.285*	0.155
	7	10.6-40.5	19.4- 896.0	0.043-0.352	0.140**	0.111
Oblada melanura	23	11.4-23.5	19.5- 165.9	0.066-0.805	0.363*	0.167
	6	18.5-23.5	71.6- 155.2	0.064-0.267	0.182**	0.077

* Akko and Haifa Bay areas
** Zarqa and Caesarea

Table II

Distribution of total mercury content ($\mu g\ g^{-1}$ wet wt.) in tissues and organs of *Diplodus sargus*, collected in Haifa Bay (12 April 1984) and Akko shore (14 February 1985) between 6-8m depth.

Total lenght cm	Weight g	Sex	Muscle	Scales	Gills	Skin	Heart	Liver	Skeleton	Stomach walls	Stomach contents	Intestines	Food from intestines	Gonads	Fat
Haifa Bay															
21.0	137.2	F	1.020	0.049	0.169	0.209	0.927	3.527	0.463	0.650	0.455	-	0.962	-	-
20.1	124.8	M	0.745	0.023	0.147	0.140	0.609	1.928	0.291	0.370	-	-	0.455	0.336	-
20.0	119.0	M	0.886	0.049	0.111	0.217	-	1.214	0.277	0.254	0.254	-	0.554	0.254	-
19.7	103.5	F	0.615	0.027	0.099	0.160	-	-	0.217	-	0.321	-	0.423	-	-
19.5	124.5	F	0.544	0.038	0.073	0.113	-	-	0.244	-	0.341	-	0.856	-	-
19.5	104.8	F	0.966	0.047	0.314	0.214	-	-	0.403	-	0.956	-	0.902	-	-
19.1	103.7	F	0.628	0.020	0.153	0.128	-	1.817	0.277	-	-	-	0.574	-	-
19.0	110.0	F	0.821	0.032	0.181	0.119	0.712	1.395	0.352	0.404	-	-	0.426	0.165	-
18.6	102.5	F	0.450	BDL	0.090	0.172	-	-	0.147	-	0.154	-	0.386	-	-
Akko															
22.5	184.3	F	0.675	0.045	0.140	0.066	0.794	0.891	0.166	0.208	0.399	0.435	0.611	0.085	0.203
17.5	89.2	F	0.353	0.023	0.114	0.047	0.360	0.537	0.096	0.105	0.116	-	-	0.103	-
16.2	60.5	F	0.228	0.020	0.049	0.013	-	0.298	0.025	-	0.069	0.025	0.069	-	-
16.0	74.5	M	0.193	0.011	0.057	0.027	0.117	0.174	0.040	0.057	0.056	0.083	0.056	0.057	-
16.0	72.8	M	0.159	0.011	0.074	0.046	0.377	0.344	0.049	0.105	0.067	0.075	0.039	0.066	-
15.5	56.4	M	0.142	0.019	0.036	0.017	0.349	0.257	0.029	0.087	0.078	0.093	0.064	0.036	-
14.5	56.0	F	0.072	0.023	0.039	0.031	0.198	0.238	0.027	0.053	0.032	0.051	0.032	0.112	-

<u>Table III</u>

Distribution of total mercury content (µg g^{-1}) wet wt.) in tissues and organs of <u>Diplodus sargus</u>, collected from Tel-Shikmona shore between 6-8m depth during April 4, 1985 and June 23, 1985.

	Total lenght cm	Weight g	Sex	Muscle	Scales	Gills	Skin	Heart	Liver	Kidney	Skele -ton	Intes- tine	Food from intes- tine	Gonads
Apr	25.0	259.5	M	0.248	0.007	0.053	0.080	0.577	0.206	0.683	0.111	0.067	0.016	-
	24.0	194.0	F	0.430	0.014	0.084	0.170	0.278	0.379	0.792	0.245	0.103	0.069	0.028
	23.5	218.4	F	0.502	0.012	0.035	0.141	0.295	0.336	0.670	0.226	0.100	0.068	0.052
	23.5	205.0	M	0.280	0.007	0.018	0.033	0.175	0.210	0.445	0.143	0.090	0.041	0.011
	22.0	140.5	F	0.170	0.009	0.030	0.043	-	0.238	0.381	0.122	-	-	-
	21,3	125.8	M	0.138	-	-	-	-	-	-	-	-	-	-
	18.5	96.5	F	0.197	0.013	0.025	0.122	-	-	0.583	0.150	0.126	0.046	0.048
	17.8	81.7	F	0.127	BDL*	0.020	0.031	0.206	0.109	0.300	0.067	0.033	0.016	0.028
	16.5	64.5	M	0.137	0.014	0.016	0.027	0.189	0.124	0.329	0.041	0.044	0.028	-
23 Jun	23.0	182.5	F	0.382	0.024	0.092	0.126	0.363	-	0.842	0.300	0.086	0.076	0.076
	22.5	163.4	M	0.215	0.007	0.022	0.007	0.111	0.130	0.631	0.037	0.060	0.044	0.080
	22.0	183.0	M	0.186	0.011	0.018	0.025	0.100	0.230	0.755	0.050	0.064	0.036	0.036
	21.5	142.0	F	0.184	0.014	0.023	0.034	0.175	0.233	-	0.071	0.101	0.047	0.027
	21.0	150.5	F	0.140	0.009	0.011	0.026	0.221	0.121	0.689	0.054	0.062	0.061	0.036
	17.0	81.5	F	0.158	0.017	0.036	0.049	0.235	0.162	0.357	0.068	0.045	0.031	0.104
	15.0	46.0	F	0.104	0.007	0.021	0.041	0.155	0.161	0.668	0.051	-	-	0.047

* Below Detection Limit

Table IV

Distribution of total mercury content (μg g^{-1}) wet wt.) in tissues and organs of *Diplodus sargus*, collected from Zarqa shore during August 21, 1984 at 7m depth.

Total length cm	Weight g	Sex	Muscle	Scales	Gills	Skin	Heart	Liver	Skele-ton	Stomach walls	Stomach contents	Intes-tines	Food from intes-tines	Gonads	Fat
20.0	120.5	M	0.170	0.015	0.039	0.071	-	-	0.085	-	-	0.069	0.092	-	-
19.6	119.4	F	0.010	BDL	0.009	0.048	BDL	0.189	0.041	-	-	0.025	0.073	BDL	-
19.5	110.0	F	0.168	BDL	BDL	BDL	-	0.033	0.009	BDL	-	0.023	0.026	0.038	0.007
19.0	104.0	M	0.147	BDL	0.022	0.039	0.222	0.240	0.184	-	-	0.102	0.133	-	-
18.5	95.8	F	0.150	BDL	0.011	0.005	-	0.229	0.064	BDL	BDL	0.024	-	BDL	-
18.5	95.6	F	0.203	BDL	0.020	0.017	-	0.209	0.098	0.039	-	0.032	0.015	0.103	-
18.0	86.8	F	0.186	0.012	0.066	0.050	0.232	0.269	0.078	-	-	-	-	0.178	-
18.0	85.6	M	0.212	BDL	0.028	0.038	-	0.258	0.092	0.060	-	0.049	-	-	-
17.0	85.5	M	0.117	BDL	0.036	0.037	0.316	0.237	0.090	-	-	0.072	-	-	0.020
16.5	74.0	F	0.075	BDL	0.019	0.008	-	BDL	BDL	0.050	-	BDL	0.043	-	-
16.0	67.5	F	0.079	BDL	0.015	0.031	-	-	BDL	-	-	-	0.041	-	-
16.0	63.0	M	0.155	BDL	0.025	0.056	BDL	0.204	0.062	0.047	-	0.030	0.048	-	-
15.6	66.5	F	0.091	0.016	0.043	0.047	0.126	0.176	0.047	-	-	0.067	-	0.046	-

Table IV. page 2

Total length cm	Weight g	Sex	Muscle	Scales	Gills	Skin	Heart	Liver	Skele -ton	Stomach walls	Stomach contents	Intes- tines	Food from intes- tines	Gonads	Fat
15.5	65.6	M	0.059	BDL	0.033	0.027	-	BDL	BDL	-	-	BDL	0.048	-	-
15.5	61.0	M	0.095	BDL	BDL	0.007	-	-	0.019	0.031	-	0.045	0.023	-	-
15.5	60.0	M	0.137	BDL	0.041	0.033	-	-	0.071	-	-	0.061	0.050	-	-
15.5	61.0	F	0.086	BDL	BDL	0.017	BDL	0.153	0.030	0.112	-	-	0.075	-	-
15.5	61.4	M	0.078	BDL	0.020	0.007	0.036	0.114	0.019	-	-	0.052	0.049	-	-
15.5	60.0	F	0.079	BDL	0.007	0.016	BDL	0.090	0.022	-	-	-	0.057	-	-
15.3	62.0	F	0.070	0.035	0.009	0.045	0.033	0.177	0.041	-	-	0.021	0.060	-	0.009

Table V

Ranges, averages and standard deviation (based on individual specimens) of total mercury content (µg g^{-1} wet wt.) in tissues and organs of Diplodus sargus.

Organ	Akko and Haifa Bay				Tel-Shikmona				Zarqa			
	n	Range	Av.	S.D.	n	Range	Av.	S.D.	n	Range	Av.	S.D.
Muscle	16	0.072-1.020	0.531	0.313	16	0.104-0.502	0.225	0.117	20	0.059-0.212	0.123	0.048
Scales	16	*BDL-0.049	0.027	0.015	15	BDL-0.024	0.011	0.006	20	BDL-0.035	0.004	0.009
Gills	16	0.036-0.314	0.115	0.070	15	0.011-0.092	0.034	0.024	20	BDL-0.066	0.022	0.017
Skin	16	0.013-0.217	0.107	0.073	15	0.0007-0.170	0.064	0.051	20	BDL-0.071	0.030	0.020
Heart	9	0.117-0.927	0.494	0.278	13	0.100-0.577	0.237	0.126	10	BDL-0.316	0.096	0.119
Liver	12	0.174-3.527	1.052	1.002	13	0.109-0.379	0.203	0.083	16	BDL-0.269	0.161	0.089
Skeleton	16	0.025-0.463	0.194	0.143	15	0.037-0.300	0.116	0.083	20	BDL-0.184	0.053	0.045
Stomach wall	10	0.053-0.650	0.229	0.194	-	-	-	-	8	BDL-0.112	0.042	0.036
Stomach contents	13	0.032-0.956	0.254	0.256	-	-	-	-	1	BDL	-	-
Intestines	6	0.025-0.435	0.127	0.153	13	0.033-0.126	0.075	0.028	16	BDL-0.012	0.042	0.028
Food from intestines	15	0.032-0.962	0.427	0.324	13	0.016-0.076	0.045	0.020	15	0.015-0.133	0.056	0.030
Gonads	9	0.036-0.336	0.135	0.100	12	0.011-0.104	0.048	0.027	6	BDL-0.178	0.061	0.069
Fat	1	0.203	-	-	-	-	-	-	3	0.007-0.020	0.012	0.007
Kidney	-	-	-	-	14	0.300-0.842	0.580	0.183	-	-	-	-

* Below Detection Limit

Oblada melanura lives in the vicinity of vegetated bottoms in coastal waters, often close to the surface, and feeds on small invertebrates (crustaceans). None of the specimens examined had sand in the gut contents. Most of the specimens had eaten various forms of Crustacea, especially Decapoda. The crustacean remains included cheliped claws of small crabs as well as isopod and amphipod remains. A small number had consumed algae.

Diplodus sargus lives on muddy sand as well as on vegetated bottoms close to the shore and is most abundant between 5 and 20m depth. The gut contents of the specimens examined are given in Table VI. One specimen had about 40% (by volume) sand, while very little or no sand was found in the others. Digested material was found in some specimens. Molluscan shell fragments formed the greater portion of the recognizable food contents, with small amounts of crustacean fragments. Polychaeta bristles, echinoderm spicules and red algae (mostly corallina) fragments were also found in the gut contents.

Table VI

Gut contents (percent occurrence) of three species of sparids from the Mediterranean shore of Israel.

No. of specimens examined	14	24	20
Gut contents	Pagrus ehrenbergii	Oblada melanura	Diplodus sargus
POLYCHAETA	21	4	10
MOLLUSCA	21		
Chitonidae			10
Gastropoda			15
Scaphopoda			8
Bivalvia			25
Cephalopoda		4	
CRUSTACEA	36	46	20
Copepoda		17	
Mysidacea		12	
Isopoda	7		
Amphipoda		21	
Macrura	14		
Euphasia		4	
Decapoda		4	15
ECHINODERMATA		38	5
ALGAE		21	20
Sand			40
Digested matter	50	33	45
Unidentified			15

The species described are of limited migratory habits and feed near or on the bottom. This explains the higher mercury content in the gut contents of specimens from polluted areas.

3.1.2 Mercury distribution in tissues and organs

In general, fish specimens taken in the Haifa Bay and Akko area have higher values of mercury in the flesh than specimens of the same species taken at Zarqa and Caesarea (Table I). For comparison we used, whenever possible, the same number of individuals of the same size range (Table I).

There are indications that mercury levels increase with fish size (weight and length). Figures 2-4 show better correlations between mercury concentration in flesh and size for specimens from the unpolluted sites. The same was found for L. mormyrus in a previous study conducted by Hornung et al. (1985). In twelve trawl species (Hornung et al., 1980), mercury concentrations increased with size in all of the species at all sampling locations. Mercury content and size have been correlated in studies by Grieg and Krzynawek (1979), Van Den Broek and Tracey (1981), Bernhard (1978, 1985), Bernhard and Andreae (1984), and Kohler et al. (1986).

The highest mercury levels were recorded in livers (0.67-4.34 μg g^{-1}) of fish from the Akko and Haifa Bay areas. The mercury values in livers of fish from unpolluted sites lay within the range of levels recorded in muscle tissues (0.149-0.407 μg g^{-1}). E. alexandrinus, E. guaza and P. ehrenbergii show a highly significant correlation (at 1% level) of the mercury content in the muscle tissue to that in the liver (Fig. 5).

Mean mercury values in muscle, liver and gut can be seen in Figure 6.

The highest mercury levels in all of the tissues and organs of D. sargus were found in specimens from Akko and Haifa Bay (Fig. 7), with much lower values in specimens from Tel-Shikmona and Zarqa.

Of the organs analyzed in D. sargus, the livers had the highest and most variable mercury concentrations in fishes from Haifa Bay. At Tel-Shikmona, the highest range was detected in the kidneys, followed by the heart and muscle. (No kidneys were examined in fish from Haifa Bay and Zarqa). The highest mercury content in specimens from Zarqa was found in the livers, followed by muscle and heart. A marked downward trend in mercury concentration with distance from the pollution source (a chlor-alkali plant in Haifa Bay) is clearly distinguished in Figure 7. By comparing the 3 populations of D. sargus, a positive linear regression and a highly significant correlation between the mercury concentration in muscle and body weight were found (Table VII): $r = 0.745$, $p<0.01$ (Haifa Bay), $r = 0.688$, $p<0.01$ (Tel-Shikmona), and $r = 0.581$, $p<0.01$ (Zarqa). Furhtermore, a very high significant correlation between mercury content in muscle and total length was evident in all the fish from the 3 areas sampled (Fig. 8). From this investigation, it appears that the mercury in D. sargus increases as their size increases. The relationship between the other tissues and body weight and length which is highly significant in all species of fish from Akko and Haifa Bay, at Tel-Shikmona it becomes less significant, and at Zarqa only the muscle tissue correlates well with both weight and length.

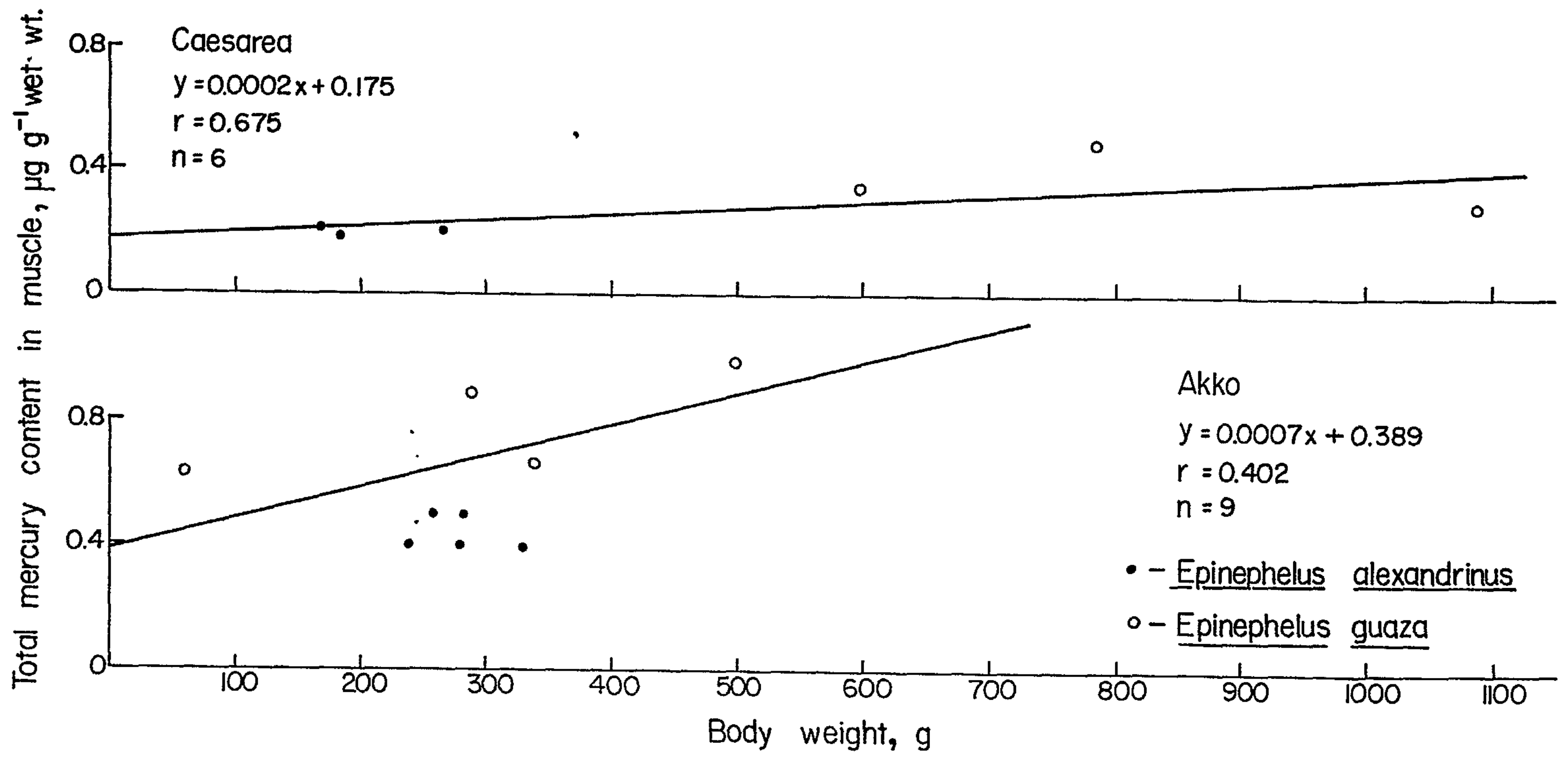

Fig. 2 Relationship between mercury levels in muscle tissues and body weight

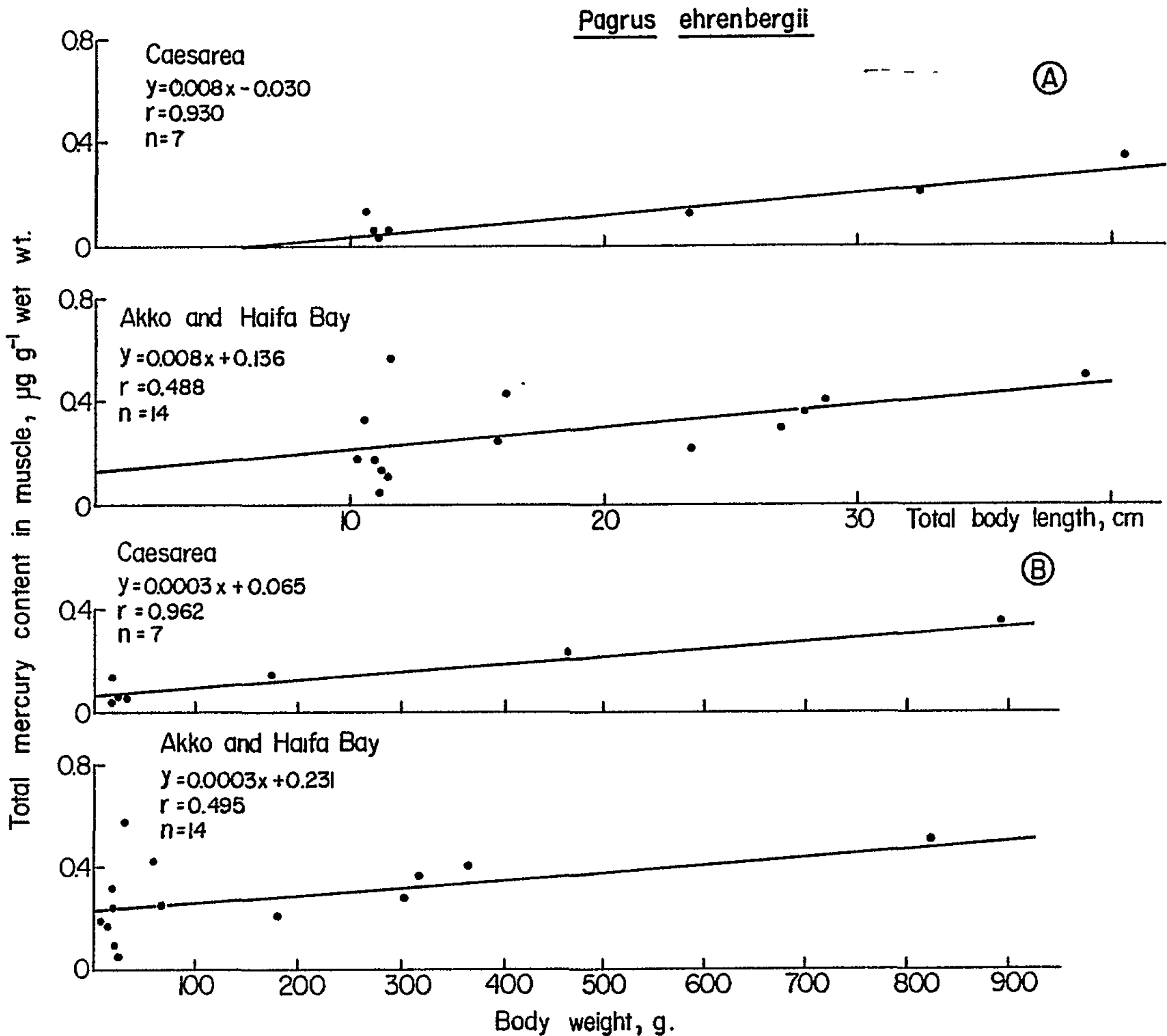

Fig. 3 Relationship between mercury levels in muscle tissues vs. total lenght (A) and vs. body weight (B)

The distribution and accumulation of mercury in the tissues and organs shows a similar pattern in specimens from both polluted and unpolluted areas (Fig. 7).

The bioaccumulation is different in different organs, being highest in the liver and kidney. The size of the fish has been found to be of importance for the accumulation pattern. A similar distribution of mercury in organs of fish was found by Dawson (1982), Salanki et al. (1982) and Jothy et al. (1983).

In five species of bottom fish, Ohta et al. (1982) found the highest mercury levels in the livers, followed by muscle, stomach and intestines, in decreasing order. Knauer and Martin (1972) found the highest levels of mercury in the livers of Engraulis mordax (anchovy) followed by muscle, gills, gonads and skin. Distribution of mercury

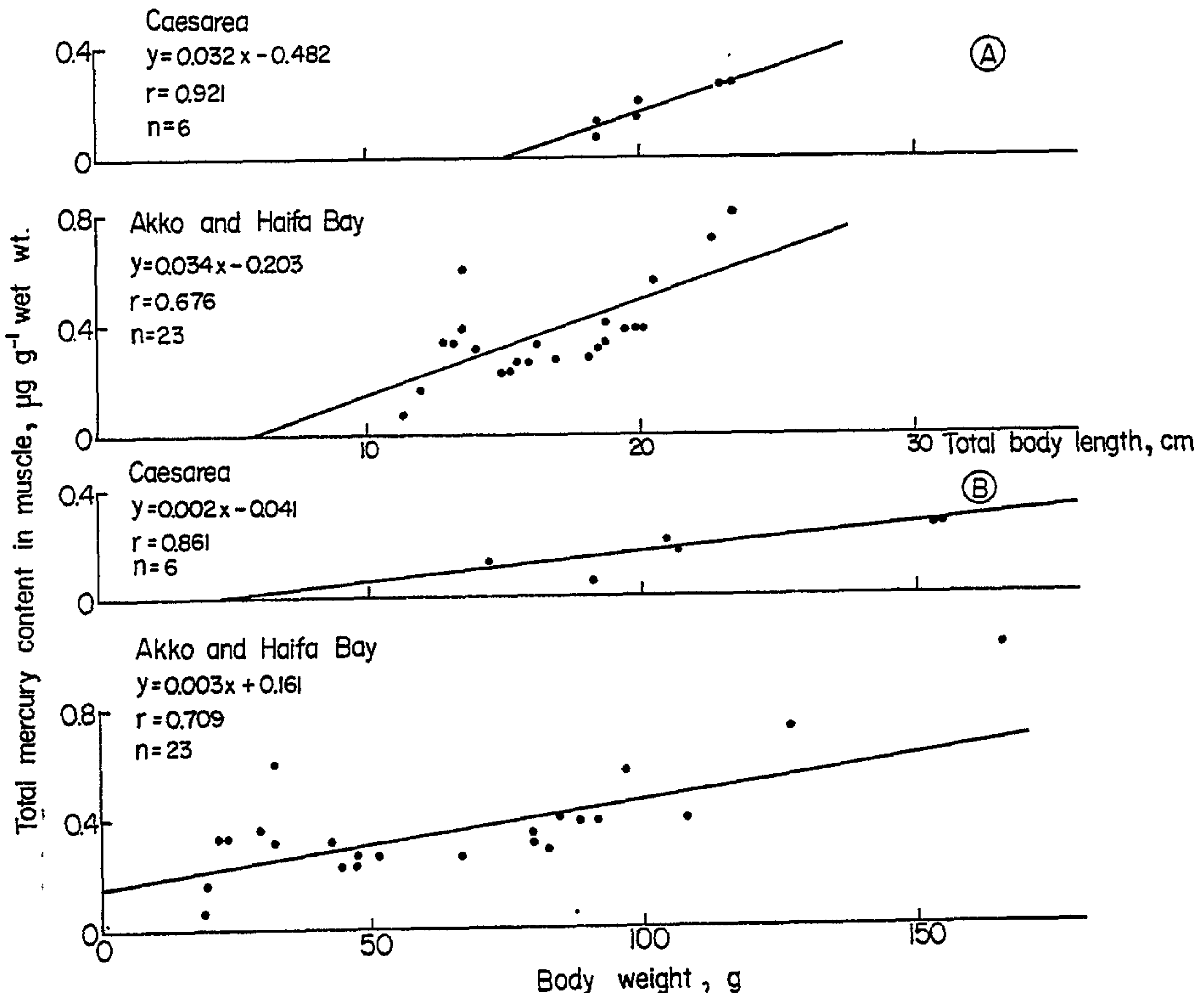

Fig. 4 Relationship between mercury levels in muscle tissues vs. total length (A) and vs. body weight (B)

among body organs of the clupeid Brevoortia tyrannus from three estuaries of the eastern USA (Cocoros et al., 1973) showed the highest levels detected in the spleen, pyloric caeca and liver, with relatively low concentrations in most of the other organs. These data indicate mercury accumulation in this species with age through the digestive system.

Comparison of the present data on D. sargus to those of other investigators is not possible due to lack of equivalent data. However, comparable studies in the Mediterranean region (Table VIII) carried out on other species show high mercury levels in fish from industrially polluted waters.

Table VII

Correlation of total mercury in tissues and organs of *Diplodus sargus* with body weight and total length*

Organ	Akko and Haifa Bay		Tel-Shikmona		Zarqa	
	Body weight	Total length	Body weight	Total Length	Body weight	Total length
Muscle	0.745(16)	0.866(16)	0.688(16)	0.660(16)	0.581(20)	0.691(20)
Scales	0.621(16)	0.647(16)	0.062(15)	0.090(15)	-0.049(20)	-0.094(20)
Gills	0.490(16)	0.600(16)	0.421(15)	0.452(15)	0.053(20)	0.069(20)
Skin	0.532(16)	0.697(16)	0.362(15)	0.355(15)	0.237(20)	0.204(20)
Heart	0.842(9)	0.901(9)	0.536(13)	0.455(13)	0.380(10)	0.392(10)
Liver	0.602(12)	0.709(12)	0.578(13)	0.604(13)	0.275(16)	0.332(16)
Skeleton	0.632(16)	0.760(16)	0.487(15)	0.521(15)	0.444(20)	0.488(20)
Stomach wall	0.600(10)	0.705(10)	-	-	-0.665(8)	-0.647(8)
Stomach contents	0.543(13)	0.657(13)	-	-	-	-
Intestines	0.982(6)	0.964(6)	0.365(13)	0.442(13)	0.168(16)	0.169(16)
Food from intestines	0.754(15)	0.832(15)	0.305(13)	0.420(13)	0.346(15)	0.322(15)
Gonads	0.391(9)	0.519(9)	-0.237(12)	-0.247(12)	-0.377(6)	-0.210(6)
Kidney	-	-	0.539(14)	0.511(14)	-	-

* The number of data points used is given in parenthesis

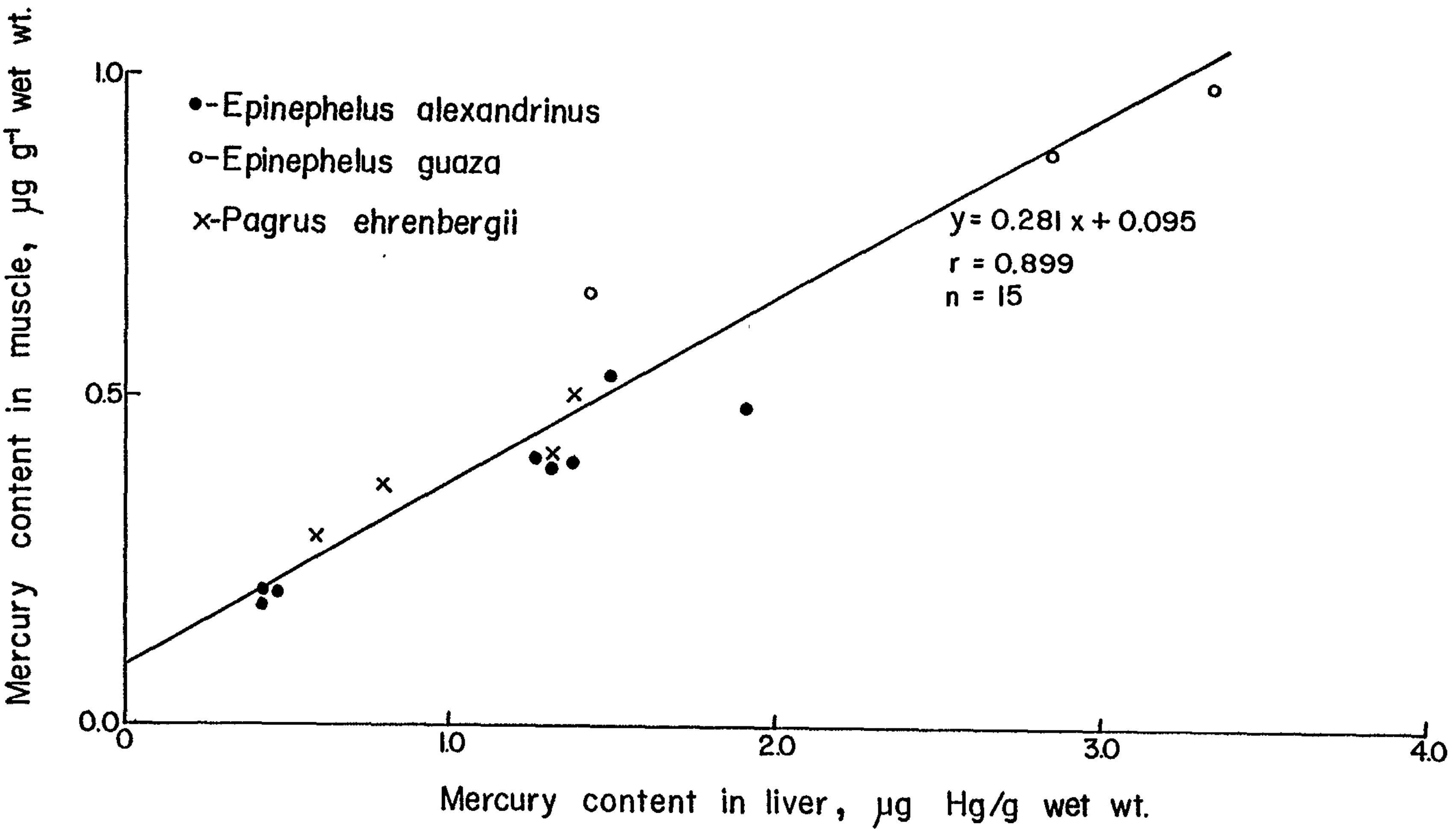

Fig. 5 Relationship between mercury levels in muscle tissues and in livers

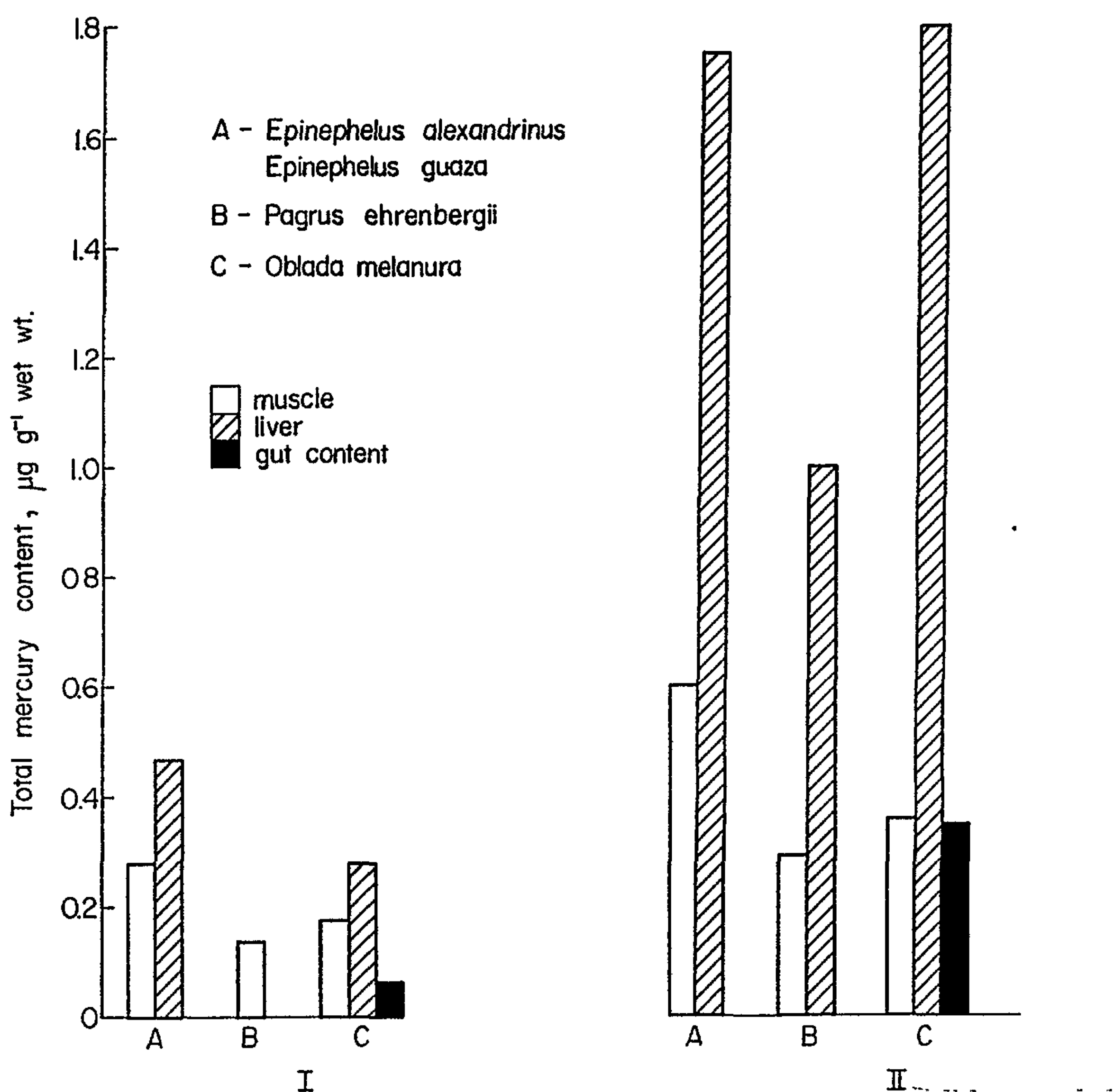

Fig. 6 Mean mercury levels in muscle, liver and gut content of inshore fish species from unpolluted (I) and polluted (II) habitats

The distribution of total mercury in tissues and organs of Upeneus moluccensis is presented in Table IX. Averages and ranges based on individual specimens are shown in Table X and Figure 9, and correlations of total mercury with body weight and total length are recorded in Table XI. U. moluccensis accumulates mercury in its tissues (Hornung et al., 1980; Tuncel et al., 1980; Balkas et al., 1982; UNEP, 1983; Salihoglu and Yemenicioglu, 1986). It inhabits sandy and muddy bottoms of coastal waters, usually at depths not exceeding 100m, and feeds on small bottom living invertebrates. The stomach contents of the fish used in this report included small decapods (Brachyura), parts of small fishes, and tissues and shells of gastropod molluscs (Rhinoclavis kochii). The distribution of mercury in organs and tissues of 19 specimens was investigated in this study (Table IX). The highest mercury range was observed in the livers (0.205-1.40 $\mu g\ g^{-1}$) followed by muscle (0.194-1.12 $\mu g\ g^{-1}$), heart (0.150-0.615 $\mu g\ g^{-1}$) and kidney (0.080-0.626 $\mu g\ g^{-1}$) (Table X).

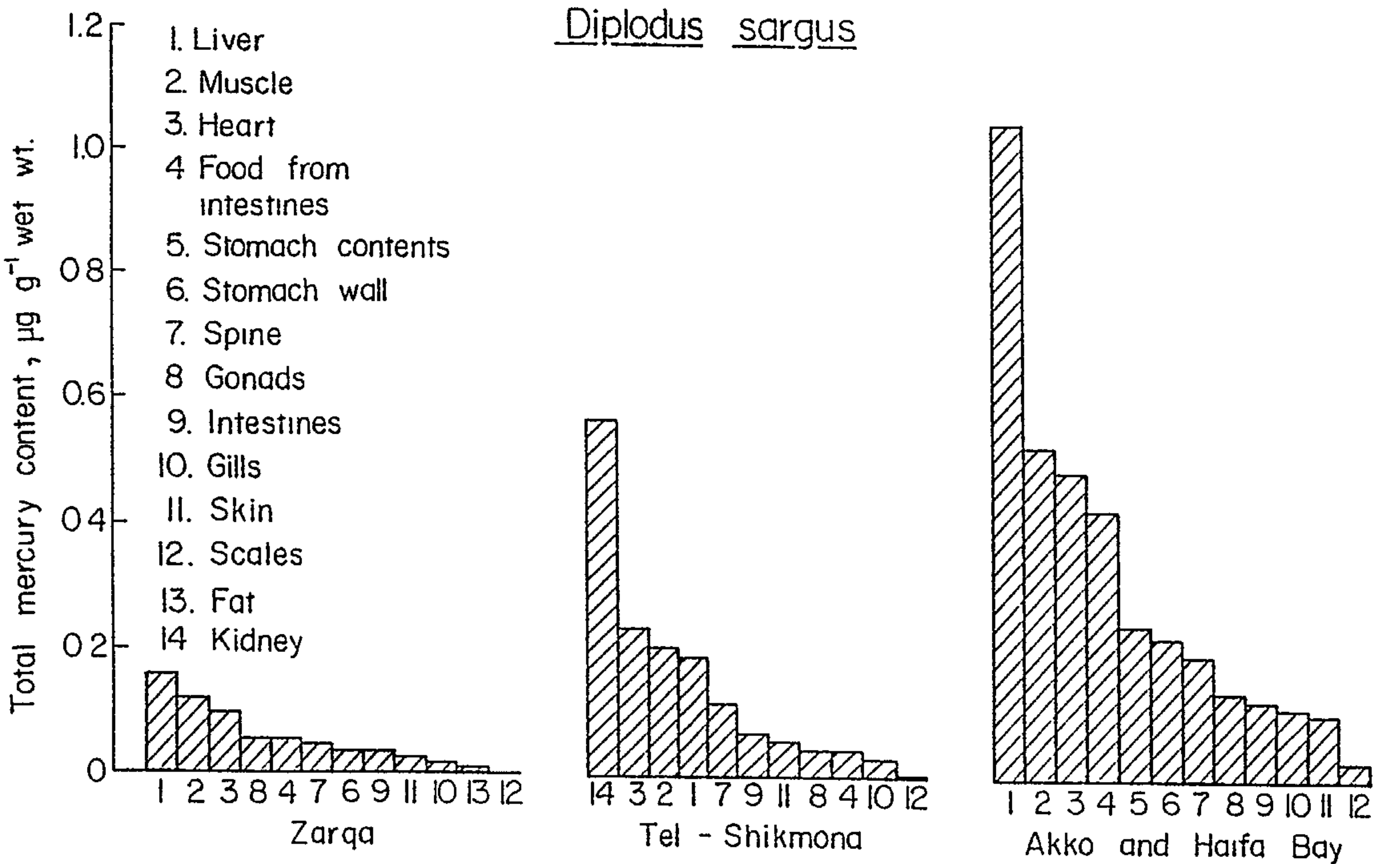

Fig. 7 Mean mercury levels in tissues and organs of <u>Diplodus sargus</u> from 3 sampling sites

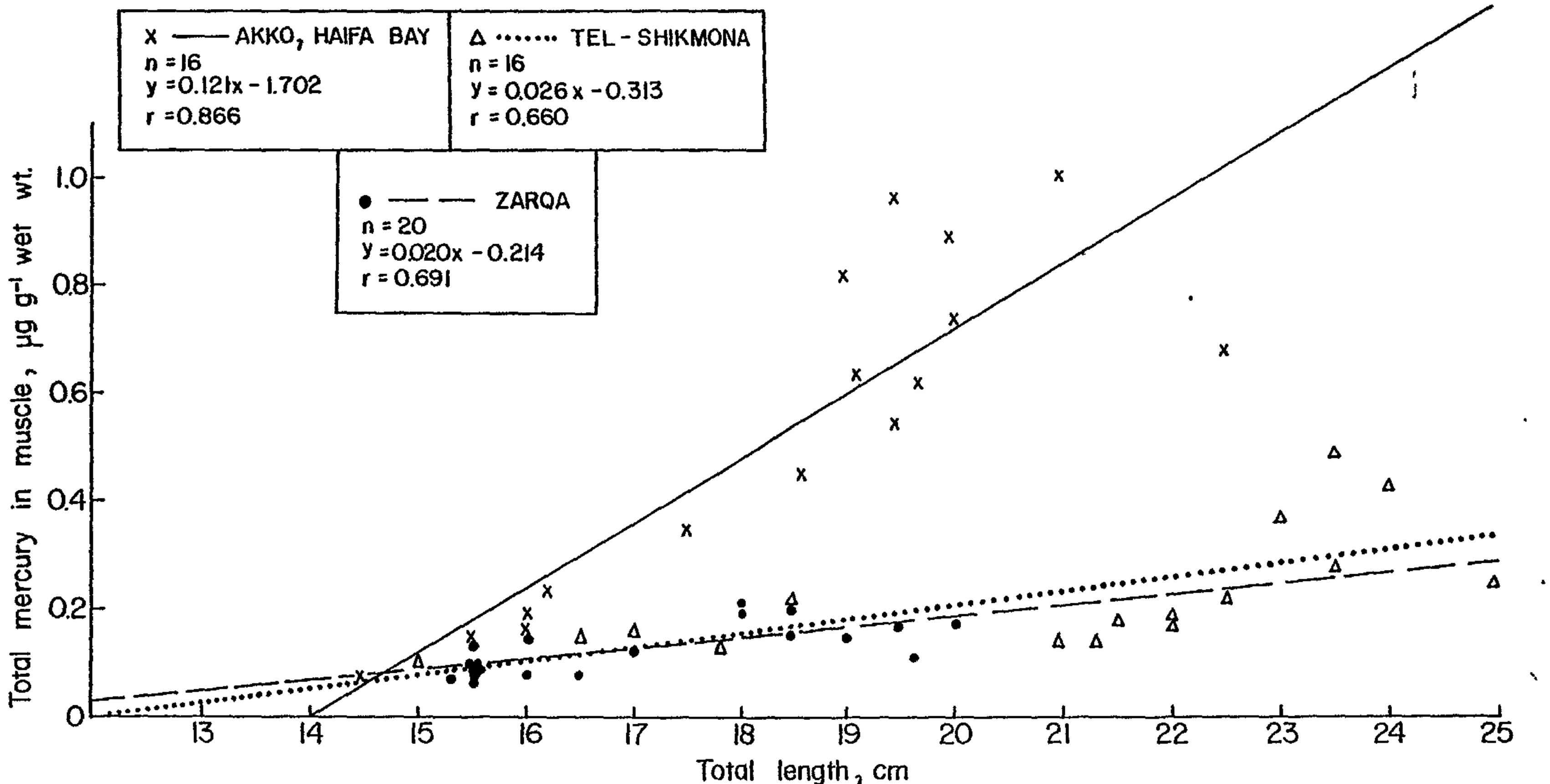

Fig. 8 Total mercury concentration in the muscle of Diplodus sargus versus total lenght

Table VIII

Total mercury content ($\mu g\ g^{-1}$ wet wt.) in tissues and organs of fish caught in various regions of the Mediterranean Sea.

Species, Locations	Muscle	Liver	Stomach	Gonads	Skin	Gills	Skeleton	Reference
Pagellus erythrinus	0.43	0.50			0.16		0.11	Papadopoulou et al., 1973
(Keratsini and Rhodes)	0.26	0.39			0.12		0.09	Papadopoulou et al., 1973
Boops salpa	0.11	3.81						Renzoni et al., 1973
Diplodus vulgaris	1.90	17.00						Renzoni et al., 1973
Scorpaena porcus	2.61	4.26						Renzoni et al., 1973
Serranus scriba	4.64	11.60						Renzoni et al., 1973
(Tyrrhenian coast)								
Scomber scombrus	0.150	0.170						Stoeppler and Nurnberg, 1979
Thunnus thynnus	0.520	0.400		0.440				Stoeppler and Nurnberg, 1979
(northwest coast, Italy)								
Mullus surmuletus	1.295	3.168	1.308		0.380	0.660	0.727	El Sokkary, 1980
Mullus surmuletus	1.494	2.043	1.500		0.818	0.993	1.060	El Sokkary, 1980
(El-Max, Alexandria)								
Gobius niger								
(Varna Bay)	0.070	0.153						Apostolov et al., 1985
(Saronikos Gulf)	0.040	0.090						Apostolov et al., 1985
(Petalion Gulf)	0.023	0.057						Apostolov et al., 1985
Solea vulgaris	0.931	2.333						El Sokkary, 1985
Solea vulgaris	1.412	3.230						El Sokkary, 1985
Mullus surmuletus	0.830	1.940						El Sokkary, 1985
Mullus surmuletus	1.425	2.435						El Sokkary, 1985
(El-Max, Alexandria)								
Diplodus sargus								
(Haifa Bay, Akko)	0.531	1.052	0.229	0.135	0.107	0.115	0.194	Present study
(Tel-Shikmona)	0.225	0.203	0.075	0.048	0.064	0.034	0.116	Present study
(Zarqa)	0.123	0.161	0.042	0.061	0.030	0.022	0.053	Present study
Upeneus moluccensis	0.372	0.565	0.138	0.121	0.167	0.063	0.167	Present study
(Mediterranean coast)								

Table IX

Distribution of total mercury content (µg g^{-1} wet wt.) in tissues and organs of *Upeneus moluccensis* collected by trawl in Haifa Bay between 30 and 60 m depth on November 25, 1985.

Total length cm	Weight g	Sex	Muscle	Scales	Gills	Skin	Heart	Liver	Kidney	Skele-ton	Stomach wall	Stomach contents	Gonads
9.0	78.1	F	1.12	0.043	0.159	0.238	0.615	1.40	0.463	0.372	0.210	0.188	0.306
18.0	63.2	F	0.466	BDL*	0.006	0.152	0.236	0.513	0.139	0.196	-	0.038	0.105
17.5	58.0	F	0.388	BDL	0.049	0.133	0.256	0.579	0.193	0.221	0.103	0.132	0.102
17.5	55.3	F	0.410	BDL	0.080	0.208	0.226	0.730	0.626	0.271	-	empty	0.113
17.5	53.1	F	0.384	BDL	0.059	0.188	0.341	0.637	0.235	0.194	0.116	0.085	0.140
17.0	52.3	F	0.334	0.008	0.078	0.215	0.351	0.730	0.301	0.093	0.164	0.148	0.124
16.6	49.4	F	0.300	0.030	0.071	0.117	0.322	0.497	0.125	0.184	0.129	0.092	0.122
16.5	51.0	F	0.321	0.006	0.049	0.136	-	0.205	0.169	0.143	-	empty	0.174
16.5	47.2	M	0.348	BDL	0.043	0.178	0.219	0.474	0.158	0.176	-	empty	0.104
16.5	46.2	M	0.447	0.007	0.072	0.192	0.311	0.662	0.323	0.196	-	empty	0.155
16.5	45.7	F	0.271	BDL	0.076	0.139	0.150	-	0.438	0.138	0.086	empty	0.061
16.0	46.8	M	0.316	0.032	0.059	0.134	0.368	0.635	0.205	0.092	0.130	0.087	0.127
16.0	44.8	F	0.293	BDL	0.057	0.187	0.252	0.706	0.144	0.150	0.135	empty	0.146
15.8	43.0	F	0.196	0.061	0.046	0.131	0.166	0.487	0.280	0.129	0.119	empty	0.073
15.7	39.0	F	0.262	BDL	0.026	0.128	0.235	0.375	0.154	0.092	0.183	0.184	0.050
15.6	42.6	M	0.378	0.007	0.084	0.200	0.347	0.295	0.191	0.229	-	empty	0.127
15.5	38.8	M	0.316	0.007	0.092	0.141	0.214	0.449	0.135	0.139	-	empty	0.070
15.3	40.6	F	0.194	BDL	0.051	0.150	0.209	0.409	0.182	0.078	-	empty	0.098
15.0	34.7	M	0.328	BDL	0.049	0.198	0.604	0.391	0.080	0.085	0.143	0.114	0.125

* Below Detection Limit

The relationships between mercury concentration in the muscle and fish size (weight and length) are highly significant ($p<0.01$). The same is true for the liver ($p<0.01$), skeleton <$p<0.01$), gonads ($p<0.01$) and kidney ($p<0.05$) (Table XI, Fig. 10). As in D. sargus, highest mercury levels were detected in the fish livers, showing that this organ accumulates mercury to a greater extent than do the other tissues and organs.

Mean ratios of mercury levels in liver to those in muscle were in D. sargus from Haifa Bay 2:1, at Tel-Shikmona 1:1, at Zarqa 1:1 and in U. moluccensis 2:1. A similar ratio of 2:1 was found by El Sokkary (1981) in specimens of Mullus surmuletus from El Mex (along the Alexandrian coast).

Table X

Ranges, averages and standard deviation (based on individual specimens) of total mercury content ($\mu g\ g^{-1}$ wet wt.) in tissues and organs of Upeneus moluccensis collected by trawl in Haifa Bay.

Organ	n	Range	Av.	S.D.
Muscle	19	0.194-1.12	0.372	0.195
Scales	19	BDL*-0.061	0.011	0.018
Gills	19	0.006-0.159	0.063	0.031
Skin	19	0.117-0.238	0.167	0.036
Heart	18	0.150-0.615	0.301	0.129
Liver	18	0.205-1.40	0.565	0.257
Kidney	19	0.080-0.626	0.239	0.139
Skeleton	19	0.078-0.372	0.167	0.074
Stomach wall	11	0.086-0.210	0.138	0.036
Stomach contents	9	0.038-0.188	0.119	0.049
Gonads F	13	0.050-0.306	0.124	0.065
Gonads M	6	0.070-0.155	0.118	0.029

* Below Detection Limit

4. CONCLUSIONS

The data presented suggest that D. sargus and U. moluccensis, which are bottom feeders, ingest mercury directly from the sediment and through the organisms on which they feed. Mercury shows spatial variation with respect to muscle tissue in both species, inshore and offshore, and shows a clear increase with size (year class).

The amount of mercury in the organs is dependent on the quality of the food eaten by the fish. Mercury is accumulated through the ingested food and associated with the feeding habits. The prey habitat in both species is benthic.

The results indicate that careful analysis of environmentally controlled patterns in the species used for monitoring purposes, as well as an appropriate statistical treatment of the data gained from chemical analysis, are essential for assessing the contaminant concentrations in organisms in relation to the pollution state of their habitat.

Table XI

Correlation of total mercury in tissues and organs of Upeneus moluccensis with body weight and total length.

Organ	Body weight	n	Total length
Muscle	0.817	(19)	0.740
Scales	0.233	(19)	0.117
Gills	0.414	(19)	0.341
Skin	0.359	(19)	0.356
Heart	0.303	(18)	0.205
Liver	0.738	(18)	0.706
Kidney	0.463	(19)	0.517
Skeleton	0.781	(19)	0.766
Stomach wall	0.380	(11)	0.278
Stomach contents	0.083	(9)	0.030
Gonads	0.684	(19)	0.567

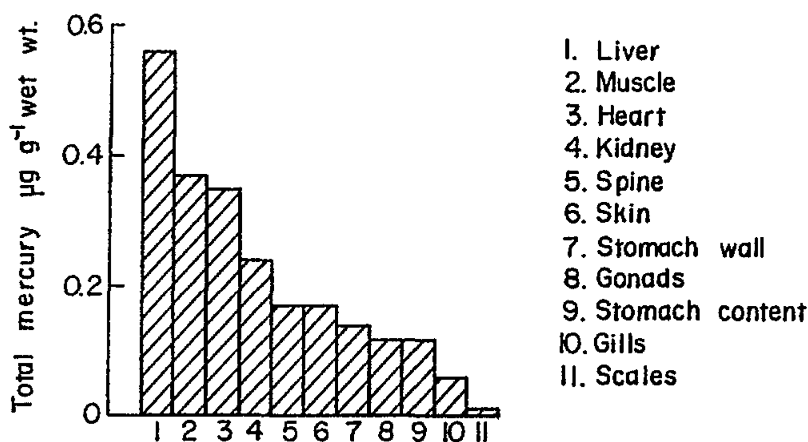

Fig. 9. Mean mercury levels in tissues and organs of Upeneus moluccensis.

Fig. 9 Mean mercury levels in tissues and organs of Upeneus moluccensis

5. ACKNOWLEDGEMENTS

This study was carried out within the framework of the MED POL Phase II program (UNEP) and supported by the Mediterranean Trust Fund. Our thanks are due to G. Brokman for collecting the fish samples, H. Bernard for the drawings and K. Diskin for correcting and typing this paper.

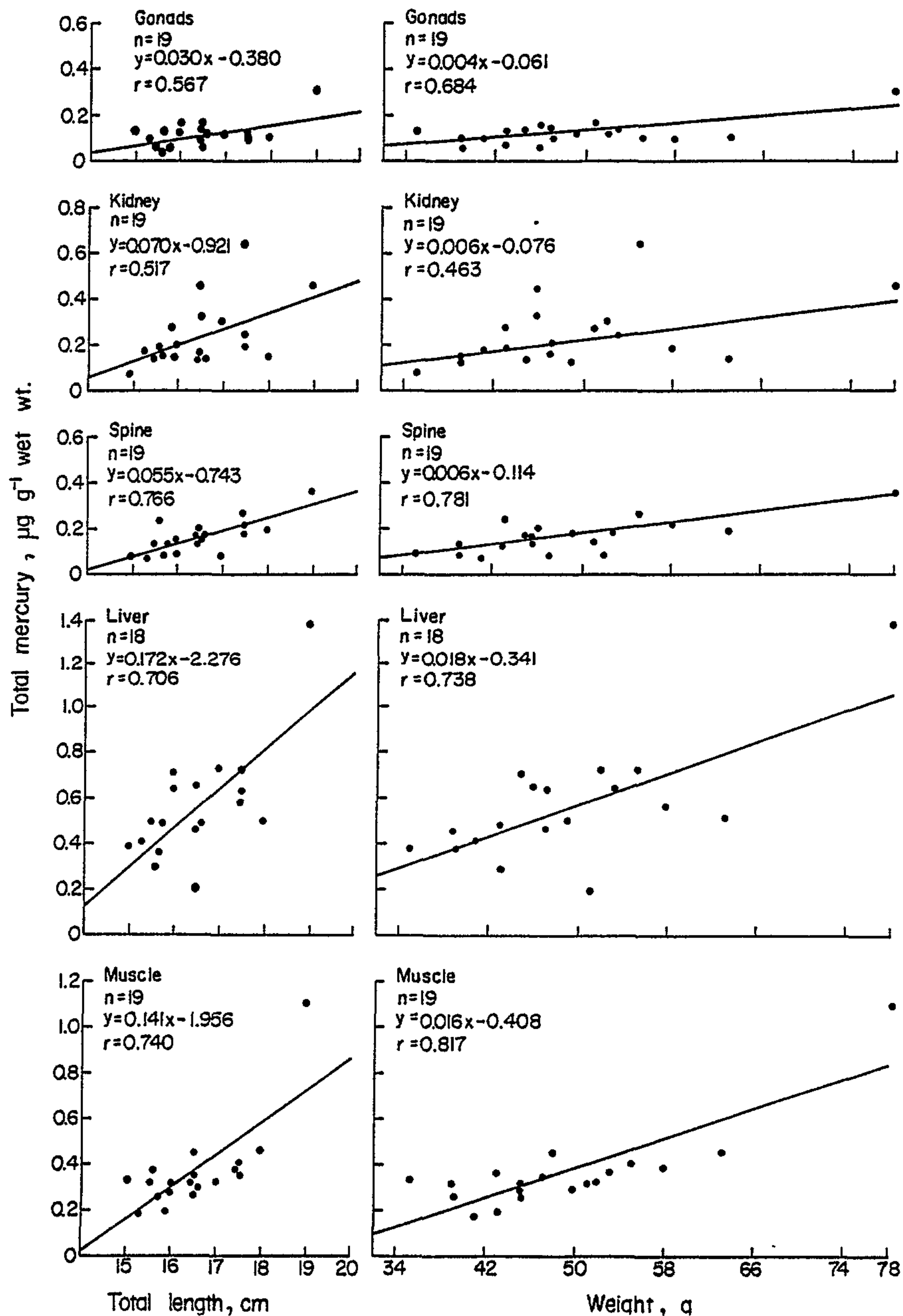

Fig. 10 Relationship between mercury concentration in various organs of Upeneus moluccensis and total length of body weight

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BLUEFIN TUNA OF THE WESTERN MEDITERRANEAN: MERCURY BODY BURDEN AND LIFE PATTERNS

by

B. MORALES-NIN * and A. CRUZADO **

* Institut d'Estudis Avancats de les Illes Balears
** Centre d'Estudis Avancats de Blanes

A B S T R A C T

As part of the research activities of the MED POL Phase II programme a study of the mechanisms by which the populations of some tuna accumulate mercury was undertaken. The present document reflects the results of the project and underlines the main findings obtained.

Fish otolith microstructure and composition contain a record of an individual fish growth, environment, migration and contact with environmental perturbations (Radtke et al., 1987). Recent structural studies on fish otoliths have confirmed the possibility of daily growth increments being used to determine the growth histories of many fish species (Radtke, 1984; Gutierrez and Morales-Nin, 1987). Otoliths are not susceptible to resorption (Mugiya and Watabe, 1977) and their chemical composition reflects the temperature and water quality along the fish life span (Radtke et al., 1987).

The proposed research objective was to study the bluefin tuna populations off the Catalan coast using otolith characteristics as a key to understand their population dynamics. Otolith chemical and structural properties were determined as well as mercury body burden.

The work carried out on 1988 followed the studies started in the previous research project phase and developed new applications. The main objectives were to complete the studies of the juvenile bluefin tuna otolith samples and to obtain and study adult bluefin tuna samples.

1. INTRODUCTION

Mediterranean bluefin tuna, Thunnus thynnus, spawn mainly from the beginning of June through August (Arena, 1979) between the Balearic Islands and Sicily. The adults migrate into the Mediterranean during May-June. Right after spawning the fish leave through the Straits of Gibraltar (Rodriguez-Roda, 1969; Sara, 1973). The majority of juvenile fish leave the area during the fall of their first year (Rey, 1979). Intermediate size fish (between 50 and 150 kg weight) are caught all year around in Tyrrhenian, Aegean and Black Seas, while in other areas are only caught in summer-fall.

The captures of bluefin tuna off the Catalan coast show a clear increase in the second half of the year, due principally to the bigger captures of the southern part (Tarragona to Sant Carles). Giant fish are caught in the South during fall while in the northern part they are caught in spring. Also bigger juvenile catches are made in fall (Recasens pers.comm) (Fig. 1).

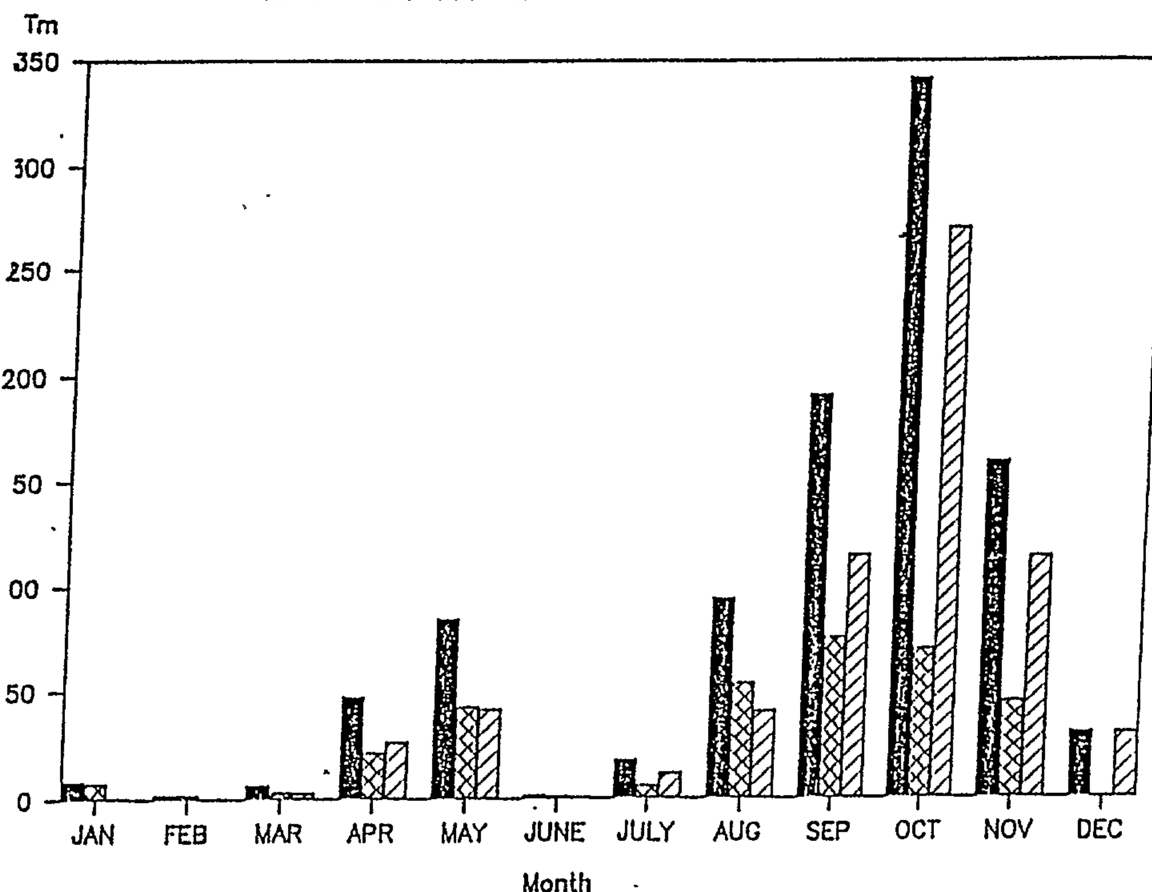


Fig. 1 Monthly bluefin tuna catches off the Catalan Coast averaged from 1975 to 1987. Solid bar total catch, single stripped bar captures in the Southern part, doubled striped bar catches in the Northern part

Mediterranean bluefin tuna show higher mercury levels relative to counterparts in other areas of the world oceans, particularly in the North Atlantic Ocean where they originate. Mercury concentrations in fish caught in the Mediterranean range from 20 to 6290 $\mu g\ kg^{-1}$ wet weight (UNEP/FAO/WHO, 1987), while in Atlantic bluefin tuna they range

from 20 to 800 $\mu g\ kg^{-1}$ wet weight (Cumont et al., 1972). In an attempt to explain the reason for this important differences in mercury levels in muscle of tuna caught in the Mediterranean and the Atlantic, Renzoni et al. (1979) postulated the existence of two bluefin tuna populations in the Mediterranean Sea: a) tuna that spend longer periods in the Mediterranean with high mercury body burdens (up to 6000 $\mu g\ kg^{-1}$) showing a positive correlation between mercury concentration and body weight; b) tuna that migrate from the Atlantic into the Mediterranean for spawning and then return to the Atlantic. These tuna have lower mercury body burdens and do not show a clear correlation between mercury concentration and body weight. Mercury is accumulated tropically (Bernhard, 1985).

Chemical differences in hard parts of the body have been used for the identification of fish stocks (Lapi and Mulligan, 1981). X-ray microanalysis allows to identify elements localized in diverse structures, in fish only such as fish scales (Lapi and Mulligan, 1981) and otoliths (Morales-Nin, 1981; Morales-Nin and Fortuño, 1990). The method is non-destructive and can simultaneously analyze a wide range of elemental concentrations. It compares favorably to other analytical methods.

Otoliths are a part of the labyrinth system and are composed of calcium carbonate crystals laid down in a protein matrix of otolin (Degens et al., 1969). Their crystalline lattice structure allows for the inclusion of other elements during their growth. Otolith microstructure and composition contain a record of an individual fish growth, environment, migration and contact with environmental perturbations (Radtke, 1987). Otoliths are not susceptible to resorption (Mugiya and Watabe, 1977) and their chemical composition reflects the temperature and water quality along the fish life span (Radtke et al., 1987). Recently Radtke and Morales-Nin (1989) studied the Mediterranean juvenile bluefin tuna life history patterns by means of the Sr/Ca relationship and structural characteristics of their otoliths while Morales-Nin and Fortuño (1990) studied the otolith elemental composition.

The elemental composition of otoliths allows stock identification and offers the means to determine the age of bluefin tuna. This method offers advantages over that based on the study of the chemical composition of bluefin tuna vertebrae, that has been used to characterize eastern and western Atlantic stocks and their degree of intermingling (Calaprice, 1985).

Additional information for the delineation of bluefin tuna populations, adult giant and juvenile bluefin tuna caught in the northwestern Mediterranean along the Catalan coast (northeast of Spain), were studied using otolith characteristics and body tissue chemistry as a key to understand their population dynamics.

2. MATERIALS AND METHODS

Bluefin tuna is not a target of the local fishery; the giant bluefin tuna is only captured occasionally during their spawning migrations, while juvenile fish are caught during their migration to the Atlantic. The catches off the Catalan coast (Fig. 1) depend on

the fishing techniques employed, which determine the absence of intermediate sizes in the catches (Recasens, Instituto de Ciencias del Mar, pers.comm.).

The paucity of captures and their high price made very difficult to obtain otolith and flesh samples of giant fish. This problem was solved through the cooperation of the Central Fish Market in Barcelona, where giant bluefin tuna from the Catalan coast are sold on pieces and the heads rejected. Some of these heads which were properly documented were collected to obtain the required samples.

The weight and length of the sampled fish was calculated from head size according to the relationship between weight and length and head size determined by Rodriguez-Roda (1983). A total of 29 fishes were sampled although only 19 pairs of otoliths were collected (Table I).

Juvenile fish collected within the framework of the National Monitoring Programme were also studied (Table I). Fish length and weight were recorded and the otoliths and flesh samples retained for analysis (Morales-Nin et al., 1988).

The sagittal otoliths were documented for the presence of growth structures using a compounds microscope. Then, they were attached to aluminum stubs, ground and polished. The sections were etched for 10-20 minutes in 8% EDTA (Ethylene Diamine Tetracetic Acid, adjusted to pH 8 with NaOH) or with HCl 0.1 N for 45 seconds, and vacuum-coated with gold-palladium for viewing in a Scanning Electron Microscope (SEM). Increments were counted from SEM micrographs of the sagitta surface, beginning at the rostrum (anterior) and proceeding to the origin (center). A microincrement was defined as an unbroken incremental zone with discontinuous zones as boundaries (Morales-Nin, 1987).

The crystalline composition of otoliths was determined by X-ray diffraction. A subsample of 0.1 g weight of dehydrated and pulverized otoliths was submitted to the action of the X-ray beam in a Philips PW 1001 diffractometer. The diffraction pattern was recorded by means of Cu radiation.

The elemental composition of adult (n=13) and juvenile fish (n=3) otoliths was qualitatively analyzed by energy dispersive X-ray analysis using a Kevex x-ray detector coupled to the SEM. Transversal sections of sagittal otoliths were obtained using standard methods (Radtke and Morales-Nin, 1989). The sections, washed in distilled water several times, were mounted on carbon stubs and attached with colloidal graphite for the analysis. The analyses were carried out sequentially along the otolith. The primary beam was set at 20 Kv. The microscope stage was tilted about 30° toward the X-ray detector, to obtain the optimum for our instrument. The analysis was carried out in the scanning area at 600 magnifications. The results were tested with spot analyses. The spectrum acquisition time was 200 seconds, longer acquisition periods (up to 3,000 seconds), did not change the results. The background was substracted from the spectra and overlapping lines deconvoluted, using standard software. The analytical procedure allows to detect low concentrations of elements ranging from ^{11}Na, to ^{99}Es. The first step of the analytical procedure was to determine the degree of repeatability of the process, two samples were analysed four times and the results contrasted. The analytical error amounts to 5%. The otolith elemental composition was analyzed by correspondence analysis.

Table I

Data related to the studied Bluefin tuna caught along the Spanish Mediterranean coast.

Giant fish caught in May 1988

Ref. No	head size cm	body weight kg	age** yr	total mercury content $\mu g\ kg^{-1}$ wet weight
1	41.2	75	-	2580
2	44.5		9	-
3	40.5	72	-	2090
4	46.5		8	-
5	47.5	107	9	2790
6	44.0	87.5	-	2350
7	43.5	115	9	2070
8	45.0		8	-
9	37.0		-	-
10	42.0	77.5	9	2820
11	48.0	112	8	850*
12	48.5		8	-
13	48.0			
14	45.0	95	8	1590*
15	38.0	65	-	2910
16	45.0	95	8	2040
17	46.0	100	6	1950
18	46.0	100	9	2070
19	42.5	80	8	2010
20	48.0		11	-
21	51.0	145	10	1360*
22	56.0	195	-	2740
23	62.0	247	14	2480
24	50.5	140	-	2830
25	50.5	140	-	2870
26	55.0	175	-	3000
27	45.5	175	8	-
28	43.0	78	7	2090
29	45.5	98	7	2270

Juvenile fish caught on 1986

Ref.	fork length cm	body weight kg	age yr	total mercury content $\mu g\ kg^{-1}$ wet weight
1	53.0	3.41	1	1040
2	53.0	3.35	1	1590
3	39.0	1.25	0	870
4	39.0	1.10	0	585
5	34.0	0.75	0	415
6	40.0	1.28	0	545
7	41.0	1.20	0	595

* Low mercury content fish
** Age determined by means of annual rings in the otoliths

The flesh samples were frozen with liquid nitrogen and total mercury levels in the muscle were determined following standard techniques (Dr. M. Bernhard, La Spezia).

3. RESULTS

3.1 Otolith composition

The crystallographic studies confirmed that the bluefin tuna otoliths are composed of aragonite (Fig. 2). The anomalous formations detected in the surface of some otoliths (Fig. 3) might be composed of very small quantities of calcite although it was not confirmed by the analytical procedure perhaps due to their smallness.

The elemental composition of the otoliths is shown in Table II. The main component in all the otoliths was Ca, Sr and Cl and were found in all the otoliths while S was present only in juvenile fish otoliths. Na was very abundant in all the otoliths except in the low mercury body burden fish, which were characterized by the presence of Al. The differences in the composition were pointed out by the correspondence analysis. Juvenile fish were grouped together near the origin of the axis, while the otoliths corresponding to low mercury body burden fish, showed a similar composition. The otoliths with high mercury body burden were more randomly distributed (Fig. 4).

3.2 Otolith microstructure

Giant bluefin tuna otoliths showed very clear external features when observed with SEM (Fig. 5). The presence of major increments which appeared as ridges in the surface (Fig. 6) can be attributed to annual growth cycles. When the otoliths were polished the increments composing these ridges showed that they were laid down with annual periodicity. This was supported by the number of daily increments observed (mean 344, s.d. 32). The mean length-at-age obtained from the interpretation of these annual rings (Table I) compared favorably to the mean length-at-age reported for bluefin tuna (Compean-Jimenez and Bard, 1983). However, the scarce data and the clustering of the samples at the age extremes do not allow to determine bluefin tuna growth parameters.

Each microincrement is composed of a protein matrix with calcium carbonate crystals deposited within the matrix. Etching with EDTA dissolves the aragonite crystals leaving areas with a higher protein content to form discernible increments (Fig. 7). Etching times (changing with the area of the otolith) can cause the protein ridges to collapse and prevent counting of the microincrements (Fig. 8). Thus, etching times were critical to the acquisition of viewable increments.

The increment width ranged from 0.2 μm to 1.5 μm in giant bluefin tuna, while in juvenile the average width was from 1.5 to 4 μm (Figs 9 and 10). The thickest increments were laid down in the nuclear area in a zone corresponding to the first 3-4 months of life. These thick increments, corresponding to a characteristic growth phase, are common in Scombrid fish (Brothers _et al._, 1983).

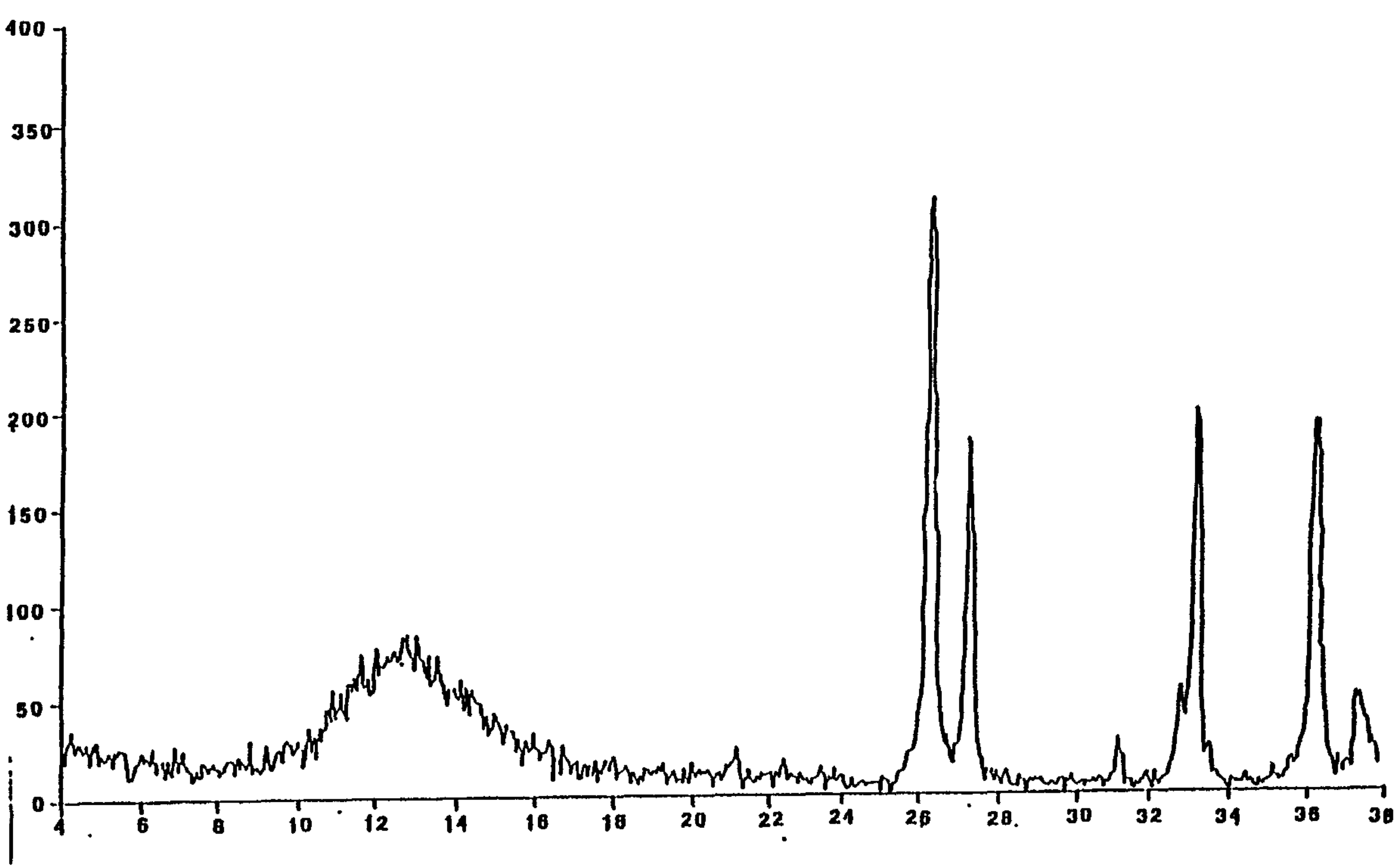


Fig. 2 X-ray diffraction pattern of a bluefin tuna otolith showing the aragonite typical pattern.

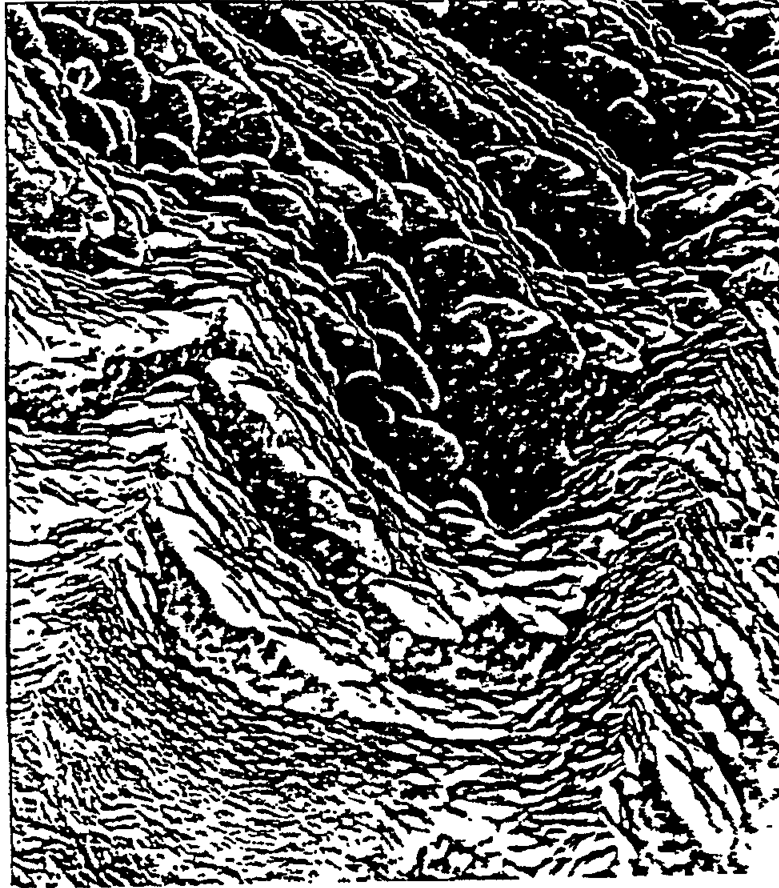


Fig. 3 Small anomalous crystals laid down in the surface of a giant bluefin tuna otolith.

Table II

Mean elemental otolith composition in percent obtained by X-ray analysis.

	Element						
	Na	Cl	Ca	Sr	Si	S	Al
high mercury bluefin tuna							
adult fish	7.515	2.555	88.855	0.830	0.490	0	0
juvenile fish	6.089	2.355	90.255	0.847	0.420	0.420	0
low mercury bluefin tuna							
adult fish	0	1.711	96.310	0.645	0.934	0	0.400
juvenile fish	4.460	2.189	92.706	0.645	0	0	0

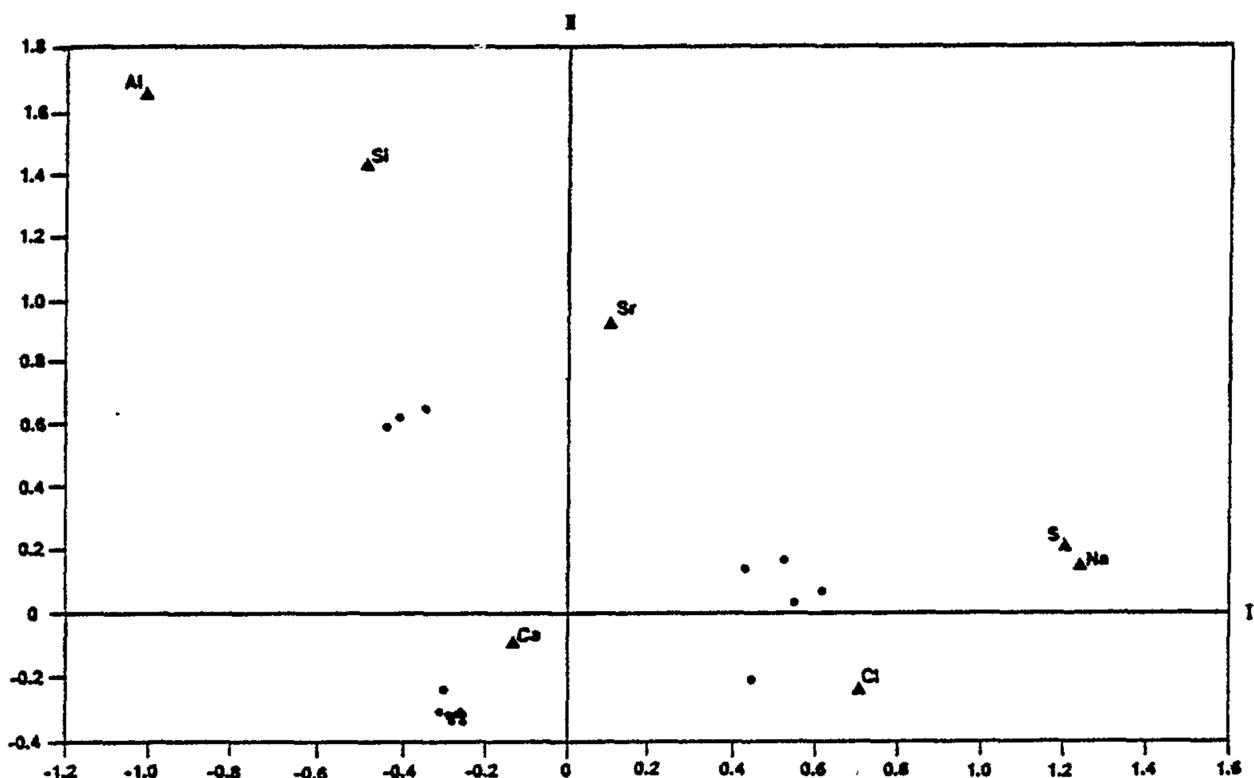


Fig. 4 Correspondence analysis of the elemental otolith composition. Circles low mercury content fish, squares juvenile fish

Growth patterns in adult fish were very variable with frequent growth zones with dense crystalline structure followed by zones of relatively thin increments (0.8 µm in average, s.d.=1.69 µm) appearing as grooves. Probably these zones had a lower aragonite content reacting more intensely to the etching. The variability of the increments composing such zones (7 to 40 increments) made very difficult to assess their periodicity and its meaning. Rhythmic growth patterns of 7, 14, 24, 28, 40 regularly laid down increments were also detected (Fig. 11). The temporal significance of these patterns might be multiple (e.g. monthly, migratory, reproductive). In some otoliths 2-4 sub-daily units of the increments were found.

3.3 Mercury body burden

The mercury level determined in the samples is shown in Table I. The correlation coefficients between mercury level and body weight are rather variable depending on the fish size interval considered (Table III). The lack of medium size fish with the consequent discontinuity in the data produced a poor fit when all the individuals were analyzed

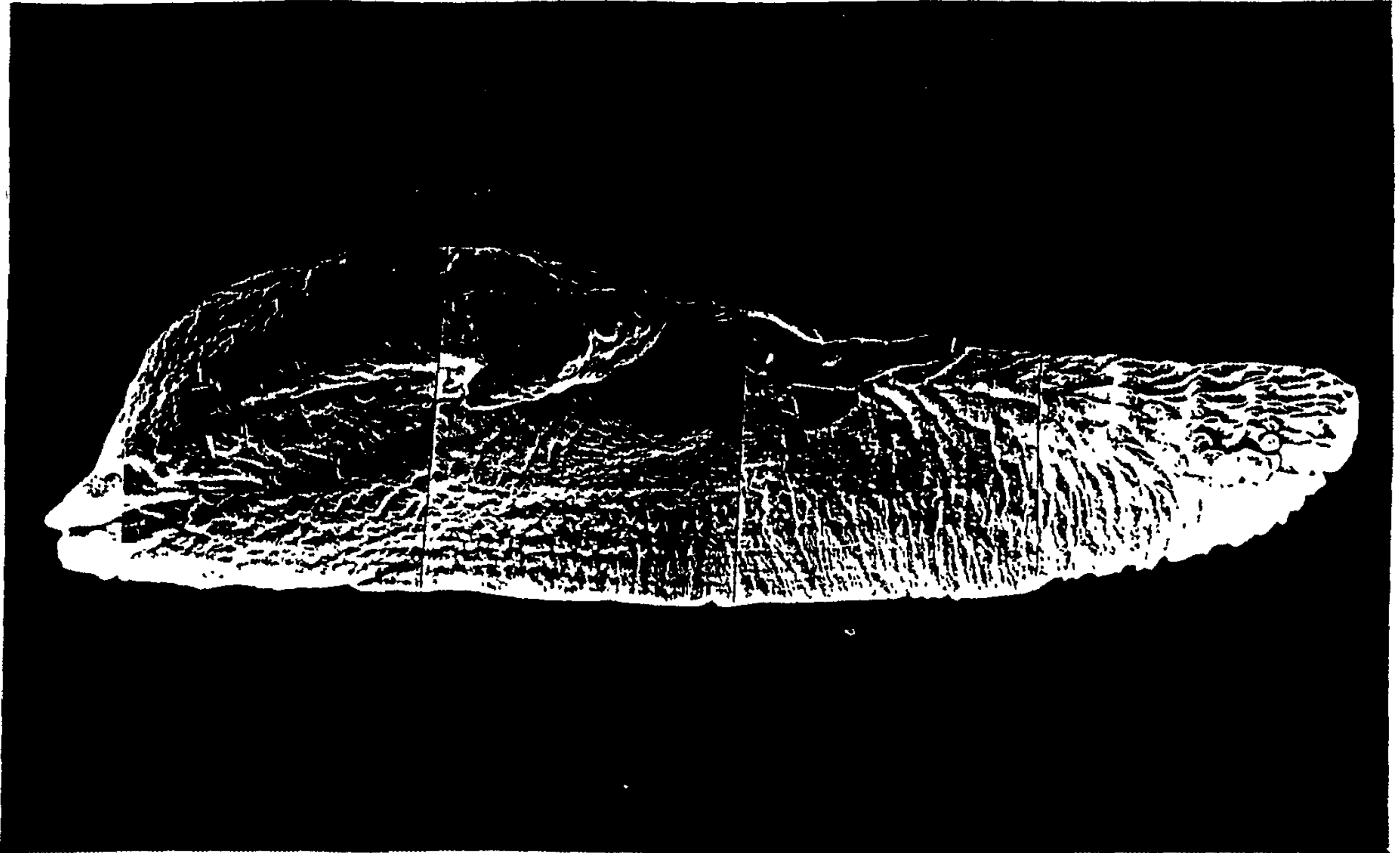


Fig. 5 Bluefin tuna otolith external morphology

together (Fig. 12). In juvenile fish the plot of the residuals (Fig. 13a) showed an increase of mercury variability with weight. This tendency was not found in adult fish (Fig. 13b).

The Mediterranean high mercury body burden population is characterized by their positive correlation between mercury content and body weight (Renzoni et al., 1979). The poor correlation between mercury body burden and weight and the high dispersion of the mercury content found in giant fish, suggested the presence of two mixed populations in the studied group. However, few fish showed a lower mercury body burden, which is considered as typical of an Atlantic dwelling tuna.

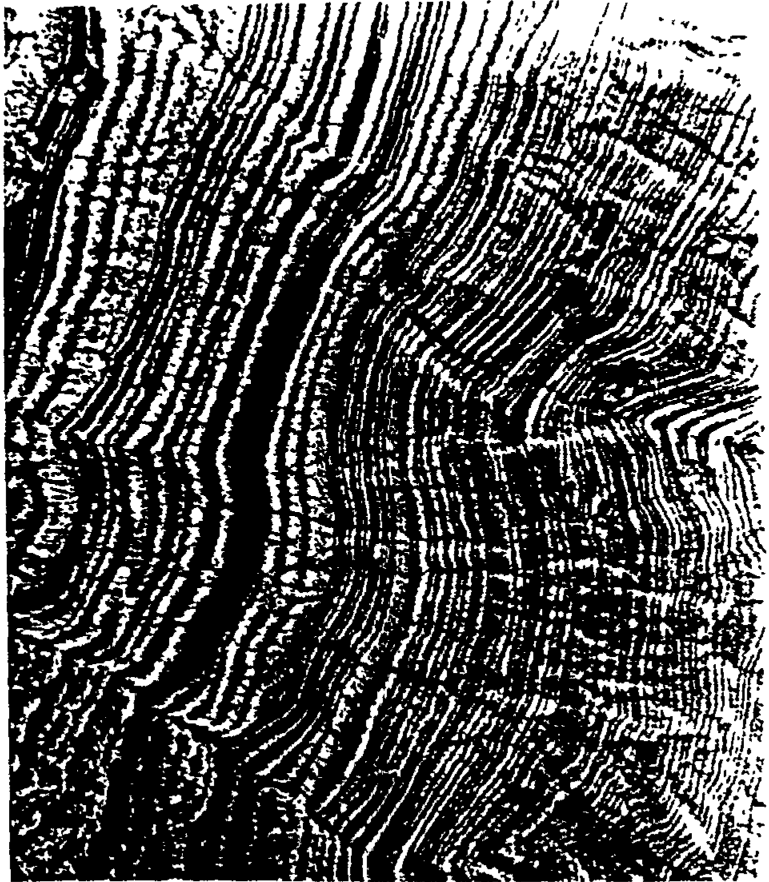


Fig. 6 Daily growth increments laid down

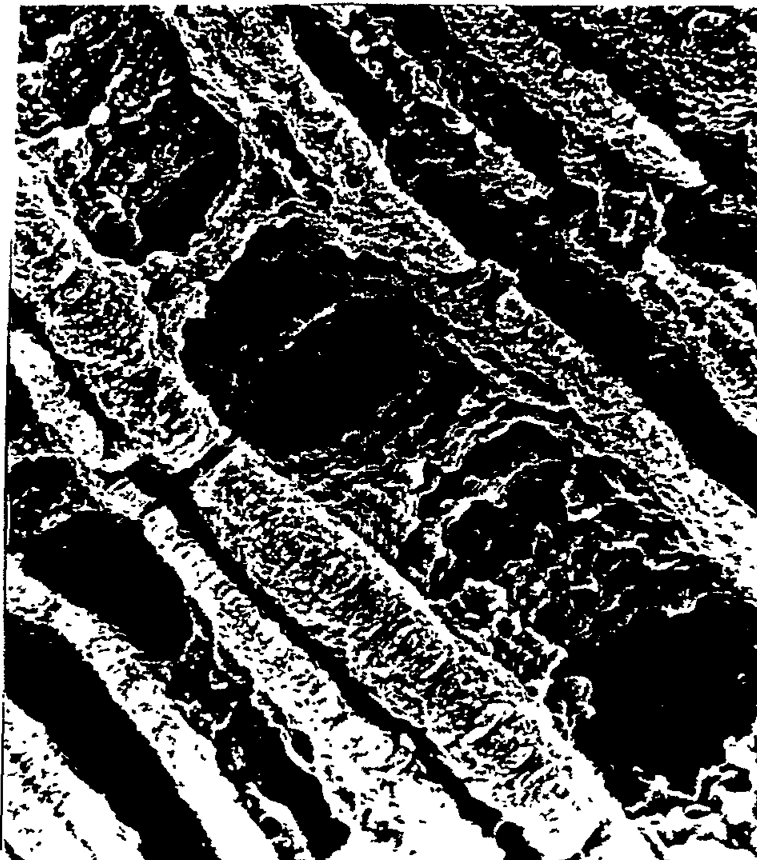


Fig. 7 Protein ridges of microincrements laid down in a bluefin tuna otolith. Strands of protein can be seen to interconnect the ridges. Scale bar 12 μm

Fig. 8 Ridges showing only protein strands and without microincrements due to uneven otolith etching. Scale bar 12 μm

Fig. 9 Daily growth increments laid down in a juvenile fish showing rhythmic growth patterns. Scale bar 30 μm

Fig. 10 Thin increments laid down in a giant bluefin tuna. Scale bar 30 µm

Fig. 11 Rhythmic patterns in the increments. Scale bar 20 µm

Table III

Correlation parameters determined for the relationship between mercury content and body weight

Parameter	Estimate	Standard error	T value	Probability level
Adult bluefin tuna		$r^2=0.1825$		
intercept	2019.88	337.459	5.98556	$9.2587e^{-6}$
slope	$2.2269e^{-3}$	$2.7512e^{-3}$ 0.80943	0.4282	
Juvenile bluefin tuna		$r^2=0.8695$		
intercept	-43.8866	211.985	-0.20702	0.8441
slope	0.4006	0.1017	3.93797	0.0109
All studied fish		$r^2=0.7724$		
intercept	1148.97	173.041	6.63987	$7.2225e^{-7}$
slope	$9.8922e^{-3}$	1.6603^{-3} 5.95788	$3.7794e^{-6}$	

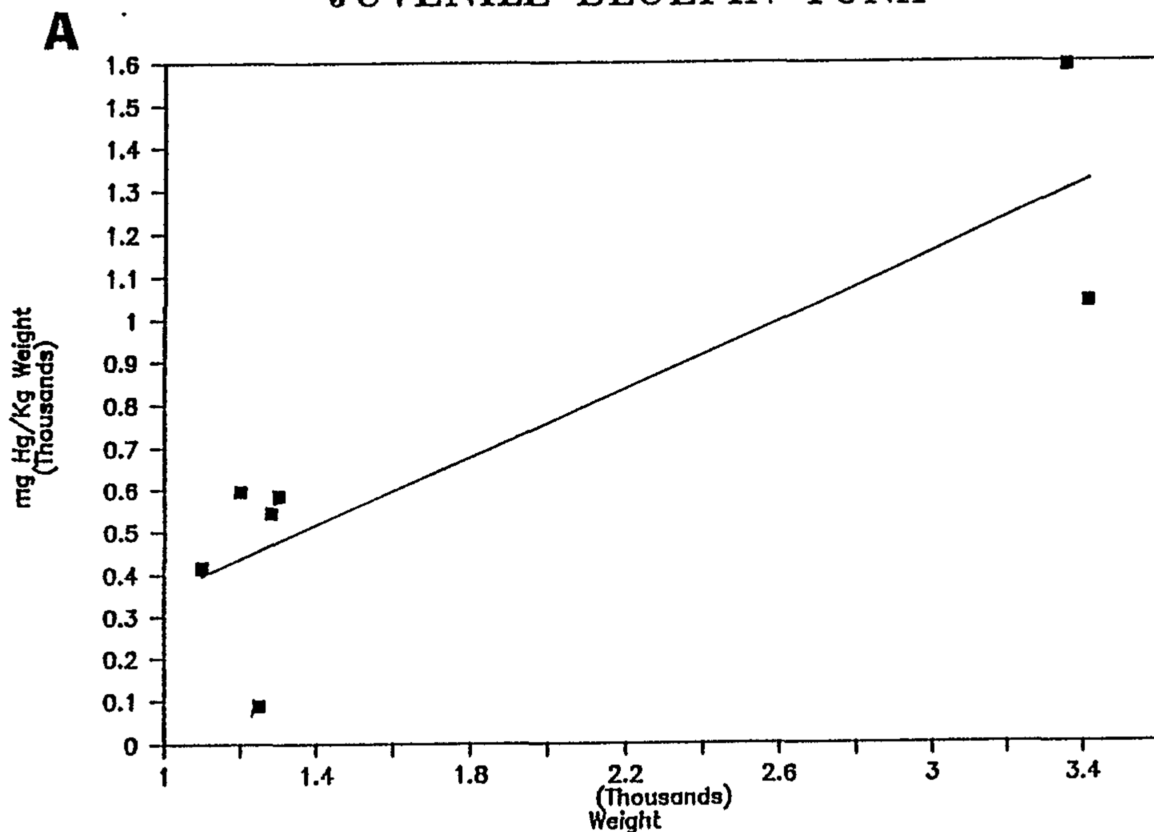
4. DISCUSSION

Mercury levels in the flesh of the studied fish ranged widely. In juvenile fish were high and significant, showing the dependence of mercury level on fish size. The amount of mercury increases notoriously in the older fishes but is poorly correlated to body weight.

Otolith characteristics offered some information about the fish life. The otolith microstructure was very variable, allowing only to differentiate the life phase from juvenile to adult fish. The microincrement width was very variable and probably a function of the time of the year when it was deposited. Observations of increment width suggests that wide increments were deposited during favorable conditions, while thin increments corresponded to unfavorable periods (migrations, spawning, winter). The average width of the increments in giant bluefin tuna when compared to juvenile increment width, reflects the lower growth rate in adult fish.

The marginal otolith structures of giant fish showed that the majority of fish (55%) were actively growing during their migration. The formation of discontinuous structures in the otoliths might be related to the physiological status, spawning and migration.

The elemental composition of fish otoliths has been shown to reflect pollution (Papadopoulou et al., 1980), geographical effects (Calaprice, 1971), and temperature (Gauldie et al., 1980) of the environment in which the fish has been living. Our results showed that it also reflects fish age (S presence in juvenile fish). Apparently, the otoliths corresponding to the lower mercury body burden were characterized by a more uniform composition, while the high mercury body burden fish showed more variability in their composition. The possible causes of this variability are unknown, genetic variability



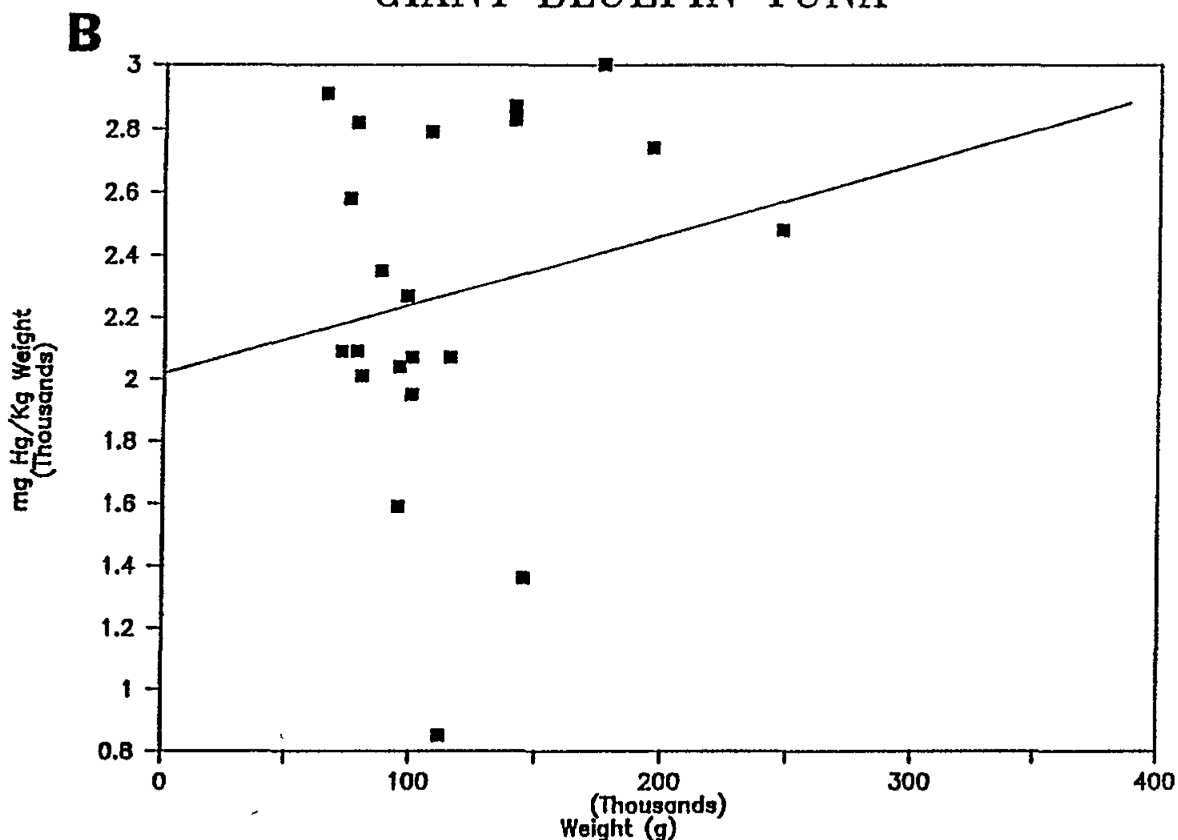
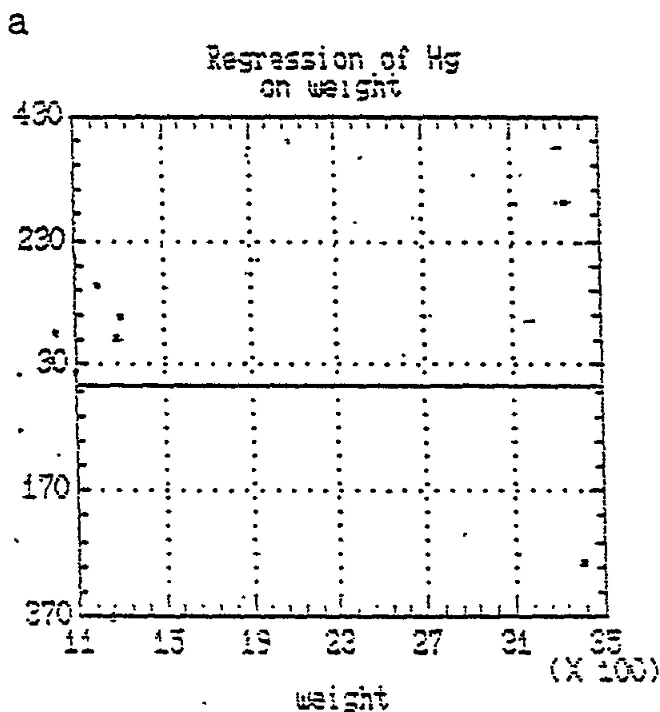


Fig. 12 Correlation between mercury body burden and weight for all the studied bluefin tuna. A: juvenile fish, B: adult fish.

a



b

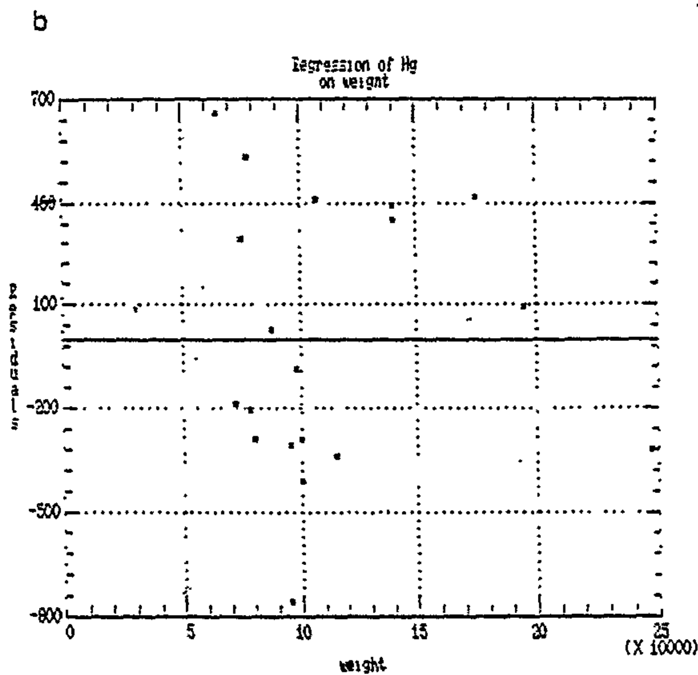


Fig. 13 Plot of the residuals of the regression between mercury body burden and fish weight for a: juvenile fish and b: adult fish

seems unlikely to be the cause of changes in elemental otolith composition (Behrens-Yamada et al., 1987). The otolith elemental composition might reflect ambient water, dietary differences or water temperature differences. This might suggest a more changing environment experienced by the high mercury body burden fish. This is not surprising since tuna feed in frontal zones in which horizontally varying conditions promote primary production and therefore pelagic fish on which tuna feed proliferate.

The results obtained in this contribution are a first attempt to differentiate the two postulated tuna populations in the Mediterranean. The difficulty in obtaining representative samples covering all the fish size range, have limited the meaningfulness of the method unless a proper sampling scheme is set up. However, such a scheme cannot be implemented by a single institution, it requires a concerted effort of various laboratories around the Mediterranean region. On the other hand, the elemental composition of the otoliths might offer some additional evidence for the characterization of the Mediterranean bluefin populations.

5. ACKNOWLEDGEMENTS

Dr. J. Obiols (Institut Quimic de Sarria, Barcelona, Spain) and Dr. M. Bernhard (ENEA, La Spezia, Italy) determined the mercury concentrations in muscle of juvenile and adult fish, respectively. Mr. J. Marti Cap del Mercat del Peix (MERCABARNA) assisted in the obtention of the giant fish samples. Mr. J.M. Fortuño (ICM, Barcelona, Spain) made the X-ray elemental composition analyses. J. Biosca (ICM, Barcelona, Spain) processed the scanning electron micrographs. All of them are thanked for their cooperation.

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STUDY OF THE TOXICITY AND BIOACCUMULATION OF SOME HEAVY METALS IN THE CRAYFISH Procambarus clarkii (GIRARD, 1852) OF THE ALBUFERA LAKE OF VALENCIA, SPAIN

by

J. MEDINA*, J. DIAZ-MAYANS**, F. HERNANDEZ*, A. PASTOR*
J. DEL RAMO** and A. TORREBLANCA**

* Environmental Laboratory, University College of Castellón, P.O. Box 224, Castellón (University of Valencia)

** Animal Physiology Laboratory, Department of Animal Biology, Faculty of Biological Sciences, Dr. Moliner 50, 46100-Burjassot (Valencia) (University of Valencia)

ABSTRACT

The American red crayfish Procambarus clarkii appeared in Lake Albufera near Valencia (Spain) in 1978 and presently, without adequate sanitary controls, is being fished commercially for human consumption. Lake Albufera and the surrounding ricefield waters are subjected to very heavy loads of sewage and toxic industrial residues (including heavy metals and pesticides) from the many urban and wastewaters in this area.

The degree of toxicity of Cr, Cd, Hg and Pb on crayfish Procambarus clarkii at various temperatures has been studied. Crayfish were obtained from Lake Albufera and after an appropriate acclimation period, were treated with different concentrations of heavy metals for 96 h under static conditions. The results show that Hg is the most toxic of the four metals tested, while Cr presented very low toxicity. The toxic effects of all metals studied increased when temperature increased.

The content of heavy metals in several tissues of this crayfish after sublethal Cr, Cd, Hg and Pb exposure was determined by A.A.S. The control crayfish showed detectable amounts of the four heavy metals analyzed. This could be indicative of heavy metal contamination of Albufera waters. For all metals studied, the metal content in all tissues increased with increasing metal concentration in the water, except Hg content in muscle and Cd and Pb content in antennal glands. Gills and antennal glands have the highest content in heavy metals, followed by midgut gland and muscle.

1. INTRODUCTION

The american red crayfish Procambarus clarkii is native to the Lousiana marshes (USA). In the 70's, this crayfish was introduced into Spain through the Guadalquivir river swamps (Librero, 1980). In 1978, the crayfish appeared in Lake Albufera near Valencia and in the surrounding rice fields. Presently the crayfish have reached a high density due to its natural resistance, rapid adaptation, and growth, producing ecological and agricultural-economic problems in rice crops.

Without adequate sanitary control, the crayfish is presently being subjected to heavy loads of sewage and toxic industrial residues (including heavy metals and pesticides) from many urban and wastewaters in this area (Dafauce, 1975; Roselló, 1983).

Chromium, an essential trace element for humans and animals, is involved in normal carbohydrate metabolism (Mertz, 1969; Anderson et al., 1983). It has been suggested that chromium may have an essential function in the regulation of glycogen metabolism of the crab Podopthalmus vigil (Sather, 1967); however, at higher concentrations it is toxic and causes histopathological and ultrastructural changes in several tissues of shrimp Palaemonetes pugio (Doughtie and Rao, 1984).

Cadmium is a ubiquitous, non-essential element which possesses high toxicity to both humans (Haguenoer and Furon 1981) and aquatic organisms (Lalande and Pinel-Alloul, 1984; Lake et al., 1979).

In recent years, cadmium and cadmium compounds have been used extensively by various industries, and this has produced sharp increases in contamination of air, water and soil. Cadmium has been described as a perfect example of a trace metal which is very widespread in the biosphere, accumulated by plants and animals, and which induces acutely and chronically deleterious effects in organisms (Schroeder, 1974).

The European Economic Community in 1975 included cadmium on its "black list" of substances requiring priority attention (Laxen, 1984). The US Environmental Protection Agency has also recently proposed water quality criteria as low as 0.5 ng l^{-1} (not to be exceeded) for soft waters (hardness <400 μequiv l^{-1}), with concentrations expressed in terms of total metal (Laxen, 1984).

Aquatic pollution with mercury and its resulting uptake and accumulation by crustaceans and other aquatic animals has been studied for a long time. Aquatic ecosystems are particular concern since many animals in these environments concentrate rapidly large amounts of mercury (Friberg and Vostal, 1972; Ackefors et al., 1970). Mercury is used widely throughout industry and has been found to be highly toxic to aquatic organisms. Woolrich (1973) found that mercury exists in the environment at an overall concentration of approximately 0.2 ppm.

A considerable volume of work has been done on toxicity of mercury and mercury compounds on marine and fresh water crustaceans (Ahsanullah, 1982; Del Ramo et al., 1987; Doyle and Klauning, 1976). The sublethal effects of mercury ions on respiration and metabolic activity have also been extensively studied (Corner and Sparrow, 1956; Chinnaya, 1971; Depledge, 1984). A few studies, however, have been performed on the mercury accumulation and distribution among organs or tissues on crustaceans.

Lead is a widespread non-essential element that is highly toxic to both humans (Haguenoer and Furon, 1981) and animals (Baudouin and Scoppa, 1974). It has become particularly important due to its relative toxicity and increased environmental contamination via automobile exhaust and highway runoff. In spite of lead salts having a low solubility in water, lead compounds may pose a hazard problem to the aquatic organisms.

There are several investigations on the toxic effects and bioaccumulation of lead in fish (Reichert et al., 1979), moluscs (Martincic et al., 1984), and crustaceans (Anderson, 1978; Gilles and Pequeux, 1983). Recently, Tulasi et al. (1987) studied the lead uptake in fresh water field crab, Barytelphusa guerini after 30 days of lead-exposure.

Mercury and cadmium are two elements of first priority, recommended by FAO to carry out environmental pollution studies; chromium and lead are also of great interest; it is very important to control the levels of these metals, since they are frequently found in the area of study due to industrial activity.

The purpose of the present study is to evaluate the degree of toxicity of various heavy metals (chromium, cadmium, mercury and lead to freshwater crayfish Procambarus clarkii of Lake Albufera of Valencia, Spain.

Since the temperature is an environmental variable which could affect strongly the toxicity of heavy metals (Green et al., 1976), the effects of temperature on the degree of toxicity of heavy metals are studied.

A second issue in the present investigation is the study of accumulation of heavy metals after 96h Cr, Cd, Hg and Pb exposure and their distribution in several tissues (gills, midgut gland, antennal gland and muscle) of this crayfish.

2. MATERIALS AND METHODS

Adult intermolt specimens of the crayfish Procambarus clarkii were collected in Lake Albufera (Valencia, Spain) and taken immediately to the laboratory where they were maintained to 300-L aquaria and for 15 days, at 20°C with a daily diet of pork liver.

2.1 Toxicity

Groups of ten crayfish were kept in tap water at several metal concentrations, each group in a 15-l experimental aquarium. Ten more crayfish used as controls were kept in 15-l clean water. Only crayfish weighing between 15 and 20 g were used.

Desired chromium concentrations were obtained by addition of appropriate amounts of stock solutions, which were prepared using tap water and $Na_2CrO_4.4H_2O$ (E. Merck).

Reagent grade $CdCl_2.H_2O$ and $HgCl_2$ (E. Merck) were made up to a stock solutions of 1 mg Cd^{++}/ml and 0.1 mg Hg^{++}/ml, respectively. Aliquots of these solutions were added to each test aquaria to achieve the appropriate concentrations.

Water quality in each aquaria was monitored daily for changes in pH and oxygen concentration. Other variables were determined at the beginning of each experiment: alkalinity, hardness and chloride concentration.

All tests have been conducted under "static conditions". In preliminary experiments the most suitable metal concentration ranges for acute toxicity tests were determined. Toxicity tests were carried out in thermostated (± 1°C) water baths. No food was added during the experiments to avoid adsorption and/or chemical interactions of metal ions. Animals to be used in the toxicity tests were acclimated to the test conditions for at least two days before they were exposed to the metal. During acclimation and toxicity test period all aquaria were aerated. The 96 h period was recommended as more suitable in the literature (US EPA, 1975).

The lack of movement by the pleopods and antennae when gently prodded was used as the criterion for animal death. Although pleopod movement is often taken by workers as the sole criterion, our experience is that it is not always reliable; pleopods may remain stationary for considerable periods in animals close to death whereas antennal movement can often still be stimulated. Animals were observed twice every day. Dead animals were removed after observation in order to avoid cannibalism.

The percentages of mortality were calculated in each concentration after 96 h exposure and converted to probits (Fisher and Yates, 1982); the metal concentrations were converted to Logs. The concentrations causing 50% mortality of the test animals, the LC_{50}'s and their 95% confidence limits and the slope of the probit line were calculated using the method described by Litchfield and Wilcoxon (1949).

2.2 Metal accumulation

The different tissues of the control and treated crayfish were dissected using plastic instruments in order to avoid metal contamination. Prior to analyses, the different tissues were lyophilized and homogenized. Digestion was carried out with concentrated HNO_3. Reagents used were of high purity appropriate for those metal analyses and, to avoid contamination.

Fifty crayfish ranging in weight 17.5 to 34.8 g were divided into five groups of 10 animals each. These were kept in 15 l experimental aquaria containing 10, 37, 136 and 500 mg l^{-1} Cr(VI) as Na_2CrO_4 (Merck). 10 more crayfish served as control and were kept in 15 l of clear water. After 96 hours of Cr-exposure at 19.5°C, the animals were transferred to clean water, free of any contamination, and kept there for an additional 5 hours.

Digestion was carried out as follows: 0.01-1 g of lyophilized tissue were introduced into the reaction flask and 10 ml of concentrated HNO_3 were added. The samples were digested on a hot plate at a temperature of about 80°C until nitrous vapours disappeared (approximately 12 hours). After cooling, solutions were quantitatively transferred and diluted with twice-distilled water to a final volume of 25 ml. The high number of samples makes the procedure of digestion in teflon reactors under pressure very tedious. Therefore, were preferred to use open flasks, which allows us to work comfortably with a large number of samples. Precision (expressed as relative standard deviation) and accuracy of the latter method, were determined from six

replicates of a homogenized sample of Mytilus galloprovincialis used for intercalibration (Coordinator Center: Escuela Nacional de Sanidad, Madrid). Analyses of chromium were carried out by flameless AAS, obtaining a precision of 14,3% and an accuracy of 8% for a content of 2,12 $\mu g\ g^{-1}$ dry weight. These values were similar to those obtained by carrying out the digestion with teflon reactors under pressure.

In most analyses, it was necessary to use the whole sample for the digestion due to the little amount of sample available. Therefore, repeated analyses of a single sample could not be carried out. In control samples and in most low Cr-concentrations treated samples (especially in muscle), the Cr content was lower than the applicability range flame for flame AAS. Thus, in order to avoid the use of two different methods, depending on the chromium level to be determined, we have chosen the HGA technique because it allows one to analyze all the samples (when Cr concentration was higher than 500 ng ml^{-1}, an aliquot of the sample was diluted with 4:10 HNO_3).

On the other hand, recoveries of three standards of Cr(VI) subjected to wet digestion were found to be as follows:

40 ng ml^{-1} - 93.8%, 200 ng ml^{-1} - 105.5%, 400 ng ml^{-1} - 102.6%.

These results show that during wet digestion no losses of chromium occurred.

A Perkin-Elmer Atomic Absorption Spectrophotometer 2380, equipped with a recorder 561, a deuterium background corrector, and a HGA 400 Heated Graphite Atomizer was used to measure atomic absorption. Determination of chromium was carried out at 357.9 nm with drying, charring and atomization temperature of 120, 1100 and 2500°C, respectively, using argon as purging gas.

Forty crayfish ranging in weight from 15.3 to 28.5 g were divided into four groups of 10 animals each. These were kept in 15-l experimental aquaria containing tap water. The cadmium stock for all experiments was reagent grade $CdC_{12}.H_2O$ (E. Merck); a stock solution of 1000 mg l^{-1} of cadmium was prepared. Aliquots of this stock were added to each test aquaria to bring the Cd concentrations to the desired levels of 3.2, 10, 32 and 100 $\mu g\ l^{-1}$. After 96 h of Cd-exposure at 20°C, the animals were transferred to clean water, free of any contamination, and kept there for an additional 5 h.

The digestion procedure was as follows: 0.005-1.5 g of lyophilized tissue was introduced into a 100-ml Erlenmeyer flask and 10 ml of conc. HNO_3 was added. The samples were digested on a hot plate at a temperature ranging between 80-90°C for approximately 14 h. In some cases, particularly in muscle and midgut gland, it was possible to make several digestions for Cd analysis. A mean precision (expressed as relative standard deviation) of 12.3% was obtained in these cases. After cooling, solutions were quantitatively transferred to a 25-ml beaker and diluted with water to the mark. In all experiments several blanks were processed to ensure that contamination was not occurring. Using this procedure of digestion, we have proven that no losses of cadmium occurred; the recoveries of three standards of Cd(II) being obtained: 4 ng ml^{-1}, 105.2%; 20 ng ml^{-1}, 95.5%; 40 ng ml^{-1}, 95.8%. An accuracy of 6.5% was obtained for the method used by comparing the results obtained from six replicates of a standard sample of Mytilus galloprovincialis (Escuela Nacional de Sanidad, Madrid).

Analyses of cadmium were made by direct comparison with aqueous standards, and also by the standard additions method, to demonstrate the matrix interference in the four tissues examined. Three different calibration curves were prepared (0-5, 5-20, 20-50 μg of cadmium l^{-1}) depending on the range of concentrations of metal in each sample. Standard solutions of Cd(II) and sample solutions were put in the same conditions of acidity ($HNO_3$4+10).

Absorbance measurements were made on a Perkin-Elmer model 2380 atomic absorption spectrophotometer equipped with a model 561 recorder, a deuterium background corrector, and an HGA 400 Heated Graphite Atomizer. Determination of cadmium was performed at 228.8 nm with drying, charring and atomization temperatures of 120, 250 and 1100°C respectively, using argon as the purging gas. A final cleaning step at 2700°C was also used.

Thirty crayfish ranging in weight from 13.7 to 29.5 g were divided into three groups of 10 animals each. These were kept in 15-l experimental aquaria containing tap water. The mercury stock for all experiments was reagent grade $HgCl_2$ (E. Merck); a stock solution of 1000 mg l^{-1} of mercury was prepared. Aliquots of this stock were added to each test aquaria to bring the mercury concentrations to the desired levels of 50, 100 and 250 μg l^{-1}. After 96 h of Hg-exposure at 20°C, the animals were transferred to clean water, free of any contamination, and kept there for an additional 5 h.

Digestion was carried out as follows: 0.01-0.4 g of lyophilized tissue were introduced into teflon reaction flasks and 4 ml of concentrated HNO_3 were added. The samples were digested under pressure at a temperature of 140°C (1 hr). After cooling solution were quantitatively transferred and diluted with twice-distilled water to a final volume of 25 ml. This digestion system under pressure was chosen to avoid any mercury losses which could occur using digestions in opened glass reaction flasks.

On the other hand, recoveries of three standards of Hg(II) subjected to wet digestion under pressure were found to be as follows: 101.2% (250 μg Hg), 98.7% (2.5 μg Hg). These results show that during this digestion no losses of mercury occurred.

Analyses of mercury were carried out by AAS cold-vapour technique. Absorbance measurements were made on a Perkin-Elmer model 5000 atomic absorption spectrophotometer equipped with a model 561 recorder and mercury hydride system (MHS-10). Determination of mercury was carried out at 253.7 nm with $NaBH_4$ as reductor agent and argon as purging gas, with 5 ml of sample. In some cases, due to the high mercury concentration, the sample was diluted from 5 to 100 times. Blanks subjected to digestion and blanks of the calibration curves gave similar absorbance values, and always lower than 0.020 units.

Three groups of eight crayfish were kept in 15-l experimental aquaria. Eight more crayfish served as a control. Only crayfish weighing between 15 and 20 g were used. Desired lead concentrations were obtained by addition of appropriate amounts of stock solutions, which were prepared using $Pb(NO_3)$ (E. Merck). After 96 h of Pb-exposure (10,50 and 100 mg of Pb l^{-1}) at 22°C, the animals were transferred to clean water (free of any contamination) and kept there for an additional 5 h.

Digestion was performed as follows: 0.01-1 g of lyophilized tissue were introduced into a 100 ml erlenmeyer flask and 10 ml of concentrated HNO_3 were added. The samples were digested on a hot plate with temperature of 80-90°C until nitrous vapours disappeared. After cooling, solutions were quantitatively transferred to a 25 ml beaker and diluted with twice-distilled water to the mark. Recoveries of three standards of Pb(II) (subject to an analogous wet digestion in open flasks) being obtained: 1 $\mu g\ ml^{-1}$, 98.3%; 2 $\mu g\ ml^{-1}$, 103.2%; 4 $\mu g\ ml^{-1}$, 101.5%. In the same way, the addition of 4 μg of lead l^{-1} in a sample of muscle tissue (subject to the same process of digestion) shows a recovery of 101.8%. These results show that during the wet digestion no losses of lead occurred in open flask.

Absorbance measurements were made on a Perkin-Elmer model 5000 atomic absorption spectrophotometer equipped with a model 561 recorder, a deuterium background corrector for flame and an HGA 400 Heated Graphite Atomizer with Zeeman 5000 background corrector. Determination of lead was performed by flame method for all samples except the control tissues. These were analyzed with graphite furnace using the direct method with $(NH_4)_2HPO_4$ as matrix modifier (May and Brumbaugh, 1982; Medina _et al._, 1987). Measure conditions were: 283.3 nm with drying, charring and atomization temperatures of the 120, 500 and 1400°C, respectively (Medina _et al._, 1986) for graphite furnace using direct and standard additions methods. The measure conditions when 0.5% $(NH_4)_2HPO_4$ was used as matrix modifier were as follows: drying 120°C, charring 800°C and atomization 2400°C. In both cases argon was used as the purging gas. A final cleaning step at 2700°C was also used.

The values of lead content in the tissues studied were analyzed by one-way analysis of variance and multiple comparation test (Tukey-test) among treatments.

3. RESULTS AND DISCUSSION

3.1 Toxicity

Preliminary experiments on the toxicity of chromium showed that after 96 h a concentration of 0.5 g Cr(VI) l^{-1} caused the death of only 40% of the population. Such elevated concentrations did not occur in the natural medium (Roselló, 1983) and we did therefore not perform the acute toxicity for chromium.

A summary of the acute toxicity results for cadmium and mercury at various temperatures is given in Table II, showing the 96 h LC_{50} values with 95% confidence limits. It must be emphasized that all LC_{50}'s are based on the initial amount of metal added to the dilution water and that after 96 hours the concentrations may have been somewhat less than those indicated.

Figures 1 and 2 show the % mortality (converted to probits) after 96 h of mercury and cadmium exposure with respect to the metal concentrations at three temperatures (20, 24 and 28°C). Regression curves were fitted by the least squares method. Slopes and intercepts of the individual regression lines is given in Table I.

The responses of crayfish to mercury and cadmium was further investigated with respect to different exposure times (Figs 3 and 4). In general the increase in percent mortality was related to both time and metal concentration, with the highest mortality occurring after 48 h of metal exposure. However, in the case of mercury at the highest temperatures (24 and 28°C), the highest mortality occurred between 24 and 72 h for 24°C and between 24 and 48 h for 28°C.

Results from this series of 96 hr-LC_{50} tests suggest that mercury has the most toxic effect on the P. clarkii followed by cadmium and chromium. These findings are in agreement with other authors. For example, Eisler and Hennekey (1977) reported the acute toxicity of cadmium, chromium, mercury, nickel and zinc to six estuarine macrofaunal species. In general, the rank order of toxicity of metals tested was Hg>>Cd>>Zn>Cr>Ni.

Ahsanullah (1982) studied the acute toxicity of chromium and mercury to the amphipod Allorchestes compresa. His results suggest that mercury has the most toxic effect, followed by chromium. Papathanassiou (1983) studied the effects of cadmium and mercury ions

Table I

Slopes and intercepts of the individual regression lines for mercury and cadmium (X = % mortality, Y = log, concentration in mg l^{-1}).

Mercury	Cadmium
20°C: $y = 4.50\ x - 7.85$; r^2=0.94	20°C: $y = 2.96\ x - 0.23$; r^2=0.99
24°C: $y = 2.34\ x - 0.94$; r^2=0.99	24°C: $y = 5.24\ x - 3.08$; r^2=0.99
28°C: $y = 2.71\ x - 0.81$; r^2=0.98	28°C: $y = 2.15\ x + 2.28$; r^2=0.97

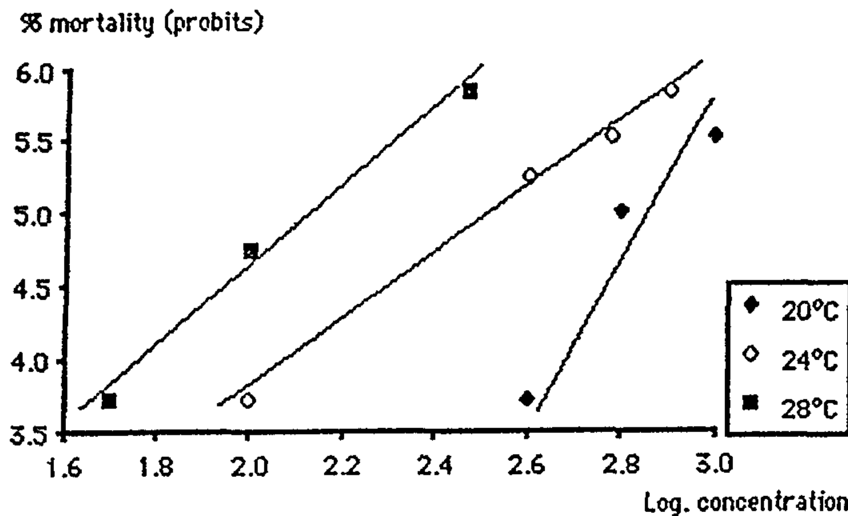

Fig. 1 Mortality/Mercury-concentration relations for Procambarus clarkii at 20, 24 and 28°C

Table II

The 96 h LC_{50} values (mg l^{-1}) and 95% confidence limits for mercury and cadmium at 20, 24 and 28°C with Procambarus clarkii. Each 96 h LC_{50} value represents the mean of 3 replicates.

Temperature	Mercury	Cadmium
20	0.79 (0.58-1.08)	58.5 (41.8-81.9)
24	0.35 (0.21-0.56)	34.8 (28.1-43.2)
28	0.14 (0.08-0.23)	18.4 (10.7-31.6)

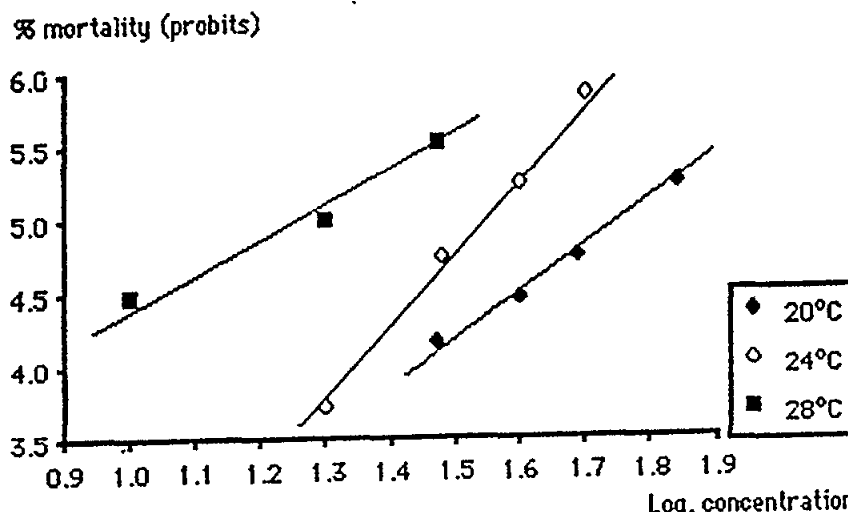

Fig. 2 Mortality/Cadmium-concentration relations for Procambarus clarkii at 20, 24 and 28°C

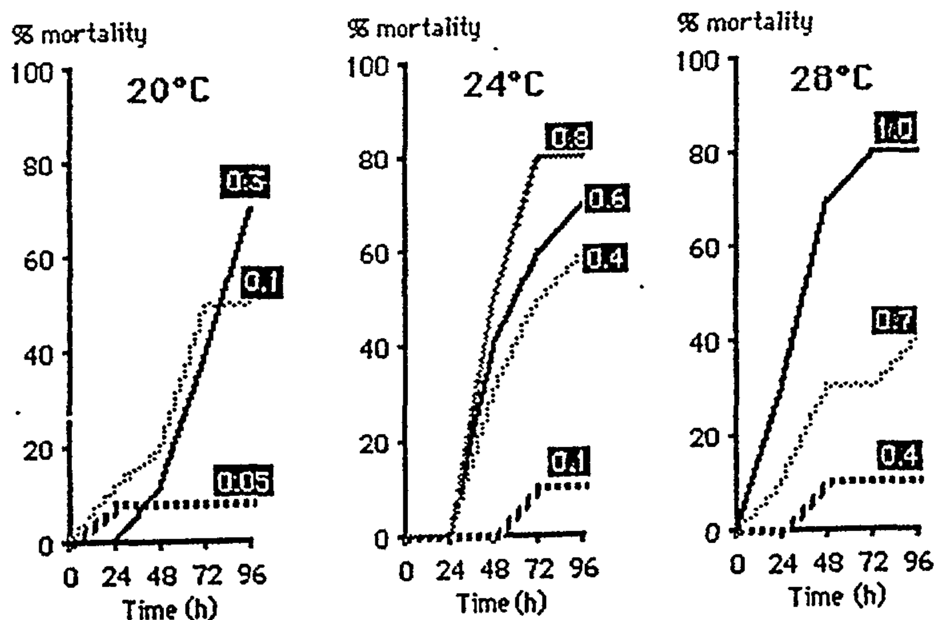

Fig. 3 Percent mortality versus exposure time for crayfish exposed to various concentrations (black squares) of mercuric chloride (mg l^{-1}) at three temperatures

upon the longevity of Palaemon serratus and suggested that mercury is more toxic than cadmium. Our results compared with literature data presented by Ahsanullah (1982) and Lake et al. (1979) show that Procambarus clarkii has very high LC_{50} for all heavy metals tested, except for mercury; that Orconectes limosus shows a 20°C 96 h LC_{60} = 1 mg Hg l^{-1} (Doyle and Klauning, 1976); and that in the case of cadmium Uca pugilator present a 20°C 96 h LC_{50} = 6.6 mg Cd l^{-1} (O'Hara, 1973).

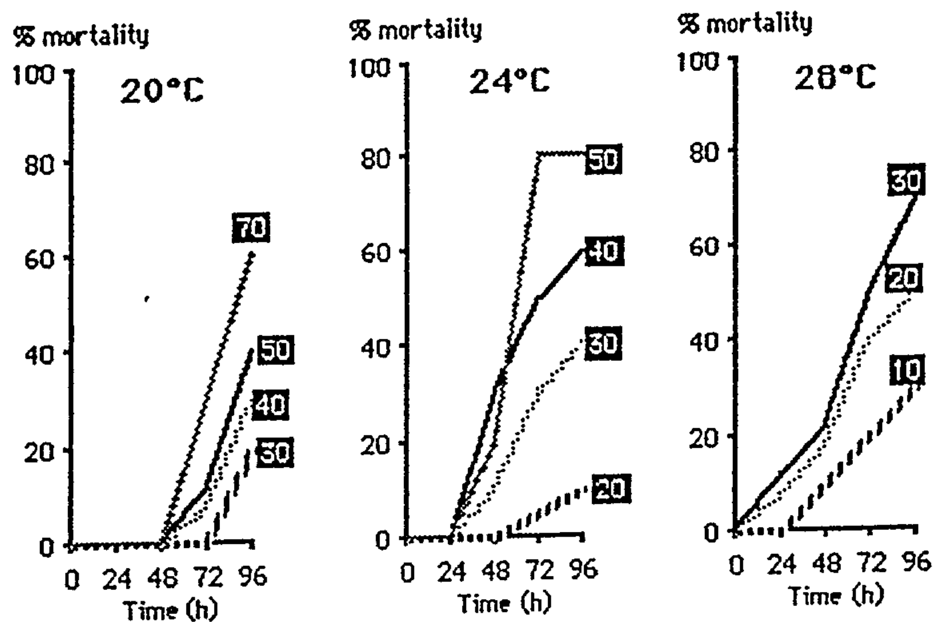

Fig. 4 Percent mortality versus exposure time for crayfish exposed to various concentrations (black squares) of cadmium chloride (mg l^{-1}) at three temperatures

In comparison with the other metallic salts commonly found in polluted waters, mercuric chloride is far more toxic than all of them (Cairns et al., 1975).

In the present study we have found that the toxic effects of cadmium and mercury increased in parallel to the increase temperature. The effect of temperature on the toxicity of mercury was more marked than in the toxicity of cadmium. So when the temperature increases from 20 to 24°C the mercury LC_{50} decreased to 56% whereas the cadmium LC_{50} decreased to 40% and, when the temperature increased from 24 to 28°C, the LC_{50} decrease was 60% and 47% for mercury and cadmium, respectively.

A marked influence of temperature on the toxicity of heavy metals has been demonstrated for different aquatic animals. In general, the higher the temperature is, the more toxic the compounds will be (Cairns et al., 1975).

The relationship between heavy metals toxicity and temperature variation illustrates that physiological stresses lower the tolerance of organisms to environmental pollutants (O'Hara, 1973).

In conclusion, the Procambarus clarkii from Albufera Lake, present a high resistance to the heavy metals pollution. In previous reports, we have shown that this crayfish accumulated great amounts of chromium (Hernandez et al., 1986) and cadmium (Diaz-Mayans et al., 1986) after exposure to these metals. The importance of metallothioneins in the detoxification events of heavy metals is well known (Ridlington and Fowler, 1979; Engel and Brouwer, 1984). These kinds of mechanisms are probably related to the resistance and accumulation ability of heavy metals in Procambarus clarkii.

3.2 Chromium accumulation

Results obtained by direct method (direct calibration with aqueous standards of chromium in the same conditions of acidity than that of samples) and standard additions method have been compared. Results obtained in the first case were always lower than those of standard additions method. Mean differences of 32.5% (midgut gland), 34.8% (muscle), 19.6% (gland), and 18.9% (gills) indicated that an important matrix interference occurs. Consequently, the standard additions method is the most adequate to perform this study. Nevertheless, when Cr concentration was higher than 500 ng ml^{-1}, flame AAS was also applied to compare the results; using the direct method, concentration of Cr in all tissues analyzed by flameless AAS were always lower than those analyzed by flame AAS, with a difference of about 30%. However, the results obtained by flame AAS (using the direct method) and those obtained by flameless AAS (using the standard additions method) were more similar; a mean difference of 10% was obtained.

Chromium levels of the control and treated crayfish tissues, exposed for 96 hours to 10, 37, 136 and 500 mg l^{-1} of Cr(VI) are presented in Table III. The control crayfish showed chromium levels ranging from 0.4 ± 0.2 $\mu g\ g^{-1}$ dry weight in muscle to 38.2 ± 5.0 $\mu g\ g^{-1}$ dry weight in antennal glands.

The relative mean Cr level in control tissues were: antennal glands>gills>midgut gland>muscle. It is important to indicate that the control animals showed amounts of Cr about 38 $\mu g\ g^{-1}$ in antennal glands and 13 $\mu g\ g^{-1}$ in gills. This can be indicative of Cr contamination in Albufera waters.

Table III

Chromium levels ($\mu g\ g^{-1}$ dry weight) in some tissues of crayfish after 96 h of Cr (VI)-exposure at several concentrations.

mg Cr(VI) l^{-1}	Gills	Midgut gland	Antennal G.	Muscle	Total
Control	13.1±1.6	1.0±0.4	38.2±5.0	0.4±0.2	52.7
10	67.2±17.0	20.3±3.5	37.5±9.2	1.8±0.4	126.8
37	89.4±13.3	55.9±25.0	147±42	3.9±1.2	296.2
136	230±69	189±99	286±88	7.3±1.5	712.3
500	541±125	462±102	1170±202	32±3	2205.0

After 96 h of Cr exposure, the Cr levels in all examined tissues increased with increasing Cr concentration in the water.

A one-way analysis of variance (ANOVA) indicated significant Cr-concentration effect on Cr levels in all tissues examined ($p<0.001$). The highest accumulation occurred in muscle. Figure 5 shows the % accumulation in tissues after 96 h of Cr-exposure, with respect to the total chromium detected in crayfish. 70% of Cr was present in glands of the control crayfish, whereas, Cr-content in muscle of animals treated with 500 mg l^{-1} of Cr(VI) was only 2%. Relative % mean chromium levels in tissues of treated crayfish were as follows: antennal glands>gills>midgut gland>muscle, as occurring in the control tissues.

Regression lines were fitted to the data presented in Table III, for each of the tissues, using the general expression: $y = a + bx$ where y = chromium levels ($\mu g\ g^{-1}$ d.w.), and x = mg l^{-1} of Cr(VI) in water. The following expressions were derived: Gills, $y = 52.45+1.02x$ ($r=0.86$); Antennal glands, $y = 30.61+2.23x$ ($r=0.93$); muscle, $y = 0.72+0.06x$ ($r=0.96$); midgut gland, $y = 18.60+0.91x$ ($r=0.87$).

Chromium concentration in tissues, expressed on a dry weight basis, increases linearly when increasing the chromium concentration of the test solution. Animals at the higher Cr-concentrations continue to accumulate chromium.

As it has been demonstrated, the crayfish P. clarkii presented a high capacity for chromium accumulation, which is not dependent upon the size and sex of animals ($p>0.05$).

Amounts of chromium as high as 38 $\mu g\ g^{-1}$ were found in antennal gland of the control animals. This is probably indicative of Cr contamination in Lake Albufera waters. We highly recommend the use of sanitary conditions for raising these crustaceans since they are being utilized for human consumption.

3.3 Cadmium accumulation

Results obtained by the application of two methods used were very different, always being lower by applicating the direct method. Mean differences of 60.3% (antennal glands), 57.3% (gills), 55.4% (midgut gland) and 42.5% (muscle) indicated that an important matrix interference occurs, which would result in errors if the direct method was used as method of analysis. These high differences have also been observed when analyzing some marine organisms for cadmium content (Medina et al., 1986) and are much higher than those observed when analyzing for chromium in the crayfish P. clarkii (Hernandez et al., 1986). Consequently, the standard additions method is the most adequate for present study.

In very few cases, it was possible to measure absorbances by flame atomic absorption spectroscopy (only in gills and in some midgut gland of the crayfish treated with 100 μg of cadmium l^{-1}). In these instances, using the direct method, results obtained by flame AAS were higher than those of flameless AAS, but much lower than those obtained by the standard additions method and flameless AAS (mean differences of 50.4% for gills, and 43.8% for midgut gland).

Cadmium levels in the gills, midgut gland, antennal glands and muscle of the control and the crayfish exposed for 96 h to 3.2, 10, 32 and 100 μg of Cd(II) l^{-1} are presented in Tables IV, V, VI and VII respectively.

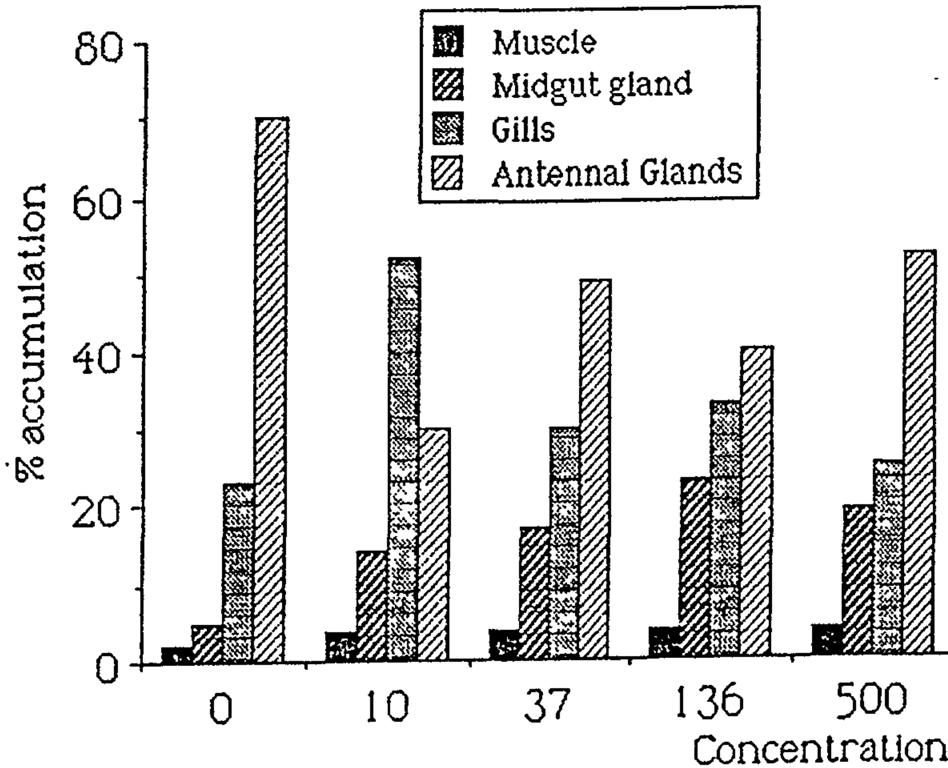

Fig. 5 Percent of chromium accumulation (with respect to the total chromium) in muscle, midgut gland, gills, and antennal glands of the control and the treated crayfish

The control crayfish showed cadmium levels ranging from 0.02 $\mu g\ g^{-1}$ dry weight in muscle to 3.08 ± 0.82 $\mu g\ g^{-1}$ dry weight in antennal gland. It is important to indicate that the control animals showed total amounts of Cd about 2 ppm localized in the four tissues examined. This can be indicative of a cadmium contamination in Albufera waters.

After 96 h, Cd(II)-exposure, the cadmium levels in all examined tissues increased with increasing cadmium concentration in the water.

A one-way analysis of variance (ANOVA) indicated significant concentration effects on cadmium levels in gills, midgut gland and muscle ($p<0.01$). However, there were not significant treatment effects on the antennal gland concentration of cadmium ($p>0.05$).

Regression curves were fitted, by the least squares method, to the data presented in Tables IV, V and VII corresponding to gills, midgut gland and muscle, respectively. The regression to the gills data was linear, whereas it was exponential for the midgut gland and muscle data.

Table IV

Cadmium levels ($\mu g\ g^{-1}$ dry weight) in gills of crayfish after 96 h Cd-exposure at several water concentrations.

μg Cd(II) l^{-1} of water				
0	3.2	10	32	100
1.93*	1.83	4.56	8.71	44.80
1.33*	1.26*	4.44	8.94*	31.18
1.41	1.14	3.43*	19.18	44.04
0.63	1.59*	2.81	6.20	27.85
1.16	1.10	2.57	20.16	41.25
1.22	2.00	4.75*	10.12	27.57
1.00*	2.13*	3.87	18.73	42.25
—	—	5.46	9.74	29.57
—	—	—	13.08	26.25
—	—	—	—	31.29
1.24 ± 0.40	1.58 ± 0.42	3.98 ± 1.00	12.76 ± 5.26	37.35 ± 10.56
F=87.51; df=4.36; p<0.01 Linear regression: r^2=0.90; y=1.04 + 0.33x				

Unless otherwise stated, each value corresponds to one sample.
(*) Pooled sample of gills from two animals.

Table V

Cadmium levels ($\mu g\ g^{-1}$ dry weight) in midgut gland of crayfish after 96 h Cd-exposure at several water concentrations.

μg Cd(II) L^{-1} of water				
0	3.2	10	32	100
0.65*	0.22*	0.15	0.45*	5.30
0.69	0.42*	0.40	0.33	0.91
0.44*	0.32	0.59*	0.39	4.86
0.37*	0.35*	0.33*	1.37*	0.79
0.44	0.63*	0.68	0.40	1.29*
0.42*	0.54	0.75	0.43	0.82
—	—	0.55*	1.38	3.25
—	—	—	1.05	2.63
—	—	—	—	1.83
0.50 ± 0.13	0.41 ± 0.15	0.49 ± 0.21	0.72 ± 0.46	2.41 ± 1.74
F=7.08; df=4.31; p<0.01 Exponential fit: r^2=0.56; y=0.40 $e^{0.01\ x}$				

Unless otherwise stated, each value corresponds to one sample.
(*) Pooled sample of midgut gland from two animals.

Table VI

Cadmium levels ($\mu g\ g^{-1}$ dry weight) in antennal glands of crayfish after 96 h Cd-exposure at several water concentrations.

µg Cd(II) l^{-1} of water				
0	3.2	10	32	100
2.31**	5.68**	1.96**	3.72*	4.07
3.00**	1.57**	0.87	1.41**	4.48*
4.23**	0.67	0.75	1.23*	5.88*
2.78	3.10**	1.43**	1.95	7.45
--	--	1.63*	1.18	2.91
--	--	--	2.13	13.37
--	--	--	--	1.59
--	--	--	--	1.67
3.08 ± 0.82	2.75 ± 2.19	1.33 ± 0.51	1.94 ± 0.95	5.17 ± 3.87
F=2.55; df=4.22; p>0.05				

Unless otherwise stated, each value corresponds to one sample
(*) Pooled sample of antennal gland from two animals
(**) Pooled sample of antennal gland from three animals.

Table VII

Cadmium levels ($\mu g\ d^{-1}$ dry weight) in muscle of crayfish after 96 h Cd-exposure at several water concentrations.

µg Cd(II) l^{-1} of water				
0	3.2	10	32	100
0.01	0.03	0.08	0.51	1.46
0.03**	0.04*	0.10*	1.00	0.40
0.03*	0.03**	0.11*	0.26	0.75
0.02**	0.03	0.07	0.71	0.54
0.02	0.03	0.05*	0.80*	1.50
--	0.04*	0.16	0.90	0.60
--	--	0.14	0.37	0.83
--	--	--	0.17	1.57
--	--	--	0.68	1.26
--	--	--	--	0.89
0.02 ± 0.01	0.03 ± 0.01	0.1 ± 0.04	0.60 ± 0.28	0.98 ± 0.43
F=19.67; df=4.32; p<0.01 Exponential fit: r^2=0.66; $y=0.05\ e^{0.03\ x}$				

Unless otherwise stated, each value corresponds to one sample
(*) Pooled sample of muscle from two animals
(**) Pooled sample of muscle from three animals.

Figure 6 shows the % accumulation in tissues after 96 h of cadmium exposure, with respect to the total amount of cadmium detected in crayfish. In controls, 62% of cadmium was present in antennal glands, whereas cadmium content in muscle was only 5%. In crayfish treated with 10,32 and 100 μg Cd l^{-1}, near 80% of cadmium was present in gills.

Relative % mean Cd levels in tissues of control and treated crayfish with low cadmium concentration (3.2 μg l^{-1}) were as follows: Antennal gland>Gills>Midgut gland>Muscle; in tissues treated with high cadmium concentrations, they were as follows: Gills>Antennal glands>Midgut gland>Muscle.

The experiments with cadmium suggested that the concentration of this metal in the tissues was a function of metal concentration in the water. Similar results have been obtained for a number of crustaceans and other invertebrates (Ahsanullah, 1982; Nimmo et al., 1977).

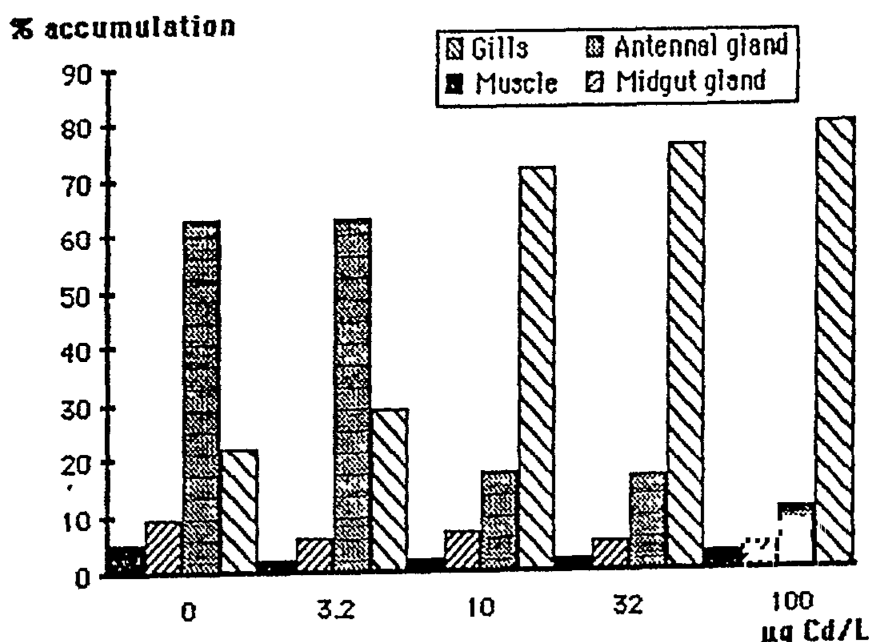

Fig. 6 % Accumulation of Cadmium with respect to the total cadmium amount in muscle, midgut gland, antennal glands and gills of the control and treated crayfish

O'Hara (1973) found that in Uca pugilator, the midgut gland and gills were important sites of cadmium accumulation when animals were exposed to 15 mg of cadmium l^{-1} in water, both these tissues reached a maximum cadmium concentration of approximately 110 ppm after 48 h; although the bioaccumulation was highest in the green gland tissue, with maximum concentrations of 380 ppm in tissue from crabs exposed to 25 ppm of cadmium. Gillespie et al. (1977), observed a great accumulation rate in Orconectes propinquus, where a mean of 18 ppm of cadmium was accumulated over a period of 190 h from water containing 10 ppb Cd. Our results show variations in the accumulation of Cd in different tissues. We found 4 and 37 ppm in gills from waters containing 10 and 100 ppb, respectively, whereas in muscle the accumulation was of 0.1 and 1 ppm, respectively, from the same waters.

As it has been demonstrated, the crayfish P. clarkii presents a high capacity for cadmium accumulation. Since these animals are ingested directly by man, a potential human health hazard exists.

3.4 Mercury accumulation

Determinations carried out for each organ by direct calibration method and standard addition method did not offer any mean differences and therefore we used the direct method. Due to this fact it was not necessary the use of deuterium background corrector.

Tissue mercury levels of the control and the treated crayfish exposed for 96 hours to 0.05, 0.10 and 0.25 mg of Hg l^{-1} are presented in Table VIII. The control crayfish showed very low mercury levels. Mercury content in the antennal glands were not detectable.

The relative mean mercury level in control tissues were: Gills>midgut gland>muscle>antennal glands. After 96 hours of Hg-exposure, the mercury levels in all examined tissues increased with increasing Hg-concentration ($p<0.01$). Regression lines were fitted to the data presented in Table VIII, for each of different tissues, using the general expression $y=a+bx$ were y=mercury tissues levels ($\mu g\ g^{-1}$ d.w.) and x=mg l^{-1} of Hg in water. The following expressions were derived: $y=5.1 + 971.9\ X$, $r=0.86$ (Gills), $y=-1 + 56.6\ X$, $r=0.75$ (Midgut gland), $y=-64 + 2932.2\ X$, $r=0.86$ (Antennal glands), $y=0.16 + 13.54\ X$, $r=0.89$ (Muscle).

Table VIII

Mercury levels ($\mu g\ g^{-1}$ weight) in some tissues of crayfish after 96 h of Hg-exposure at several concentrations.

mg Hg l^{-1}	Gills	Midgut gland	Ant. glands	Muscle	Total
0	0.93±0.51	0.08±0.06	---	0.02±0.01	1.03
0.05	69.8±24.1	1.09±0.61	40.1±9.2	1.28±0.10	112.27
0.10	83.7±18.8	2.6±1.4	122±153	0.80±0.03	209.12
0.25	249.3±66.9	13.68±5.85	697.4±194.5	3.59±0.52	963.97

Figure 7 shows the % accumulation in tissues after 96 hours of Hg-exposure, with respect to the total mercury amount detected in crayfish. The highest relative content of mercury in control animals was found in gills (90%). In treated crayfish, the % of mercury in gills decrease with increasing mercury concentration in water. In this way, 62%, 38% and 25% was found in gills of crayfish exposed to 0.05, 0.1 and 0.25 mg Hg l^{-1}, respectively.

In treated crayfish, the relative content of Hg in the antennal gland increase when increasing mercury concentration in water, corresponding 35, 56 and 69% to the glands of crayfish exposed to 0.05, 0.1 and 0.25 mg Hg l^{-1}, respectively. The % accumulation of muscle and midgut gland of control and treated crayfish remained in low levels.

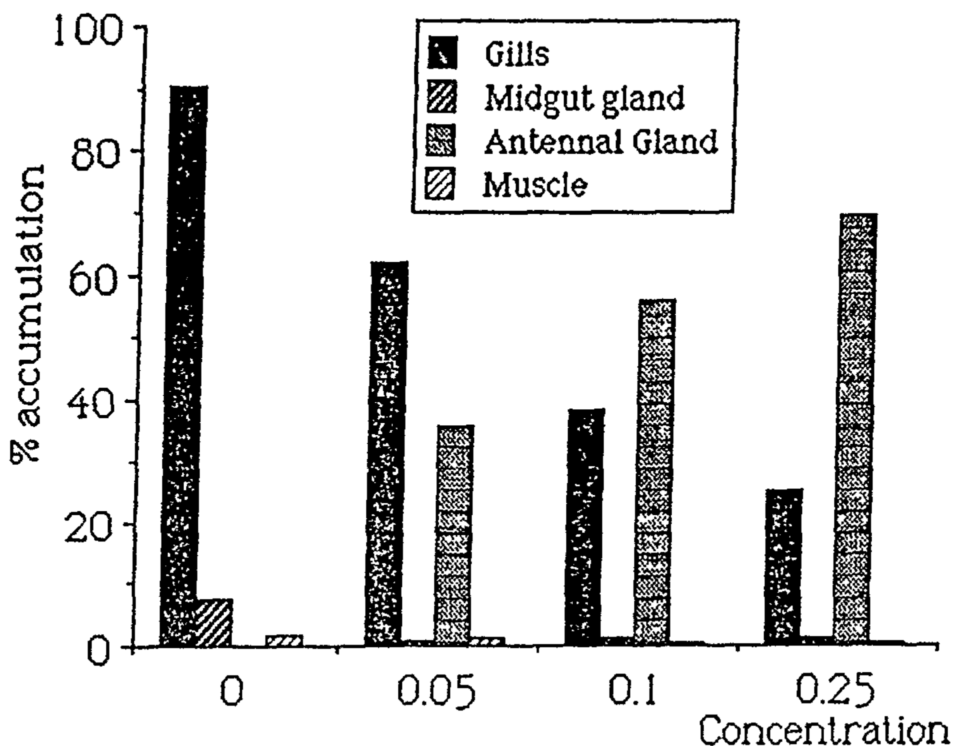

Fig. 7 Accumulation percentage of mercury (with respect to the total mercury amount) in gills, midgut gland, antennal glands and muscle of the control and treated crayfish

Figure 8 shows the relative Hg-accumulation rate (μg Hg g^{-1} d.w. day-1 mg Hg l^{-1}) of gills, midgut gland, muscle and antennal glands of crayfish treated with several Hg-concentrations.

In gills, the relative accumulation rate decreases when Hg-concentration in water increased from 0.05 to 0.1 mg l^{-1} and remains without apparent variation until the maximum Hg-concentration tested. On the other hand, the rate of uptake in antennal glands steadily increases for all Hg-concentrations tested. The relative accumulation rates of midgut gland and muscle show a tendency to become equal when the Hg-concentrations in water increase.

Since Hg-content was not detected in antennal glands of control crayfish and there is a gradual increase of Hg-content in this tissue of treated animals when Hg-concentration in water increases the antennal glands could play an important role on the Hg-elimination process in P. clarkii.

Our results demonstrate that the P. clarkii presents a high capacity for mercury accumulation. An increase in the relative accumulation rate has been found, especially in antennal gland, when the Hg-concentration increased which supports the statement of the previous sentence. This may be in accordance with the high capacity of mercury (especially $HgCl_2$) to pass across membranes. $HgCl_2$ passes across membranes more than a million times faster than ions such as Na^+ (Gutknecht, 1981; Simkiss, 1983).

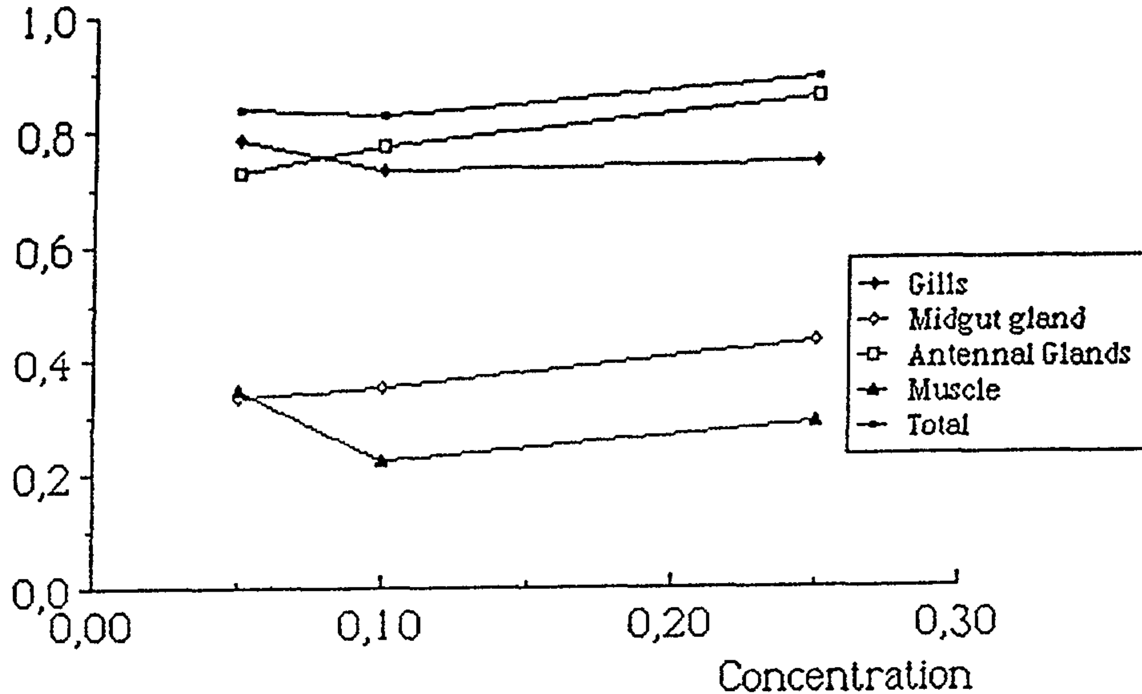

Fig. 8 Relative Hg-accumulation rate of gills, midgut gland, antennal glands, muscle and total of the crayfish treated with several Hg-concentrations

There are very few studies on the mercury accumulation and its distribution among tissues on crustaceans. On red crayfish P. clarkii, Heit and Fingerman (1977) found that the highest Hg-content were present in gills of crayfish exposed to 1 ppm of mercury for 7 days ($HgCl_2$). Unfortunately, they did not studied Hg-content in antennal glands. As it has been previously indicated, noticeable Cr and Cd contents have been found in tissues of this crayfish after 15 days of depuration on clean water. On the contrary, the contents in all tissues of control crayfish are very low. This could be due to the rapid elimination of metal by the antennal glands.

These results could indicate that the antennal gland plays an important role on the mercury elimination. However further investigation in this field is needed to elucidate the mechanism lying behind mercury elimination.

3.5 Lead accumulation

The determination of lead by flame AAS has been realized for all tissues exposed of lead since its content (considering the necessary dilutions) has been the adequate to make use of this method. The direct and standard additions methods have been applied for all the tissues. Significant differences have not been observed between these methods, therefore the direct method has been used.

In relation to control tissues, the lead content of the gills can be determined by flame whereas the levels of lead in midgut gland and muscle are found near the detection limit, although significant measures have been realized. In the case of antennal glands (with a limited quantity of available sample) the determination of lead by flame cannot be performed. For this control tissues the determination of lead has been realized basically by graphite furnace, and the direct and standard additions methods, so as the direct method with 0.5% $(NH_4)_2HPO_4$ as matrix modifier have been applied. The results obtained in this comparative study, as well as the results obtained by flame, are shown in Table XIII. It must be emphasized that the values obtained by flame and graphite furnace (with standard addition or in the presence of modifier) can be considered similar. Significant differences have also been found between the results obtained by graphite furnace using the direct and standard additions methods since a mean diminution of 45% has been obtained using the direct method. For this reason the determination of lead in the control samples has been performed by graphite furnace and direct method in the presence of 0.5% $(NH_4)_2HPO_4$.

An accuracy of 8.5% was obtained with graphite furnace and matrix modifier by comparing the results obtained from six replicates of the standard sample of Mytilus galloprovincialis (International Atomic Energy Agency, Monaco. Standard sample MA-M-2/TM). The precision, expressed as relative standard deviation, was 12.2%.

Lead concentrations in the gills, midgut gland, muscle and antennal glands of the control and crayfish exposed for 96 h to 10, 50, and 100 mg Pb l^{-1} are presented in Tables IX, X, XI and XII respectively.

Table IX

Lead levels (ppm of Pb) in gills of crayfish after 96 h Pb-exposure at several concentrations.

mg Pb(II) l^{-1} of water			
0	10	50	100
153	3940	12330	27039
184	3070	56470	53113
95	1730	36270	22338
211	970	20520	30969
125	1400	52200	13409
233	6600	4570	59053
410	4060	---	40486
370	---	---	---
223±113	3110±1966	30393±21357	35199±16547
F=13.8; df=3.24; p<0.001			

The control crayfish showed lead levels ranging from 6.9±2.8 ppm dry weight in midgut gland to 261±114 ppm dry weight in gills. This may be indicated of high lead contamination of Lake Albufera.

As can be seen in Tables IX, X, XI and XII the four analysis of variance show a significative effect of lead concentration, but they are different according to tissue. Two groups can be considered: effect on gills and the rest of tissues. In gills two groups of lead exposure can be considered: a Tuckey test shows that mean values of lead levels are different among control-10 mg Pb l^{-1} and 50-100 mg Pb l^{-1}. The other tissues, however, show only significant differences among control-10-50 mg l^{-1} and 100 mg Pb l^{-1}. There are not significant differences among the three different treatments (control-10-50 mg l^{-1}).

Table X

Lead levels (ppm of Pb) in midgut gland of crayfish after 96 h Pb-exposure at several concentrations.

mg Pb(II) l^{-1} of water			
0	10	50	100
6.1	380	70	150
5.0	140	850	250
4.2	160	430	300
9.6	70	830	1500
12.4	410	630	350
6.5	220	60	600
8.4	270	90	--
3.1	--	80	--
6.9±3.1	236±126	380±351	525±501
f=3.9; df=3.25; p<0.05			

In control and treated crayfish the highest % accumulation (with respect to the total amount lead detected) was present in gills. Near to 90% of lead was present in gills of crayfish treated with 100 mg of Pb l^{-1} whereas the lead content in other tissues, as midgut gland and muscle was less than 1%.

Similar results have been obtained in other crustaceans by several authors. Anderson (1978) found that gills were the most important site of lead accumulation when Orconectes virilis was exposed to several concentrations of lead; lead accumulation in muscle and midgut gland were lower than in gills. Crayfish tend to accumulate lead primarily in exoskeleton and gills (Anderson, 1978). Dickson et al. (1979) examined the concentration of lead in tissues of two species of crayfish obtained from natural water that contained only 2.3 ppm of lead. They found the highest lead concentration in gills and antennal glands and the lowest in midgut gland and muscle. Tulasi et al. (1987) show that the lead content in gills was very higher than lead content in muscle and midgut gland of crabs after 4 days of lead exposure.

Table XI

Lead levels (ppm of Pb) in muscle of crayfish after 96 h Pb-exposure at several concentrations.

0	10	50	100
16.2	40	30	710
12.8	24.1	120	220
9.0	9.9	250	70
11.1	6.3	80	320
14.2	19.0	220	200
25.1	13.1	70	70
8.6	15.3	40	100
13.2	22.7	40	—
15.8±6.1	35±16	106±85	241±226
F=6.08; df=3.27; p<0.01			

Table XII

Lead levels (ppm of Pb) in antennal glands of crayfish after 96 h Pb-exposure at several concentration.

mg Pb(II) l^{-1} of water			
0	10	50	100
162	2640	1870	990
78	1170	8350	2180
155	3390	1860	1240
55	1370	1510	8180
104	3780	1600	990
154	5050	2660	7670
82	4060	—	—
135	4520	—	—
110±38	3249±1418	2975±2664	3542±3427
F=3.62; df=3.23; p<0.05			

The crayfish P. clarkii has a high capacity for lead accumulation. The gills are the most important tissue of lead accumulation, as evidenced by increasing lead concentrations in the gills with increasing water concentrations. Anderson (1978) postulated that there was some type of physiological compensation and crayfish are able to acclimate the metal concentration by compensating for decreased gill efficiency. In a previous study we have found that high concentrations of lead caused some decrease in the oxygen consumption, so as histopathological alterations in gill tissue (Torreblanca et al., 1986).

Table XIII

Values of lead concentration (ppm) in control tissues by flame and graphite furnace.

TISSUES	FLAME	GRAPHITE FURNACE		
		Direct method	Standard additions	0.5% $(NH_4)_2HPO_4$
Gills	93 190 365	63 99 226	100 188 382	95 184 370
MIdgut gland	4.4 8.6 11.7	2.3 5.1 7.1	4.1 8.8 11.9	4.2 8.4 12.4
Antennal glands	— — —	41 71 85	84 110 138	78 115 154
Muscle	10.3 16.7 22.4	4.9 8.3 13.9	9.1 15.5 23.3	9.9 15.3 24.1

In the present study we have used sublethal lead concentrations, but that were near the LC_{50} value (96 h LC_{50}=127 ppm at 22°C). This fact allowed us to find the lead saturation levels in antennal gland of this crayfish (see Table XII). This is in accordance with the excretory function of antennal gland.

Since the crayfish used as controls in this study appear to be able to accumulate large quantities of lead without apparent lethal consequences, these animals may be potentially toxic and harmful in human and natural food chains.

9. CONCLUSIONS

a. According to LC_{50} 96h values of heavy metals, the American red crayfish Procambarus clarkii of Albufera Lake waters presents a high resistance to these elements. Mercury and cadmium are the most toxic of the heavy metals studied.

b. The toxicity of heavy metals (cadmium and mercury) was affected by temperature. The toxicity increases notably with increasing temperature.

c. After sublethal heavy metal exposure, Procambarus clarkii accumulate important amounts of chromium, cadmium, mercury and lead.

d. The heavy metal distribution among several tissues of the crayfish is a function of the heavy metal concentration used. Commonly the gills and antennal glands present a high content, whereas the muscle is the organ which accumulates lower amounts of metals.

e. As it has been demonstrated, the crayfish Procambarus clarkii has both a high resistance and high capacity for heavy metal accumulation. Since these animals are consumed directly by man, a potential human health hazard exists.

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STUDY OF THE BIOGEOCHEMICAL CYCLE OF ORGANOPHOSPHORUS PESTICIDES IN THERMAIKOS GULF, GREECE

by

K. FYTIANOS and V. SAMANIDOU

Environmental Pollution Control Laboratory,
University of Thessaloniki, Greece

ABSTRACT

Malathion and parathion are the most extensively used organophosphorus pesticides.

Their application for the control of pests is clearly an important source of these chemicals in the environment. The object of this project was to investigate the seasonal variations and the distribution of the organophosphorus compounds in the Thermaikos Gulf (N. Greece). For the determination of these compounds, water and sediment samples were taken every three months from four sampling stations in the gulf, for a total period of one year.

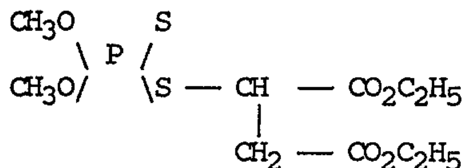
From the obtained data of this study we can conclude that the levels of the examined organophosphorus compounds are relatively close to those found for slightly polluted areas.

However particularly high concentrations were observed at the estuaries of Axios River and at the area where untreated municipal and industrial sewage are respectively discharged.

1. INTRODUCTION

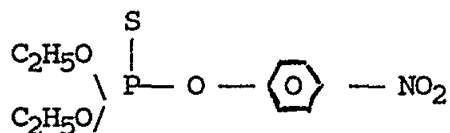
Malathion and Parathion (chemical forms I, II, respectively) are extensively used organophosphorus pesticides.

In 1972, agricultural, home and garden uses of these compounds accounted for approximately two-thirds of the USA domestic use. The remaining one-third was used for industrial, commercial and governmental purposes.



S-(1,2-Dicarbethoxyethyl) 0,0-dimethyl phosphorodithioate

I



0,0-Diethyl 0-p-nitrophenyl phosphorothioate

II

Malathion was one of the earliest organophosphorus compounds to be developed as an insecticide and is registered for use on more than 130 crops against a wide spectrum of insects and mites.

Malation and Parathion, like many other organophosphate insecticides, are used widely to control crop pests, flies and mosquitoes, presumably because they are degradable. Even though they decompose at high temperatures and with increasing alkalinity, they may be highly toxic to target and non-target organisms alike.

Malathion and Parathion act as a nerve poison by blocking synaptic transmission in the cholinergic parts of the nervous system. The distribution of nerve impulse transfers, is caused by excessive accumulation of the neurotransmitter acetylcholine (ACh). Malathion binds the active side of the acetylcholine esterase (AChe) and prevents breakdown of ACh, Coppage and Matthews (1974).

No evidence for the natural occurrence of these compounds was found in the literature reviewed, possibly due to the fact that it appears to be anthropogenic in origin. The application of malathion and parathion for the control of pests is clearly an important source of these chemicals in the environment.

Malathion is soluble in water at approximately 145 mg l^{-1} at 25°C, while the solubility of parathion in water is 24 mg l^{-1} at 25°C.

Their stability in solution is a function of pH. Malathion is hydrolyzed more rapidly in the presence of alkali than acid. The alkaline hydrolysis, under properly controlled conditions, results in quantitative yields of 0,0-dimethyl phosphorodithioate salts.

Parathion is quite stable in neutral or acid aqueous systems, but hydrolyzes under alkaline conditions, Badaway and El-Dib (1984). The hydrolytic decomposition of parathion proceeds via dearylation with loss of p-nitrophenol and yields to 0,0 diethyl monothiophosphoric acid, Weber (1976); Pritchard et al. (1987). Exposure to UV light results in some decomposition.

Later it was found that the metabolites of hydrolysis of malathion are non-toxic, Bourquin (1975).

Malathion is rapidly degraded in vitro by salt-marsh bacteria to malathion-monocarboxylic acid, malathion-dicarboxylic acid and various phosphothionates as a result of carboxyesterase cleavage. In addition, some expected phosphatase activity produces desmethylmalathion, phosphomono- and dithionates, 4-carbon dicarboxylic acids and the corresponding ethyl esters, Cowart et al. (1971).

Malathion and parathion are rapidly adsorbed from the digestive system after ingestion by mammals. Distribution is general; very low concentrations are found in many tissues, Mulla et al. (1981); Gile and Gillett (1981).

Both compounds are active against a broad range of insects. The acute oral LD_{50} (rats) is about 2500 mg kg^{-1} for malathion and 4 mg kg^{-1} for parathion.

The scope of this project was to investigate the seasonal variations and the distribution of the organophosphorus compounds in the Thermaikos Gulf.

2. MATERIALS AND METHODS

2.1 Thermaikos Gulf

In this area we have actually three zones. Thessaloniki Bay is in the direct vicinity of Thessaloniki City, Thessaloniki Gulf and Thermaikos Gulf both of which communicate with the North Aegean Sea.

Thessaloniki Bay and Gulf are characterized by their interesting geological shape. They actually consist of two basins communicating with each other and one of them with the open sea, by narrow and shallow deltas. About 120.000 m^3/day of untreated sewage water from the city of Thessaloniki, with a population of 1.000.000 inhabitants, are directly poured into the Bay of Thessaloniki. An amount of about 25.000 m^3/day of treated or partially treated diverse industrial effluents are discharged on the north-western coast of the Gulf, where the industrial zone is located.

The Axios river, which originates in Yugoslavia where approximately 650.000 m^3/day of untreated or partially treated domestic sewage and industrial effluents are discharged, flows also into the Thermaikos Gulf.

For the determination of the organophosphorus compounds malathion and parathion), water and sediment samples were taken every three months (time covered: 1.1.88-31.12.88) from 4 sampling stations in the Thermaikos Gulf as shown in the map (Fig. 1).

Station 1 was located in the area where industrial and municipal sewages are discharged.

Station 2 near the estuaries of Axios River, in order to estimate the wash out amount of pesticides by rain water.

Station 3 was located in a swimming area and Station 4 in the centre of the gulf.

Special samplers were used for seawater and sediments in order to avoid any contamination, (Sarkar and Gupta, 1986).

Sediment samples were kept at -25°C until analysis, Kjolholt (1985). Two liter samples of seawater were collected in bottles previously cleaned with 15% methylene chloride in n-hexane (v:v).

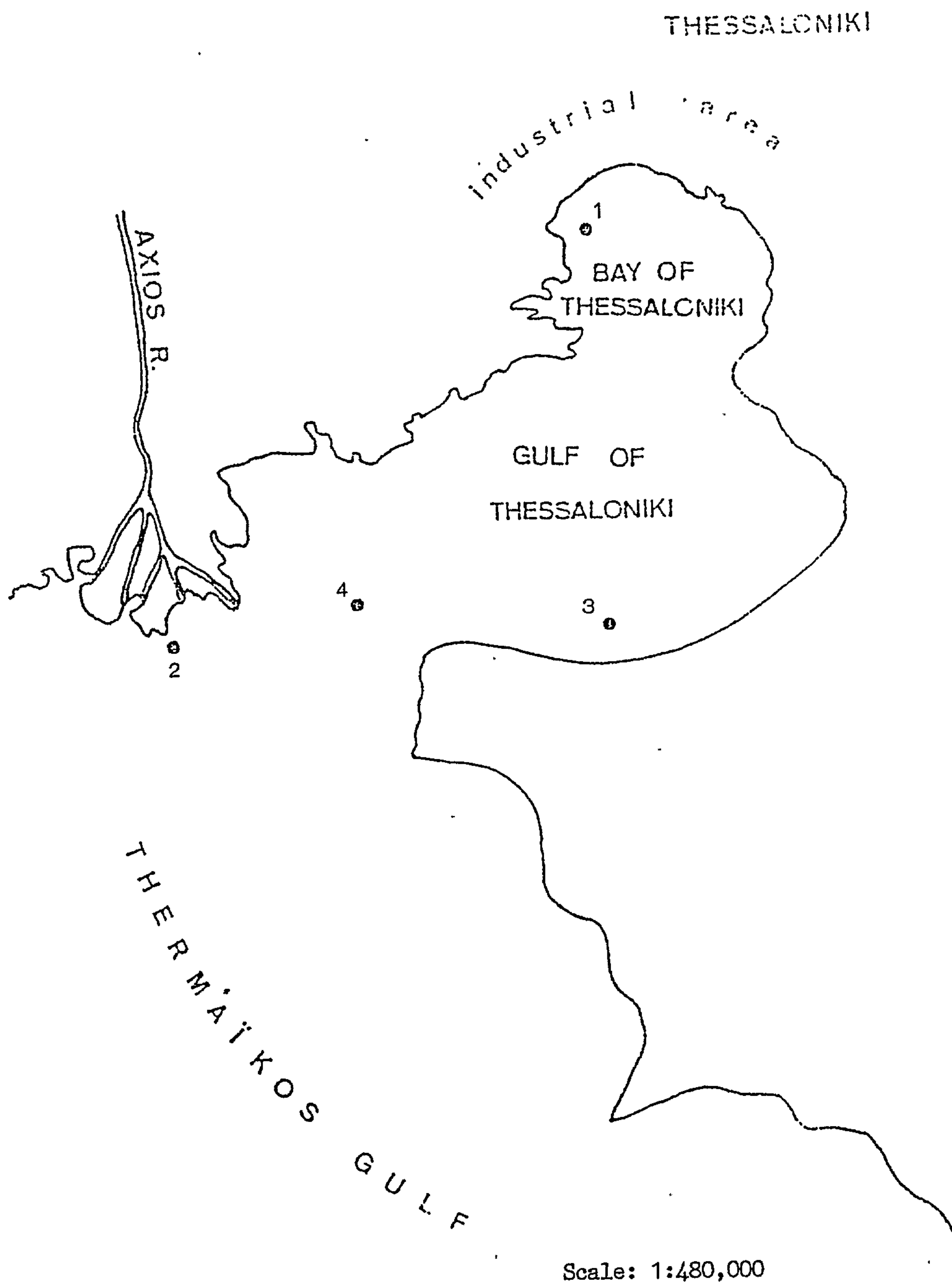


Fig. 1 Map of the Thermaikos Gulf with the sampling stations

They were stored in amber glass bottles at 4°C prior to extraction, normally within 24h of collection.

All glassware used in the analyses was heated at 250°C up to 40 min and repeatedly washed with solvents in order to remove all the pesticides traces. The solvents used were suitable for pesticide residue analysis, while the glass wool was silanized to avoid contamination, (Sherma, 1987; Lawrence, 1987).

For the organophosphorus insecticides one liter sample of water was extracted with 140 ml of a mixture of 15% methylene chloride in hexane (v:v) in sealed jars and placed horizontally on a Fisher-Kahn shaker (280 oscillations/min) for 15 min, Albanis et al. (1986). The extracts were removed by decanting and the water extracted two additional times by shaking for 15 min with 140 ml of 30% methylene chloride in n-hexane.

The three extracts were combined in a 500 ml bottle containing 5gr of anhydrous Na_2SO_4. Next the extracts were evaporated in a rotary evaporator and concentrated down to 4 ml (40°C water bath). For hydrolysis to take place 2 ml 10% methanolic KOH was added to methylene chloride extracts and the mixture was left to be hydrolyzed at room temperature. Next the solution was acidified and extracted with benzene (2x50ml). The combined extracts were passed through a 10g Na_2SO_4 column and concentrated in a rotary evaporator as previously.

As a clean up system for organophosphorus insecticides a microcolumn (14x0.5cm) was used filled with Florisil stored at 130°C. Two fractions were obtained after elution with 10ml portions of 6 and 15% ethyl ether in petroleum ether. The first elution fraction was discarded.

In order to assess the possible losses during the above procedures a number of tests were run with blanks containing a known amount of standard pesticide mixtures. Blanks containing Varian Supplied mixtures and spiked in the G.C. showed losses between 8-16% after the above procedure of extraction concentration and clean up.

Gas chromatographic analyses were carried out mainly on a varian 3300 G.C. equipped with a Ni^{63} E.C.D. Different columns were used as 6% QF-1 plus 4% SE-30 and 4% SE-20 plus 6% OV-210, both on 80/100 chromosorb W for organophosphorus insecticides and 3% OV-17 on 100/120 HP chromosorb W.

The temperatures set in the column, the injector and the detector of the aeriograph were 210-270 and 300°C respectively. All samples were run in duplicate and their concentrations determined by direct comparison with pure analytical standards and mixtures of them.

The sediment sample (ca 25g) was acidified with hydrochloric acid and extracted by Soxhlet overnight with 200 ml of acetone: hexane (4:1). The extract was reduced to ca 25ml on a rotary evaporator an in the case of presence of elemental sulphur in the sediment the solution was shaken with 10 ml of 0.1 M solution of tetrabutylammonium sulphite in order to remove this compound.

Both phases arising from this procedure were transferred to a separating funnel with 25ml of methylene chloride 100 ml of distilled water were added and pH adjusted to approx. 5-6.

The aqueous phase was partitioned with a total of 3x25ml of methylene chloride that were dried with anhydrous sodium sulphate and evaporated to almost dryness after addition of 0.5ml propylene glycol as "Keeper".

The residue was dissolved in 5ml of ethyl acetate, transferred to a glass column containing 10g of the mixture for the adsorption chromatographic separation with 120ml of ethyl acetate saturated with water and eluted with further 150ml of ethyl acetate: acetone: toluene (1:1:2) at 5-6ml/min. The total eluate was collected and evaporated to dryness.

The residue was finally dissolved in 1ml of cyclohexane.

3. RESULTS AND DISCUSSION

Results presented in Tables I and II show the residue levels of organophosphorus compounds (Malathion and Parathion) found in the surface seawater and sediments of the examined Thermaikos Gulf in Northern Greece.

Plotting the monthly values of organophosphorus concentrations for St.2 (estuaries of Axios River) versus time, a maximum at autumn and during spring and a minimum at summer was observed.

The peak concentrations were probably partially a result of heavy rainfall washing out the organophosphorus pesticides. About the same seasonal fluctuations were also observed for the other sampling stations in the Thermaikos Gulf.

No obvious seasonal fluctuations were observed in the examined organophosphorus pesticides for the surface sediments.

At St. 3 and 4 (near swimming and in the middle of the gulf) low concentrations of Parathion and Malathion were observed in the water samples during summer. (The concentrations were under the detection limits).

The analyses showed that the organophosphorus pesticides, parathion and malathion, were present in each sample with concentrations ranging from 2 to 46 ng l^{-1} for the water samples and from 3 to 35 ng g^{-1} for the surface sediments.

Comparing the concentrations of the examined compounds for water and sediment samples we can conclude that high values were observed at the stations 2 (estuaries of Axios river) and 1 (area where industrial and municipal sewages are discharged without any purification).

The high concentrations in the Axios estuaries indicate the use of organophosphorus pesticides due to the extensive agricultural cultivation in this area.

Table I

Concentrations of malathion and parathion in water samples from Thermaikos Gulf (ng l^{-1}).

	WINTER		SPRING		SUMMER		AUTUMN	
Station	Malathion	Parathion	Malathion	Parathion	Malathion	Parathion	Malathion	Parathion
St. 1	5	19	5	24	2	8	3	14
St. 2	9	32	16	46	4	15	5	37
St. 3	3	8	5	10	nd	nd	2	5
St. 4	nd	6	6	12	nd	4	4	7

nd = not determined

Table II

Concentrations of malathion and parathion in sediment samples from Thermaikos Gulf (ng g^{-1}).

	WINTER		SPRING		SUMMER		AUTUMN	
Station	Malathion	Parathion	Malathion	Parathion	Malathion	Parathion	Malathion	Parathion
St. 1	11	19	16	20	8	12	7	19
St. 2	23	35	27	33	12	23	29	40
St. 3	3	9	8	15	4	5	7	13
St. 4	4	9	10	14	4	7	9	12

The Thermaikos Gulf has a small mean depth and communicates with the open sea by a narrow and shallow mouth, which do not permit the streams to carry off the wastes into the open sea.

Comparing the present data to those reported in the literature, (Lenardon et al., 1984; Khan, 1977), the levels of the examined organophosphorus are relatively close to those found for slightly polluted areas.

However, particularly high concentrations were observed in the Stations 2 and 1 (estuaries of Axios river and area where untreated municipal and industrial sewage are respectively discharged).

Further more, the low water exchange with the open sea may lead to a continuous accumulation of these compounds in the future.

While the values found in the other examined areas indeed show the presence of environmental organophosphorus pollutants, the concentration of these compounds has not yet reached threatening levels (16).

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STUDY OF METHODOLOGY FOR THE DETERMINATION OF TOTAL ARSENIC IN MARINE ORGANISMS

by

J. OBIOLS, J. SALAYET and A. FERRAN

Instituto Quimico de Sarria, Barcelona, Spain

ABSTRACT

The present work describes the application of atomic absorption spectrometry using hydride generation and atomization in electrically heated quartz tube, for the analysis of arsenic in marine organisms. The diverse parameters involved in the technique, as well as the mechanisms explaining the chemical processes are studied and optimized.

A wet method of digestion has been applied for the analysis of arsenic content in biological materials. The total mineralization of the different forms of arsenic present has been verified, especially that of the methylated forms. The method has been applied to 6 samples of different marine organisms, thereby verifying the fact that the proposed technique works correctly.

1. INTRODUCTION

Even though arsenic is an element which has been well known since very ancient times for its toxic properties, it is only now that its study has undergone a very important boost, especially as a consequence of two facts: the knowledge of its healing properties (in small doses), and its concentration into very specific zones.

As a result of this interest many techniques have been applied, within the last decades, for the determination of arsenic: Meyers and Osteryoung (1973); Henry and Thorpe (1980), describe electrochemical techniques (polarography); Forsberg et al. (1975); Davis et al. (1978), describe voltametry; Brukenstein and Johnson (1964) preconize the coulometry; radiochemical techniques were applied by Steinnes (1972) and Heydron and Damsgaard (1973); X-Ray fluorescence was described by Taylor and Beanish (1968) and Lindner and Seltner (1978); the chromatographic techniques were applied by Schewedt and Russel (1973), Fish and Brinckman (1982) and Francesconi (1985). More recently the techniques of emission spectrophotometry have been applied by Liversage, et al. (1984), Miyazaki et al. (1979) and the atomic absorption technique using graphite furnace by Xiao-quan and Zhe-ming (1984), Jin et al. (1983), Hagen and Lovett (1986), and using hydride generation by Feldman (1979), Arbad-Zavar and Howard (1980), and Verlinden (1982).

From all these techniques, described above, atomic absorption spectro-photometry (AAS) is the most employed one, mainly because it is the one which presents good sensitivity with moderate costs. The most frequently applied variety is that of hydride generation.

A large number of publications and bibliographic reviews have been dedicated to the analysis of arsenical traces, since Chu and Barrow (1972), describe the separation of arsenic as arsine and its atomization in an electrically heated quartz tube (Crosby, 1977; Godden and Thomerson, 1980; Kinard and Gales, 1981; Brooks et al., 1981).

This lack of definition causes various authors to point out the fact that despite the efforts made towards increasing the sensitivity and the precision in the analysis of arsenic, the exact estimation of this element in environmental samples presents a great deal of difficulties (Brzezinska and Van Loon, 1986; Webb and Carter, 1984).

In the present work, the analytical parameters involved in the technique of hydride generation have been set by a study of their influence on the signal, with a view to the improvement of the detection limit, the precision and the accuracy of the results. A method is also proposed for the analysis of the total arsenic content in biological material.

2. MATERIALS AND METHODS

All chemicals used (perchloric acid, sulphuric acid and sodium borohydride), were of reagent grade quality. However, nitric acid used for the mineralizations of biological tissues, was bi-distilled.

Standard solutions of arsenic(III), dimethylarsenic acid and monomethylarsenic acid, were obtained by dilution of the proper amount in 0.1N nitric acid. Standard of arsenic(V) was obtained by dilution in 0.1N sodium carbonate.

All of the reagents used have been checked by blank tests.

The determinations have been worked out on an atomic absorption spectrophotometer Perkin-Elmer 4000, to which a graphic register Perkin-Elmer 561 was coupled. The source of radiation was an electrodeless discharge arsenic lamp (EDL). The arsine generation was made in an accessory MHS-20 Perkin-Elmer, of automatic programmation and atomization in an electrically heated quartz cell.

The mineralization of the biological samples was made in a Herm Jos Groteklaess autoclave, with a capacity for nine teflon reactors, each one of 25 ml.

The working conditions adopted for the analysis of arsenic, by means of the hydride generator MHS20, were:

Wavelength: 193.7 nm.
Slit: 7H
Absorbance reading: peak height
Reducing agent: sodium borohidryde 3% (W/v)
solution, in 1% aqueous sodium hydroxide.
Inert carrier gas: Nitrogen at 2.5 bars
Solvent: 10 ml. of 1.5N nitric Acid

Time of the 1st purge: 25 seconds
Reaction time: 6 sec.
Time for the 2nd purge: 40 sec.
Temperature of quartz cell: 1000°C.

The method used for wet digestion was: 0.5 g of dry sample (previously lyophilized and homogenized), were weighed in each teflon reactor. Then 2 ml of concentrated NO_3H were added and allowed to stand for 12 hours pre-digesting at room temperature. Afterwards, the digestion continues in an autoclave at a temperature of 120-130°C for 4 hours. It was then cooled and the resulting solution, plus the water from the washing, were placed in a beaker and concentrated to 4 ml; 1 ml perchloric acid (70%) and a 2 ml sulphuric acid (98%) were added and heated at 250°C for 30 minutes, avoiding bringing the sample to dryness. Finally, once the solution was cooled, it was made up to 15 ml with distilled water. Aliquots of this solution were atomized in hydride generator and the signal was interpolated on a calibration curve (absorbance versus concentration) obtained with As(V) standards.

3. RESULTS AND DISCUSSION

The method of digestion proposed here, has been applied to the determination of total arsenic content in several marine organism samples, from the coastal zone of Catalonia. The analyses have been performed in triplicate and Table I gives the results obtained.

The precision of the results obtained was between 2% and 8%, which permits us to conclude the good behaviour of the digestion method as well as that for measuring the diverse unrelated samples and concentrations of arsine.

In a first stage, the different experimental parameters have been optimized for the application of the technique to standard As(III) solutions, with a view to establish optimal conditions of maximal sensitivity and precision. The different parameters have been varied individually, keeping the rest constant.

Figure 1, shows the influence of the time of the 1st purge on the signal. For periods above 30 sec, a decrease of the signal was observed; this justifies the need of oxygen traces in the cell, for the formation of arsenic atoms from the arsine (Welz and Melcher, 1981; 1983).

To carry out the analysis, a period of 25 sec is taken as the optimal, although absorbance is greater at shorter times; the amount of air is still large and so the analysis is non-reproducible, thereby arising a wider dispersion of the signal obtained.

During the time of reaction, borohydride solution is added to the reaction vessel; the amount of reducing agent is directly proportional to the time of this stage, because the borohydride is added at constant flow. In order to determine the optimum time, the variation of the signal with time of addition of reagent has been studied and

Table I

Arsenic content in three samples of selected marine organisms from the Catalonian Coast.

Organism	Location	As Content mg kg^{-1} fresh weight
Mullus surmuletus	Barcelona	85 - 80 - 83
Mullus surmuletus	Ametlla de mar	21 - 19 - 20
Aristeus antennatus	Blanes	142 - 137 - 139
Mullus surmuletus	L'Estartit	76 - 79 - 77
Mytilus galloprovincialis	Barcelona	13 - 11 - 12
Mytilus galloprovincialis	St. Carles de la Rapida	3.9 - 4.2 - 4.1

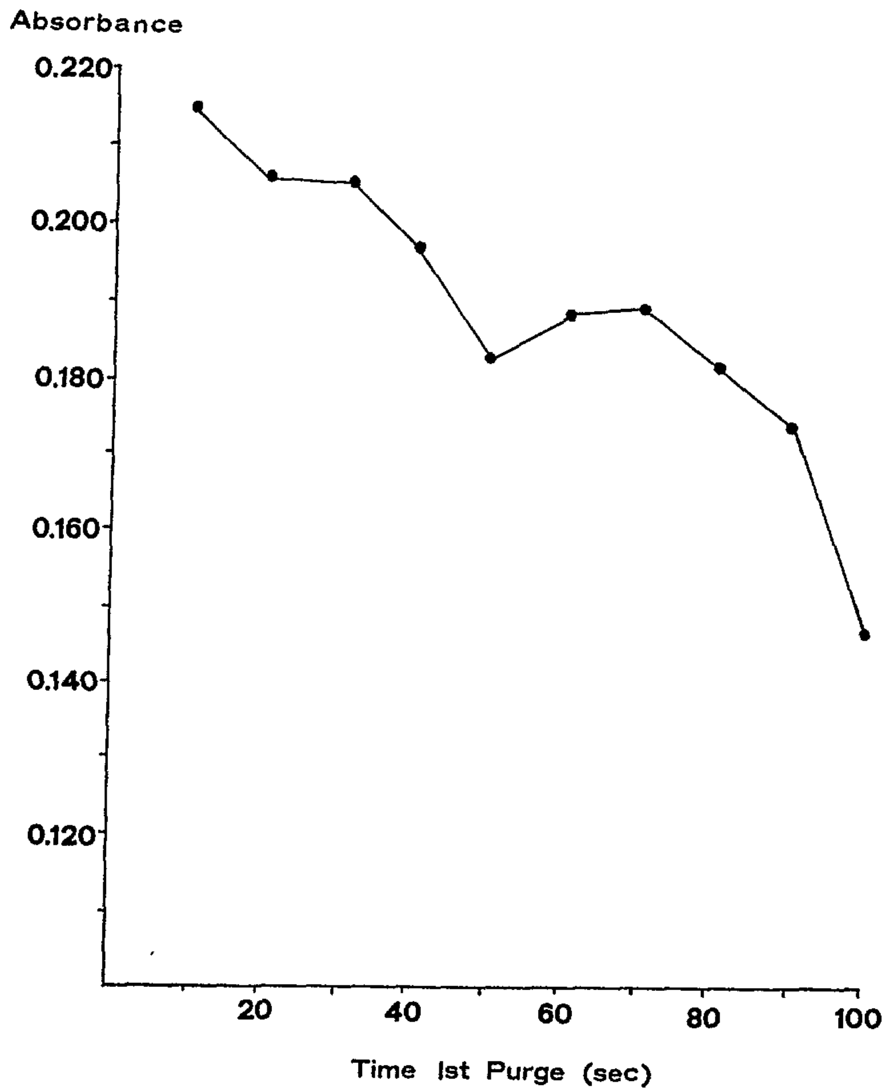

Fig. 1 Signal evolution with time of 1st purge

represented in Figure 2. It has been observed that for laps of time over 4 sec, the signal stays constant, which means the amount of reducing agent added is sufficient for the formation and removal of the arsine in the reaction vessel.

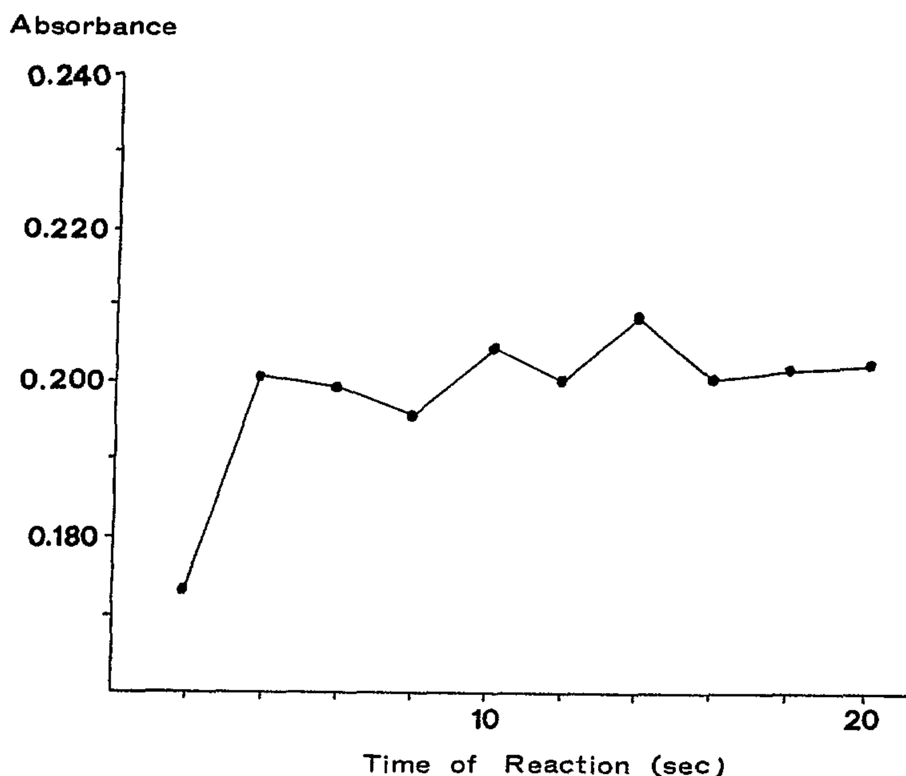

Fig. 2 Optimal time of reaction

Six seconds were taken as optimal time, because for longer laps of time there appears a double peak in the register of the signal during the measuring cycle (Fig. 3), coinciding with the beginning of the 2nd purge. This second peak is produced as a consequence of the excess of borohydride added which causes the medium to turn basic and thus preventing the total generation of arsine. As the reaction time increases, this second peak widens and becomes more irregular and non-reproducible, thereby decreasing the precision of the measurement.

The minimal time of the 2nd purge, necessary for the signal to come back to the base line and thereby, to expel all the arsine from the system was checked experimentally and 40 sec were calculated to be enough.

The temperature is a critical parameter, as can be seen from the result presented in Figure 4, from which it is verified that at temperatures below 800°C the signal decreases drastically as a consequence of the non-atomization of the arsine. This confirms the hypothesis that the atomization takes place through reactions involving radicals, instead of a thermal decomposition (Aggett and Aspell, 1976; Hinners, 1980; Welz and Melcher, 1982). In view of the previous results, as optimal temperature was taken, the one that gives a maximal signal, namely 1000°C.

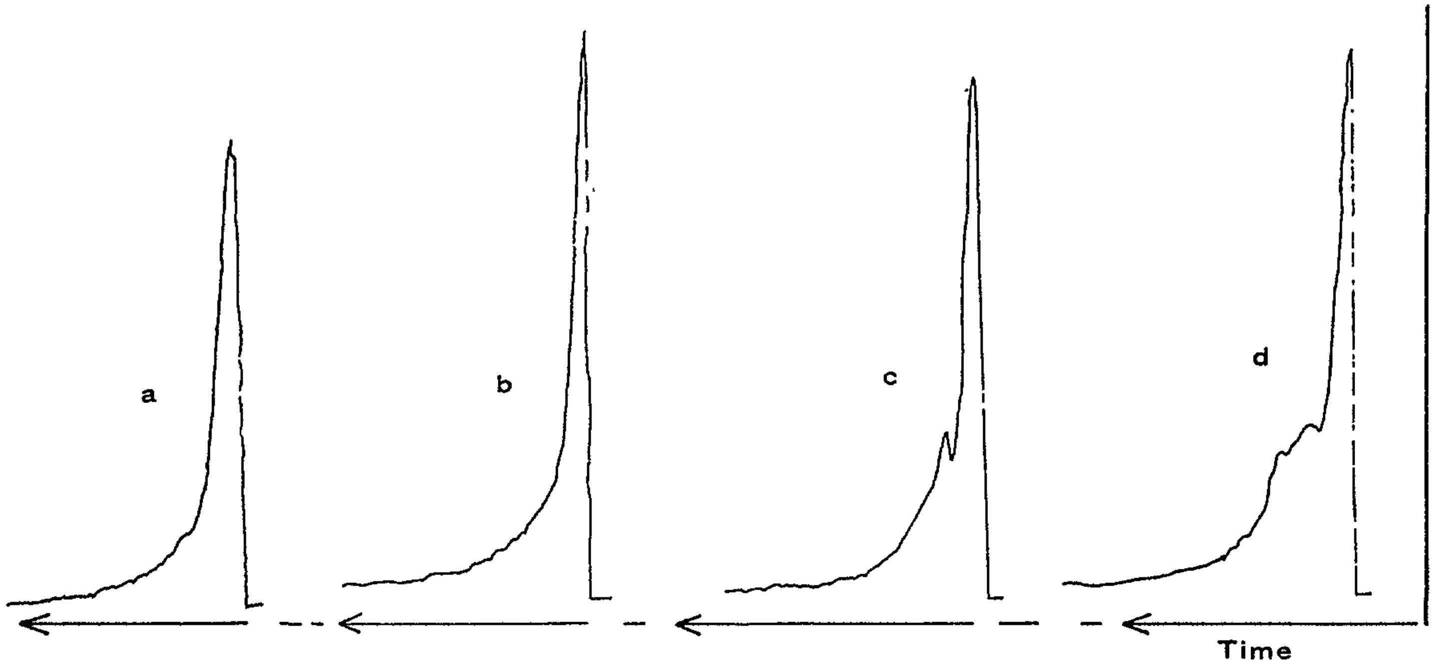

Fig. 3 Registering the signal during measuring cycles to different times of reaction. a: 2 sec; b: 4 sec; c: 8 sec; d: 14 sec

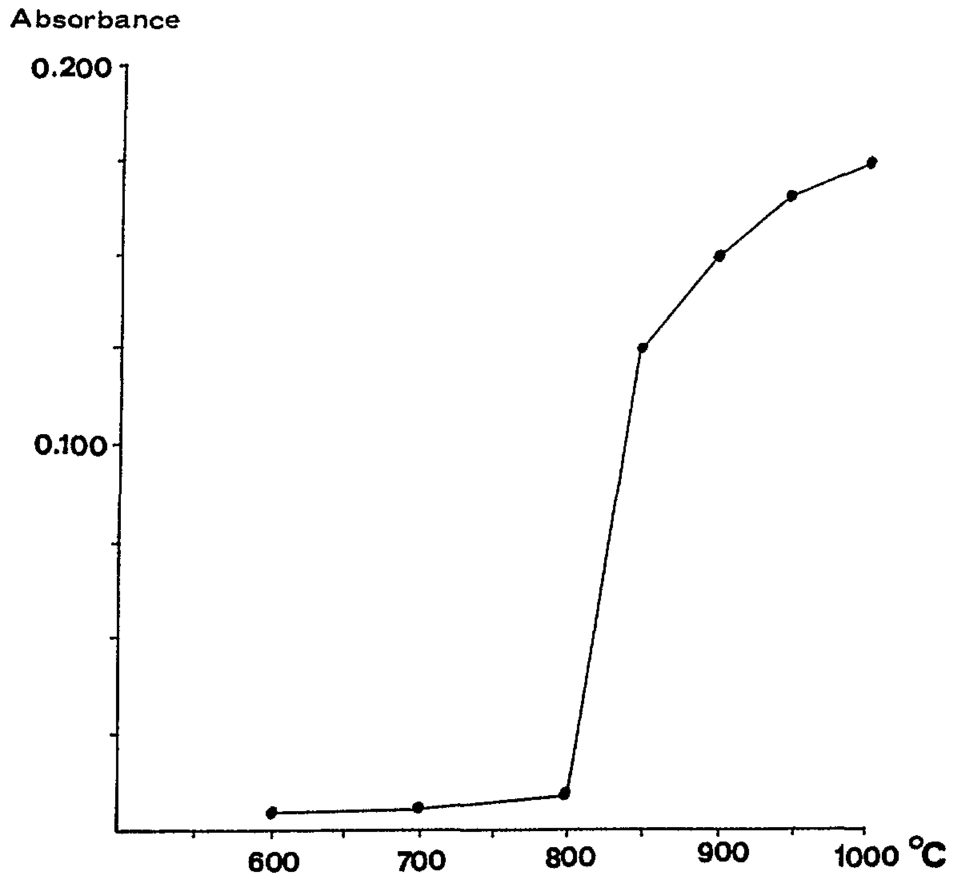

Fig. 4 Effect of temperature to signal

The determinations were made with 3% borohydride solutions, since working with lower concentrations the amount of hydrogen generated is not sufficient; the signal decreases and appears at longer times, specifically when the 2nd purge starts, thereby losing part of the cleaning efficiency of this stage. This effect is observed in Figure 5.

The concentration of the acid to be utilized as solvent for the arsine generation is related to the borohydride concentration and has a notable influence on the signal, as can be observed in Figure 6. Working with acid concentrations below 0.2N, a double peak arises in the signal, assignable to the fact that the amount of hydrogen generated is not sufficient to carry away the total measurement. The optimal margin for the performance of the analysis is placed between 1.5N and 4.5N.

With the optimized analytical parameters of the technique, Beer's law is well obeyed between 10 ng and 100 ng of As(III), as can be observed form the results in Table II and their plot in Figure 7.

The detection limit of the technique has been calculated as twice the standard deviation, from a series of 10 repetitions, from the same standard sample, with an As(III) content of 5.0 ng which is near the detection limit. The results obtained are: Absorbance mean 0.070, $\sigma \pm 0.002$, which implies a detection limit of 0.3 ng expressed as absolute amount of arsenic detectable.

The precision has been determined by means of 10 consecutive measurements of the same sample with an approximate content of 20 ng As(III). The results obtained were: Absorbance mean 0.203, $\sigma \pm 0.012$ ie 6% coefficient of variation.

Table II

Absorbance reading (peak height), for AsIII standards.

As(III) ng	Absorbance	C.V.(%)
0	0.028	15.9
10	0.111	4.1
20	0.204	2.9
30	0.302	6.4
40	0.397	7.8
50	0.456	4.1
60	0.539	2.9
70	0.644	3.2
80	0.736	0.5
90	0.810	4.6
100	0.900	3.4

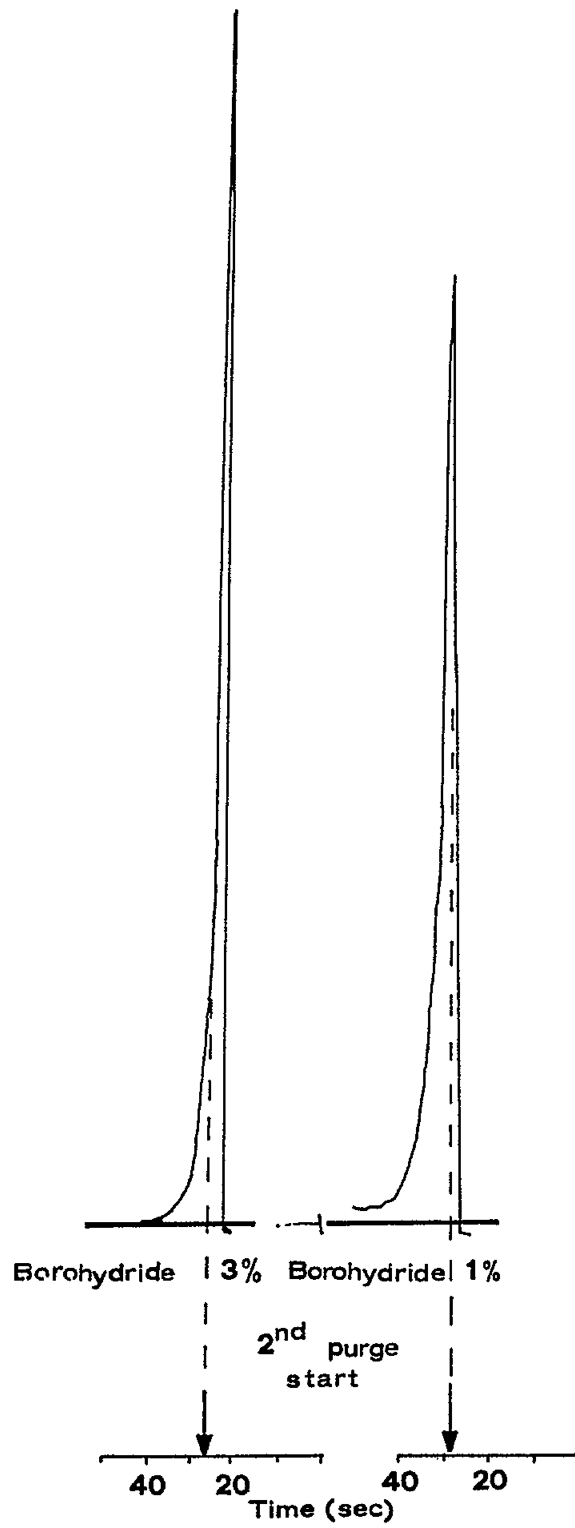

Fig. 5 Signal evolution at different borohydride concentrations

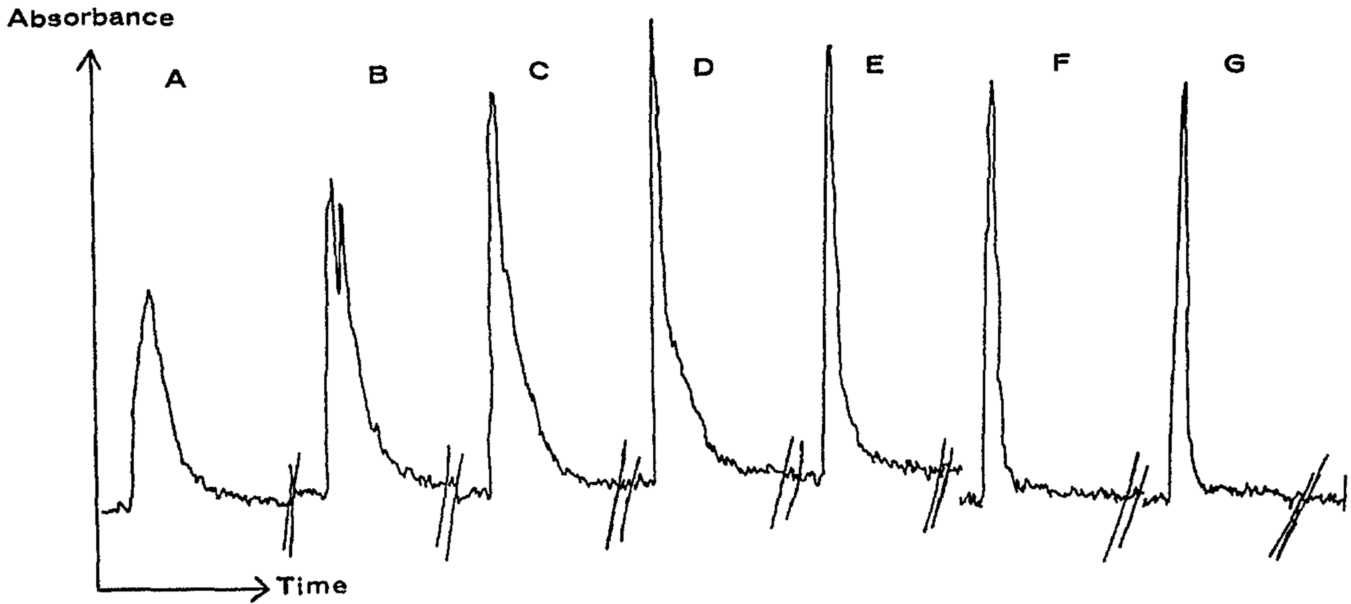

Fig. 6 Signal evolution of 20 ng As(III) at different concentration of nitric acid (A:0.05N; B:0.10N; C:0.15N; D:0.20N; E:0.25N; F:0.50N; G:0.75N)

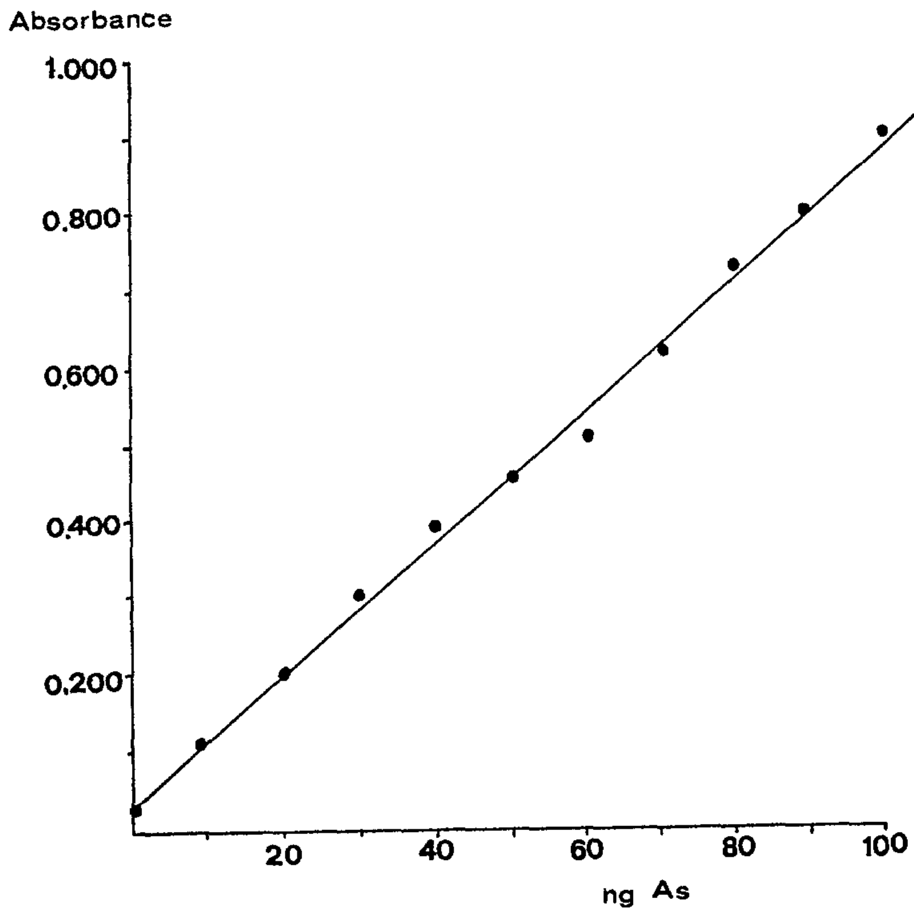

Fig. 7 Calibration curve with As(III)

3.1 Mineralization of biological samples

Recent studies have shown that the most abundant chemical forms of arsenic present in biological samples are: arsenates, arsenites, dimethylarsenic acid (DMAA), monomethylarsenic acid (MMAA) and in smaller proportions other compounds such as arsenobetaines, arsenophospholipids, etc.

The methylated forms are specially important, because they are the majority of chemical species in the biological desintoxication mechanism in mammals. Thalmi and Feldman (1975), Webb and Carter (1984) and Welz and Melcher (1985) describe the different sensitivities shown by the chemical species of arsenic (mainly the methylated forms), as regards the quantification by hydride generation technique; probably the different reduction kinetics down to arsine shown by the chemical forms. Therefore, it is necessary to use digestion processes to mineralize the methylated compounds to inorganic forms.

The incomplete degradation, as a consequence of the presence of several chemical forms of As, will result to a systematic error in the quantification against a given standard of inorganic As and so low accuracy results are obtained (Welz and Melcher 1981; 1985).

In the literature two groups of mineralization techniques are described: dry and wet mineralization. In the first one the oxidation of the organic matter is obtained by heating at high temperatures (400°-700°C), the usual oxidizing agent being atmospheric oxygen, although magnesium nitrate may be added, alone or mixed with magnesium oxide in several proportions. This technique was utilized by Evans and Bandemer (1954), Morrison and George (1969), George and Frahn (1973) and Uthe _et al._ (1974).

In the wet mineralization the temperature is lower, being limited by the boiling point of the reagents, and the oxidation is done by oxidizing agents in solution. These usually employ mixtures of nitric, sulphuric and perchloric acids alone. However, Stone (1967) uses mixture of nitric and sulphuric acids, and Brooks _et al._, 1981 use a mixture of perchloric and nitric acids. The common factor in all these methods is keeping As in As(V) form, so as to prevent losses by votalization.

In view of the excessive manipulation required by the dry mineralization and the interferences usually introduced (Ni for example), we have mainly opted for the wet digestion which is a much faster technique, easier and with less contamination risk.

For undertaking the present study, acid digestion has been chosen employing nitric acid, sulphuric acid and perchloric acid, this techniques being mostly employed in the literature (Ihnart and Thomson, 1980; Dedina and Rubesca, 1980; Welz and Melcher, 1985).

The complete mineralization requires the employment of high temperatures (above 250°C) as a consequence of the carbon-arsenic strong bonds (Henry and Thorpe, 1980; Webb and Carter, 1984). We have verified experimentally that at lower temperatures the mineralization

is incomplete, thereby low recoveries are obtained. On the other hand, the proposed mineralization technique, degrades the chemical species of arsenic down to As(V), without the existence of As(III), which would give low accuracies in the recovery test.

The well known treatment with perchloric/sulphuric acids, after mineralization with nitric acid, causes an increase in the signal (Fig. 8), which corresponds to samples of marine organisms. The increase can be assigned to the complete degradation of the methylated forms and to the elimination of the nitrogen oxides produced during the digestion in the autoclave; these being strong depressors of the signal in the measuring technique employed.

3.2 Study of interferences

With our point of view centered on the possible utilization of the technique for the measurement of As content in different materials (organisms, sediments, water, etc), it was necessary to carry out some studies of the possible components inherent to the matrix, which present some interferences with the described technique.

Although hydride generation is a technique in which the AA measurement is made after previous separation of the analyte from its matrix, it is however subjected to strong interferences by elements and compounds comparatively abundant in the sample matrix.

The possible interferences may be classified in 3 groups:

- interferences due to the acids used for mineralization
- interferences due to the presence of heavy metals
- interferences due to the generation of hydride

In Figure 9, it is shown the great interference of concentrated nitric acid used in the mineralization of the sample, as a consequence of the nitrous oxides generated. No interference is observed for sulphuric or perchloric acid.

The transition metals also present great interferences; in Table III interference caused a decrease of 10% in the signal of As(III). This interference can be eliminated, or avoided in part, by increasing the concentration of the acid used in the arsine generation.

In the case of interferences by competition in the hydride generation, selenium, which forms H_2Se, is an important interferant, since 0.6 micrograms of this element in 10 ml of solution causes a 10% decrease of the As(III) signal. This interference is reduced with the addition of a Cu salt, which will retain the H_2Se selectively.

However, in the biological samples analysed, we found good agreement in the slopes of the addition and standard representation of Beer's law (Fig. 10) which indicates a low possibility of interferences.

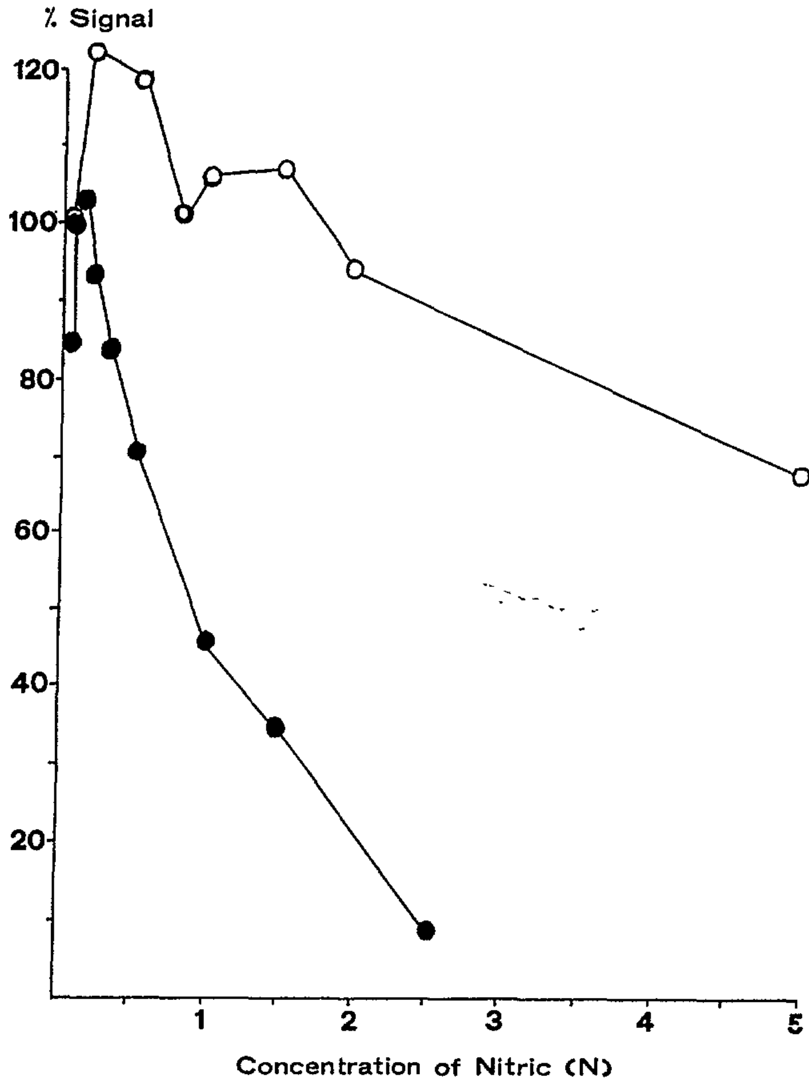

Fig. 8 Signal evolution of sample without treatment (o) and with perchloric-sulphuric acids (o)

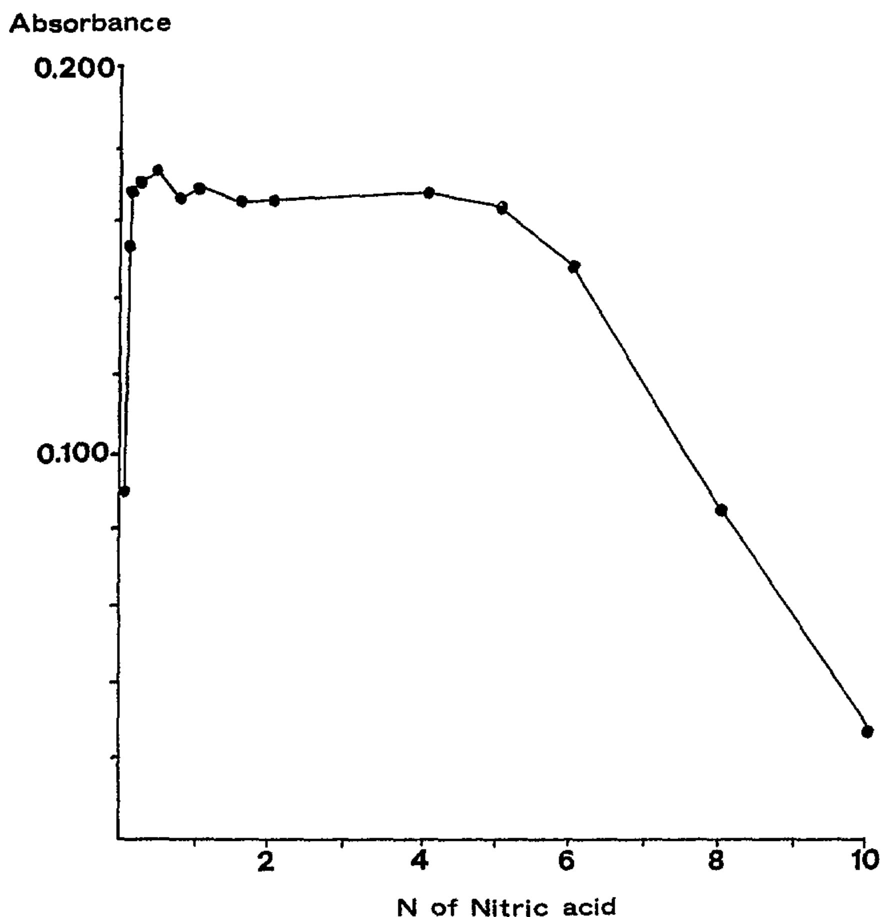

Fig. 9 Signal of 20 ng As, at different concentrations of nitric acid used in mineralization

Table III

Amount of some interferences that depress 10% the signal of As, by hydride generation technique.

Interferant	mg in 10 ml
Fe III	6.2
Ni II	0.02
Cu II	1.4
Fe II	37
Pb II	25
Co II	1.0

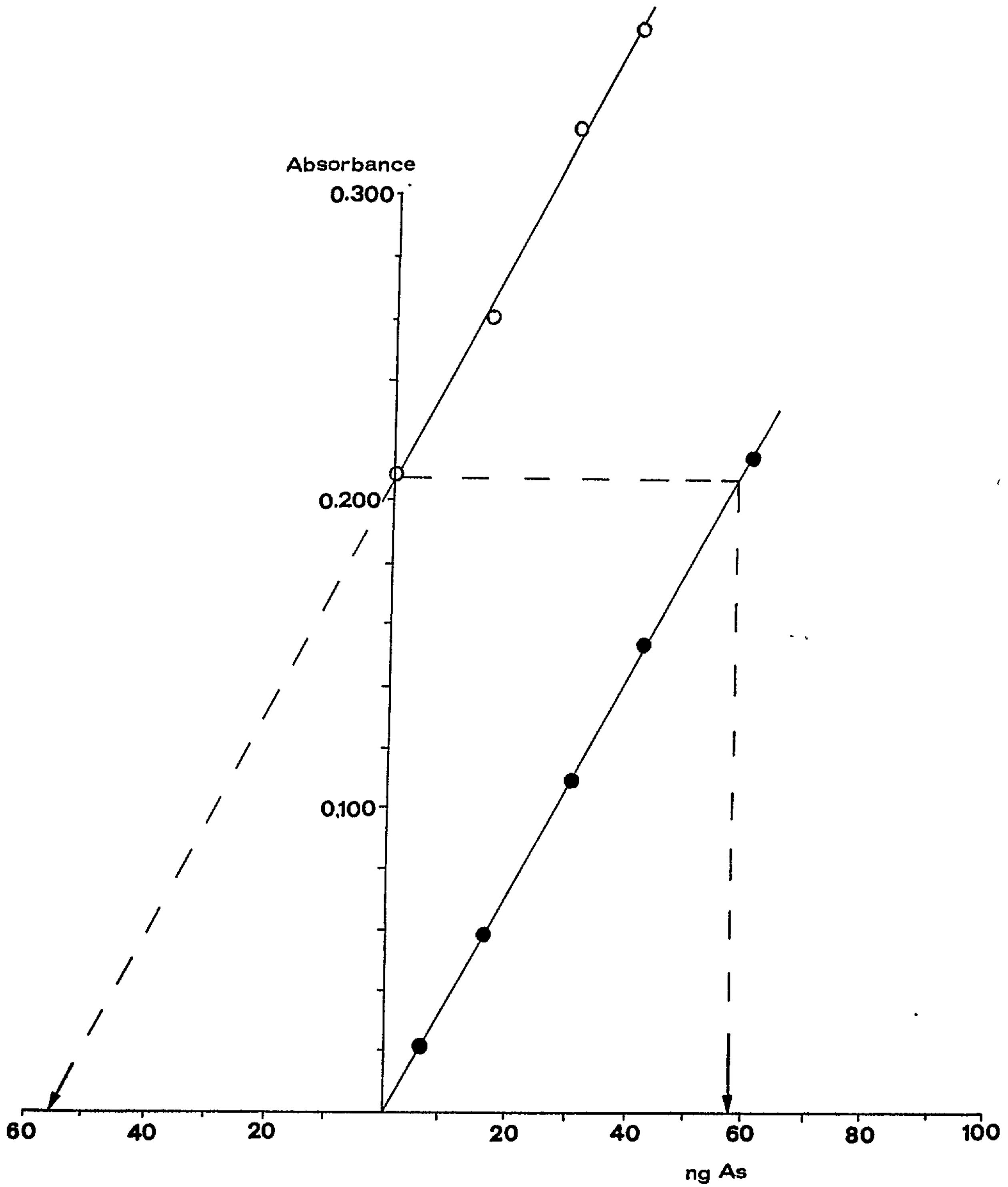

Fig. 10 Comparison between calibration curve (o) and standard additions curve (o)

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