MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

WORLD HEALTH ORGANIZATION

RESEARCH ON THE TOXICITY, PERSISTENCE, BIOACCUMULATION, CARCINOGENICITY AND MUTAGENICITY OF SELECTED SUBSTANCES (ACTIVITY G)

RECHERCHES SUR LA TOXICITE, LA PERSISTANCE, LA BIOACCUMULATION, LA CANCEROGENICITE ET LA MUTAGENICITE DE CERTAINES SUBSTANCES (ACTIVITE G)

Final reports on projectsdealing with carcinogenicity and mutagenicity

Rapports finaux sur les projets ayant trait à la cancérogénicité et la mutagénicité

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This volume is the fifty-seventh issue of the Mediterranean Action Plan Technical Report 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 Sea.

Ce volume constitue le cinquante-septième 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.

GENERAL 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 on 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 and exchange of information and assessment of the state of pollution and of 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 interdependent and provide a framework for comprehensive action to promote both the protection and the continued development of the Mediterranean ecoregion. No component is an end in itself. The Action Plan is intended to assist the Mediterranean Governments in formulating their national policies related to the continuous development and protection of the Mediterranean area and to improve their ability to identify various options for alternative patterns of development and to make choices and appropriate allocations of resources.

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 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 geographic 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.

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 1989), 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.

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 first eight volumes of the MAP Technical Reports Series present the collection of final reports of the principal Investigators who participated in the relevant pilot projects (MED POL I - MED POL VIII). The ninth volume of the MAP Technical Reports Series is the final report on the implementation of MED POL-Phase I, prepared, primarily, on the basis of individual final reports of the principal investigators with the co-operation of relevant United Nations Agencies (FAO, UNESCO, WHO, WMO, IAEA, IOC).

From the tenth volume onwards, the MAP Technical Report Series contains final reports on research projects, assessment documents, and other reports on activities performed within the framework of MED POL-Phase II, as well as documentation originating from other components of the Mediterranean Action Plan.

This fifty-seventh volume of the MAP Technical Reports Series contains the final reports of two research projects on the effects of carcinogenic and mutagenic substances on marine organisms within the framework of MED POL Phase II in Activity G - "Research on the toxicity, persistence, bioaccumulation, carcinogenicity and mutagenicity of selected substances".

INTRODUCTION GENERALE

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 y relatifs avec leurs annexes techniques pour la protection du milieu méditerranéen (élément juridique).

Tous les éléments du Plan d'action étaient interdépendants et fournissaient le cadre d'une action d'ensemble en vue de promouvoir, tant la protection que le développement continus 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 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 ait jamais été entrepris dans le bassin méditerranéen.

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'environnement, 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 Barcelone 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.

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.

Les huit premiers volumes de la Série des rapports techniques du PAM rassemblent les rapports finaux de chercheurs responsables qui ont participé aux projets pilotes correspondants (MED POL I -MED POL VIII). Le neuvième volume de cette même Série se compose du rapport final sur la mise en oeuvre de la Phase I du programme MED POL, établi essentiellement sur la base des rapports finaux individuels des chercheurs responsables avec la coopération des organismes compétents des Nations Unies (FAO, UNESCO, OMS, OMM, AIEA, COI).

A partir du dixième volume, la Série des rapports techniques du PAM, comprend des rapports finaux sur les projets de "recherche", des documents d'évaluation et d'autres rapports d'activités effectués dans le cadre de MED POL-Phase II, ainsi que de la documentation prise dans d'autres domaines du Plan d'action pour la Méditerranée.

Ce cinquante-septième volume de la Série des rapports techniques du PAM comprend les rapports finaux de deux projets de recherche sur les effets des substances cancérigènes et mutagènes sur les organismes marins menés dans le cadre de la Phase II du MED POL au titre de l'Activité G - "Recherche sur la toxicité, la persistance, la bioaccumulation, la cancérogénicité et la mutagénicité de certaines substances".

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GENOTOXIC RISK ASSESSMENT IN THE MARINE ENVIRONMENT USING INVERTEBRATES AS INDICATOR ORGANISMS

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I. INTRODUCTION

A considerable part of the effects of natural or anthropogenic pollutional on living organisms has mutagenic and/or carcinogenic consequences. In very high relation to this potential, damaging DNA alterations are found, such as: changes in helicity, in hydrogen bonding, single- and double-strand breaks, gaps, intercalations, deletions, adduct formations, intramolecular and intermolecular crosslinks, sequence changes, gene amplifications etc. DNA alterations are quite numerous and among them the strand breaks, especially the single-strand breaks, are by far the most numerous ones, even outweighing the sum of all other DNA alterations. Therefore the most promising methods for assessing possible DNA damaging potentionals would be those which pick up the most numerous consequences of them, the single-strand scissions. This was the reason to introduce and develop alkaline filter elution as a method for detection of DNA strand breaks as an biomaker of environmental genotoxicity.

The alkaline elution assay has been utilized for monitoring DNA damage in cultured mammalian cells (Kohn *et al.*, 1976, Swenberg *et al.*, 1976) and in various organs of laboratory animals treated with single doses of carcinogens (Petzold and Swenberg, 1978, Parodi *et al.*, 1978). In order to make our results comparable with those of other laboratories, the effects of benzo(a)pyrene (BaP) and aminoanthracene (AA) as indirect acting carcinogens, and 4-nitroquinoline-N-oxide (NQO) as direct acting carcinogen on FLC/F cells, were tested.

In chemical carcinogenesis testing with aquatic animals, considerable use has been made of fish, found to be extremely sensitive to some carcinogens, notable aflatoxin B_1 (Hendricks *et al.*, 1980, Sinnhuber *et al.*, 1977), benzo(a)pyrene (Zahn *et al.*, 1981, Batel *et al.*, 1985), and kerosene (Stuber and Zahn, 1985).

Information on the nature of the reactive metabolites produced, their toxic molecular effects, and the mechanisms involved, in marine invertebrates is both sparse and controversial. Induction of chromosomal aberrations occurred in gills of *M. galloprovincialis* in animals from polluted field sites (Al Sabti and Kurelec, 1985). Frequency of SCE increased in gills of *M. edulis*, caused by promutagen cyclophosphamide (Dixon *et al.*, 1985), and epithelial tumours were invoked in the gastropod *Ampullarius australis* by exposure to 3-MC (Krieg, 1972). However, mutagenic metabolites of BaP in postmitochondrial fractions of *M. galloprovincialis*, have not been detected in the Ames test (Britvic and Kurelec, 1986). Digestive gland homogenates from mussels exposed *in vitro* to BaP (23 µM) did not show any adduct or a very weak adduct spot (1 adduct per 1 to $4*10^9$ nucleotides, Kurelec *et al.*, 1988). No neoplastic diseases in the oyster *Ostrea edulis* and *C. gigas* could be correlated with the effects of the AMOCO CADIZ oil spill in Brittany, France (Berthou *et al.*, 1987). On the other hand, investigations on crustacea were limited to studies on *in vitro* and *in vivo* xenobiotic metabolism. As far as we know, there is hardly any information about DNA alterations in crabs.

Our aim was to find out correlation of DNA damage with increase in benzo(a)pyrene monooxygenase activity (BPMO) responsible for metabolic activation of BaP, by measuring single-strand breaks in the liver of the fish *Gambusia affinis*, in the haemolymph of the marine crab *Maja crispata* and in the haemolymph of the mussel *Mytilus galloprovincialis*, following exposure to BaP and some other pollutants. Further, to investigate the usefulness of mussels as test and indicators

organisms, by testing DNA damage in mussel haemolymph in naturally exposed mussels as well as in mussels injected with sediment extracts in the laboratory. This is important for selection of bioassay organisms which would be useful for the detection and evaluation of pollution. Ideally, the selected species would reflect not only the presence or absence of genotoxic pollutants, but also relative pollution levels and their periodic fluctuations, and would perhaps identify factors other than chemicals that contribute to environmental degradation.

2. MATERIALS AND METHODS

2.1 Cells

FLC/F cells were grown in plastic flasks using RPMI 1640 medium supplemented with 10% foetal calf serum, penicillin and streptomycin incubated at 37 EC. For each experiment 1.5-2*10^6 cells/ml were incubated with model substances with and without S9 mix. S9 mix was prepared according to Maron and Ames (1983). Model substances were dissolved in DMSO and concentrations range tested have been 1-5 µg/ml for benzo(a)pyrene and aminoanthracene and 0.3-10 µg/ml for 4-nitroquinoline-N-oxide. Cells were incubated for 1.5 h.

2.2 Animals and treatment

Specimens of a laboratory population of the mosquito fish *Gambusia affinis* raised in charcoal-filtered tap water at 23 EC, with 1.5-2 g body weight and a liver weight of up to 1.5 mg were used. Benzo(a)pyrene (BaP) was added to experimental tanks in a single dose of 0.1 mg/ml methanol; the control fish received the same volume of methanol. For determination of single-strand breaks in fish liver DNA caused by known carcinogens, fish received one i/p injection of Aroclor 1254 and N-methyl-N-nitrosoguanidine (MNNG) in 10 µl DMSO. Controls received only DMSO.

Adult male crabs, *Maja crispata*, Risso (Crustacea: Decapoda), weighing 66 ± 5g were collected by diving, and stored in large basins with running seawater for three weeks prior to treatment. Specimens in incipient or recently-completed ecdysis were eliminated from the experiments. After 3 weeks of adaptation to laboratory conditions, animals received a single injection of appropriate doses of BaP or MNNG in 100 µl DMSO directly into the haemolymph. Control ones received only 100 µl DMSO.

Mussels, *Mytilus galloprovincialis* L., 10g average weight, were collected from mariculture area and stored in large basins with running seawater. The DNA damage measurements were studied in animals that had been given a single injection of appropriate doses of BaP, 4-nitroquinoline-N-oxide below the mantle, or sediment extracts in DMSO directly into the pallial fluid. Controls received only DMSO.

For investigation of DNA damage caused by actual pollution, mussels were collected from locations under the direct influence of industrial runoff from a fish cannery (s-1), urban waste in the harbour (s-2), industrial and/or urban runoff from a tobacco factory (s-3), and from a clean area - Lim bay (s-4). In contrast to our previous laboratory experiments with model substances, mussels from Lim bay were taken from their natural habitat instead of from a mariculture area. Mussels were transported in basins with seawater to the laboratory and selected according to their biometric characteristics (size, volume and weight). Haemolymph was drained out within 1 hour after collection.

Benzo(a)pyrene monooxygenase determination was performed in a time dependent manner for all three organisms as well as DNA damage determination in marine invertebrate haemolymph.

2.3 Preparation of fish liver and crab and mussel haemolymph

The fish were killed and their livers removed and homogenized in 0.05 M Tris-HCl buffer. pH 7.4, with Potter-Elvejhem homogenizer. Supernatant containing cca 10 mg proteins was applied onto a filter. All preparations were done at 4 EC.

Crab haemolymph was taken from the basal region of the large leg and directly diluted 1:4 with Merchant's solution containing 0.1 M Na_2EDTA to prevent coagulation. Cells were mostly intact within 2 hours and very easy to precipitate at 10000 g, 15 min, 4 EC. Crab haemolymph has cca $6.5.10^6$ cells/ml (60 µg DNA, 400 µg RNA and 2.0 mg proteins).

Mussel haemolymph was taken from the adductor muscle and directly diluted 1:2 with Merchant's solution, pH 7.4, containing 0.1 M EDTA to prevent coagulation. Haemocytes containing 11 µg/ml DNA, 140 µg/ml RNA, and 0.6 mg/ml proteins were intact with 4 hours, as inspected by microscopy.

It was possible to take out up to 5 ml of crab haemolymph and up to 1.0 ml of mussel haemolymph from one animal without significant impairment to it. This allowed us to use the same crab or mussel for time experiments and repair studies.

2.4 Alkaline elution

Alkaline elution was performed essentially according to Kohn *et al.* (1976) and Parodi *et al.* (1978), with minor modifications: 2.10^6 cells, 10 mg fish liver supernatant, 250 µl crab haemolymph, or 2 ml mussel haemolymph were carefully deposited onto a filter (PVF, 25-mm diameter; 0.2 µm pore size, Millipore Corp., Bedford, Mass. USA) and washed with 5 ml of Merchant's solution which contained 0.1 M Na_2EDTA at a flow rate of 0.2 ml/min. Cells were then lysed at room temperature with 5 ml 0.2% sodium lauryl-sarcosinate, 2 M NaCl and 0.02 M Na_2EDTA pH 10; the filter was then washed with 10 ml 0.02 M Na_2EDTA buffered to pH 10 with NaOH. Pump tubings and filter holder bases were then dried, and 18 ml of alkaline solution (0.02 M Na_2EDTA buffered to pH 12.3 with tetraethylamonium hydroxide) were carefully added and the single-stranded DNA eluted in the dark using a peristaltic pump, at a flow rate of 0.05 ml/min. Six fractions of 3 ml (1 hour) were collected, the 7th containing the remaining alkaline solution. To recover the remaining DNA from the filter, the filter was broken up with a blender in 5 ml of alkaline solution, the filter holder and the pump lines were rinsed with 3 ml of alkaline solution. These two solutions were mixed with the 7th fraction: a 2ml aliquot of this mixture was used for determining the amount of non-eluted DNA. Three fractions of 2 ml alkaline solution were used for blank reading. The rate of fish liver DNA elution from control and treated specimens was independent of the amount of liver deposited on the filter in the range of 10-20 mg, of crab haemolymph in the range of 150-500 µl, and of mussel haemolymph in the range of 1.5-3 ml.

2.5 Spectrofluorometric determination of DNA

Eluted DNA and DNA remaining on the filter were measured in 2 ml aliquots according to Stout and Becker (1982) with bisbenzimide as fluorescent stain with minor modifications: to 2 ml of eluate 0.5 ml 7.5 µM bisbenzimide in 0.12 M NaCl, 0.012 M Na_3citrate and 0.26 M KH_2PO_4 were added. Fluorescence was read at 450 nm with excitation of 360 nm in a Farrand A4 spectroflurometer. The fluorescence reading was linear between 10 ng and 1 µg DNA.

2.6 Enzyme activity measurement

BPMO activity was determinated by the method of Nebert and Gelboin (1968), however, the 9000 g supernatant was used instead of microsomal preparations. After incubation for 15 min. at 27 EC, the fluorescence of the hydroxy products formed was measured with a fluorimeter (Farrand A-4, FOC, USA) at an excitation at 365 nm and emission at 520 nm, BPMO activity are expressed in pmol 3-OH BaP/mg prot./min. Protein was determinated by the method of Lowry *et al.* (1951) with

a bovine serum albumin as standard.

2.7 Preparation of sediment extracts

The surface layer of sediment samples was collected by Scuba divers from 5 locations. About 500 g wet sediment was frozen at -20 EC and transported to the laboratory. Dry weight was determined by heating 10 g aliquots at 105 EC for 24h. 100 g wet weight of sediments was extracted with dichloromethane-methanol (2:1) as previously described by Schiewe *et al.* (1985).

2.8 Result expression

Results are expressed as % DNA retained on the filter or as rate constant defined as a slope of linear elution curve. In some cases elution curves expressed a two phase kinetics. The first phase is approximately of the first order with respect to the elution volume and dose, depending on the length of single stranded DNA, while the second phase is volume and dose independent. This was the reason to express the results as strand scission factors (Meyn and Jenkins, 1983) estimated for the linear part of the elution curves (9 ml). A value characterizing the relative number of DNA-strand breaks, referred to as a "strand scission factor", SSF, was calculated by taking the absolute value of the $\log_{10}$ of the percentage of DNA retained in the treated sample of 9 ml eluted divided by the percentage of the DNA retained in the control sample at 9 ml eluted. Therefore, a strand scission factor 0 indicates no DNA strand breaks. Values lower than 0 indicate a relative value for DNA breaks in treated mussels. For practical reasons SSF are expressed as absolute values; the higher the value, the higher the amount of strand breaks in DNA.

3. RESULTS AND DISCUSSION

Figure 1 shows typical alkaline elution profiles of DNA from FLC/F cells incubated in different concentrations of 4-nitroquinoline N-oxide (NQO) 1.5 hour before the alkaline elution assay was performed. In the range of concentrations between 0.3 - 10 µg/ml an increase in elution rate was observed. The shape of the elution profile provides some indications of the type of the DNA damage, i.e., single-strand breaks and highly alkali-labile sites on the DNA give a first order elution (Kohn *et al.*, 1981). This was the case for two lower concentrations of NQO. For the highest concentration of 10 µg/ml the profile of elution curve was not linear. Since the amount of DNA retained on the filter was less than 20% after 9 ml eluted, we could conclude that DNA damage was too high., DNA eluted fast, and the elution rate depended on the amount of DNA on the filter instead on the DNA strand size.

The effects of the indirect mutagens benzo(a)pyrene (BaP) and aminoanthracene (AA) on FLC/F cells were also tested. Since this cell line does not possess a measurable activation system for xenobiotic transformation to reactive intermediaries, external activation S9 mix from liver of Aroclor treated rats, were added to the incubation mixture. In both cases, linear alkaline elution profiles were observed and results expressed as strand scission factors at 9 ml eluted. The strand scission factor was calculated in relation to control samples containing cells incubated with all components of S9 mix except for the cofactor - NADPH. The presence of S9 mix alone in cell cultures did not show any effect on the rate of DNA elution from the filter. Both carcinogens, BaP and AA, activated by rat liver activation system (S9 mix), gave a dose related increase in elution (Table 1). Comparable results have been reported by Swenberg *et al.* (1976), Ochi *et al.* (1986).

For determination of *in vivo* DNA damage caused by known carcinogens, fish and marine invertebrates were used as target organisms. Specimens of the fish *Gambusia affinis* in good physiological condition that come into contact with BaP showed a measurable increase in BPMO activity reaching its maximum after two days of exposure (Figure 2). A consequence of such altered BaP metabolism could be an increase in DNA damage, measured as DNA-adducts

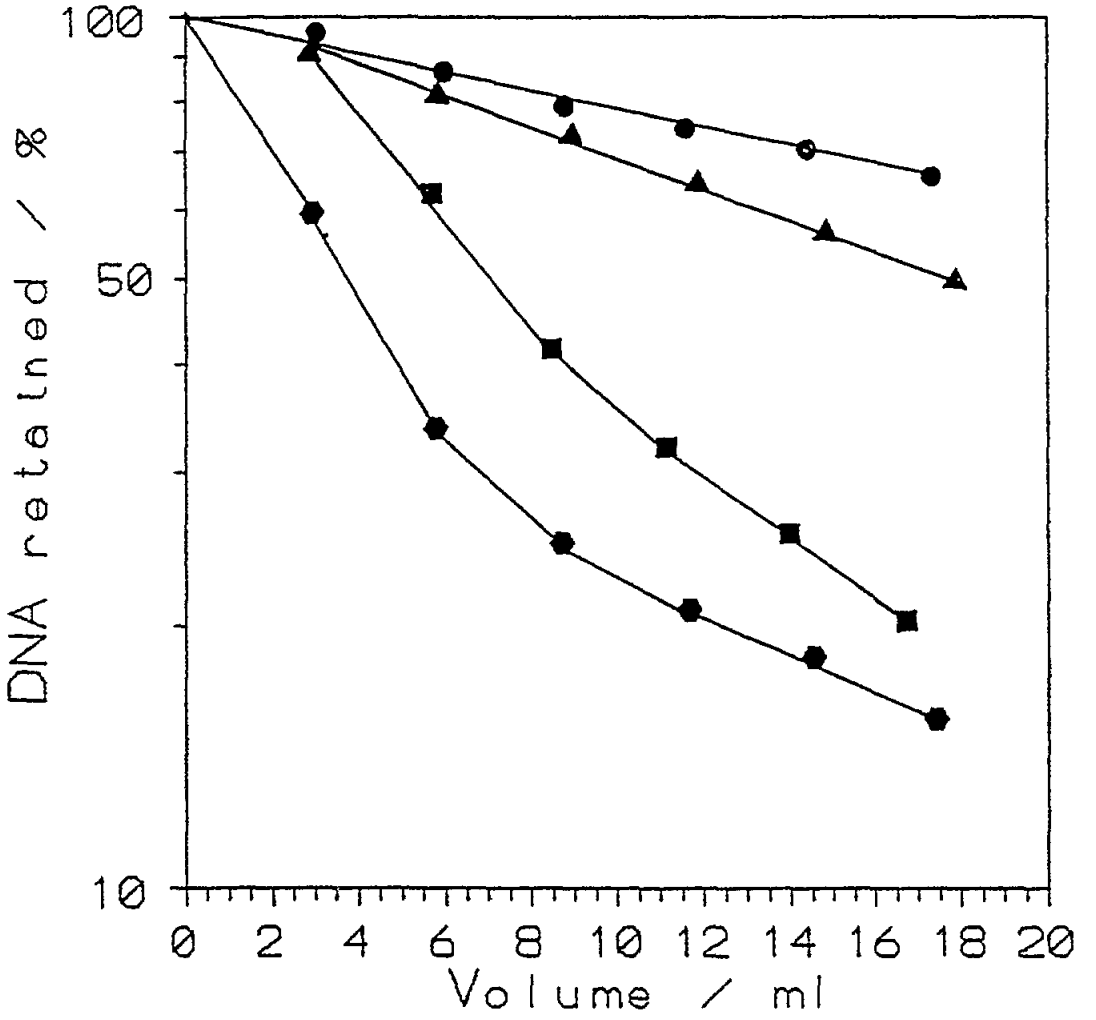

Fig. 1 Elution of DNA from FLC/F cells following exposure to 4-nitroquinoline-N-oxide 1.5 hours. ● - untreated cells, ▲ - 0.3 μg/ml, ■ - 3,0 μg/ml, ⬢ - 10.0 μg/ml

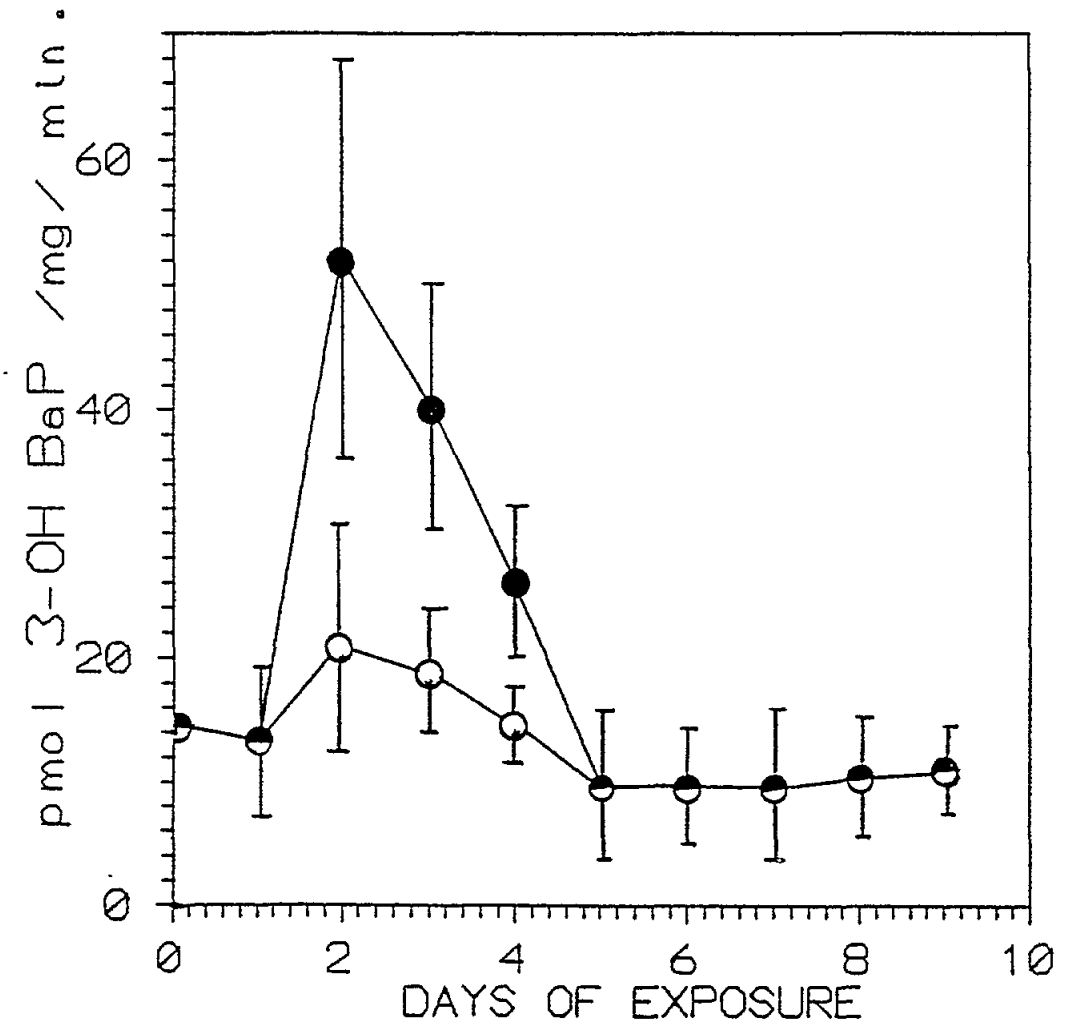

Fig. 2 Increase in liver benzo(a)pyrene monooxygenase activity of fish *Gambusia affinis* as a function of time after exposure
○ - control fish - methanol
● - exposed fish - 100 ppb BaP in water

while Aro increases the genotoxicity of direct and indirect acting carcinogens (Mendoza-Figueroa *et al.*, 1985). We illustrated these by measuring single-stranded breaks in fish liver DNA 1.5 hour after i/p injection (Figure 3). The shape of elution profiles for the chemicals tested showed that single-stranded breaks were formed in fish treated with model substances.

Although optimal activities of BPMO in *Maja crispata* hepatopancreas could be obtained at 27 EC and at pH 7.4. (Bihari *et al.*, 1984, Batel *et al.*, 1986), monooxygenase activity in crabs was lower to those observed in *G. affinis*, reaching its maximum after 4 days (Figure 4). This is related to the low level of general metabolism in marine invertebrates. In the case of mussel digestive gland, optimal conditions for measuring BPMO activity could not be obtained (Figure 4). However, the rate of BaP metabolism in mussel *Mytilus galloprovincialis*, once normalized to cytochrome P-450 concentration (BPMO specific activity) (Suteau and Narbonne, 1988) was similar to that previously reported for the mullet *Chelon labrosus* (Narbonne *et al.*, 1987).

The formation of single-stranded breaks in crab haemolymph dependent on the dose of BaP injected directly into the crab haemolymph (Table 2). The amount of haemolymph DNA eluted increased in time for a dose of 0.3 µg/g. The increased elution could be the result of accumulated damage in crab haemolymph. We expected further increase in DNA damage after four days of exposure when the maximal benzo(a)pyrene monooxygenase induction in crab hepatopancreas occurred (Batel *et al.*, 1986, Batel *et al.*, 1988), and when more than 2.0% of BaP metabolites was present in the haemolymph of crabs (James and Little, 1984). However, for higher dose of 3.0 µg/g BaP increase in elution rate after 1.5 hours was followed by drastic decrease in elution after 24 hours. This could be explained as due to processes of DNA single-strand breaks repair in crab haemolymph DNA.

The levels of single-stranded breaks and alkali-labile sites in mussel haemolymph were studied as a function of dose and time after single injections of BaP and 4-nitroquinoline-N-oxide (NQO). NQO was used as a model compound that requires metabolic activation for DNA adduct formation (Soileau, 1987), and can cause double stranded breaks in rats (Niedermuller, 1985). In the stationary phase cells of *Saccharomyces cerevisiae*, NQO is reported to be a strong mutagen that is detoxified by the monooxygenase system (Del-Carratore *et al.*, 1986).

Treatment of mussels with varying amounts of BaP and NQO, 1,5 hours before taking out the haemolymph, caused a dose-dependent increase in the elution rate of ssDNA (Figures 5, 6). DNA elution curves from untreated mussels were of the first order with respect to elution volume. On the contrary, elution curves from both BaP and NQO-treated mussels expressed two-phased kinetics. The first phase was approximately of the first order with respect to the elution volume and dose, depending on the length of ssDNA (Kohn *et al.*, 1976), while the second phase was volume and dose independent. This was the reason to express the results as strand scission factors estimated for the linear part of elution curves (9 ml). The relationship between the slopes for the linear part of curves and strand scission factors was influenced by the time of exposure, being linear and well correlated for both pollutants (Figures 7, 8).

Administration of 5 µg BaP/g mussel significantly increased DNA damage in haemolymph 1.5 h, followed by a further increase after 24 hours (Table 3). However, higher doses of BaP (10 and 20 µg/g mussel) after an initial increase at 1.5 h, caused a decline in DNA damage after 48 h, suggesting that significant repair occurred.

In the case of NQO, 5 µg/g mussel and 10 µg/g mussel caused an increase in DNA damage after 1.5 h, with a further increase after 24 h (Figure 9). DNA damage with the dose of 20 µg/g mussel drastically increased after 1.5 h, followed by a decline after 24 h, suggesting that fast DNA repair occurred. However, after 5 days of administration these mussels died.

Table 1

Dose-response relationship for DNA damage following *in vitro* exposure of FLC/F cells to indirect mutagens

Compound	µg/ml	SSF*
benzo(a)pyrene	0	0
	1.0	0.021 ± 0.013
	5.0	0.046 ± 0.009
aminoanthracene	0	0
	1.0	0.064 ± 0.009
	5.0	0.086 ± 0.006

* strand scission factors (SSF) were calculated in relation to control samples containing cells incubated with all components of S9 mix without the cofactor - NADPH, and expressed as means ± standard deviations of three independent experiments.

Table 2

Dose and time dependence of DNA damage in crab haemolymph caused by single injection of benzo(a)pyrene

Dose µg/g	1.5 h	Strand scission factor 24 h	5 days
0.0	0	0	0
0.3	0.071 ± 0.020	0.090 ± 0.024	0.038 ± 0.025
1.0	0.118 ± 0.072	0.106 ± 0.053	
3.0	0.226 ± 0.123	0.044 ± 0.019	

Results are expressed as means ± standard deviations of three independent experiments with 4 crabs each.

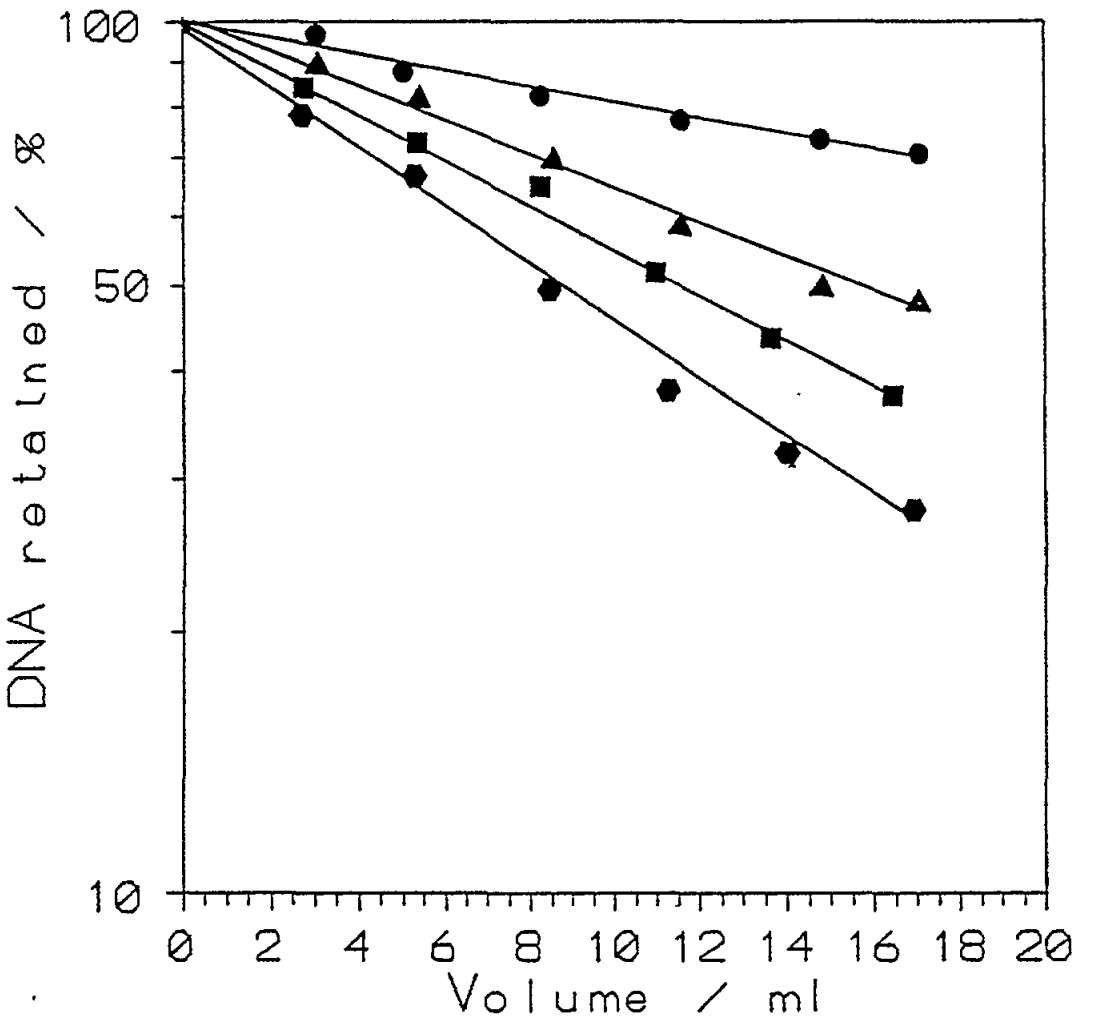

Fig. 3 Alkaline elution profiles of fish liver DNA from animals treated with different pollutants.

- ● - control (10 µl DMSO)
  y = -0.023x + 100.0; r = 0.96
- ▲ - Aroclor 1254 (Aro) - 0.5 µg/g
  y = -0.055x + 104.5; r = 0.98
- ■ - N-metil-N-nitroso-nitrogvanidin (MNNG) - 0.3 µg/g
  y = -0.074x + 100.1; r = 0.99
- ⬟ - MNNG + Aro (0.3 µg/g + 0.5 µg/g) simultaneously
  y = -0.092x + 103.8; r = 0.98

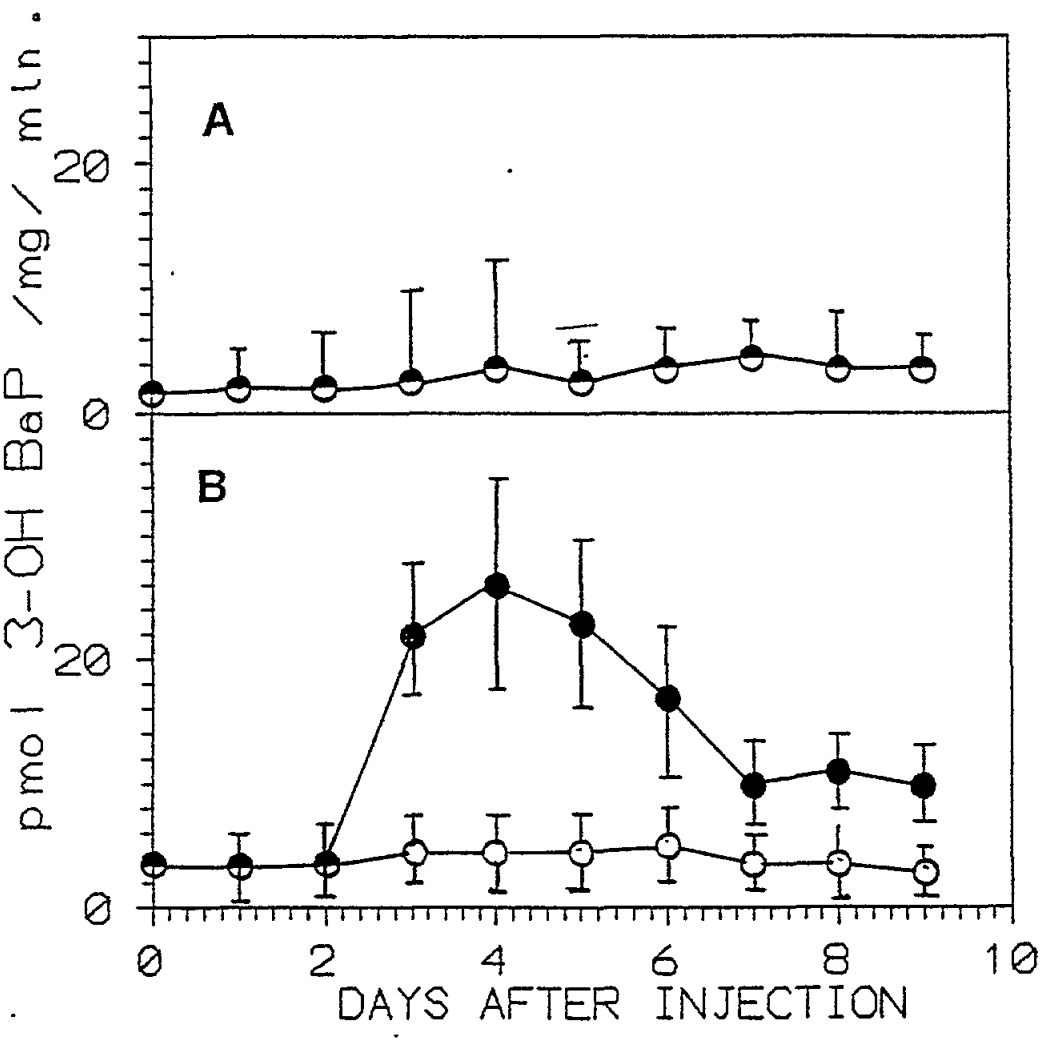

Fig. 4 Benzo(a)pyrene monooxygenase activity as a function of time of exposure in:
A - in the digestive gland of mussel *Mytilus galloprovincialis*
B - hepatopancreas of the crab *Maja crispata* and
○ - controls
● - injected with BaP (3 μg/g crab or 10 μg/g mussel)

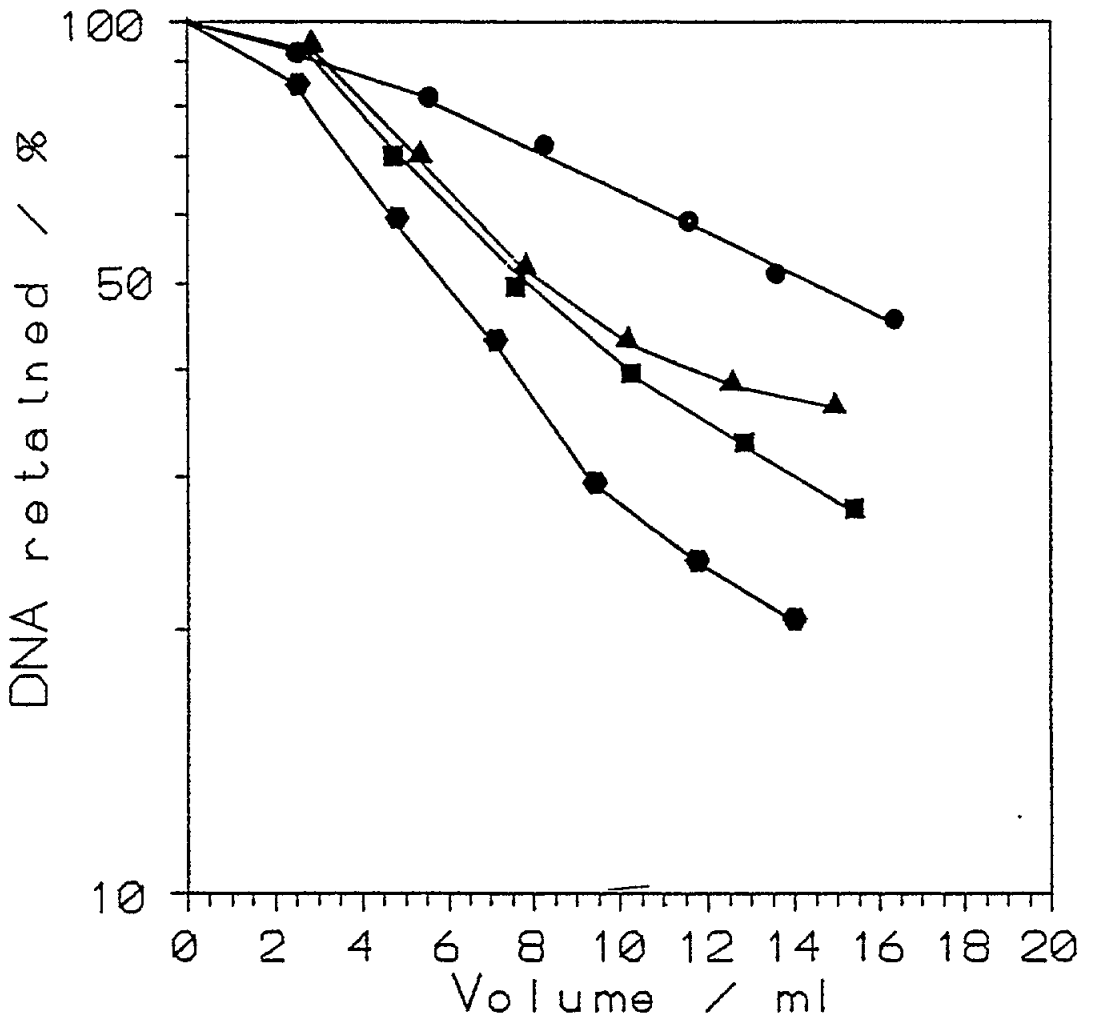

Fig. 5 Alkaline elution profiles of haemolymph DNA from BaP treated mussels 1.5 h after single injection:
● - controls (100 µl DMSO)
▲ - 5 µg/g mussel
■ - 10 µg/g mussel
⬢ - 20 µg/g mussel

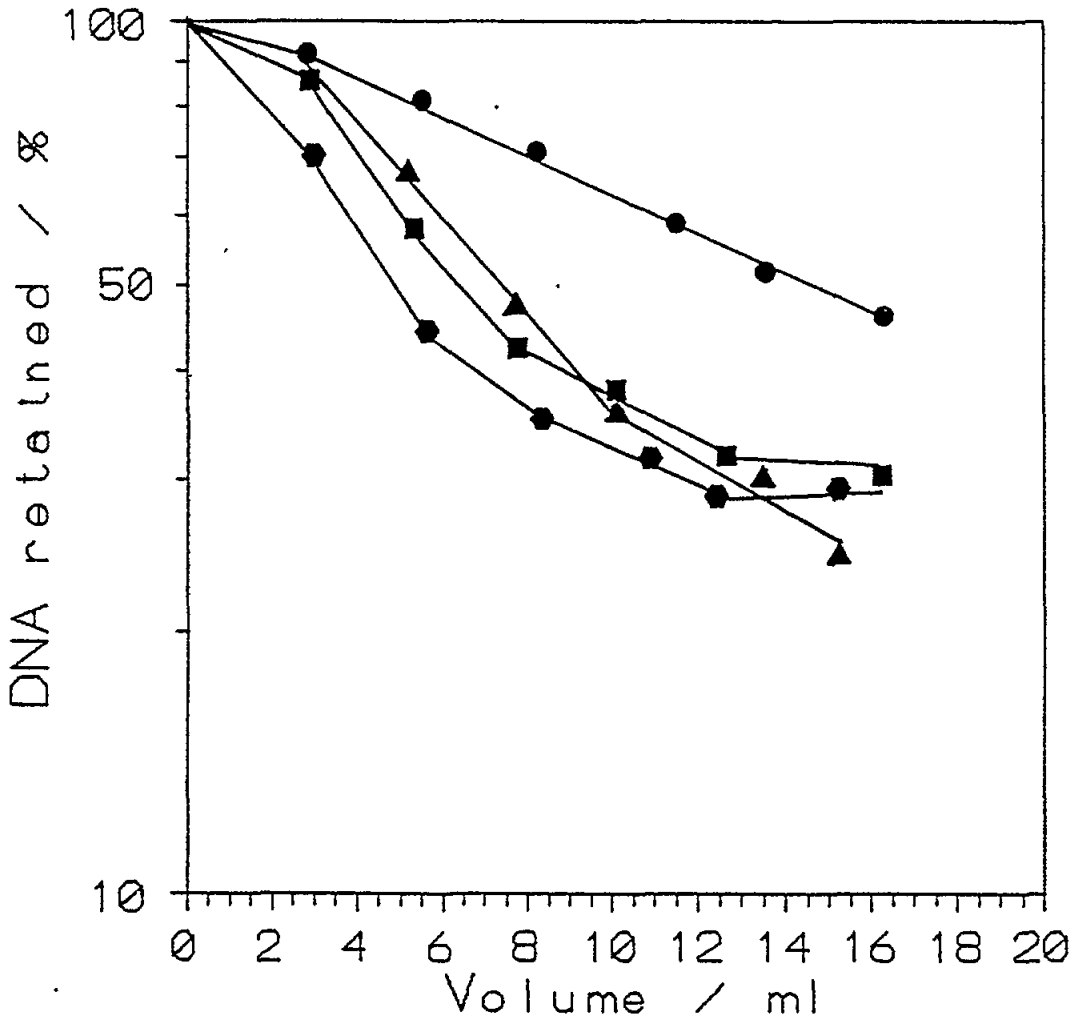

Fig. 6 Alkaline elution profiles of haemolymph DNA from NQO treated mussel 1.5 h after single injection:
● - controls (100 µl DMSO)
▲ - 5 µg/g mussel
■ - 10 µg/g mussel
⬣ - 20 µg/g mussel

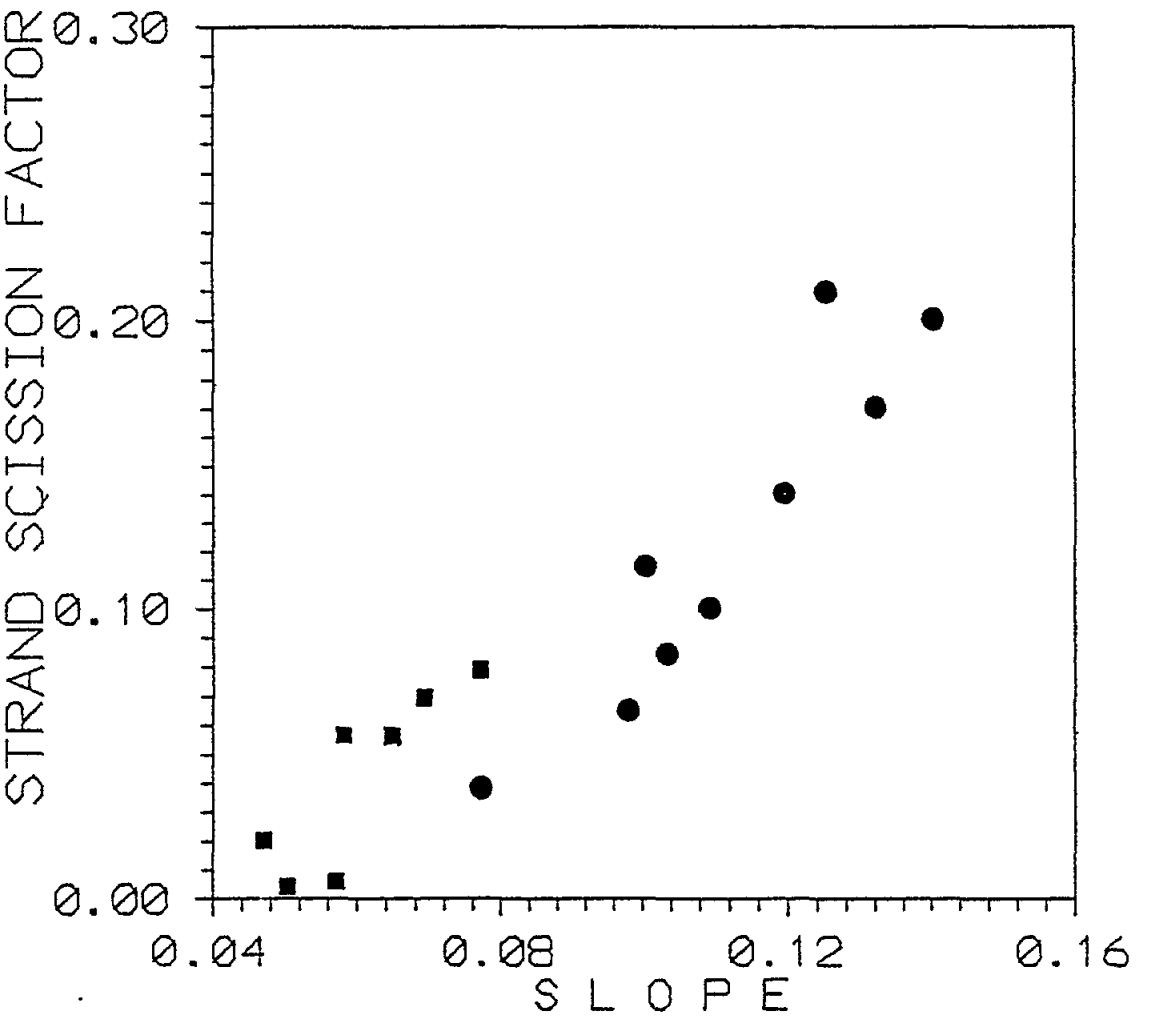

Fig. 7 The relationship between the slopes and strand scission factors for mussels treated with BaP
■ - 1.5 h (y = 2.83x - 0.19; r = 0.93),
● - 48 h (y = 2.32x - 0.11; r = 0.74)

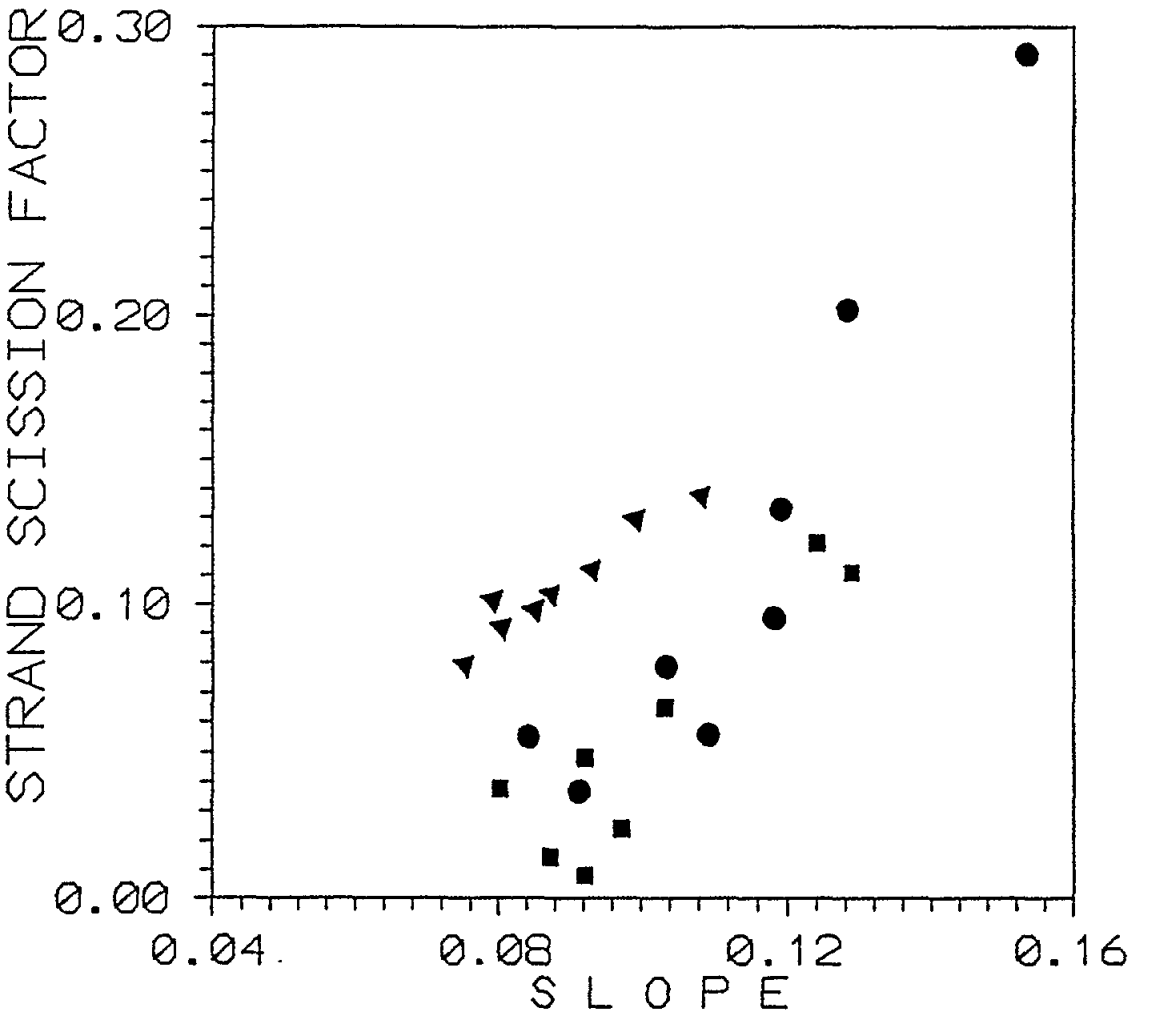

Fig. 8 The relationship between the slopes and strand scission factors for mussels treated with NQO
■ - 1.5 h (y = 3.73x - 0.30; r = 0.93),
● - 24 h (y = 1.76x - 0.05; r = 0.96),
▲ - 5 days (y = 2.28x - 0.18, r = 0.84)

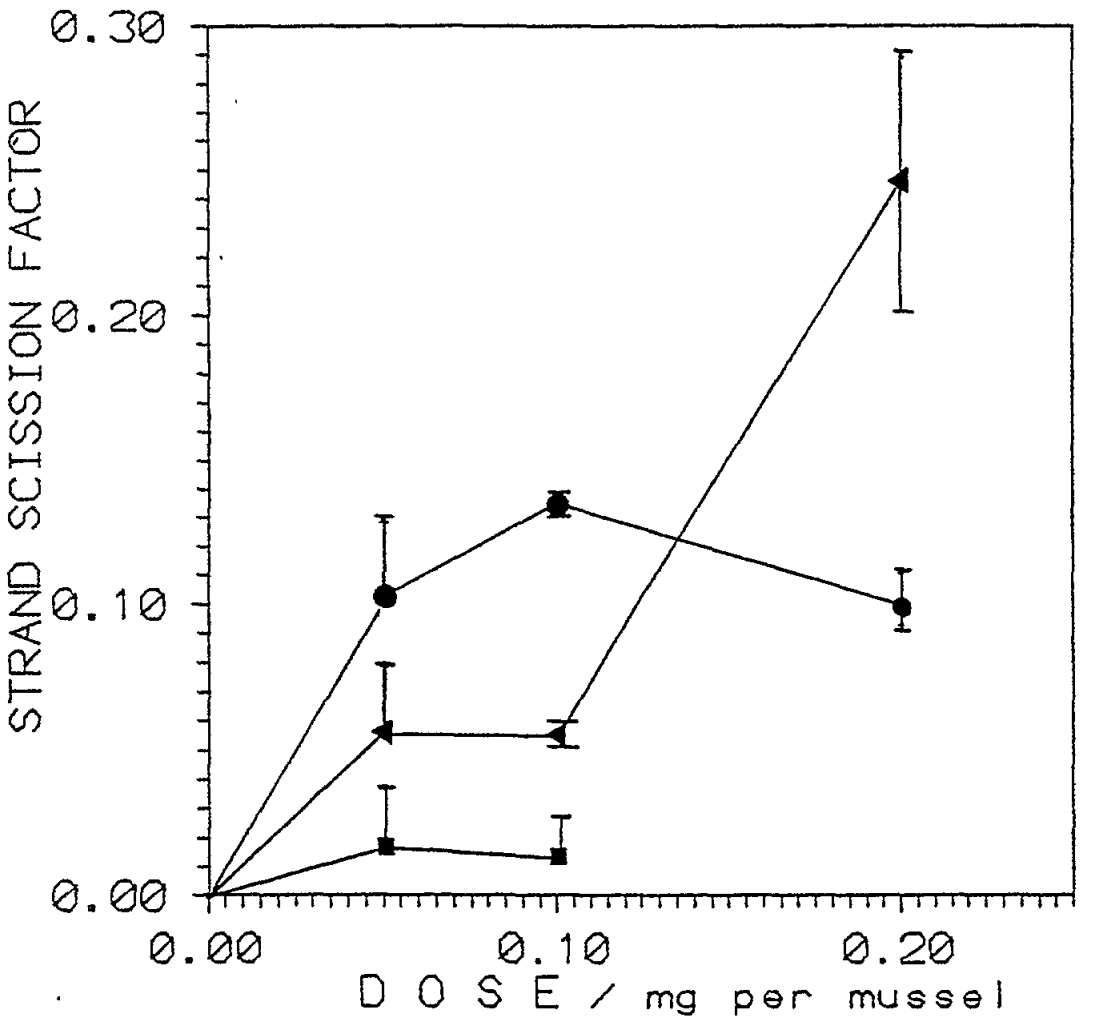

Fig. 9 Dose and time dependence of DNA damage in mussel haemolymph caused by single injection of NQO. Bars and S.E. are associated with at least 3 measurements with at least 5 mussels in each

Furthermore, in mussels treated with 5 µg/g and 10 µg/g, significant repair occurred 5 days after exposure.

These results showed that although there is a relationship between the target dose and the level of damage, repair processes can influence the damage levels in mussel haemolymph. The lower the dose of BaP applied to the mussel, the lower the DNA damage and faster the repair. In the case of NQO treatment, the rate of DNA repair seems to be faster for higher doses too. It is known that significant repair of DNA damage induced by NQO in rat occurred with 4 h and no increase in elution was detectable 24 h after administration (Petzold and Swenberg, 1978). In comparison to rapidly repairable DNA ssb detected after BaP treatment of mammalian cells (Ochi *et al.*, 1986) and in rat treated with NQO, the repair system in mussel haemolymph is significant and slower (Bihari *et al.* 1990b).

In order to confirm the usefulness of mussels as test organisms in the monitoring of actual pollution by the method of alkaline filter elution, mussels were injected with sediment extracts from different polluted locations (Figure 10). Two of the investigated locations (s-2, s-3) are under the direct influence of industrial and/or urban runoff from a tobacco factory and urban waste in the harbour, respectively, and one (s-9) is under the influence of the river Po. By using SSF as a measure of DNA damage caused by sediment extracts after 1.5 h, it was possible to distinguish the genotoxic potential of sediments from differently polluted areas (Figure 11). After 24 h an increase in DNA damage could be observed, giving further evidence of the genotoxic potential of sediment organic extracts, ranking subsequently: s-3, s-2, s-9, s-6, s-5. These results are in good agreement with the occurrence of chromosomal aberrations in gills of *Mytilus galloprovincialis* (Al Sabti and Kurelec, 1985) from these contaminated areas. Sediment extracts from location s-3 are toxic for mussels after 24 h which is in agreement with the measured toxic potential of sediments, as reported by us recently (Bihari *et al.*, 1989).

Our primary study goal was to find out, whether the method of alkaline filter elution is suitable for detecting DNA alterations resulting from unknown environmental pollution among natural mussel populations, and can thus be used for routine monitoring studies. Therefore we compared mussels from locations with different pollution load. DNA elution curves from the haemolymph of mussels living in the clean area of Lim bay (s-4), as well as from a mariculture area, were of the first order with respect to elution volume (Figure 12). On the contrary, elution curves from mussels living in polluted areas expressed two phase kinetics (Figure 13). The first phase was approximately of the first order with respect to the elution volume and dose, depending on the length of single stranded DNA, while the second phase was volume and dose independent. Since we examined at least 18 and up to 48 mussels per location, the probabilities of error became low. One explanation could be the presence of DNA-protein crosslinks. Similar effects explained by reduced transcriptional activity as part of an environmentally caused stress syndrome were described by Herbert and Zahn (1990).

A background level of DNA with low integrity (DNA with various types of structural alterations, caused by normal cellular events or processes, physical and chemical agents), may exist in the cell at any time. Fortunately, mussel haemocytes have DNA repair mechanisms, as we showed previously, that under normal circumstances efficiently eliminate DNA of low integrity. By using SSF as a measure of DNA damage (single-stranded breaks) in mussel haemolymph, it is possible to distinguish the genotoxic potential of differently polluted areas (Table 4). These results are in good agreement with the occurrence of chromosomal aberrations in the gills of *Mytilus galloprovincialis* (Al Sabti and Kurelec, 1985) from these contaminated areas.

Since mussels live in a more or less constant environment, the pollution level is fairly constant, and their DNA damage and repair is also fairly constant. In this case the method of DNA damage assessment provides a reasonable picture of the pollution status. In other cases it mirrors an integral extending into the recent pollution history. Determination of DNA damage in mussels with different pollution histories confirms the usefulness of the method as a biomarker of pollution.

Table 3

Dose and time dependence of DNA damage in mussel haemolymph caused by single injection of BaP

Dose µg/g mussel	Strand scission factor	
	1.5 h	48 h
0	0	0
5	0.052 ± 0.013	0.096 ± 0.029
10	0.091 ± 0.019	0.012 ± 0.008
20	0.180 ± 0.027	0.031 ± 0.025

Results are expressed as means ± standard deviations of three independent experiments with at least 5 mussels in each.

Table 4

Strand scission factors of mussel haemolymph related to control samples from clean area

Station	No of composite samples	SSF	No of mussel/ sample
s-4	7	0	48
s-3	3	0.065 ± 0.038	29
s-2	2	0.044 ± 0.014	18
s-1	2	0.154 ± 0.089	36

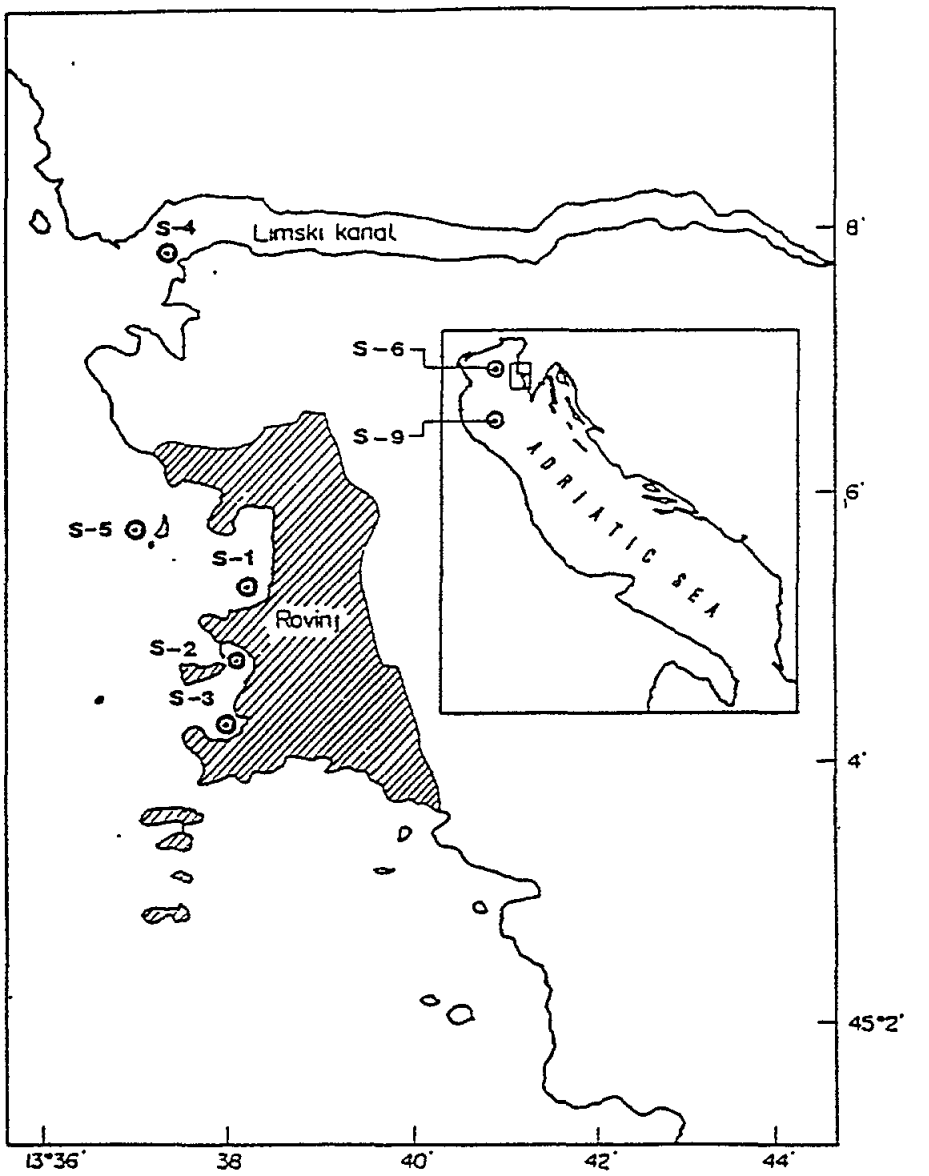

Fig. 10 Map of Rovinj Northern Adriatic showing sampling sites

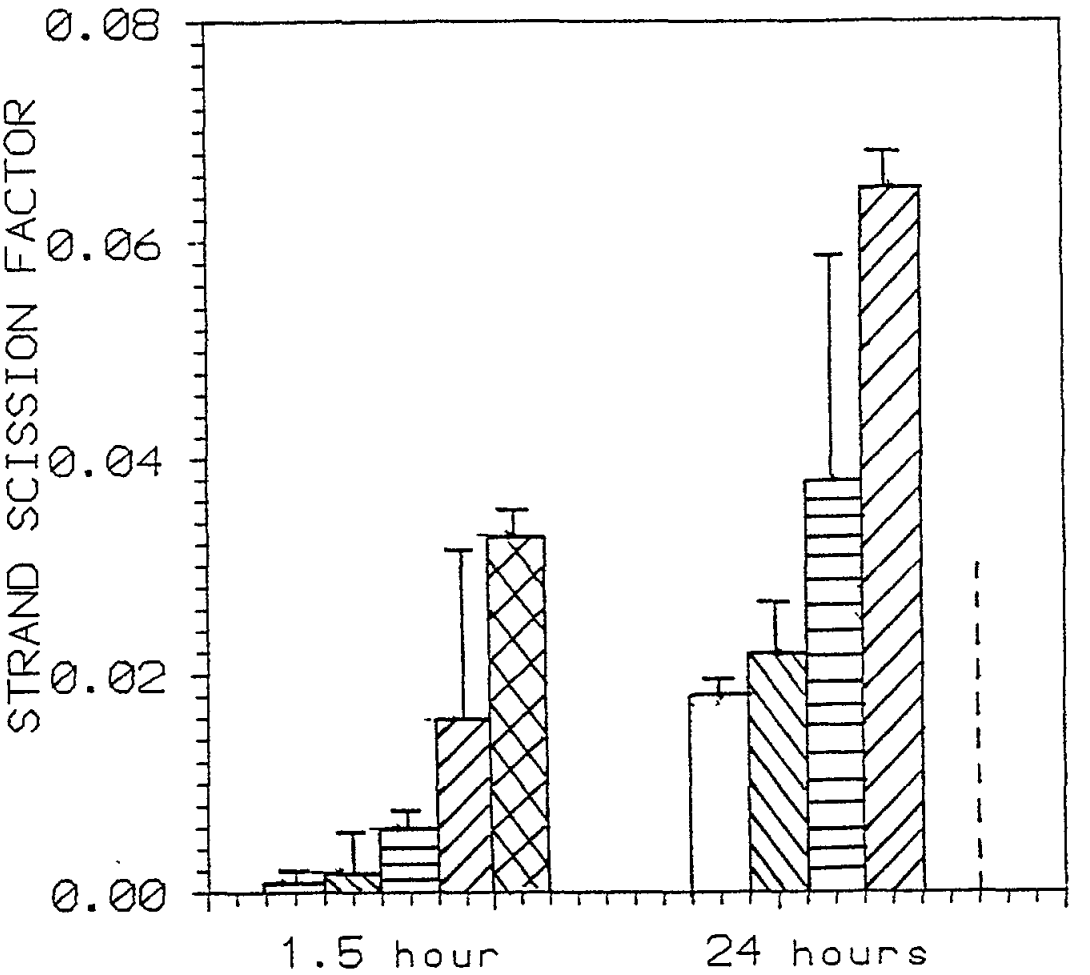

Fig. 11 Increase in DNA damage after 1.5 and 24 hours in mussels injected with sediment extracts from different locations: (▨) s-2, (▩) s-3, (□) s-5, (▧) s-6, and (▤) s-9. Mussels receiving extracts from location s-3 deceased after 24 h.
Results are expressed as strand scission factors divided by grams of dry weight of extracted sediment applied. Bars represent standard deviations of 3 independent experiments.

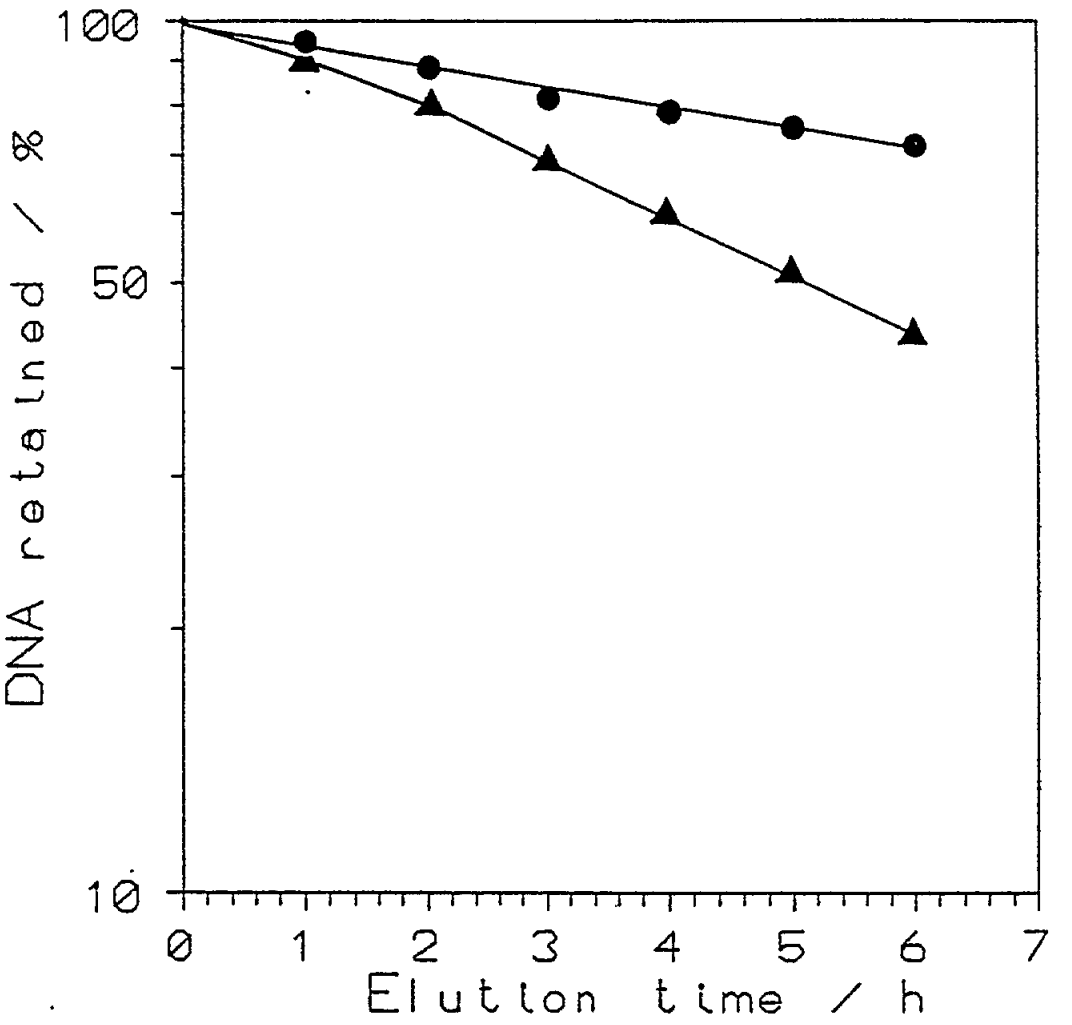

Fig. 12 Alkaline elution profiles of haemolymph DNA from natural mussel population in clean (●) and (▲) maricultured area - Lim bay

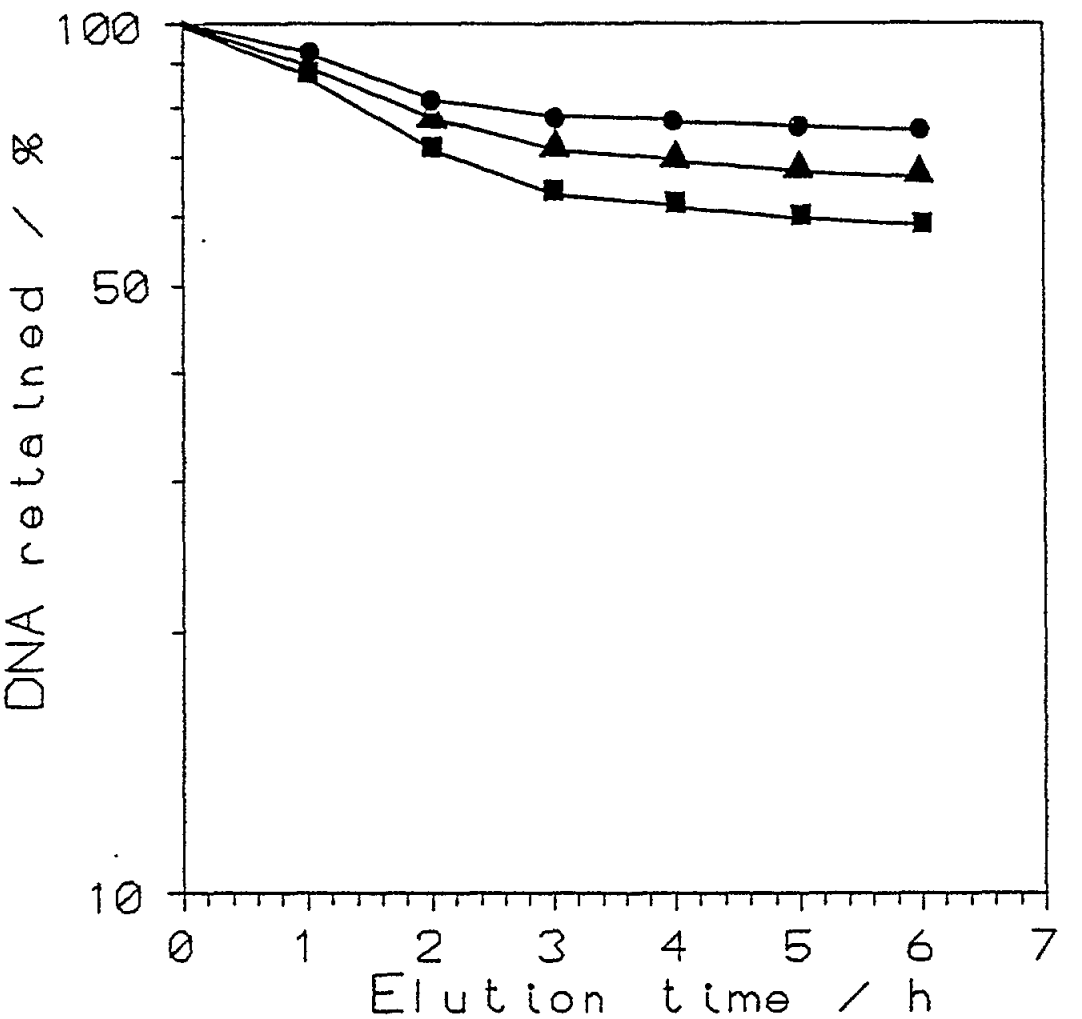

Fig. 13 Alkaline elution profiles of haemolymph DNA from natural mussel population, (■) s-1 industrial runoff from fish cannery, ● s-2 urban waste in the harbor, ▲ s-3 industrial and/or urban runoff from a tobacco factory

4. CONCLUSIONS

The alkaline elution assay represents a rapid, reproducible and inexpensive technique for detecting compounds with potential carcinogenic or mutagenic activity. The results from different groups and laboratories are comparable and this method could be easily introduced in marine laboratories.

The alkaline elution assay represents a good technique for determination of genetic damage in marine crab and mussel haemolymph caused by model pollution. This is also important in the investigation of PAH metabolism in mussels, since extrapolation from *in vitro* levels of enzyme activity to *in vivo* PAH metabolism is still a problem. The general metabolism of benzo(a)pyrene in marine invertebrates is low compared to vertebrates. Failure to detect benzo(a)pyrene monooxygenase activity does not necessarily prove its absence. Measurable DNA damage in crab haemolymph and fish liver after treatment with BaP is a consequence of increased BPMO activity. In mussel, repairable indirect DNA damage produced by free radical formation during low (in our case, undetectable) BaP metabolism exists.

The usefulness of the alkaline elution method for determination of genetic damage in mussels caused by actual pollution was established. This is important in strengthening the use of mussels as sentinel organisms when assessing environmental genetoxicity.

The use of haemolymph as a target organ has several advantages:

- it constitutes about 50% of mussel wet weight minus the shell (Martin *et al.*, 1958, Thompson *et al.*, 1978);

- it is easily drained without disturbance of the whole animal, allowing the use of the same individuals for time course studies;

- haemolymph cells are not disrupted by handling, and they do not coagulate in the time needed for manipulation.

Haemocytes are cells capable of independent movement and are distributed throughout the open vascular system and within other tissues. They can ingest particles too large to enter the cells of the digestive diverticulum, and transport them to the haemolymph. On the other hand, haemocytes reduce the amount of nutritional and toxic particles in the haemolymph (Bayne *et al.*, 1976, Jones, 1983). Therefore, one can expect elevated amounts of pollutants in the haemolymph of mussels, regardless of their solubility and their route of entry.

5. REFERENCES

AL SABTI, K., B. KURELEC (1985). Induction of chromosomal aberrations in the mussel *Mytilus galloprovincialis* watch. *Bull. Environ. Contam., Toxicol.* **35**: 660-665.

BARTSCH, H., R. TERRACINI, C. MALAVEILLE, L. TOMATIS, J. WARENDORF, G. BRUNN, B. DODET (1983) Quantitative comparison of carcinogenicity, mutagenicity and electrophylicity of 10 direct-acting alkylating agents and the initial O^6: 7-alkylguanine ratio in DNA with carcinogenic potency in rodents. *Mutat. Res.* **110**: 181-219.

BATEL, R., BIHARI, N., KURELEC, B. and ZAHN, R.K. (1985). DNA damage by benzo(a)pyrene in the liver of mosquito fish *Gambusia affinis. Sci. Tot. Environ.* **41**: 275-283.

BATEL, R., BIHARI, N., ZAHN, R.H. (1986): Purification and characterization of a single form of cytochrome P-450 from the spiny crab *Maja crispata*. *Comp. Biochem. Physiol.* **83C**: 165-170.

BATEL, R., BIHARI, N. and ZAHN, R.K. (1988): 3-Methylcholanthrene does induce mixed function oxidase activity in hepatopancreas of spiny crab *Maja crispata. Comp. Biochem. Physiol.* **90C**: 435-438.

BAYNE, B.L., THOMPSON, R.J., WIDDOWS, J. (1976): Physiology: I, II. In: *Marine mussels,* Ed. B.L. Bayne, Cambridge University Press, London, , pp. 121-260.

BERTHOU, F., BALOUET, G., BODANNEC, G., MARCHAND, M. (1987). The occurrence of hydrocarbons and histopathological abnormalities in oysters for seven years following the wreck of the *Amoco Cadiz* in Brittany (France). *Mar. Environ. Res.* **23**: 103-133.

BIHARI, N., BATEL, R., KURELEC, B., ZAHN, R.K. (1984): Tissue distribution, seasonal variation and induction of benzo(a)pyrene activity in the crab *Maja crispata*. *Sci. Tot. Environ.* **35**: 41-51.

HENDRICKS, J.D., WALES, J.H. SINNHUBER, R.O., NIXON, J.E., LOVELAND, P.M. and SCANLAN, R.A. (1980): Rainbow trout (*Salmo gairdneri)* embryos: A sensitive animal model for experimental carcinogenesis. *Fed. Proc.* **39**: 3222-3229.

HERBERT, A., ZAHN R.K. (1990): Monitoring DNA damage in *Mytilus galloprovincialis* and other aquatic animals. *Ger. J. Appl. Zool.* **76**: 13-35.

JAMES, M.O. and LITTLE, P.J.(1984). 3-Methylcholanthrene does not induced in vitro xenobiotic metabolism in spiny lobster hepatopancreas, or affect *in vivo* disposition of benzo(a)pyrene. *Comp. Biochem. Physiol.* **78C**: 241-245.

JONES, H.D. (1983): Circulatory systems of gastropods and bivalves. In: *The mollusca*, Vol 5, Physiology, Part 2., eds. A.S.M. Saleuddin and K.M. Wilbur, Academic Press, New York, pp. 189-238.

KOHN. K.W., ERICKSON. L.C., EWIG, A.G. and FRIEDMAN, C.A. (1976). Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry,* **15**: 4629-4637.

KOHN, K.W., EWIG, R.A.G., ERICKSON, L.C., ZWELLING, L.A. (1981). Measurement of strand breaks and cross-links by alkaline elution, in: DNA repair. In: *A laboratory manual of research procedures*, Vol 1, pt. B. (Friedberg and Hanawalt Eds.), Marcel Dekker Inc., New York, pp. 379-401.

KRIEG, K. (1972): *Ampullarius australis* d'Orbigni (Mollusca, gastropoda as experimental animal in ecological research. A comparison to the study of carcinogenesis in invertebrates. *Neoplasma* **19**: 41-49.

KURELEC, B., CHACKO, M., GUPTA, C. (1988). Postlabelling analysis of carcinogen-DNA adducts in mussel, *mytilus galloprovincialis*. *Mar. Environ. Res.* **24**: 317-320.

LOWRY, O.H., ROSENBERG, N.J., FARR, A.L., RANDALL, R.J. (1951): Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.

BIHARI, N., NAJDEK, M. FLORIS, R., BATEL, R., ZAHN, R.K. (1989): Sediment toxicity assessment using bacterial bioluminescence: effect of an unusual phytoplankton bloom. *Mar. Ecol. Progress Ser.* **57**: 307-310.

BIHARI, N., BATEL, R., ZAHN, R.K. (1990): The use of alkaline elution procedure to measure DNA damage in crab haemolymph treated with benzo(a)pyrene. In: Carcinogenic, Mutagenic and Teratogenic Marine Pollutants: Impact on Human health and Environment. *Adv. Appl. Biotechnol. Ser.*, **5**: pp. 121-127.

BIHARI, N., BATEL, R., ZAHN, R.K. (1990). DNA damage determination by the alkaline elution technique in the haemolymph of mussel *Mytilus galloprovincialis* treated with benzo(a)pyrene and 4-nitroquinoline-N-oxide. *Aquatic. Toxicol.* **18**: 13-22.

BRITVIC, S., KURELEC, B. (1986). Selective activation of aromatic amines to bacterial mutagens in the marine mussel *Mytilus galloprovincialis. Comp. Biochem. Physiol.*, **85C**: 111-114.

CLEAVER, J.E. (1975): Methods for studying repair of DNA, damaged by physical and chemical carcinogenesis. *Meth. Cancer Res.* **11**: 123-165.

DEL CARRATORE, R., CUNDARI, E., VELLOSI, R., GALLI, A., BRONZETTI, G. (1986). Specific inhibitors of the monooxygenase system of *Saccharomyces cerevisiae*. *Carcinogenesis* **7**: 1127-1230.

DIXON, R.D., JONES, I.M., HARRISON, F.L. (1985). Cytogenic evidence of inducible processes linked with metabolism of a xenobiotic chemical in adult and larval *Mytilus edulis. Sci. Tot. Environ.* **46**: 1-8.

GAMPER, H.B., TUNG, A.S.C., STRAUP, K., BARTHOLOMEW, J.C. and CALVIN, M. (1977). DNA strand scission by benzo(a)pyrene diol epoxides. *Science,* **197**: 671-674.

MARON, D.M., AMES, B.N. (1983). Revised methods for the *Salmonella* mutagenicity test. *Mutat. res.* **113**: 173-215.

MENDOZA-FIGUEROA, T., LOPEZ-REVILLA, R., VILLA-TREVINO, S. (1985). Aroclor 1254 increases the genotoxicity of several carcinogens to liver primary cell cultures. *J. toxicol. environ. health* **15**: 245-254.

MEYN, R.E., JENKINS, W.T. (1983). Variation in normal and tumor tissue sensitivity of mice to ionizing radiation-induced DNA strand breaks *in vivo*. *Cancer Res.,* **43**: 5668-5673.

NARBONNE, J.F., SUTEAU, P.M., DAUBEZE, M., AUDY, C. (1987). Polycyclic aromatic hydrocarbon metabolism in mullets (*Chelon labrosus*) treated by polychlorinated biphenyls. *Bull. Environ. Contam. Toxicol.*, **38**: 57.

NEBERT, D.W., GELBOIN, H.V. (1968). Substrate-inducible aryl hydroxylase in mammalian cell culture: I. Assay and properties of induced enzymes. *J. Biol. Chem.*, **243**: 6242-6249.

NIEDERMULLER, H. (1985). DNA repair during aging. *Aging*, **29**: 173-193.

NISHIMOTO, M., VARANASI, U. (1985). Benzo(a)pyrene metabolism and DNA adduct formation mediated by English sole liver enzymes. *Biochem. Pharmacol.*, **34**: 263-268.

OCHI, T., ISHIGURO, T. OHSAWA, M. (1986). Induction of alkaline-labile sites in DNA by benzo(a)pyrene and the repair of those lesions in cultured Chinese hamster cells. *Mutat. Res.* **165**: 31-38.

PARODI, S., TANINGHER, M., SANTI, L., CAVANNA, M. SCIABA, L., MAURA, A., and BRAMBILLA, G. (1978). A practical procedure for testing DNA damage *in vivo* proposed for a pre-screening of chemical carcinogens. *Mutat. Res.*, **54**: 39-46.

PETZOLD, G.L. and SWENBERG, J.A. (1978). Detection of DNA damage *in vivo* following exposure of rats to carcinogens. *Cancer Res.*, **38**: 1589-1594.

SCHIEWE, M.H., HAWK, E.G., ACTOR, D.I. KRAHN, M.M. (1985). Use of the bacterial bioluminescence assay to assess toxicity of contaminated marine sediments. *Can. J. Fish aquat. Sci.*, **42**: 1244-1248.

SINNHUBER, R.O., HENDRICKS, J.D., WALES, J.H., and PUTNAM, G.B. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann. N.Y. Acad. Sci*, **298**: 389-408.

SOILEAU, S.D. (1987). *Carcinogen DNA adducts. Introduction, literature summary, and recommendations.* EPA, Lockheed-EMSCO, Inc, Las Vegas, Nevada.

STOUT, D.L., and BECKER, F.F. (1982). Fluorometric quantitation of single-stranded DNA: A method applicable to the technique of alkaline elution. *Anal. Biochem.*, **127**: 302-307.

STUBER, J. and ZAHN R.K. (1985). Biochemical alterations induced in fish by an acute kerosine-spillage. *Arch. Hydrobiol.*, **103**: 117-127.

SUTEAU, P.M., NARBONNE J.F. (1988). Preliminary data on PAH metabolism in the marine mussel *Mytilus galloprovincialis* from Arcachon Bay, France. *Mar. Biol.*, **98**: 421-425.

SWENBERG, J.A., PETZOLD, G.L. and HARBACH, P.R. (1976). *In vitro* DNA damage/alkaline elution assay for predicting carcinogenic potential. *Biochem. Biophys. Res. Commun.* **72**: 732-738.

VARANASI, U., GMUR, D.J. (1980). Metabolic activation and covalent binding of benzo(a)pyrene to deoxyribonucleic acid catalyzed by liver enzymes of marine fish. *Biochem. Pharmacol.*, **29**: 753-761.

ZAHN, R.K., MULLER, W.E.G., KURELEC, B., RIJAVEC, M. and MICHAELIS, M.L. (1981). Single strand nicks: Cause or consequence of environmental damage or aging. *Res. Mol. Biol.*, **10**: 2054.

EFFECT OF SOLAR IRRADIATION ON MUTAGENS AND IMPACT OF SEA POLLUTION ON THE BIOTRANSFORMATION OF CARCINOGENS IN FISH LIVER

by

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1. INTRODUCTION

1.1 Foreword

This article reports the results of research activities carried out in our laboratory within the framework of MED POL Phase II Program, during the period June 1986 - October 1989. Two different studies were implemented, regarding the photodynamic activation of mutagens and carcinogens, and the effect of marine pollution on fish liver biotransformations, respectively. The latter investigation was carried out in collaboration with the Institute of Zoology of Genoa University. The results obtained have also been published in separate papers (De Flora *et al.*, 1989a and 1989b; Bagnasco *et al., 1990).*

1.2 Environmental fate of mutagens and carcinogens

Environmental pollutants, including mutagenic and carcinogenic substances, can interact with other chemicals or with physical agents. Of particular interest is the interaction between solar irradiation and molecules which are either *per se* biologically active or can be converted, in target organisms, into noxious metabolites. In certain cases sunlight can lead to photodecomposition processes, as shown, e.g., with certain PAHs and their derivatives (Fox and Olive, 1979; Valerio and Lazaretto, 1985; Stärk *et al.*, 1985; Holloway *et al.*, 1987). On the other hand, photoactivation processes can also occur. Especially if compared with the huge literature devoted to the intricate subject of the metabolism of xenobiotics and to exploration of the biochemical pathways involved, relatively less attention is commonly paid to the possibility that precursor compounds may be activated to genotoxic products following simple exposure to natural or artificial light. Clearly, this phenomenon has considerable relevance because of its consequences on the environmental spreading of genotoxic and/or carcinogenic substances.

Previous studies carried out in this and other laboratories have shown that certain promutagens or even nonmutagenic compounds can be photoactivated to derivatives exhibiting a direct mutagenicity in prokaryotic or eukaryotic target cells. These compounds include polycyclic aromatic hydrocarbons (PAHs) (Gibso *et al.*, 1978; Tu *et al.*, 1979; McCoy *et al.*, 1979; McCoy and Rosenkranz, 1980; Claxton and Barnes, 1981; Strniste and Brake, 1981; De Flora, 1982; De Flora and Badolati, 1982; Takeda *et al.*, 1984; White *et al.*, 1985), aromatic amines (McCoy *et al.*, 1979; De Flora, 1982, De Flora and Badolati, 1982; Nishi and Nishioka, 1982; Strniste *et al.*, 1985; White and Heflich, 1985; Okinaka *et al.*, 1986), and aflatoxins (Buchi *et al.*, 1982; Israel-Kalinsky *et al.*, 1982). Similar findings have been obtained by irradiating complex mixtures, such as coal-and shale-derived synthetic fuels (Selby *et al.*, 1987).

In the present study, we investigated the activation of compound solutions exposed under various experimental conditions to sunlight or to different spectra of UV and visible light produced by artificial sources. Test substances included compounds of different chemical classes, which can be categorized either as nongenotoxic carcinogens (such as organochlorine pesticides) or as indirect

carcinogens and/or mutagens (such as PAHs, aromatic amines and heterocyclic amines). The results obtained provided evidence for structure-activity relationships in the response to oxygen-dependent photoactivation. The heterocyclic amines IQ and MeIQ, which can be formed not only in the charred part of cooked foods (Sugimura, 1985) but also in other combustion process, e.g., in cigarette smoke (Yamashita *et al.*, 1986), were found to be the most sensitive compounds to photoactivation.

1.3 Metabolism of mutagens and carcinogens in fish liver, and its inducibility by marine pollutants

The mechanisms involved in the metabolic activation and in detoxication of mutagens and carcinogens have been extensively investigated in mammals. A number of studies carried out worldwide over the last two decades have provided evidence that fish species also possess the metabolic machinery needed for the biotransformation of xenobiotics. As assessed both in experimental studies and under field conditions, induction of microsomal cytochrome P-450-dependent monooxygenases or mixed-function oxidases (MFO), has been proposed as an index for water quality monitoring and as an early warning parameter for delineating sublethal effects of pollutants (see Payne *et al.*, 1987, for a review).

Our study aimed at investigating the effect of seawater pollution on the liver metabolism of mutagens and carcinogens in a marine fish species living in a harbour environment, as compared to a clean reference area. Measurement of microsomal MFO was completed with the analysis of reduced glutathione (GSH) and of several cytosolic enzyme activities. These included enzymes of the GSH cycle, i.e. GSH S-transferase, GSH peroxidase and oxidized glutathione (GSSG) reductase, and dehydrogenases involved in the hexose monophosphate shunt, i.e., glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), and in the transfer of electrons from NADH and/or NADPH to suitable acceptors, i.e., diaphorases.

In addition, liver post-mitochondrial fractions from fish living either in clean or in polluted seawater were used in order to compare the ability to activate promutagens to mutagenic metabolites as well as to decrease the potency of direct-acting mutagens. Such a methodological approach provides a more comprehensive view of the influence of environmental factors on the metabolism of biologically active xenobiotics, which cannot be inferred from the determination of individual biochemical parameters. The possible presence of mutagens in fish muscle, which had been previously detected in freshwater fish exposed to high concentrations of sodium nitrite (De Flora and Arillo, 1983), was also checked.

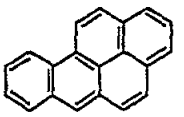
2. MATERIALS AND METHODS

2.1 Effect of sunlight on mutagens and carcinogens

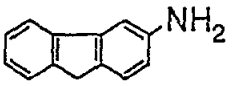
2.1.1 Test compounds

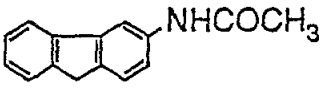
Test compounds included both mutagens and nongenotoxic carcinogens, i.e., 2-aminoanthracene (2AA), 2-acetylaminofluorene (2AAF), 1-naphtylamine (1NA) and 2-naphtylamine (2NA) (Sigma Chemical, St Louis, MO); 4-acetylaminofluorene (4AAF) (Lancaster Synthesis Ltd, Morecambe, UK); benzo[a]pyrene [B(a)P], 1-aminoanthracene (1AA) and 2-aminofluorene (2AF) (Ega-Chemie KG, Steinheim/Albuch, FRG); the pyrolysis products Trp-P-1, Trp-P-2, IQ and MeIQ (gifts from Drs T. Sugimura and K. Wakabayashi, National Cancer Center Research Institute, Tokyo, Japan). Nongenotoxic carcinogens included: p,p'-DDE (Ega-Chemie); p,p'-DDT and dieldrin (Serva Feinbiochemica, Heidelberg, FRG). The formulas of test compounds are reported in Fig. 1.

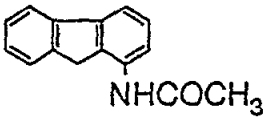
Polycyclic aromatic hydrocarbons

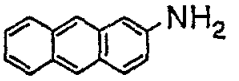
B(a)P 

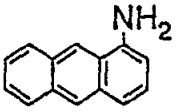
Aromatic amines

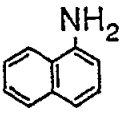
2AF 

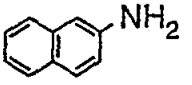
2AAF 

4AAF 

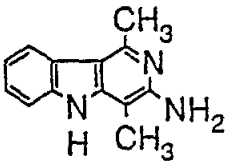
2AA 

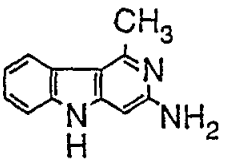
1AA 

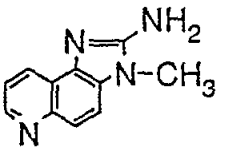
1NA 

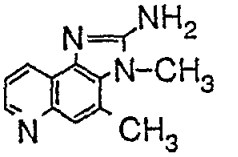
2NA 

Heterocyclic amines

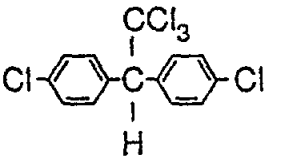
Trp-P-1 

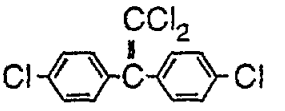
Trp-P-2 

IQ 

MeIQ 

Organochlorine pesticides

p,p'-DDT 

p,p'-DDE 

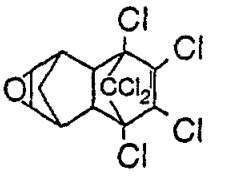
Dieldrin 

Fig.1 Chemical formulas of the 15 compounds investigated in photoactivation studies. See Table 1 for the identification of acronyms

2.1.2 Solar irradiation

Unless otherwise, specified, test compounds were dissolved in DMSO. Solutions were distributed into flame-sealed glass vials or in other containers, such as capped plastic tubes, 30mm plastic Petri dishes, or glass syringes (see "Results"), and then exposed in a thin layer to direct sunlight for varying time intervals (typically 30-60 min). The corresponding intensity of solar radiation in the 290-2800 nm spectral region, which was communicated by Dr G. Flocchini (Section of Geophysics, University of Genoa), was expressed as J/cm^2 (1 J/cm^2 corresponds to 0.239 ly or Cal/cm^2).

2.1.3 Use of light filters

In some experiments two types of light filters (both from Schott Optics Division, Mainz, FRG) were arranged on open Petri dishes containing compound solutions. Color filter glasses, models WG230, WG335, WG420 and WC830, cut various regions of infrared, visible and UV light. UV interference filters, models UV-R-250, UV-R-280 and UV-R-340, are based on a 4-time reflection of sunlight on black glasses with interference coatings, which allows transmission of specific UV light spectra.

2.1.4 Exposure to UV light and fluorescent light

Two artificial UV sources were used. The first one (A.D.P., Milano, Italy) was equipped with 4 vertical 4-Watt tubes, emitting monochromatic 254-nm radiation. The other source (Spectroline ENF-24/F, Spectronics Co., Westbury, N.Y.) was a double-tubed lamp, equipped with two independently operatable 4-Watt tubes emitting 254- and 365-nm radiation, respectively. A unit composed of two 36-Watt cool-white lamps (Thorn, Polylux, FRG) was used as a source of fluorescent light.

The radiant energy in the UV spectrum absorbed under our experimental conditions, expressed in J/cm^2, was measured by means of a radiometer (model 44XL, Photodyne Inc., Westlake Village, CA), equipped with a silicon photodiode sensor head (model 450).

2.1.5 Assessment of mutagenicity in S typhimurium

Mutagenicity of test compounds was evaluated in the *Salmonella* reversion test, according to the standard plate incorporation procedure (Maron and Ames, 1983). Aliquots of compound solutions, before and after irradiation, were assayed in triplicate plates (100 ml/plate) along with the corresponding solvents. The *S. typhimurium his*-strains TA1535, TA1537, TA97, TA98, TA100, TA102 and TA104 were used as targets of mutagenicity. The nitroreductase-deficient derivatives TA98NR, TA98/1,8-DNP_6 and TA100NR were also used in some assays with the purolysis products. In experiments aiming at comparing metabolic activation with photoactivation, the metabolic system was composed of S-9 mix containing 10% liver S-12 fractions from Aroclor 1254-pretreated Spraque-Dawley rats.

2.1.6 Assessment of DNA damage in repair-deficient *E. coli*

The direct genotoxicity of 2AF and BP DMSO-solutions, before and after exposure to sunlight or to UV light, was also assessed by evaluating their selective toxicity in repair-deficient and-proficient *E. coli* strains. Killing of WP2 (wildtype) and of CM871 (uvrA- recA- lexA-) was evaluated by measuring the diameter of the zone of inhibition of bacterial growth produced by compound solutions (10 µl poured on sterile paper discs) placed at the centre of agar plates embedding bacteria (De Flora *et al.*, 1984).

2.2 Impact of sea pollution on biotransformation of carcinogens in fish liver

2.2.1 Monitored areas

Two different areas were investigated, i.e., a clean water reference area 10 miles east of the Port of Genoa and 100 m far from the coast, and an area of the Port of Genoa located in the internal old harbour. The latter site receives a large input of many potential pollutants via wastewaters, contaminated streams, and harbour activities. Seawater appears to be polluted with oil and other contaminants even at a visual and olfactory analysis. No chemical analytical data was available for seawater, but analyses of sediment samples during the same period showed the presence of high concentrations of various metals and of organic matter (Società Servizi Ecologici, Porto di Genova, personal communication).

2.2.2 Concentration of seawater

For mutagenicity assays of seawater, 100 l samples were collected from the two areas, transferred to the laboratory and immediately subjected to concentration by means of the blue cotton procedure. This method (Hayatsu, 1990) is based on the ability of the blue pigment copper phtalocyanine trisulfonate, covalently linked to cotton or other supports, to adsorb aromatic compounds in aquatic environment and to elute them in organic solvents. Each 100 l sample was filtered through 1 g of column-packaged blue cotton (Sigma Chemical Co., St. Louis, MO), then washed with distilled water and eluted with 50 ml of methanol:ammonia (50:1). The organic eluate was dried under vacuum and finally redissolved in 2.5 ml DMSO. Clean seawater and distilled water, artificially contaminated with 2-aminofluorene (Ega-Chemie KG, Steinheim/Albuch, FRG), were used in order to check the ability of blue cotton to adsorb organic mutagens in seawater.

2.2.3 Characteristics of fish

Annular seabream (*Diplodus annularis*) was chosen for assessing the effect of seawater pollution because this species is known to be a rather stenotopic, non-migratory fish and is widespread both in polluted and clean areas. Fish were collected during the winter 1988 (December) from the polluted harbour area (13 specimens) and from the reference area (12 specimens).

The size (m ± SD) of the animals caught in the port (length: 11.6 ± 1.7cm) was similar to that of the animals caught in the unpolluted area (10.7 ± 1.4cm), but their weight was significantly ($P < 0.05$) higher (40.2 ± 17.0 g vs. 33.6 ± 12.4g). Therefore, the condition factor (CF), relating the weight (w) to the length (l) [$CF = w/l^3 \times 100$], was 2.57 and 2.74 g/cm^3, respectively. Specimens were caught alive by means of fishing nets, and immediately killed by decapitation. Liver (free of gall bladder) and portions of skeletal muscle were rapidly removed and frozen in liquid nitrogen, whereas the remaining fish body was placed in a dry ice-refrigerated container. In the laboratory, the specimens were weighed and subsequently stored at -80E C.

2.2.4 Extraction of fish muscle

Tissues were finely minced in a mortar containing liquid nitrogen. The resulting powder was homogenized (1/4, w/v) in 50 mM phosphate buffer / 200 mM NaCl, pH 7.0. Homogenates were then extracted with cyclohexane (1/4, v/v) and ethylacetate (1/1, v/v) for 18 hr at 20 EC, according to Nikunen (1985). Finally, the extracts were concentrated to a small oily residue by flask evaporation. Each concentrate, corresponding to 1.7 g of original muscular tissue, was redissolved in 2 ml DMSO and stored at -80 EC until mutagenicity testing.

2.2.5 Sub-fractionation of liver homogenates

The livers from 4-5 animals were pooled, thereby obtaining 3 pools from each one of the two experimental groups. Livers were minced and homogenized in 50 mM Tris-0.25 M sucrose, pH 7.4

(3 ml/g wet tissue), using a Potter-Elvehjem apparatus with teflon pestle. The homogenates were centrifuged twice at 12,000 x g for 20 min. The pellets were discarded and aliquots of the second supernatant were harvested at -70E C as post-mitochondrial (S-12) fractions, to be used in the mutagenicity test system. The remaining supernatant was gauze filtered and further centrifuged at 105,000 x g for 1 hr. The resulting supernatant was harvested at -70E C in small aliquots as S-105 fractions, to be used for the analysis of cytosolic biochemical parameters. The pellet was washed once and resuspended in 50mM Tris-0.1mM EDTA, pH 7.4, supplemented with 20% glycerol (0.5 ml buffer per g of original tissue), and harvested at -70E C in small aliquots, to be used for the analysis of microsomal biochemical parameters. In order to prevent GSH oxidation during processing of liver, small portions of liver pools were separately homogenized in 40 mM Tris-4 mM EDTA, supplemented with 5mM dithiothreitol, pH 6.5, and the corresponding S-12 fractions were used for GSH analysis.

2.2.6 Determination of cytochrome P-450, GSH, and microsomal or cytosolic enzyme activities

The protein content of liver S-12, S-105 and microsomal fractions was determined by the method of Hartree (1972), using bovine serum albumin as a standard. The GSH content of S-12 fractions was determined according to Saville (1958). Cytosolic enzyme activities were measured as follows: GSH S-transferase according to the method of Habig *et al.* (1974), using 1-chloro-2,4-dinitrobenzene as a substrate; GSH peroxidase according to the method of Beutler (1975); oxidized glutathione (GSSG) reductase according to the method of Veiga Salles and Ochoa (1950); glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) according to the method of Rudack *et al.* (1971); diaphorase activities, using either NADH or NADPH as electron donors and 2,4-dichlorophenolindophenol as electron acceptor, and their dicoumarol-inhibitable activity, i.e. DT diaphorase or NAD(P)H-quinone oxidoreductase, according to the method of Ernster *et al.* (1962). Cytochrome P-450 was determined in microsomal fractions by means of the method of Omura and Sato (1964), evaluating CO binding to cytochrome reduced with dithionite. Arylhydrocarbon hydroxylase (AHH) was measured in the same fractions as described by Sabadie *et al.* (1980).

2.2.7 Mutagenicity assays of seawater and fish muscle extracts

The *Salmonella*/microsome reversion assay was used for checking the mutagenicity of concentrated seawater (*S. typhimurium* strains TA98 and TA100) and of fish muscle extracts (TA97, TA98, TA100 and TA102). In particular, 100 µl amounts of concentrated seawater and muscle extracts, either undiluted or diluted 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 in DMSO, were assayed in triplicate according to the plate incorporation test procedure, both in the presence and in the absence of S-9 containing 10% liver S-12 fractions from Aroclor-treated Sprague-Dawley rats (Maron and Ames, 1983).

2.2.8 Biotransformation of mutagens by fish liver S-12 fractions

Fish S-12 fractions from liver pools were used in order to comparatively assess their ability to activate promutagens or to decrease the potency of direct-acting mutagens. Promutagens included aflatoxin B_1 [AFB_1] (Sigma Chemical Co., St. Louis, MO), benzo(a)pyrene[B(a)P] (Ega-Chemie KG, Steinheim/Albuch, FRG), benzo(a)pyrene-trans-7,8 diol [B(a)P-7,8-diol] (National Cancer Institute, Bethesda, MD), and 3-amino-1-methyl-5H-pyrido(4,3)indole[Trp-P-2] (gift from Dr. T. Sugimura, National Cancer Center, Tokyo, Japan). Direct-acting mutagens included sodium dichromate [SDC] (Merck-Schuchardt, Munich, FRG) and 2-methoxy-6-chloro-9-[3-(2-choroethyl) aminopropylamino]acridine[ICR 191] (Polysciences Inc., Warrington, PA). *Salmonella* tester strains included TA97 (ICR 191), TA98 (Trp-P-2), and TA100 [AFB_1, B(a)P, B(a)P-7,8-diol, and SDC].

Each of the liver S-12 fraction pools (or their buffer as a control) was incorporated into S-9 mix, in amounts corresponding to 1 mg protein/plate, mixed with test compounds and bacteria and processed according to the plate incorporation test procedure (Maron and Ames, 1983). In the case

a rotary shaker, prior to mixing with bacteria and top agar. Liver S-12 fractions from Aroclor-treated rats were assayed in order to check the efficiency of the test system used. Each mixture was assayed in triplicate plates. The results are expressed by calculating the relative metabolic efficiency (RME) index, which in the case of promutagens was obtained by dividing the mean number of revertants observed in the presence of each S-12 fraction by the mean number of revertants observed in the absence of S-12 fractions. The reverse calculation was made for direct-acting mutagens. In such a way, the RME index is scored by a scale of values, which is suitable for statistical analysis. The higher the number, the higher is the metabolic efficiency for both direct and indirect mutagens, whereas 1 indicates no activation or deactivation.

Table 1

Effect of exposure to sunlight of 15 compounds (see their formulas in Fig. 1), dissolved in DMSO, on their mutagenicity in strain TA98 of *S. typhimurium*

Chemical class Compound (abbreviation)	Amount per plate (μg)	Number of revertants[a]	
		Dark	Sunlight[b]
Polycyclic aromatic hydrocarbons (PAHs)			
Benzo[a]pyrene (B[a]P)	100	32 ± 9	Toxic
	10	25 ± 8	68 ± 12
	1	29 ± 3	37 ± 8
Aromatic amines			
2-Aminofluorene (2AF)	1000	114 ± 23	Toxic
	100	63 ± 11	730 ± 68
	10	34 ± 5	164 ± 25
2-Acetylaminofluorene (2AAF)	1000	24 ± 7	236 ± 31
	100	25 ± 3	74 ± 15
	10	31 ± 8	39 ± 11
4-Acetylaminofluorene (4AAF)	1000	26 ± 5	24 ± 6
	100	21 ± 9	29 ± 6
	10	28 ± 4	25 ± 5
1-Aminoanthracene (1AA)	1000	Toxic	Toxic
	100	34 ± 7	28 ± 6
	10	27 ± 5	31 ± 9
2-Aminoanthracene (2AA)	1000	86 ± 18	69 ± 20
	100	64 ± 11	54 ± 8
	10	35 ± 7	43 ± 12
1-Naphtylamine (1NA)	1000	41 ± 8	45 ± 11
	100	29 ± 5	26 ± 6
	10	27 ± 3	34 ± 9
2-Napthtylamine (2NA)	1000	39 ± 6	35 ± 8
	100	31 ± 9	36 ± 11
	10	26 ± 5	23 ± 7
Heterocyclic amines			
3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1)	10	23 ± 5	26 ± 4
	1	22 ± 7	28 ± 6
	0.1	26 ± 4	24 ± 7

(cont.)

Table 1 (cont.)

Chemical class Compound (abbreviation)	Amount per plate (μg)	Number of revertants[a]	
		Dark	Sunlight[b]
3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)	10	22 ± 8	35 ± 9
	1	28 ± 5	29 ± 4
	0.1	25 ± 4	21 ± 5
2-Amino-3-methylimidazo-[4,5-f] quinoline (IQ)	10	98 ± 18	TNTC[c]
	1	54 ± 11	1057 ± 72
	0.1	37 ± 5	209 ± 18
2-Amino-3,4-dimethylimidazo-[4,5-f] quinoline (MeIQ)	10	123 ± 24	TNTC[c]
	1	60 ± 16	1528 ± 63
	0.1	29 ± 8	246 ± 45
Organichlorine pesticides			
4,4'-Dichloroduphenyl-trichloroethane (p,p'-DDE)	1000	19 ± 8	27 ± 9
	100	28 ± 5	33 ± 11
	10	26 ± 6	29 ± 8
1,1-Dichloro-2,2-bis(p-chloro-pheny)ethylene (p,p'-DDE)	1000	25 ± 6	26 ± 5
	100	31 ± 8	29 ± 8
	10	29 ± 5	25 ± 6
1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4-endo-5,8-dimethanonaphtalene (Dieldrin)	1000	26 ± 9	23 ± 6
	100	24 ± 3	28 ± 5
	10	30 ± 8	31 ± 12

[a] Mean ± SD of triplicate plates. Underlines values indicate at least a doubling of revertants, as compared with unirradiated samples

[b] Intensity corresponding to 109 to 163 J/cm^2

[c] Too numerous to count

3. RESULTS

3.1 Effect of sunlight on mutagens and carcinogens

3.1.1 Photodynamic effects on various mutagens

Exposure to sunlight of the solvents used, i.e., dimethylsulfoxide (DMSO), acetone, methanol and ethanol, never had any influence on the number of spontaneous revertants.

Table 1 reports the results of a comparative assay with 15 compounds, each assayed at 3 doses either in the dark or following exposure to sunlight. As confirmed in several separate experiments, irradiation of B(a)P produced toxic effects at high doses and mild mutagenic effects at sublethal doses in both strains TA98 (Table 1) and TA100 (not shown).

Structure-activity relationships were detected in the case of aromatic and heterocyclic amines (see Fig. 1 and Table 1). In particular, of the aromatic amines tested, 2AF exhibited a direct, dose-dependent mutagenicity following activation by sunlight. The presence of the acetylamino group instead of the amino group resulted in a decrease of photoactivation when the substitution was in the same position (2AAF), and in a complete loss of photoactivation when the substitution was in position 4 (4AAF). On the other hand, neither 1AA nor 2AA (differing from 2AF

for the fluorene structure) were photoactivated. The 2-ring compounds 1NA and 2NA were also unaffected by sunlight activation. Of the two pairs of heterocyclic amines, IQ and MeIQ, carrying the amino group in the position 2 of the molecule, were found to be very sensitive to photoactivation, which contrasted with the complete insensitivity of the two tryptophan pyrolysis products Trp-P-1 and Trp-P-2.

Assay of 2AF and B(a)P in the bacterial DNA-repair test provided evidence that solar irradiation also produced direct-acting derivatives yielding an enhanced lethality in the repair-deficient strain CM871 (data not shown).

3.1.2 Influence of the solvent on photoactivation

Most assays were carried out by using DMSO as a solvent of test compounds to be irradiated. Photoactivating effects of the same order of magnitude, or even more pronounced, were observed by dissolving compounds in acetone, which however was less frequently used than DMSO due to its higher volatility.

In some assays, DMSO-solutions were further diluted 1:10 with other solvents miscible with DMSO and then irradiated. Photoactivation to mutagenic derivatives was rather poor using methanol and ethanol, and negligible using distilled water. Also, no effect could be detected when compounds were exposed to sunlight under crystalline form, before solubilization in DMSO (data not shown).

3.1.3 Time dependence of photoactivation

The formation of direct-acting mutagens was related to the time of exposure and, consequently, to the intensity of solar light. Appearance of mutagenicity was very rapid, the optimal intensity of solar light being in the 50-200 J/cm^2 range, corresponding to less than 1 hr exposure under our experimental conditions. Overexposure resulted in a progressive decrease of mutagenicity of the irradiated compounds solutions.

3.1.4 Stability of photoproducts

When stored in the dark, after exposure to an optimal intensity of solar light, the direct-acting photoactivated mutagens were remarkably stable. For instance, MeIQ was dissolved in DMSO (10 mg/ml), exposed to sunlight (134 J/cm^2), and then distributed into small aliquots which were kept in the dark at -20E C, +4E C or room temperature (20 to 25E C). Every week or, after the first month, every 2 months, a sample for each temperature was assayed for direct mutagenicity in strain TA98. The results obtained during 2 years and 3 months of storage showed that the levels of mutagenicity were fairly constant (in the range between 1300 and 1800 revertants/100 µl) and, within each assay, no significant variation of activity was ever observed among the samples kept at the 3 different temperatures (data not shown).

3.1.5 Oxygen requirement for photoactivation

In order to check the role of oxygen in photoactivation, irradiation assays were carried out by filling 10-ml glass syringes, containing 1 ml DMSO or DMSO-solutions of test compounds, with either air or pure O_2 or pure N_2. The syringes were sealed with gas-taps and then exposed to sunlight. A set of corresponding syringes was kept in the dark.

The results of one such experiment are shown in Fig. 2. It is evident that conversion into mutagenic derivatives was negligible when exposure to sunlight was carried out in a N_2 atmosphere, whereas photoactivation of promutagens was further magnified when air was replaced with pure O_2. None of these atmospheres had any effect on DMSO (either in the dark or irradiated) or on the compound solutions kept in the dark (not shown).

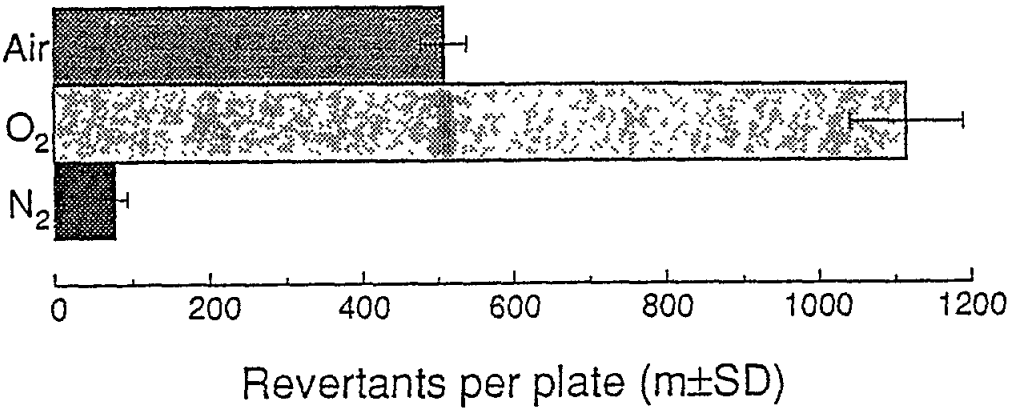


Fig 2. Direct mutagenicity of 2AAF (40 μg/plate) in strain TA98 of S. typhimurium following exposure of sunlight (136 J/cm^2) either in air, pure oxygen,or nitrogen atmosphere

3.1.6 Light wavelengths responsible for photoactivation

Special colour or interference filters were used in order to detect the range of wavelengths of solar irradiation responsible for activation of IQ and MeIQ. Interposition of a filter transmitting only infrared radiation (WG830) prevented photoactivation of both IQ and MeIQ. Note also that heating at 56° C of the same solutions, kept in the dark for a time corresponding to irradiation periods, had no effect on the lack of direct mutagenicity of these compounds.

A very weak mutagenic response was observed by interposing filter WG420, cutting most UV radiation wavelengths. Conversely, IQ and MeIQ were efficiently photoactivated by allowing transmission, together with infrared and visible light, of UV wavelengths. Mutagenicity was only slightly enhanced by using filter WG335, as compared to WG230, which indicates that the bulk of photoactivation can be ascribed to the near-UV wavelengths range.

A similar indication was provided by the comparative use of interference filters. In fact, the most pronounced photoactivation occurred by interposing the UV filter having the maximum internal transmittance at 340 nm.

3.1.7 Effect of artificial light sources

The organochlorine pesticides p,p'-DDE and dieldrin, but not p,p'-DDT, acquired a weak mutagenicity following exposure to an artificial 254-nm UV source. Such mutagenic activity was observed in both strains TA98 and TA100, and tended to be decreased in the presence of S-9 mix.

Several experiments were carried out by exposing 2AF and B(a)P DMSO-solutions to 254- or 365 nm UV sources for varying time intervals. The results of these assays can be summarized as follows:(a) as already reported for sunlight, the production of direct-acting mutagenic derivatives was higher with 2AF than with B(a)P; (b)2AF was activated considerably better by 365- than by 254-nm UV; (c) in contrast, B(a)P was better activated by 254-nm light radiation; (d) the appearance of a direct mutagenicity following exposure of 2AF and B(a)P to UV light was accompanied by a slight decrease in their S-9 mediated mutagenicity.

At variance with the untreated compounds, which were devoid of toxicity towards both repair-deficient and -proficient *E. coli*, 2AF and B(a)P exposed to 254 nm UV light selectively killed the triple mutant CM871 (data not shown).

3.1.8 Effect of fluorescent light

Exposure of MeIQ solutions to fluorescent light also resulted in the appearance of a direct mutagenicity, which was related to exposure times (data not shown).

3.1.9 *Comparison of genetic mechanisms of aminocompounds metabolites and photoproducts*

The sensitivity of 10 *S. typhimurium his*-strains, which are selectively reverted by mutagens working through different mechanisms, was investigated in order to check whether photoactivated IQ and MeIQ display the same spectrum of genetic activity like metabolically activated compounds. Both IQ and MeIQ, either metabolically activated or photoactivated (which clearly required higher concentrations of the two compounds) reverted strains Ta1537, TA97, TA98, TA100, TA104 and, with very limited effects, TA102. Of the standard strains, only TA1535 was insensitive. The most useful indications were provided by the assay of nitroreductase-deficient strains. In fact, TA100NR was less sensitive than TA100, but only following photoactivation of IQ and MeIQ. TA98NR behaved like TA98, whereas a considerable loss of mutagenicity, following both photoactivation and metabolic activation, occurred with TA98/1,8-DNP_6 (Fig. 3).

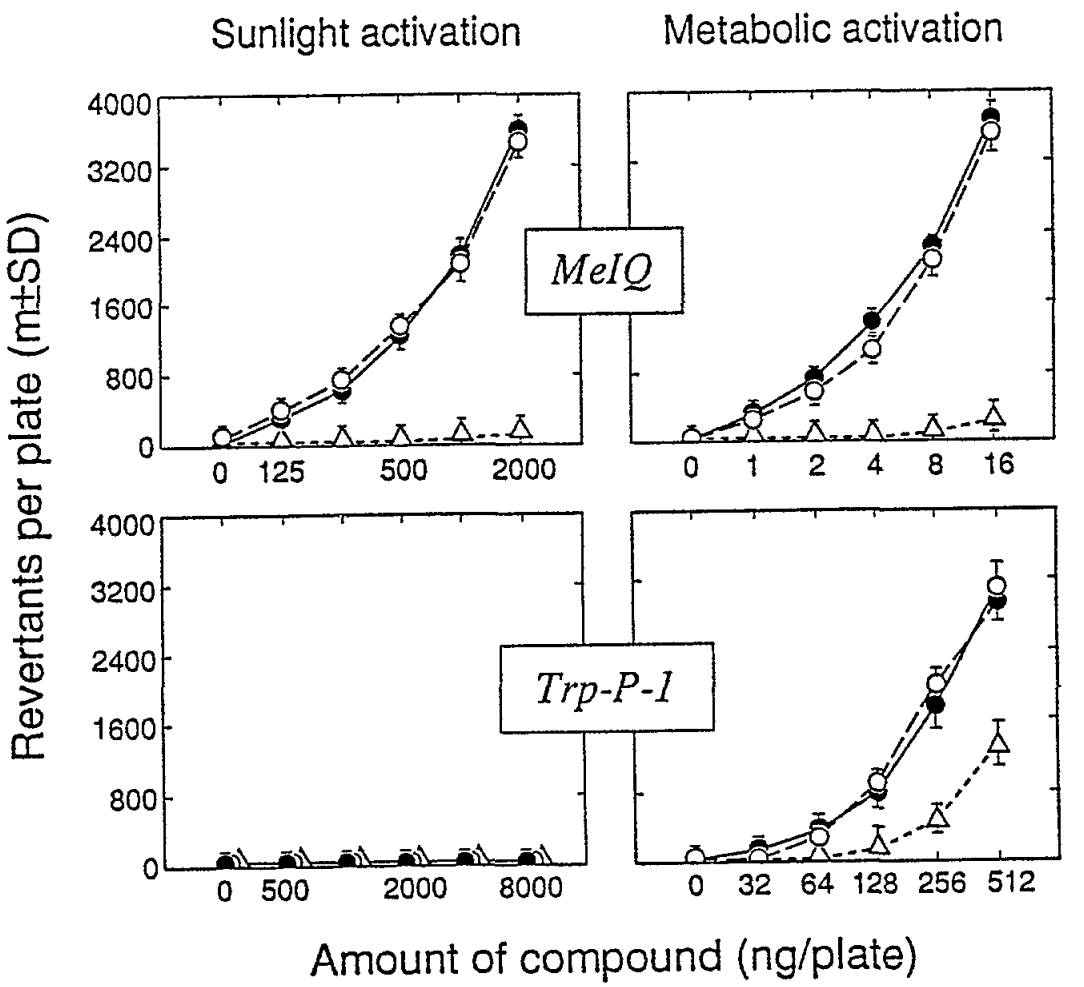


Fig.3 Mutagenicity of varying amounts of MeIQ and Trp-P-1, activated either by exposure to sunlight (159 J/cm^2) or by S-9 mix, in S. typhimurium strains TA98 (full circles), TA98NR (empty circles), and TA98/1,8-DNP_6 (triangles)

Also in the case of 2AF, the levels of mutagenicity were not significantly different in TA98 and TA98NR, whereas TA98/1,8-DNP_6 responded like TA98 and TA98NR to other mutagens, such as metabolically activated B(a)P. The liver metabolites of Trp-P-2 and especially of Trp-P-1 which, as reported in section 3.1.1., are not photoactivatable, also underwent a decrease of mutagenicity in TA98/1,8-DNP_6, but not to such an extent as metabolically activated IQ and MeIQ (Fig. 3).

3.2 Impact of sea pollution on the biotransformation of carcinogens in fish liver

3.2.1 Analysis of seawater mutagenicity

Preliminary assays provided evidence that the blue cotton procedure is suitable for concentrating organic mutagens from seawater. In particular, the mutagenicity of the aromatic amine 2AF in strain TA98 of *S. typhimurium*, in the presence of rat liver S-12 fractions, was very similar when the mutagen (0.5 µg/ml) was subjected to a theoretical ten-fold concentration from distilled water (286.6±14.5 revertants/plate) or seawater (298.3±11.1).

No mutagenic activity could be detected when seawater concentrates from the two monitored areas were tested in strains TA98 and TA100, either in the presence or in the absence of rat liver S-12 fractions. In these assays, seawater underwent a theoretical x 40,000 concentration, and the DMSO-concentrates were assayed in amounts ranging between 62.5 ml and 4 l of the original seawater sample (data not shown).

3.2.2 Analysis of the mutagenicity of fish tissues

Irrespective of their source, none of the muscle extracts prepared from the 25 analyzed fish specimens enhanced the spontaneous mutagenicity to a significant extent, either in the absence or in the presence of rat liver S-12 fractions, in strains TA97 (spontaneous revertants: 176.4 ± 23.7 without S-12; 184.9 ± 15.3 with S-12), TA98 (26.7 ± 8.5; 34.3 ± 10.6), TA100 (119.5 ± 24.3; 127.3 ± 12.7), or TA102 (192.4 ± 15.4; 211.7 ± 23.5).

Similarly, all liver S-12 fraction pools used for assessing the metabolism of mutagens (see section 3.2.5) did not significantly affect the spontaneous mutagenicity of the Salmonella tester strains (data not shown).

3.2.3 Fish liver weight

A highly significant correlation existed between fish body weight and liver weight both in the reference area ($r = 0.96$) and in the harbour area ($r = 0.93$). The Somatic Liver Index (SLI = liver weight/body weight x 100) was significantly higher in fish from the polluted area (1.5 ± 0.3) than in fish from the clean area (1.07 ± 0.2). No statistically significant correlation was found between body weight and SLI, which indicates that variations in SLI are independent from the fish size.

3.2.4 Influence of seawater pollution on microsomal and cytosolic biochemical activities

The fish source had significant effects on liver microsomal functions. In particular, exposure to polluted seawater produced a significant ($P<0.05$) shift on the left of mean cythochrome P-450 peaks, i.e., from 450.3 + 0.3 nm to 448.5 ± 0.9 nm. Total P-450 levels were almost doubled, but such an increase was not statistically significant, whereas a significant, 5-fold induction was recorded for AHH activity.

Of the monitored cytosolic enzyme activities, G6PD and 6PGD were significantly stimulated (more than doubled) by seawater pollution. NADPH- and NADH-dependent diaphorase, DT diaphorase and GSSG reductase were only slightly and nonsignificantly enhanced. GSH peroxidase and GSH S-transferase were conversely significantly decreased. GSH concentrations in S-12 fractions were very similar in the two series of specimens (Fig. 4).

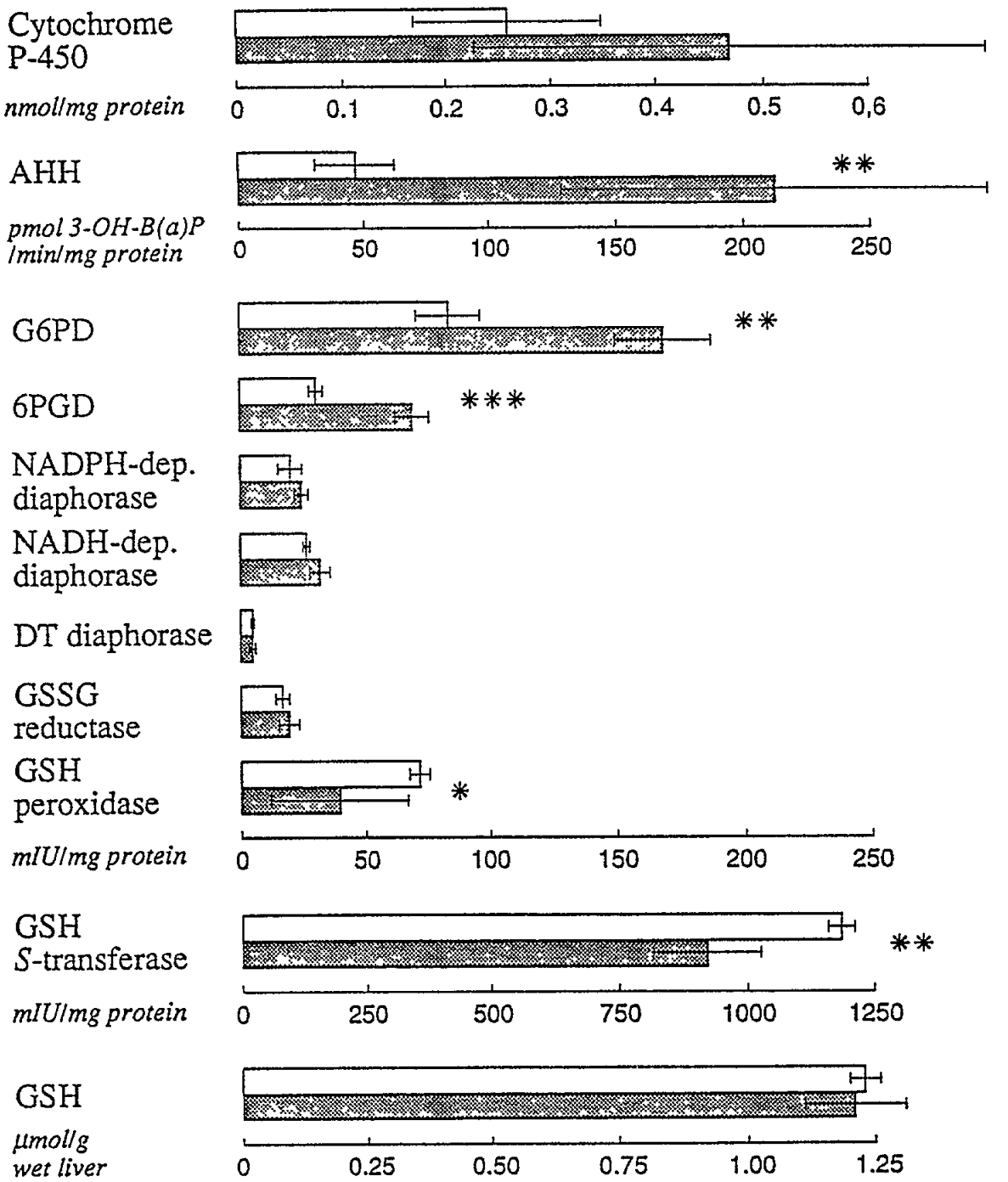


Fig.4 Comparison of biochemical parameters in liver microsomal, cytosolic or post-mitochondrial fractions of the fish *Diplodus annularis* living either in clean seawater (empty columns) or in a polluted port environment (dotted columns). Asterisks indicate significant differences at $P < 0.05$(*), $P < 0.01$(**), or $P < 0.001$(***).

3.2.5 Influence of seawater pollution on the biotransformation of mutagens by liver S-12 fractions

The results of these experiments are summarized in Fig. 5. Irrespective of the source of specimens, the liver S-12 fractions of *Diplodus annularis* failed to activate AFB_1 and B(a)P to mutagenic metabolites detectable in the test system used. Samples of fish caught from polluted water were significantly and considerably more efficient than those from clean water in activating B(a)P-7,8-diol and the heterocyclic amine Trp-P-2, as shown at 3 dose levels of this tryptophan pyrolysis product. On the other hand, fish from polluted seawater was significantly less efficient in detoxifying the direct-acting mutagen ICR 191. Liver samples from both sources efficiently decreased the mutagenicity of the hexavalent chromium salt SDC (Fig. 5).

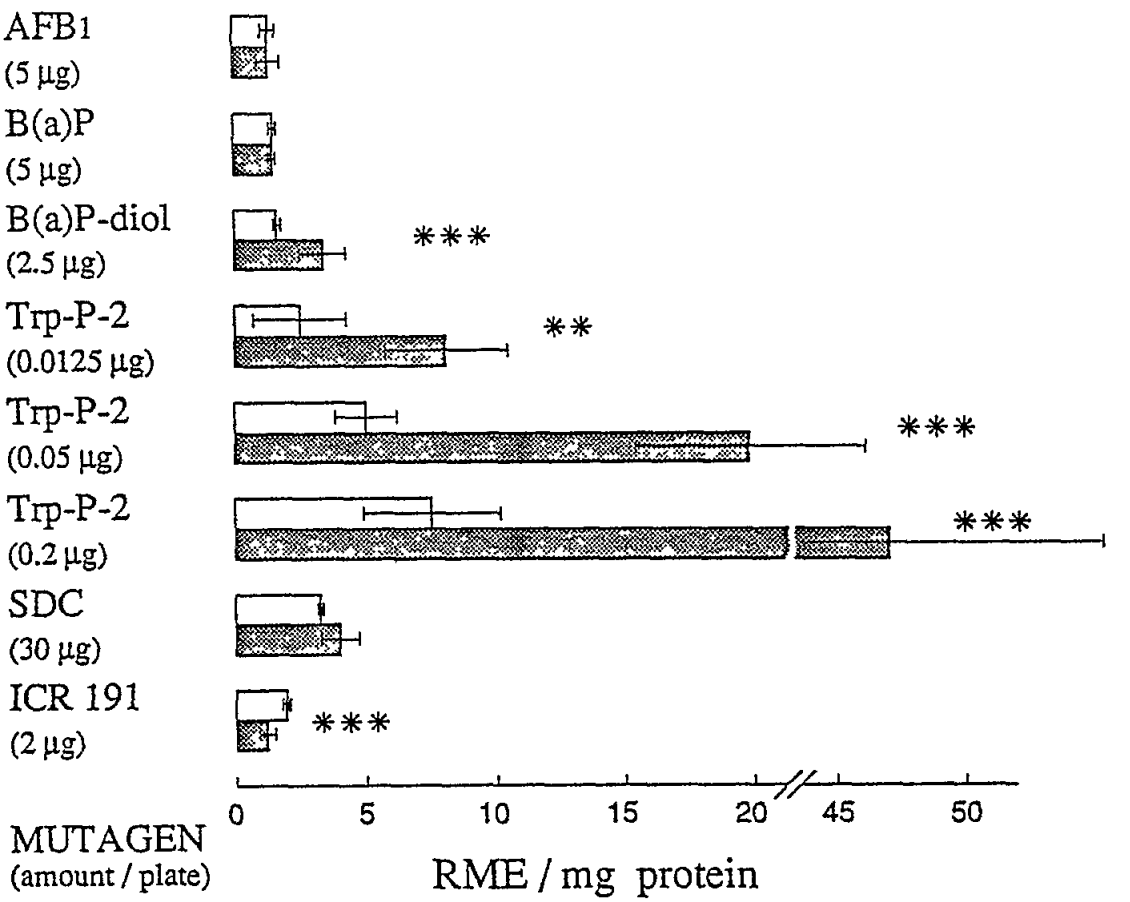


Fig. 5. Comparison of the post-mitochondrial fractions of the fish *Diplodus annularis*, living either in clean seawater (empty columns) or in a polluted port environment (dotted columns), in activating promutagens, i.e., AFB_1, B(a)P-7,8-diol and Trp-P-2(at 3 dose levels), or in decreasing the potency of direct acting mutagens, i.e., SDC and ICR 191. See section 2.2.8 for calculation of the RME (relative metabolic efficiency) index. Asterisks indicate significant differences at $P<0.01$(**), or $P<0.001$(***)

4. DISCUSSION

4.1 Photoactivation of mutagens

The most striking photodynamic effect discovered in this study was the light- and oxygen-mediated activation of promutagens, acquiring a direct mutagenicity just following exposure to sunlight. The moderate photoactivation of B(a)P is consistent with previous findings concerning the light-mediated conversion of this and other PAHs into direct-acting mutagens (Gibso *et al.*, 1978; Tu *et al.*, 1979; McCoy *et al.*, 1979; McCoy and Rosenkranz, 1980; Claxton and Barnes, 1981; Strniste and Brake, 1981; De Flora, 1982; De Flora and Badolati, 1982; Takeda *et al.*, 1984; White *et al.*, 1985), DNA-binding derivatives (Prodi *et al.*, 1984) and skin carcinogens (Santamaria *et al.*, 1966), presumably through formation of endoperoxides (Pitts, 1971; Evans, 1982).

Structure-related effects were observed in the case of aminocompounds. The aminoimidazoquinoline compounds IQ and MeIQ were by far the most sensitive molecules to photoactivation, which contrasted with the insensitivity to light of the tryptophan pyrolysis products Trp-P-1 and Trp-P-2. Of the aromatic amines, the fluorene derivatives 2AF and 2AAF which, like IQ and MeIQ, carry the amino-group in the position 2 of the molecule, were readily photoactivated to direct-acting mutagens. This confirms similar data reported from this and other laboratories (McCoy *et al.*, 1979; De Flora, 1982; De Flora and Badolati, 1982; Nishi and Nishioka, 1982; Strniste *et al.*, 1985; White and Heflich, 1985; Okinaka *et al.*, 1986). On the other hand, no photoactivation could be observed when the acetylamino-substitution was in the position 4 of fluorene (4AAF), or when the amino-group, irrespective of the position, was inserted in the anthracene (1AA and 2AA) or naphtalene (1NA and 2NA) molecules.

The irradiation experiments performed in different atmospheres and those using optical and UV interference filters clearly indicated that the mechanism of photoactivation involves an interaction between near-UV wavelengths and molecular oxygen, resulting in the generation of reactive oxygen species. Presumably, absorption of these radiations elevates dioxygen to singlet oxygen, which can oxidize certain molecules, as it has been postulated in the case of some PAHs (McCoy and Rosenkranz, 1980). It is however noteworthy that photoactivation effects were also produced by exposure to 254-nm UV, for instance in the case of the pesticides p,p'-DDE and dieldrin and of B(a)P which, at variance with 2AF, was better activated by 254-nm than by 365-nm UV light. Far-UV light can photodissociate O_2 into its constituent oxygen atoms, oxidizing in turn other O_2 molecules to produce ozone (Pitts *et al.*, 1980), which can convert promutagens into direct-acting mutagens, as shown, e.g., with B(a)P (Fridovich, 1974) and diesel exhaust particle extracts (Claxton and Barnes, 1981). Ozone is a very reactive substance which attacks, with special vigor, compounds bearing carbon-carbon double bonds. This may explain the selective effect of far-UV radiation of dieldrin and p,p'-DDE, as compared to p,p'-DDT, which lacks the double bond. It is of interest that, as previously shown with chrysene (McCoy and Rosenkranz, 1980), certain radiation spectra can even photoactivate chemicals which fail to be activated by metabolic systems, such as organochlorine pesticides, which are typically classified as nongenotoxic carcinogens (IARC, 1974). Note that, one the other hand, sunlight and UV light are capable of decreasing the potency of certain mutagenic pesticides, such as captan (De Flora and Badolati, 1982), or of other direct-acting genotoxic substances, such as oil dispersants (De Flora *et al.*, 1985).

The metabolic activation of both aromatic and heterocyclic amines is triggered by an N-hydroxylation process, which depends on the same cytochrome P-450I isozymes, i.e. $P\text{-}450_{\beta NF\text{-}B}$ and $P450_{ISF\text{-}G}$ (Guengerich, 1988). Analytical and mutagenicity data are consistent with the conclusion that oxidation of the exocyclic nitrogen group also provides the primary mechanism involved in the photoactivation of the same compounds. In fact, recent studies provided evidence three of which, i.e. 2-nitrosofluorene, 2-nitrofluorene and 2-nitrofluoren-9-one, exhibited a directmutagenic activity (Strniste *et al.*, 1988). Formation of N-oxidized photoproducts of 2AF following exposure to sunlight and cool-white light was also supported by the decrease of mutagenicity observed in the nitroreductase-deficient derivatives of TA98, i.e. TA98NR and especially TA98/1,8-DNP_6 (White and

Heflich, 1985). This was confirmed by our results, showing similar levels of mutagenicity in TA98 and TA98NR but a sharp decrease of activity in TA98/1,8-DNP_6 following not only sunlight activation but also metabolic activation.

In order to clarify the chemical nature of the mutagenic photoproducts of aminoimidazoquinolines, we collaborated with the National Cancer Center Research Institute and the Ochanomizu University in Tokyo. The results of these additional studies, which have been just published (Hirose *et al.*, 1990), demonstrated that MeIQ is converted by solar irradiation into the corresponding nitroderivative, i.e., NO_2-MeIQ. Preliminary data generated by the same study show that IQ is also converted into NO_2-IQ. Both nitroderivatives are direct-acting mutagens in *S. typhimurium*.

Photoactivation of promutagens is expected to lead to an environmental spread of direct-acting genotoxic chemicals, which can by-pass crucial metabolic pathways in living cells and exert noxious effects in exposed tissues. In this connection, it is noteworthy that photoactivation is a dynamic process. In fact, time course assays clearly showed that exposure to light results in the formation of active derivatives, which are then degraded by extending the irradiation times. Nevertheless, once formed and shielded from light, photoproducts are considerably stable, as previously reported with 2AF and PAHs (McCoy *et al.*, 1979; De Flora, 1982; White and Heflich, 1985) and as confirmed with photoactivated aminoimidazoquinoline compounds, which even at room temperature kept their mutagenic potency unvaried for years.

Further studies are needed in order to clarify the applicability of laboratory findings to environmental conditions. In particular, all investigations on photoactivation so far reported in the literature used DMSO-solutions of promutagens. In the present study, photoactivation was even more pronounced by exposing acetone-solutions, but it was considerably attenuated by using other solvents, such as ethanol and methanol, and became undetectable either by using DMSO-solutions diluted in water or by directly irradiating compound crystals before solubilization. It would be of interest to know whether photoactivation may occur when promutagens resulting from pollution sources are distributed among environmental matrices, possibly adsorbed onto solid substrates (Korfmacher *et al.*, 1980) and generally in the form of complex mixtures, where photosensitizers may favour the reaction of oxygen with suitable acceptors (Pitts, 1971). Formation of direct-acting mutagens from PAHs in the environment may also result from exposure to ionizing radiation (Gibso *et al.*, 1978) and from chemical reactions, e.g., with ozone (Claxton and Barnes, 1981; Pitts *et al.*, 1980) or with nitrogen oxide (Pitts *et al.*, 1978; Hisamatsu *et al.*, 1986).

4.2 Impact of sea pollution on the biotransformation of mutagens and carcinogens in fish liver

The biotransformation of mutagens and carcinogens in mammals is well known to be subjected to considerable interspecies, interstrain and interindividual variations. Likewise, large interspecies differences occur in microsomal MFO activities and other biochemical parameters monitored in fish liver (Funari *et al.*, 1987; James *et al.*, 1988). In analogy to the situation occurring in other animal species, these differences can be ascribed both to genetic features and to exogenous factors.

An evident induction of AHH activity in fish caught from the port area was accompanied by a less pronounced increase in the concentration of total cytochromes P-450. These patterns are in agreement with the results of in several other field trials investigating marine environments exposed to hydrocarbon pollution or to mixed organic pollution (Payne *et al.*, 1987). Of particular interest was the significant shift on the left of the Soret peak of cytochromes, which typically occurs after induction of rodents with polycyclic aromatic hydrocarbons, and has been also observed after experimental administration of 3-methylcholanthrene to estuarine fish species (James *et al.*, 1988). It is noteworthy that, as shown in coastal waters receiving the discharge of a petrol refinery in the Adriatic

Sea, induction of MFO in fish liver occurs throughout the year and therefore is not a seasonal phenomenon (Rijavec *et al.*, 1981).

The polluted marine environment also stimulated the two dehydrogenase activities involved in the hexose monophosphate shunt, i.e. G6PD and 6PGD, which are of special relevance in supplying buildings blocks for nucleotide synthesis and in the generating NADPH for drug metabolism or lipid synthesis. Although GSH concentrations were not affected in the liver of exposed fish, a moderate yet significant decrease was observed for two protective enzymes involved in the GSH cycle, i.e., GSH peroxidase and GSH-S-transferase. The latter enzyme was not stimulated in the liver of various fish species, 5 days after an i.p. MC injection (James *et al.*, 1988), and was only marginally increased in the liver of rainbow trout, 2 weeks after injection of the MC-like inducer ß-napthoflavone (Andersson *et al.*, 1985). The parallel induction of activating enzymes and depression of detoxifying enzymes, triggered by seawater pollution in *Diplodus annularis*, provide evidence for the independent regulation of microsomal MFO and of cytosolic enzymes involved in the GSH cycle.

The results of biochemical analyses are in agreement with the parallel findings of mutagenicity assays, showing that the liver of exposed fish is more efficient in activating promutagens to mutagenic metabolites and at the same time is less capable of decreasing the potency of direct-acting mutagens. Therefore, although MFO enzyme induction may be also regarded as a primary detoxification response (Payne *et al*., 1987), the overall consequence appreciable in the mutagenicity test system is an enhanced production or lack of detoxification of genotoxic molecules. In particular, such a conclusion could be inferred from the experiments carried out with the promutagens B(a)P-7,8-diol, a proximal metabolite of B(a)P, and Trp-P-2, a carcinogenic food pyrolysis product (Sugimura, 1982), whose metabolism is stimulated not only by chemical inducers but also by liver infections, such as hepatitis B virus infection (De Flora *et al.*, 1989c). In contrast, the acridine compound ICR 191, whose metabolic deactivation by various rodent tissue preparations is chemically inducible (De Flora *et al.*, 1982a), was better detoxified by liver preparations from fish living in clean seawater. This indicates a slightly depressed detoxification capacity following exposure to polluted seawater, which parallels the decrease of enzymes involved in the GSH cycle. As previously observed with rainbow trout (*Salmo gairdneri*) liver (De Flora *et al.*, 1982b), liver preparations of seawater fish from both sources efficiently decreased the mutagenicity of sodium dichromate, which is due to composite intracellular mechanisms (De Flora and Wetterhahn, 1989). Together with other processes, such as reduction by fish skin and gill mucus, depending on protein-bound sulfhydryl groups (Arillo and Melodia, 1990), these mechanisms are expected to protect the fish in water contaminated with hexavalent chromium.

At variance with the results obtained in *Salmo gairdneri* (De Flora *et al.*, 1982b), liver S-12 fractions from *Diplodus annularis* failed to bioactivate B(a)P and AFB_1 (Hsieh *et al.*, 1977) are known to be carcinogenic in various fish species, the results obtained suggest that *Diplodus annularis*, both uninduced and induced, is not particularly efficient in activating these procarcinogens. More probably, due to the observed induction of B(a)P-hydroxylase in microsomes and of B(a)P-7,8-diol activation to the ultimate mutagenic metabolite, the findings of the mutagenicity test system can be interpreted in the sense that seawater pollutants in the monitored area did not alter the balance between activating and detoxifying (e.g., epoxide hydrolases) enzyme activities specifically involved in B(a)P and AFB_1 metabolism.

In the evaluation of both biochemical and mutagenicity parameters it should be also taken into account the considerable increase in the somatic index values displayed by exposed fish. Liver enlargement is generally considered as a result of exposure to chemical pollutants (Sherwood and Mearns, 1977; Poels *et al.*, 1980; Slooff *et al.*, 1983). It is evident that this circumstance renders even more pronounced the differences recorded in terms of specific activity for parameters evaluating metabolic activation, whereas it attenuates and probably eliminates the differences related to detoxification. A similar trend to a concomitant enlargement of fish liver and increase in specific enzyme activities has been also observed in other field trials. Enzyme induction can be considered

a "fast" adaptive response, while liver hyperplasia is a "slow" adaptive response, depending on the extent of seawater contamination (Payne *et al.*, 1987).

The modulatory effects produced by seawater pollution on fish liver metabolism are in apparent contrast with the lack of mutagenicity of seawater not only in the reference area but also in the polluted port environment. However, this is not surprising, because hardly predictable interactions occur in complex mixtures. For instance, B(a)P mutagenicity was eliminated in the presence of crude oil extracts, irrespective of the addition of oil dispersants (Petrilli *et al.*, 1980). It is also noterworthy that no mutagenicity could be detected in samples of seawater artificially contaminated with a combination of pollutants or with crude oil (Rijavec *et al.*, 1981), or in concentrated effluents and sediment samples at the site of discharge of a petrochemical industry (Nikunen, 1985). It should be mentioned, however, that the presence of direct-acting frameshift mutagens was detected in a seawater sample from another Italian port (Migliore *et al.*, 1989).

It is a controversial issue whether pollution of seawater and sediments from anthropogenic sources may have toxicological consequences on marine organisms (Payne and Rahimtula, 1989). Even more questioned is the hypothesis of toxicological consequences on human health resulting from the marine environment. The role of natural pollutants has been also pointed out, and molecular epidemiology techniques applied to freshwater and seawater fish revealed that formation of adducts occurs in liver DNA irrespective of the extent of water pollution (Kurelec *et al.*, 1989). It is rather unlikely that mutagens may be present in fish muscle in amounts detectable in a biological test system, although in a previous study we could demonstrate an evident mutagenic response in muscle extracts of rainbow trout housed in freshwater supplemented with high amounts of sodium nitrite (De Flora and Arillo, 1983). In the present study muscle extracts from *Diplodus annularis* caught both from the polluted harbour area and the reference area did not contain detectable mutagens, whose presence might have raised questions on the safety of the fish used for human consumption.

4.3 Concluding remarks

The results of the studies described in the present report provide information on some toxicological aspects which may bear relevance in the marine environment.

Photoactivation of promutagens, which are *per se* biologically inert molecules, is of particular importance. In fact, it may lead to an environmental dissemination of direct-acting mutagens, capable of attacking target organisms, tissues and cells without any need to undergo further metabolic transformation. In such a way, photodynamically activated compounds are not only expected to pose more hazards in higher organisms equipped with a well developed metabolic machinery, but can also exert direct effects in organisms having no detectable MFO activity. For instance, it has been shown that B(a)P can bind the DNA of sponges only in the presence of light (Zahn *et al.*, 1981). The hypothesis has been raised that the light irradiating the sea surface may convert B(a)P to sufficiently stable derivatives, which are then transported and react with the macromolecules of the sponge in the dark (Zahn *et al.*, 1982).

Certainly, a dynamic balance between photoactivation and photodecomposition processes is likely to occur in nature. We have not available sufficient element to extrapolate our laboratory data to field conditions and in particular to the marine environment. Nevertheless, the frequent detection of direct frameshift mutagens in environmental samples (e.g., water or air samples), which is supported by the available literature, is consistent with the view that exposure to light represents an important mechanism contributing to the activation of mutagenic and potentially carcinogenic compounds.

As to the metabolism of xenobiotics in fish liver, the comparison of biochemical and mutagenicity end-points in annular seabream living either in a clean reference area or in a polluted portual area confirmed that the microsomal MFO analysis in the liver represents a sensitive index of fish exposure to polluted seawater. In addition, they provided evidence for the sensitivity and

reliability of other inducible parameters reflecting the biotransformation patterns of xenobiotics, such as cytosolic enzyme activities or the overall capacity to activate or detoxify standard mutagens. Therefore, irrespective of unassessed toxicological consequences on marine organisms or on human health, the observed metabolic changes appear to provide an appropriate indicator of mixed organic pollution of seawater, even in the absence of a detectable mutagenicity in unfractionated seawater concentrates.

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6. REFERENCES

ANDERSSON, T., PESONEN M. and JOHANSSON C. (1985): Differential induction of cytochrome P-450-dependent monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyl transferase activities in the liver of rainbow trout by b-naphthoflavone or clophen A50, *Biochem. Pharmac.*, **34**: 3309-3314.

ARILLO, A., and MELODIA F. (1990): Protective effect of fish mucus against Cr(VI) pollution. *Chemosphere*, **20**: 397-402.

BAGNASCO, M., CAMOIRANO A.., DE FLORA S., MELODIA F. and ARILLO A. (1990): Enhanced liver metabolism of mutagens and carcinogens in fish living in polluted seawater. *Mutation Res.*, submitted.

BEUTLER, E. (1975): *Red Cell Metabolism. A Manual of Biochemical Methods. Ed. 2.* New York: Grune & Stratton, pp. 69-73.

BUCHI, G., FOWLER K.W. and NADZAN A.M. (1982): Photochemical epoxidation of aflatoxin B1 and sterigmatocystin: synthesis of guanine-containing adducts. *J. Am. Chem. Soc.*, **104**: 544-547.

CLAXTON, L.D., and BARNES H.M. (1981): The mutagenicity of diesel-exhaust particle extracts collected under smog-chamber conditions using the *Salmonella typhimurium* test system. *Mutat. Res.*, **88**: 255-272.

DE FLORA, S. (1982): Biotransformation and interaction of chemicals as modulators of mutagenicity and carcinogenicity, in: T. Sugimura, S. Kondo and H. Takebe (Eds.), *Environmental Mutagens and Carcinogens*, University of Tokyo Press, Tokyo / Alan R. Liss, New York, pp. 527-541.

DE FLORA, S., and BADOLATI G. (1982): Activation of mutagens by sunlight and ultraviolet light. *Proceedings 13th International Cancer Congress, Seattle, WA,* p. 591.

DE FLORA, S., and ARILLO A. (1983): Mutagenic and DNA-damaging activity in muscle of trout exposed in vivo to nitrite. *Cancer Lett.*, **20**: 147-155.

DE FLORA, S., and WETTERHAHN K.E. (1989): Mechanisms of chromium metabolism and genotoxicity. *Life Chem. Reports*, **7**: 169-244.

DE FLORA, S., MORELLI A., ZANACCHI P. BENNICELLI C. and DE FLORA A. (1982a): Selective deactivation of ICR mutagens as related to their distinctive pulmonary carcinogenicity. *Carcinogenesis*, **3**: 187-194.

DE FLORA, S., ZANACCHI P., BENNICELLI C. and ARILLO A. (1982b): Influence of liver S-9 preparations from rats and rainbow trout on the activity of four mutagens. *Toxicol. Lett.*, **10**: 345-349.

DE FLORA S., ZANACCHI P., CAMOIRANO A., BENNICELLI C., G. and Badolati (1984): Genotoxic activity and potency of 135 compounds in the Ames reversion test and in a bacterial DNA-repair test. *Mutat. Res.*, **133**: 161-198.

DE FLORA, S., DE RENZI G.P., CAMOIRANO A., ASTENGO M., BASSO C., ZANACCHI P. and BENNICELLI C. (1985): Genotoxicity assay of oil dispersants in bacteria (mutation, differential lethality, SOS DNA-repair) and yeast (mitotic crossing-over). *Mutation Res.*, **158**: 19-30.

DE FLORA, S., CAMOIRANO A., IZZOTTI A., D'AGOSTINI F. and BENNICELLI C. (1989a): Photoactivation of mutagens. *Carcinogenesis*, **10**: 1089-1097.

DE FLORA S., ZANACCHI P., BENNICELLI C., CAMOIRANO A., BASSO C., BAGNASCO M., IZZOTTI A. and BADOLATI G.S. (1989b): Genotoxicity, biotransformations and interactions of marine pollutants, as related to genetic and carcinogenic hazards: In: *Carcinogenic, Mutagenic and teratogenic Marine Pollutants Impact on Human Health and the Environment (World Health Organization Regional Office for Europe and United Nations Environment Programme)*, Advances in Applied Biotechnology Series, Vol. 5, The Portfolio Publ. Co., The Woodlands, Texas, pp. 3-31.

DE FLORA S., HIETANEN E. BARTSCH H., CAMOIRANO A., IZZOTTI A., BAGNASCO M. and MILLMAN I. (1989c): Enhanced metabolic activation of chemical hepatocarcinogens in woodchucks infected with hepatitis B virus. *Carcinogenesis*, **10**: 1099-1106.

ERNSTER, L., DANIELSON L. and LJUNGGREN M. (1962): DT-diaphorase. I. Purification from the soluble fraction of rat-liver cytoplasm, and properties. *Biochim. Biophis. Acta*, **58**: 171-188.

EVANS, J.V. (1982): The sun's influence on the earth's atmosphere and interplanetary space. *Science*, **216**: 467-474.

FOOTE, C.S. (1968): Mechanisms of photosensitized oxidation, *Science*, **162**: 963-969.

FOX, M.A., and OLIVE S. (1979): Photooxidation of anthracene on atmospheric particulate matter. *Science*, **205**: 582-583.

FRIDOVICH, I. (1974): Superoxide and evolution, in: E. Quagliarello, F. Palmieri, and T.P. Singer (Eds.), *Horizons Biochem. Biophys*, Addison-Wesley Publishing Company Inc., Massachusetts, pp. 1-37.

FUNARI, E. ZOPPINI A., VERDINA A., DE ANGELIS G. and VITTOZZI L. (1987): Xenobiotic-metabolizing enzyme systems in test fish. *Ecotoxicol. Environ. Safety*, **13**: 24-31.

GIBSO, T.L. SMART V.B. and SMITH L.L. (1978): Non-enzymic activation of polycyclic aromatic hydrocarbons as mutagens. *Mutation Res.*, **49**: 153-161.

GUENGERICH, F.P. (1988): Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer therapy. *Cancer Res.*, **48**: 2946-2954.

HABIG, W.H., PABST M.J. and JAKOBY W.B. (1974): Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.*, **249**: 7130-7139.

HARTREE, E.F. (1972): Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.*, **48**: 422-427.

HAYATSU, H. (1990): Blue cotton. Broad possibility in assessing mutagens.carcinogens in the environment, in: G. Obe (Ed.). *Advances in Mutagenesis Research, Vol. 1*, Springer-Verlag, Berlin, FRG, pp. 1-26.

HIROSE, M., WAKABAYASHI K., GRIVAS S., DE FLORA S., ARAKAWA N., NAGAO M. and SUGIMURA T. (1990): Formation of a nitro derivative of 2-amino-3,4 dimethylimidazo-[4,5-f]quilonine by photo-irradiation. *Carcinogenesis*, **11**: 869-871.

HISAMATSU, Y., NISHIMURA T., TANABE K. and MATSUSHITA H. (1986): Mutagenicity of the photochemical reaction products of pyrene with nitrogen dioxide. *Mutation Res.*, **172**: 19-27.

HOLLOWAY, M.P., BIAGLOW M.C., MC COY E.C., ANDERS M., ROSENKRANZ H.S. and P.C. HOWARD (1987): Photochemical instability of 1-nitropyrene, 3-nitrofluoranthene, 1,8-dinitropyrene and their parent polycyclic aromatic hydrocarbons. *Mutation Res.*, **187**: 199-207.

HSIEH, D.P.H., WONG J.J., WONG Z.A., MICHAS C. and RUEBNER B.H. (1977): Hepatic transformation of aflatoxin and its carcinogenicity, in: H.H. Hiatt, J.D. Watson and J.A. Winsten (Eds.), *Origin of Human Cancer*. Cold Spring Harbor Laboratory, NY, Book B, pp. 697-707.

IARC (1974): Some organochlorine pesticides, in: *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Vol. 5*. International Agency for Research on Cancer, Lyon, pp. 83-156.

ISRAEL-KALINSKY, H., TUCH J., ROITELMAN J. and AVISHAY-ABRAHAM S. (1982): Photoactivated aflatoxins are mutagens to *Salmonella typhimurium* and bind covalently to DNA *in vitro*. *Carcinogenesis*, **3**: 423-429.

KORFMACHER, W.A., NATUSCH D.F.S., TAYLOR D.R., MAMANTOV G. and WEHRY E.L. (1980): Oxidative transformations of polycyclic aromatic hydrocarbons adsorbed on coal fly ash. *Science*, **207**: 763-765.

KURELEC, B., GARG A., KRCA S., CHACKO M. and GUPTA R.C. (1989): Natural environment surpasses polluted environment in inducing DNA damage in fish. *Carcinogenesis*, **10**: 1337-1339.

JAMES. M.P., HEARD C.S. and HAWKINS W.E. (1988): Effect of 3-methylcholanthrene on monooxygenase, epoxide hydrolase, and glutathione S-transferase activities in small estuarine and freshwater fish. *Aquat. Toxicol.*, **12**: 1-15.

MARON, D.M. and AMES B.N. (1983): Revised methods for the *Salmonella* mutagenicity test. *Mutation Res.*, **113**: 173-215.

MCCOY, E.C., and ROSENKRANZ H.S. (1980): Activation of polycyclic aromatic hydrocarbons to mutagens by singlet oxygen: an enhancing effect of atmospheric pollutants? *Cancer Lett.*, **9**: 35-42.

MCCOY, E.C., HYMAN J. and ROSENKRANZ H.S. (1979): Conversion of environmental pollutant to mutagens by visible light. *Biochem. Biophys. res. Commun.*, **89**: 729-734.

MIGLIORE, L., DI MARINO F., SCARPATO R., BARALE R. and COGNETTI-ALFINITO G. (1989): Detection of mutagenic/carcinogenic compounds in the marine environment. In: *Carcinogenic, Mutagenic and Teratogenic Marine Pollutants Impact on Human Health and the Environment (World Health Organization Regional Office for Europe and United Nations Environment Programme)*, Advances in Applied Biotechnology Series, Vol. 5, The Portfolio Publ. Co., The Woodlands, Texas, pp. 111-120.

NIKUNEN, E. (1985): Toxic impact of effluents from petrochemical industry. *Ecotoxicol. Environ. Safety*, **9**: 84-91.

NISHI, K. and NISHIOKA H. (1982): Light induces mutagenicity of hair-dye p-phenylenediamine. *Mutat. Res.*, **104**: 347-350.

OKINADA, R.T., NICKOLS J.W., WHALEY T.W. and STRNISTE G.F. (1986) 1-nitropyrene: a mutagenic product induced by the action of near ultraviolet light on 1-aminopyrene. *Mutation Res.*, **173**: 93-98.

OMURA, T. and SATO R. (1964): The carbon monoxide binding pigment of liver microsomes, J. *Biol. Chem.*, **239**: 2370-2378.

PAYNE, J.F. and RAHIMTULA A. (1989): Monitoring for mutagens and carcinogens in the aquatic environment. In: *Carcinogenic, Mutagenic and Teratogenic Marine Pollutants Impact on Human Health and the Environment (World Health Organization Regional Office for Europe and United Nations Environment Programme)*, Advances in Applied Biotechnology Series, Vol. 5, The Portfolio Publ. Co., The Woodlands, Texas, pp. 227-248.

PAYNE, J.F., FANCEY L.L. RAHIMTULA A.D. and PORTER E.L. (1987): Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. *Comp. Biochem. Pysiol.*, **86C**: 233-245.

PETRILLI, F.L., DE RENZI G.P. and DE FLORA S (1980): Interaction between polycyclic aromatic hydrocarbons, crude oil and oil dispersants in the *Salmonella* mutagenesis assay. *Carcinogenesis*, **1**: 51-56.

PITTS, J.N. (1971): The role of singlet molecular oxygen in the chemistry of urban atmospheres, in: C.S. Tuesday (Ed.), *Chemical Reactions in Urban Atmospheres*. American Elsevier Publishing Company, Inc., New York, pp. 3-33.

PITTS, J.N., VAN CAUWENBERGHE K.A., GROSJEAN D. SCHMID J.P., FITZ D.R., BELSER W.L., KNUDSON G.B. and HYNDS P.M. (1978): Atmospheric reactions of polycyclic aromatic hydrocarbons: facile formation of mutagenic nitro derivatives. *Science*, **202**: 515-519.

PITTS, J.N., LOKENSGARD D.M., RIPLEY P.S., VAN CAUWENBERGHE K.A., VAN VAECK L., SHAFFER S.D., THILL A.J. and BELSER W.L. (1980): "Atmospheric" epoxidation of benzo[a]pyrene by ozone: formation of the metabolite benzo[a]pyrene-4,5-oxide. *Science*, **210**: 1347-1349.

POELS, C.L.M., VAN DER GAAG M.A. and VAN DE KERKHOFF J.F.J. (1980): An investigation into the long-term effects of Rhine water on rainbow trout, *Water Res.*, **14**: 1029-1035.

PRODI, G., GRILLI S., MAZZULLO M., COLACCI A. and ARFELLINI G. (1984): Comparison between photo-induction and microsomal activation of polycyclic hydrocarbons with different oncogenic potency. *Toxicol. Pathol.*, **12**: 185-188.

RIJAVEC, M., PROTIC M. and KURELEC B. (1981): A study of some biological (sublethal and mutagenic) effects of the waste waters of the INA Petrol Refinery, Rijeka. *Thalassia Jugosl.*, **17**: 237-243.

RUDACK, D., CHISHOLM E.M., and HOLTEN D. (1971): Rat liver glucose 6-phosphate dehydrogenase. Regulation by carbohydrate, diet, and insulin. *J. Biol. Chem.*, **246**: 1249-1254.

SABADIE, N., MALAVEILLE C., CAMUS A.M., and BARTSCH H. (1980): Comparison of the hydroxylation of benzo(a)pyrene with the metabolism of vinyl chloride, *N*-nitrosomorpholine, and *N*-nitroso-*N'*-methylpiperazine to mutagens by human and rat liver microsomal fractions. *Cancer Res.*, **40**: 119-126.

SANTAMARIA, L., GIORDANO G.G., ALFISI M. and CASCIONE F. (1966): Effects of light on 3,4-benzpyrene carcinogenesis. *Nature*, **210**: 824-825.

SAVILLE, B. (1958): A scheme for the colorimetric determination of microgram amounts of thiols. *Analyst*, **83**: 670-672.

SELBY, C.P., CALKINS J., ENOCH H.G., WRIGHT C.W. and WILSON B.W. (1978): Chemical basis for photomutagenicity in synthetic fuels and correlations with carcinogenicity. *Mutat. Res.*, **188**: 287-299.

SHERWOOD, M.J. and MEARNS A.J. (1977): Environmental significance of fin erosion in southern California demersol fishes. *Ann. N.Y. Acad. Sci.*, **288**: 177-189.

SLOOFF, W., VAN KREIJL C.F. and BAARS A.J. (1983): Relative liver weights and xenobiotic-metabolizing enzymes of fish from polluted surface waters in the Netherlands. *Aquatic Toxicol.*, **4**: 1-14.

STARK, G., STAUFF J., MILTENBURGER H.G. and STUMM-FISHER I. (1985): Photodecomposition of 1-nitropyrene and other direct-acting mutagens extracted from diesel-exhaust particulates. *Mutation Res.*, **155**: 27-33.

STRNISTE, G.F., and BRAKE R.J. (1981): Cytotoxicity in human skin fibroblasts induced by photoactivated polycyclic aromatic hydrocarbons, in: M. Cooke, A.J. Dennis and G.L. Fisher, G.L. (Eds.), *Polynuclear Aromatic Hydrocarbons*. Battelle Press, Columbus, pp. 109-118.

STRNISTE, G.G., NICKOLS J.W. and OKINAKA R.T. (1985): Photochemical oxidation of 2-aminofluorene: correlation between the induction of direct-acting mutagenicity and the formation of nitro and nitroso aromatics. *Mutation Res.*, **151**: 15-24.

STRNISTE, G.F., NICKOLS J.W., OKINAKA R.T. and WHALEY T.W. (1986) 2-nitrofluoren-9-one: a unique mutagen formed in the photo-oxidation of 2-aminofluorene. *Carcinogenesis*, **7**: 499-502.

SUGIMURA, T. (1982): Mutagens, carcinogens and tumor promoters in our daily food. *Cancer*, **49**: 1970-1984.

SUGIMURA, T. (1985): Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutation Res.*, **150**: 33-41.

TAKEDA, N., TERANISHI K and HAMADA K. (1984): Mutagenicity of sunlight exposed sample of pyrene in *Salmonella typhimurium* TA98. *Bull. Environ. Contam. Toxicol.*, **33**: 410-417.

TU, M.H., PERRY D., and CHEN C. (1979): Mutagenic effect of 7,12-dimethylbenz[a]anthracene-epidioxide on *Salmonella typhimurium*. *Molec. Pharmacol.*, **15**: 189-191

VALERIO, F. and LAZZAROTTO A. (1985): Photochemical degradation of polycyclic aromatic hydrocarbons (PAH) in real and laboratory conditions. *Intern. J. Environ. Anal. Chem.,* **23**: 135-151.

VARANASI, U., NISHIMOTO M., REICHERT W.L. and LE EBERHART B.-T. (1986): Comparative metabolism of benzo(a)pyrene and covalent binding to hepatic DNA in English sole, starry flounder, and rat. *Cancer Res.*, **46**: 3817-3824.

VEIGA SALLES, J.B., and OCHOA S. (1950): Biosynthesis of dicarboxylic acids by carbon dioxide fixation. *J. Biol. Chem.*, **187**: 849-861.

WHITE, G.L. and HEFLICH R.H. (1985): Mutagenic activation of 2-aminofluorene by fluorescent light. *Teratogenesis, Carcinogenesis, and Mutagenesis*, **5**: 63-73.

WHITE, G.L. FU P.P. and HEFLICH R.H. (1985): Effect of nitro substitution on the light-mediated mutagenicity of polycyclic aromatic hydrocarbons in *Salmonella typhimurium* TA98. *Mutat. Res.*, **144**: 1-7.

YAMASHITA, M. WAKABAYASHI K., NAGAO M., SATO S., YAMAIZUMI Z., TAKAHASHI, M., KINAEN., TOMITA I. and SUGIMURA T. (1986): Detection of 2-amino-3-methylimidazo[4,5-f]quinoline in cigarette smoke condensate. *Jpn. J. Cancer Res. (Gann)*, **77**: 419-422.

ZAHN, R.K., ZAHN G., MULLER W.E.G., KURELEC B., RIJAVEC M., BATEL R. and GIVEN R. (1981): Assessing consequences of marine pollution by hydrocarbons using sponges as model organisms. *Sci. Total Environ.*, **20**: 147-169.

ZHAN, R.K., KURELEC B., ZAHN-DAIMLER G., MULLER W.E.G., RIJAVEC M., BATEL R., GIVEN R. and BEYER R. (1982): The effect of benzo(a)pyrene on sponges as model organisms in marine pollution. *Chem.-Biol. Interactions*, **39**: 205-220.

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