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Marine molluscs and fish as biomarkers of pollution stress in littoral regions of the Red Sea, Mediterranean Sea and North Sea

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Abstract The intensive development of industry and urban structures along the seashores of the world, as well as the immense increase in marine transportation and other activities, has resulted in the deposition of thousands of new chemicals and organic compounds, endangering the existence of organisms and ecosystems. The conventional single biomarker methods used in ecological assessment studies cannot provide an adequate base for environmental health assessment, management and sustainability planning. The present study uses a set of novel biochemical, physiological, cytogenetic and morphological methods to characterize the state of health of selected molluscs and fish along the shores of the German North Sea, as well as the Israeli Mediterranean and Red Sea. The methods include measurement of activity of multixenobiotic resistance-mediated transporter (MXRtr) and the system of active transport of organic anions (SATOA) as indicators of antixenobiotic defence; glutathione S-transferase (GST) activity as an indicator of biotransformation of xenobiotics; DNA unwinding as a marker of genotoxicity; micronucleus test for clastogenicity; levels of phagocytosis for immunotoxicity; cholinesterase (ChE) activity and level of catecholamines as indicators of neurotoxicity; permeability of external epithelia to anionic hydrophilic probe, intralysosomal accumulation of cationic amphiphilic probe and activity of non-specific esterases as indicators of cell/tissue viability. Complete histopathological examination was used for diagnostics of environmental pathology. The obtained data show that the activity of the defensive pumps, MXRtr and SATOA in the studied organisms was significantly higher in the surface epithelia of molluscs from a polluted site than that of the same species from control, unpolluted stations, providing clear evidence of response to stress. Enhanced frequency of DNA lesions (alkaline and acidic DNA unwinding) and micronucleus-containing cells was significantly higher in samples from polluted sites in comparison to those from the clean sites that exhibited genotoxic and clastogenic activity of the pollutants. In all the studied molluscs a negative correlation was found between the MXRtr levels of activity and the frequency of micronucleus-containing hemocytes. The expression of this was in accordance with the level of pollution. The complete histopathological examination demonstrates significantly higher frequencies of pathological alterations in organs of animals from polluted sites. A strong negative correlation was found between the frequency of these alterations and MXRtr activity in the same specimens. In addition to these parameters, a decrease in the viability was noted in molluscs from the polluted sites, but ChE activities remained similar at most sites. The methods applied in our study unmasked numerous early cryptic responses and negative alterations of health in populations of marine biota sampled from the polluted sites. This demonstrates that genotoxic, clastogenic and pathogenic xenobiotics are present and act in the studied sites and this knowledge can provide a reliable base for consideration for sustainable development.

Key words Molluscs · Fish · Red Sea · Mediterranean Sea · North Sea

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Introduction

The enormous urbanization and industrial development along riverbanks and seashores have introduced into the water masses large and diverse quantities of biologically active substances, including thousands of organic and inorganic man-made chemical compounds (xenobiotics). These xenobiotics stress the natural ecosystems, causing

diseases of selected biota, enhanced lethality and often leading to elimination (extinction) of the more sensitive taxa and the dominance of more resistant and opportunistic organisms, such as various algal and bacterial species. Such degradation is well documented in the northern seas as well as in coral reefs in the tropics, concomitant with the decline in economically important fish stocks in the more northern global regions due to overfishing and environmental pollution. Alarmed by these facts, decision-makers have started to ask ecologists for solutions offering a more reasonable and sustainable development. The conventional ecological practice and single biomarker methods cannot provide, in fact, the basic answers as to what is the actual state of health in the natural populations and, owing to this, cannot predict the future of such biota. The frequency of sick animals in a population can increase dramatically in a polluted environment. To detect and assess early responses and the state of health of populations, it is necessary to introduce in ecology a special new set of methods derived from cytochemistry, cytophysiology, cytogenetics and pathology (Fishelson 1995; Bresler et al. 1998; Fishelson et al. 1999). These novel biochemical, cytochemical, cytophysiological, cytogenetic and cytopathological methods enable us to detect cryptic early responses and early prepathological alterations in exposed organisms before enhanced disease and mortality rates occur or population shifts take place (Adams 1990; Roy and Hänninen 1993; Bresler and Fishelson 1994; Hansen 1995; Bresler et al. 1998; Fishelson et al. 1999). Such methods can therefore offer an effective early warning system in monitoring biota of aquatic environments. For example, altered activity of several antixenobiotic defence mechanisms, such as membrane export pumps, multixenobiotic resistance-mediated transporter (MXRtr), the system of active transport of organic anions (SATOA) and enzyme-mediated detoxification and conjugation of xenobiotics, reflects early defensive responses of organisms to environmental polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins (Yawetz et al. 1992; Bresler and Fishelson 1994; Bresler et al. 1998; Fishelson et al. 1999). Altered glutathione S-transferase (GST) activity also indicates defensive response of the organism to enhanced pollution stress (Roy and Hänninen 1993). Numerous methods employing molecular biology, biochemistry, cytochemistry and cytogenetics are also used to detect various genotoxic (DNA adducts formation, single-stranded DNA breaks, mutations) and clastogenic (chromosome breaks, chromosome aberrations, aneuploidy, enhanced sister chromatide exchange) effects of pollution (Jensen and Leary 1990; Scarpato et al. 1990; Erbes et al. 1997; Nepomuceno et al. 1997; Ralph and Petras 1997; Torous, et al. 1998; Wakata et al. 1998). Recently, DNA-unwinding assays and micronucleus tests have been developed as prospective methods for mass monitoring of genotoxicity and clastogenicity (Darzynkiewicz 1993, 1994; Hansen et al. 1996; Bresler et al. 1998; Herbert and Hansen 1998). The inhibition of cholinesterase activity has been also suggested as an effective parameter to detect the toxic influence of organophosphates, carbamates, some heavy metals and surfactants (Galgani et al. 1992; Silva de Assis 1998). Finally, cyto- and histopathological examination permits us to detect early pre-pathological and pathological alterations, and to unmask target species, specimens, organs, cells and subcellular structures as well as to assess etiology and pathogenesis of these alterations (Cotran et al. 1989; Schwaiger et al. 1997).

Taking these facts into consideration and using a novel methodology, our study focused on the assessment of the health in littoral biota of marine environments affected by anthropogenic activities. The state of health was

Table 1 Sites of collection, dates of sampling and species collected along the Israeli Mediterranean shore

Sites of collection	Collected species	Dates of collection
Frutarom	Main: *Donax trunculus* (374) Additional: *Mactra corallina*	12.11.95; 17.04.96; 3.07.96; 19.12.96; 2.01.97; 17.09.97; 4.03.98
Na'aman River	Main: *Donax trunculus* (212) Additional: *Mactra corallina*	12.11.95; 17.04.96; 2.01.97
Maagan Michael (Taninim River)	Main: *Donax trunculus* (212), *Patella coerulea* (75) Additional: *Mactra corallina*, *Monodonta turbinata*	17.04.96; 3.07.96; 19.12.96; 2.01.97; 4.03.97; 14.04.97; 8.05.98
Shemen Beach	Main: *Patella coerulea* (107)	12.11.95; 17.04.96; 3.07.96; 19.12.96; 14.04.97; 30.04.97; 4.03.98; 8.05.98
Achziv	Main: *Patella coerulea* (55) Additional: *Monodonta turbinata*	17.04.96; 3.07.96; 4.03.98; 8.05.98
Akko	Main: *Patella coerulea* (85) Additional: *Monodonta turbinata*	17.04.96; 14.04.97; 30.04.97; 4.03.98; 8.05.98
Sdot Yam	Main: *Patella coerulea* (70) Additional: *Monodonta turbinata*	3.07.96; 14.04.97; 4.03.98; 8.05.98
Tel Shikmona	Main: *Patella coerulea* (25) Additional: *Monodonta turbinata*	14.04.97; 30.04.97; 4.03.98

studied in selected biota along the Israeli Mediterranean shores, the Gulf of Aqaba (Eilat, Red Sea) and the German North Sea. The presented comparison of related data from three such different seas validates and demonstrates the objectivity of the methodology. The data relate to selected bioindicative taxa, common in the sampling sites, which offer evidence of the state of health in the studied ecosystems. To determine the health characteristics, we developed a novel method of vital light microscopy, particularly contact fluorescent microscopy and microfluorometry. Marker fluorescent probes and quantitative fluorescent microscopy enabled us to study in precise detail the cellular organization, cell functions, metabolic pathways and pathological deviations.

Materials and methods

Collection sites and studied species

The Israeli Mediterranean coast (Fig. 1, Table 1)

The Mediterranean coast of Israel is approximately 180 km long and ecologically can be clearly divided into two types of ecosystems: (1) shallow water sandy habitats and (2) intertidal rocky habitats, composed mainly of the special sand beachrock termed "kurkar" (Fishelson et al. 1996a,b). The community of this rocky intertidal habitat includes the typical Mediterranean Littorinidae (Gastropoda)-Balanidae (Cirripedia) assemblage of species, but the most dominant is the gastropod *Patella coerulea* (Mannelis and Fishelson 1989). The subtidal sandy habitats in the shallow water are populated by the *Dotilla* (Bivalvia)-*Nassa* (Gastropoda) assemblage of species, dominated by *Donax trunculus* in the most shallow subtidal area, in some sites occurring in densities of 2000/m^2 (Fishelson et al., 1996b). Because of the prevalence of and accumulated knowledge on *D. trunculus* and *P. coerulea*, these two species of molluscs were selected as indicator species and collected along the coast from stations with different pollution histories: along Haifa Bay on Shemen Beach and at Akko, further north at Achziv and south at Sdot Yam, Tel Shikmona and Maagan Michael. Pollution is especially heavy in Haifa Bay, where industry includes oil refineries, petrochemical plants, plastic, paint and fertilizer factories, and the Haifa harbour (Hornung et al. 1991; Yawetz et al. 1992; Herut et al. 1993, 1994; Bresler and Fishelson 1994; Fishelson et al. 1996a). Shemen Beach in Haifa Bay is the most polluted station. Maagan Michael, a settlement situated along an open shore, 25 km south of Haifa Bay, is a comparatively clean site (Yawetz et al. 1992). Another group of the MARS1 project (B. Herut and N. Kress) has conducted chemical analyses of seawater, sediment and biota taken from the same sampling sites along the Israeli Mediterranean shore and these data will appear in this issue.

The Israeli Red Sea Coast (Fig. 2, Table 2)

The Gulf of Aqaba forms the most northern extension of the Red Sea and the Israeli area extends along 12 km of the western-northwestern shore. A part of the seashore along this site is rocky, mostly formed of beachrock formations, paralleled in the sea by fringing coral formations. Pollution in the area was originally caused by phosphate loading in the local harbour (Fishelson 1971, 1973), later enhanced by frequent small oil spills at the oil terminal. On these intertidal substrates the dominant assemblages are from the Littorinidae-Balanidae taxa, but the most common organism of this habitat is the gastropod *Cellana rota*, the closest relative of the Mediterranean *Patella coerulea*. The sandy habitats extend mostly along the northern part of the shore, which is also the most polluted due to hotel developments, urban effluents and cage-based fish farming (Angel et al. 1995). These sandy habitats also harbour specific species assemblages (Fishelson 1971), of which the most common in the shallow waters, and used in our study, were the mussels *Callista florida* and *Dosinia histrio*, relatives of the Mediterranean mussel *Donax trunculus.*

The German North Sea shore (Fig. 3, Table 3)

The North Sea sampling was carried out from a tidal–subtidal rocky habitat populated by growths of the bivalve *Mytilus edulis* which were collected by picking them directly from the hard bottom (rocks) and from onshore estuary piers. The flatfish *Platichthys flesus* and dab *Limanda limanda* (17–25 cm total length) were caught by a bottom trawl from the vessels "Alkor" and "Walther Herwig III". The sampling stations were located at Cuxhaven at the mouth of the Elbe; at the estuary of the Eider; off the island Helgoland; and in some cases on offshore stations in the southern North Sea. The grade of pollution of the three main sites is as fol-

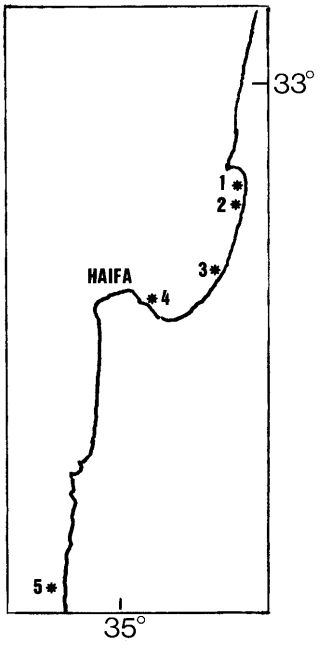

Fig. 1 Sampling sites along the Israeli Mediterranean shore. *1* Na'aman River; *2* Frutarom; *3* Kiryat Yam; *4* Shemen Beach; *5* Maagan Michael

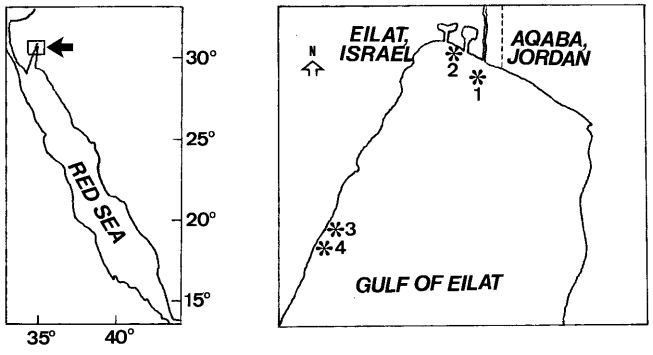

Fig. 2 Sampling sites along the Israeli Red Sea shore, the Gulf of Aqaba. *1* Ardag, fish cages; *2* Ardag, rocks of North Beach; *3* Marine Biological Laboratory (MBL) of Inter-University Institute

Table 2 Sites of collection, dates of sampling and species collected along the Israeli Red Sea shore (Gulf of Eilat). *MBL* Marine Biological Laboratory of Inter-University Institute

Sites of collection	Collected species and number of specimens	Dates of collection
Ardag	Main: *Callista florida* (102), *Dosinia histrio* (80), *Cellana rota* (82) Additional: *Lucinia dentifera*, *Circa* sp., *Pteria aegipta*, *Anadara antiquata* and others	7.11.95; 11.01.96; 24.03.96; 14.07.96; 1.12.96; 4.05.97
MBL (light tower)	Main: *Callista florida* (83), *Dosinia histrio* (93), *Cellana rota* (83) Additional: *Lucinia dentifera*, *Circa* sp., *Pteria aegipta*, *Anadara antiquata* and others	7.11.95; 11.01.96; 24.03.96; 14.07.96; 1.12.96; 4.05.97

Table 3 Sites and characteristics of samples of molluscs and fish along the German North Sea

Sites of collection	Collected species and number of specimens	Dates of collection
Elbe/Cuxhaven	*Platichthys flesus* (126); *Limanda limanda* (50)	09.95; 01.96; 07.96; 10.96; 04.97; 09.97
Eider	*Platichthys flesus* (114) *Limanda limanda)* (25)	09.95; 01.96; 07.96; 10.96; 04.97; 09.97
Helgoland/Tiefe Rinne	*Platichthys flesus* (124) *Limanda limanda* (25)	09.95; 01.96; 07.96; 10.96; 04.97; 09.97
Spiekeroog	*Platichthys flesus* (45) *Mytilus edulis* (37)	04.97; 09.97
Stations No	*Platichthys flesus* (30)	01.06; 07.96; 04.97; 09.97
Elbe/Cuxhaven	*Mytilus edulis* (201)	01.96; 07.96; 04.97; 09.97
Eider	*Mytilus edulis* (123)	04. 96; 07.96; 04.97; 09.97
Helgoland/Dune	*Mytilus edulis* (168)	01.96; 07.96; 04.97; 09.97

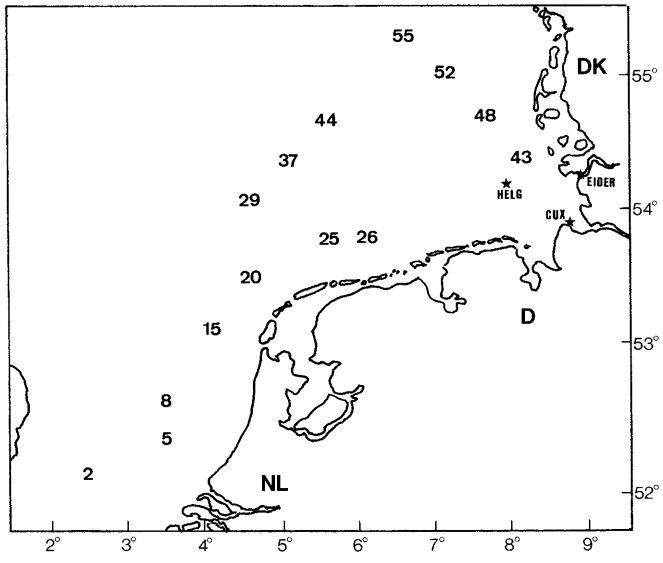

Fig. 3 Sampling sites in the German North Sea. *CUX* Cuxhaven; *HELG* Helgoland. Other sites are marked by *numbers*

lows: Cuxhaven >Eider>Helgoland, Tiefe Rinne >Helgoland, the harbour >Spiekeroog (Karup 1994).

Sample treatment

The collected live molluscs were transported in special containers to the laboratory and kept for up to 14 days in aerated aquaria with circulating and filtered artificial seawater, at 25°C. The animals were used for vital biophysical, cytophysiological and cytochemical examinations. For all assays with acridine orange tissues, samples were fixed in cold absolute methanol and for counting of micronucleated cells and histopathological examinations in 5% formaldehyde solution in seawater. Samples fixed in methanol were washed the following day in 80% ethanol and stored in clean 80% ethanol at 4°C. Collected organisms from the North Sea were immediately fixed in 5% formaldehyde solution in seawater or in absolute methanol, and transported to Tel Aviv University. There they were re-fixed in methanol, washed in 80% ethanol and stored at 4°C in clean 80% ethanol. After preparation and homogenization, tissue samples were transported at –20°C to the laboratory in insulated boxes for biochemical examinations.

Analytical methods (Table 4)

Multidrug resistance-mediated transporter (MXRtr) and system of active transport of organic anions (SATOA)

The use of specific fluorescent substrates, non-fluorescent competitive inhibitors and microfluorometry allows in vivo and in

Table 4 Main parameters (indicators) of environmental health and corresponding fluorescent microscopic methods for their determination

Parameter	Method
Metabolic state of mitochondria in living cells and tissues in situ	Microfluorometry of inherent blue and green fluorescence of NADH and FAD
Content of DNA, RNA and other cell constituents	Quantitative fluorescent cytochemistry
Enzyme activity in living cells in situ: non-specific esterase activity	Fluorogenic substrates, specific inhibitors and microfluorometry
Permeability of plasma membranes, epithelial layers and histohematic barriers	Fluorescent markers of permeability and microfluorometry
Carrier-mediated transport systems for xenobiotic elimination: SATOA and MXR	Fluorescent substrates, specific inhibitors and microfluorometry
Xenobiotic-binding proteins	Fluorescent analogues of ligands and microfluorometry
Intra- and extracellular depot for xenobiotic accumulation and storage	Fluorescent analogues of xenobiotics and microfluorometry
State of lysosomes and cell viability	Vital test with acridine orange or neutral red and microfluorometry
Functional state of nuclear chromatin and cell cycle phases	Staining with acridine orange and microfluorometry at 530 and 590 nm
Cytogenetic examination	Functional activity of nuclear chromatin, one-stranded DNA break, apoptosis and micronucleus test
Complete histopathological examination	Staining of tissue blocks and contact fluorescent and epimicroscopy

vitro estimation of the activity of these transport systems and their main kinetic parameters (Bresler et al. 1990, 1998; Bresler and Fishelson 1994; Bresler and Yanko 1995a,b; Haugland 1996; Schreiber et al. 1997).

Rhodamine B (Rh B) and the specific inhibitor Verapamil (Ver) were used to estimate MXRtr export activity in different tissues of molluscs and fish. The shells of live *D. trunculus* were opened by cutting the adductor muscles and the two valves, each with intact gills, mantle and part of the foot, were carefully separated. One valve with its soft tissues was incubated at 25°C for 1 h in aerated artificial seawater with 0.5 µM Rh B, and the other valve was incubated for 1 h in seawater with 0.5 µM Rh B and 10 µM Ver. Similarly, small pieces of living fish gills, kidney and liver were also incubated with Rh B only or Rh B + Ver.

Red fluorescence of accumulated Rh B in the tissues was measured by contact microfluorometry (excitation 510–560 nm, dichroic mirror 575 nm, barrier filter >590 nm) with an objective LC25×0.75 and mirror (25 µm in diameter) measuring diaphragm. Twenty separate sites were measured along each tissue preparation. The mean difference between Rh B fluorescence with and without inhibitor is proportional to the amount of Rh B eliminated by MXRtr per hour, i.e. MXRtr activity in arbitrary units (a.u.).

To estimate SATOA activity in the gills of molluscs or fish, one valve of the molluscs or fragment of fish gills with its intact soft tissues was incubated for 10 min in seawater with 1 µM fluorescein diacetate, and the green fluorescence of liberated fluorescein (FLU) was measured by contact microfluorometry (excitation 450–490 nm, dichroic mirror 505 nm, barrier filter >520 nm) for 20 separate filaments along the gills. The same preparation was then incubated for 15 min in clean artificial seawater (washing) and FLU content was measured again. The other valve with its intact soft tissues was preloaded with 1 mM of a known competitive inhibitor of SATOA, probenecid, and FLU liberation and its possible runout was then examined as described above. Because probenecid produced full inhibition of FLU runout from the gills, the difference between FLU level after incubation with fluorescein diacetate and after washing for 15 min in clean artificial seawater reflects SATOA activity in arbitrary units (a.u.). To determine SATOA activity in the renal proximal tubules and liver bile canalicules of fish, incubation of small living pieces of corresponding tissues with marker substrate, FLU, contact fluorescent microscopy and microfluorometry were used as described previously (Bresler and Nikiforov 1977, 1981; Schreiber et al. 1997).

Non-specific esterase (NSE) activity

NSE is a widely distributed group of enzymes inhibited by the same pollutants as ChE. The level of activity of these cytoplasmic enzymes is a sensitive marker of cell viability. Fluorescein diacetate (final concentration from 0.1 to 10 µM), the permeable fluorogenic substrate of non-specific esterases, was used to examine enzyme activity in the different tissues of molluscs and fishes or to load the cells by fluorescein. The level of liberated fluorescein (FLU) in the tissue was determined microfluorimetrically.

Cholinesterase activity

Cholinesterase (ChE) is widely used to estimate neurotoxic impacts of pollutants on the cellular level of marine organisms (Galgani et al. 1992). Inhibition of ChE activity has been suggested as a parameter to detect effects of organophosphates, carbamates, some heavy metals and surfactants (Escartin and Porte 1997; Silva de Assis 1998). ChE activity was measured in soluble fraction from brain and muscle tissue of *Platichthys flesus* and gills and hemolymph of *Mytilus edulis*. Approximately 0.2–0.3 g of the corresponding tissues was homogenized in 2 ml phosphate buffer (0.1 M, pH 7.4 for fish, 8.0 for mussels). After centrifugation of the homogenates at 10.000 *g* (10 min), the supernatant was divided into two fractions, one for ChE, one for GST, frozen and stored in liquid N_2. ChE activity was measured colorimetrically according to Ellmann et al. (1961), using a photometer (Dynarec, SLT ATTC 340) at 414 nm wavelength and a temperature of 20±2°C (Herbert et al. 1996). The protein content of the samples was determined according to Bradford (1976).

Cytochemical measurement of catecholamines

Formaldehyde-induced fluorescence of catecholamines was measured to estimate their concentration in nerve fibres (neuropile) of

molluscan nerve ganglia. The level of these biogenic monoamines, especially dopamine and serotonine, reflects their response to such chemicals as neurotoxic pesticides (Gardner and Brady 1977). Soft tissues of molluscs were exposed to formaldehyde vapour in a hermetically closed jar containing 10% formaldehyde for 24 h at room temperature and the induced fluorescence was measured by contact microfluorometry at excitation/emission wavelength 400–420/470–500 nm.

Glutathion4-S-transferase (GST) activity

GST conjugates xenobiotics or their metabolites with glutathione and so makes them less toxic and more amenable to elimination by SATOA (Roy and Hänninen 1993). Induced GST activity indicates an adaptation of the organism to enhanced pollution stress. The GST activity was measured in hepatic cytosol fractions of flounder and in gills of mussel. The liver cytosol was kindly provided by the Biologische Anstalt Helgoland and diluted 1:16 with phosphate buffer (0.1 M, pH 6.5). For detection of gills GST 1 ml of supernatant was diluted 1:2 with phosphate buffer. The colorimetric measurement was conducted at 340-nm wavelength, at 25°C.

Plasma membranes and epithelial layers permeability

Intact plasma membranes, tight junctions of epithelial layers and histohematic barriers show very low permeability to hydrophilic anionic organic compounds, particularly the fluorescent acid dye, fluorescein (FLU). Injuries to cell junction or plasma membranes increase this permeability, allowing distinction between intact and damaged cells and epithelial layers (Bresler and Fishelson 1994; Bresler and Yanko 1995 a,b; Haugland 1996). FLU, contact fluorescent microscopy and microfluorometry allow us to distinguish and measure permeability through cell junction (paracellular permeability) and focal permeability through damaged epithelial area (anomalous permeability), as described previously by Bresler and Fishelson (1994) and Schreiber et al. (1997).

Intralysosomal accumulation of neutral red (NR)

Various amphiphilic organic cationic compounds with high pK_a are actively accumulated by lysosomes and related structures. Cationic dye, NR, is a well known fluorescent analogue of such compounds. Intralysosomal accumulation of NR is an energy-dependent process and each cellular injury decreases its level (Saito et al. 1991; Bresler and Yanko 1995 a,b; Haugland 1996). Tests with NR are often used in ecotoxicology to distinguish viable, damaged and dead cells or organisms. We incubated living tissues of sampled molluscs in artificial seawater with NR (0.01%) and its accumulation in the tested tissues was measured by microfluorometry.

Phagocytic activity

The defensive phagocytic activity of blood cells is strongly inhibited by xenobiotics. The phagocytosis test was conducted by measuring the following parameters: the density of hemocytes, fluorescence of ingested yeast in a volume of 100 µl, and phagocytosis index. The hemocyte density of hemolymph from each mussel was measured by using a Thoma-Zeiss counting chamber and a light-optical microscope. The relative fluorescence unit (rfu) value reflects the amount of fluorescein isothiocyanate (FITC) conjugated yeast ingested by hemocytes contained in 100 µl volume of hemolymph. Because of different hemocytes concentration in the100 µl hemolymph pipetted into each well, the phagocytosis index was calculated for each sample as $rfu/5.10^5$ hemocytes to compare the phagocytic activity of different samples.

Samples of hemolymph of *Mytilus edulis* were withdrawn from the posterior adductor muscle sinus of the mussels using a one-way 2-ml hypodermic syringe. The yeast cells *Saccharomyces cerevisiae* were conjugated with FITC (Sigma F7250) and stored in aliquots at a concentration of 10^9/ml at –70°C.

A microtiter plate assay was performed for phagocytosis of mussel hemocytes according to Hansen (1992) and Anderson and Mora (1995). Phagocytosis of FITC-yeast by hemocytes was performed during incubation in the dark for 30 min, at room temperature. The fluorescence signals of extracellular non-ingested FITC yeast were quenched by trypan blue in a working solution of 1 mg/ml using 30 µl per well for 8–10 min. The fluorescence of ingested FITC yeast was measured using a microtiter plate fluorometer (Dynatech, Fluorolite 1000) by an extinction wavelength of 485 nm and emission wavelength of 535 nm. The difference in fluorescence signals between samples with and without hemolymph could be precisely calculated as relative fluorescence unit per millilitre of hemolymph (Anderson and Mora 1995).

Alkaline DNA-unwinding assay

DNA damage in liver tissue of fish and in gills of mussels was investigated by alkaline denaturation and hydroxyl apatite elution as described by Herbert and Hansen (1998) after separation and elution of single (ss)- and double-stranded (ds)-DNA fraction from the homogenized tissues. The relationship of ds-DNA fraction to total DNA (ss+ds) was calculated as F-values. The negative logarithm of F (-log F) is proportional to the relative average number of DNA-unwinding points and thus to the degree of DNA damage.

Fluorescent cytochemical measurement of DNA, RNA and cell cycle phases by acridine orange (AO)

Darzynkiewicz (1993) introduced a precise method to determine cell cycle phases from DNA versus RNA plot after AO staining. For DNA/RNA staining by AO, blocks of fixed tissue, cell suspensions or cell smears were treated by a cold solution containing 20 µM AO, 1 mM EDTA (sodium salt), 150 mM NaCl, and citric acid-phosphate buffer, pH 6.0. The samples were then measured for fluorescence in a microfluorometer between 5–10 min after addition of the AO solution. Fluorescence was excited at 450–490 nm and dichroic mirror 505 nm; the DNA-associated green fluorescence was measured at 520–550 nm, the RNA-associated red fluorescence was measured at 590–620 nm. Contact objective Fluor 45×1.0 was used for measurements of the tissue blocks and Nikon objective Fluor 40×0.76 was used to measure cell suspensions and cell smears. The amount of cells in different cell cycle phases was calculated from DNA versus RNA plot.

Acidic DNA-unwinding assay

Analysis of DNA denaturability in situ was made according to Darzynkiewicz (1993, 1994) with small modifications. Fixed total preparations of gills were washed for 1 h in Hanks' buffered salt solution (HBSS), transferred in HBSS containing 100 units of RNAse A, and incubated at 37°C for 1 h. RNAse-treated preparations were transferred in 10 ml HBSS mixed with 5 ml of 100 mM HCl at room temperature. After 80 s 20 ml of 20 µM AO solution in phosphate-citric buffer, pH 2.6, was added. Control pieces of the same gill preparation were stained without incubation in HBSS-HCl. Stained total preparations were used for two-wavelength contact microfluorometry between 5 and 10 min after addition of the AO solution.

Two-wavelength microfluorometry

For two-wavelength microfluorometry we used a Nikon epifluorescent microscope with a stabilized super high-pressure mercury lamp, equipped with a measuring head with mirror measuring diaphragms, changeable barrier interference filters, a highly sensitive

from 180 to 850 nm end-on photomultiplier, current pre-amplifier and the readout (Oriel, Stratford, Connecticut, USA). Excitation of AO was made by blue light (450–490 nm). Intensity of the double-stranded DNA-associated green fluorescence (520–540 nm) and red fluorescence (590–620 nm) of AO bound with one-stranded DNA or RNA was determined in each nucleus by using two-wavelength microfluorometry with contact objective Fluor 43×1.0. The microfluorometer was calibrated using microcuvettes as described by Bresler et al. (1990) and multispectral fluorescence microscopy standard kit (MultiSpeck, Molecular Probes, Eugene, Oregon, USA).

Counting of micronuclei-containing cells

Two methods were used to reveal the micronucleus-containing cells:

1. Total preparations of the molluscan mantles fixed in methanol were washed for 1 h in 150 mM NaCl with phosphate-citric acid buffer, pH 6.0, and stained in a solution containing 20 µM AO, 1 mM EDTA-Na, 150 mM NaCl, and citric acid-phosphate buffer, pH 6.0
2. Total preparations of the mantle fixed by formaldehyde were washed for 12 h in running water, for 1 h in 0.1% sodium citrate and stained with 5 mM ethidium bromide solution in 0.1% sodium citrate.

Fluorescent microscopy with contact objective Fluor 43×1.0 was used to detect and count micronuclei-containing hemocytes in the tissue blocks. For each preparation we examined microscopically 3000 hemocytes and calculated the frequency of micronuclei-containing hemocytes per 1000. Conventional fluorescent microscopy was used to count micronuclei-containing erythrocytes in blood smears fixed with methanol and stained with AO.

Cyto- and histopathological examination

The literature of ecotoxicology is rich in examples of tissue and cell alterations in organisms from polluted habitats. Therefore, we studied these parameters in our sampled organisms. Contact fluorescent and contact epimicroscopy, conventional light and electron microscopy were used for rapid complete cyto- and histopathological examinations of different organs of animals from the clean and polluted sites. Samples of living or fixed tissue blocks were investigated in toto through their natural surfaces or through surfaces of sections. These blocks were stained with AO for fluorescent and with Giemsa for contact fluorescent and contact epimicroscopy. Conventional histological slides of molluscan organs were stained with hematoxylin-eosin or PAS-alcyan blue. Transmitted electron microscopy studies were carried out ultrathin sections of gills and the siphons. We quantified the detected pathological alterations in samples using a special protocol and calculated frequency of pathological alterations within populations collected from different sites. The observed alterations were compared with levels of MXRtr or SATOA in the same samples.

Statistics and calculations

For all data obtained, the main statistical parameters used were: mean, standard deviation, standard error, 95% confidence limit and coefficient of variance (CV), calculated using computer program Sigma Plot50. As in a DNA unwinding assay in vitro (Nacci and Nelson 1992; Meyers-Schone et al. 1993), fraction of double-stranded DNA (Fds) in mean nucleus can be calculated from the ratio of green fluorescence of double-stranded DNA (G) to summary fluorescence of total DNA or green plus red (R) fluorescence: Fds=G/(G+R). The negative log of Fds (-log Fds) is proportional to the relative number of DNA-unwinding points. The relative number of strand breaks per unwinding units (*n* value) we calculated as *n*=–log Fds.in/-log Fsd.ref, where the index in indicates values from the investigated site and ref from the reference site. In this case, the *n* in the reference site is 1. The ratio of red fluorescence to green fluorescence intensities (R/G ratio or coefficient α) was also calculated. The same program was used to calculate ANOVA, MANOVA. To create graphs, the computer program Slide was used.

Biomarker Index

A comprehensive assessment of all biomarker results was conducted using the biomarker index (BI) according to Narbonne et al. (1998). This method is based on the subdivision of results into different classes by means of the Tukey test (Lienert 1978; Narbonne et al. 1998). Sites with lowest pollution (e.g. reference) have the highest BI, whereas sites with high pollution (e.g. low ChE activities, high DNA fragmentation) have the lower Bis.

Results

Mediterranean, Red Sea and North Sea bivalves

MXRtr and SATOA activities in surface epithelia

MXRtr and SATOA activities were detected in surface epithelia of all the investigated molluscs. The highest levels of activities were always presented in the gill epithelium and the lowest in the foot epithelium. Significant seasonal variations were not detected. MXRtr activity in the gills of freshly collected *Donax trunculus*, *Callista florida*, *Dosinia histrio* and other species ("initial" activity) from the polluted sites increased after 3 days in the clean seawater ("maximal" activity). The observed data suggest that these differences between "initial" and "maximal" activities are mediated by competitive inhibition. Mediterranean and Red Sea bivalves from the clean sites (Maagan Michael and MBL) demonstrated the lowest mean MXR activity in the gills (4490.3±304.2 a.u.) and its highest variability. In contrast, molluscs from the polluted sites demonstrated a higher mean MXRtr activity in the gills (6392.9±153.2 a.u. in Frutarom and 5634.1±230.0 a.u. in Na'aman River) and the lowest variability. Figure 4 shows significant differences in mean gill MXRtr activity in *D. trunculus* collected from different sites along the Israeli Mediterranean shore.

In a similar manner, the mean SATOA activities in the gills of *D. trunculus* from the clean sites (296.2±9.3 a.u. in Maagan Michael) were significantly lower than in gills of molluscs from the polluted sites – 348.6 a.u. in Frutarom and 334.2±5.9 a.u. in Na'aman River (Fig. 5). The Red Sea bivalves *C. florida* and *D. histrio,* also demonstrated similar alterations of MXRtr and SATOA activity.

Paracellular permeability of surface epithelia to FLU

In living bivalves (*D. trunculus, C. florida* and *D. histrio*) paracellular permeability of surface epithelia to FLU was the lowest in specimens from the clean sites

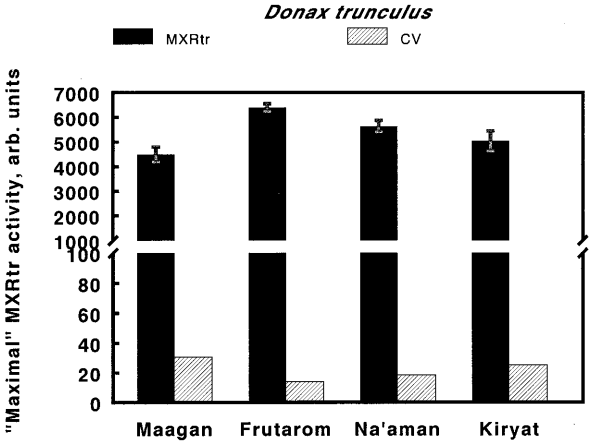

Fig. 4 Mean "maximal" MXRtr activities in gills of *Donax trunculus* from different sampling sites along the Israeli Mediterranean shore. Number of specimens: Maagan Michael 80; Frutarom 120; Na'aman River estuary 80; Kiryat Yam 40. *CV* Coefficient of variation

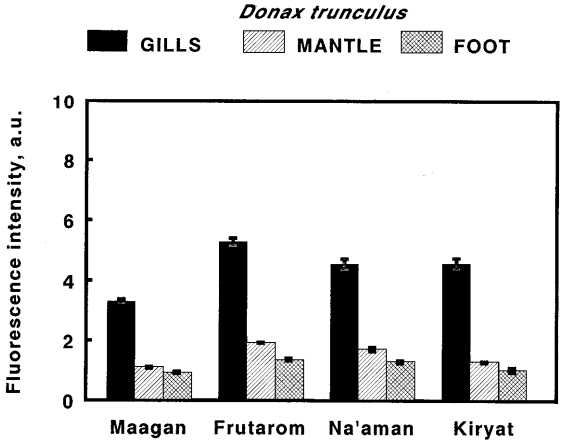

Fig. 6 Paracellular permeability of gill and mantle epithelia of *D. trunculus* from different sampling sites along the Israeli Mediterranean shore. Number of specimens: Maagan Michael 20; Frutarom 35; Na'aman River estuary 20; Kiryat Yam 10

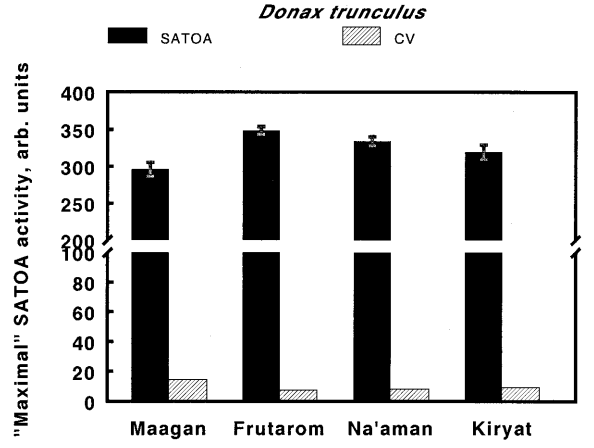

Fig. 5 Mean "maximal" SATOA activities in the gills of *D. trunculus* from different sampling sites along the Israeli Mediterranean shore. Number of specimens: Maagan Michael 80; Frutarom 80; Na'aman River estuary 80; Kiryat Yam 40. *CV* Coefficient of variation

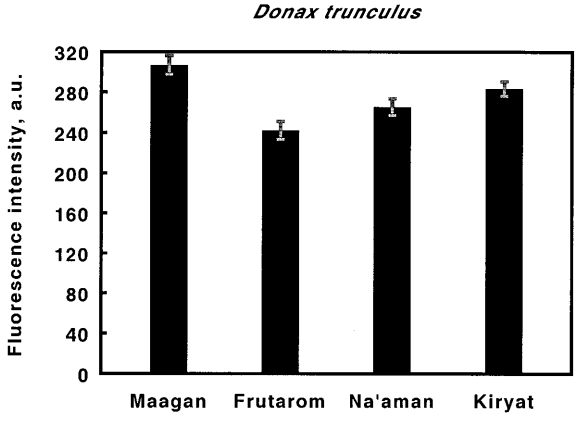

Fig. 7 Intralysosomal neutral red (NR) accumulation in gill epithelium of *D. trunculus* from different sampling sites along the Israeli Mediterranean shore. Number of specimens: Maagan Michael 10; Frutarom 10; Na'aman River estuary 10; Kiryat Yam 10

and highest in specimens from the polluted sites, which exhibited a significant increase in permeability (Fig. 6). Significant seasonal variations were not detected.

Intralysosomal accumulation of NR in gill epithelium

In *D. trunculus*, *C. florida* and *D. histrio*, NR accumulation was the lowest in bivalves from the polluted sites (Fig. 7). Significant seasonal variations were not detected.

ChE activity

In gills of *D. trunculus* the ChE ranged from 9.1±3.3 nmol·min^{-1}·mg^{-1} protein in specimens from Maagan Michael to 16.1±5.1 nmol·min^{-1}·mg^{-1} protein in specimens from Kiryat Yam. No significant decrease in ChE activity in gills of *D. trunculus* from Frutarom was detected (Fig. 8). In *M. edulis* the ChE activity in the gills exhibited marked seasonal variability and was significantly higher in hemolymph than in the gills. Only in September 1997 did specimens from Spiekeroog demonstrate significantly higher ChE activity both in the gills and hemolymph as compared to those from other sampling sites (Table 5).

Non-specific esterase

The non-specific esterase (NSE) activities in epithelial cells of gills, mantle and hepatopancreas of *D. trunculus* from the Mediterranean and *C. florida* and *D. histrio* from the Red Sea decreased significantly in the

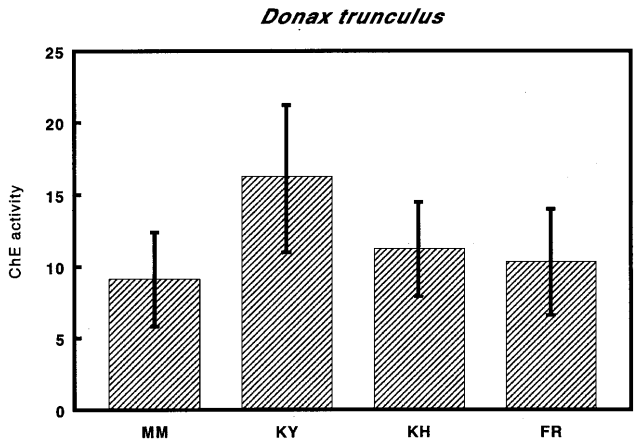

Fig. 8 Cholinesterase (ChE) activity in supernatant fraction from gills of *D. trunculus* from different sampling sites along the Israeli Mediterranean shore. Activity presented as mean±SD, in nmol·min^{-1}·mg^{-1} protein, 10 specimens from each site. *MM* Maagan Michael; *KY* Kiryat Yam; *KH* Kiryat Haim; *FR* Frutarom

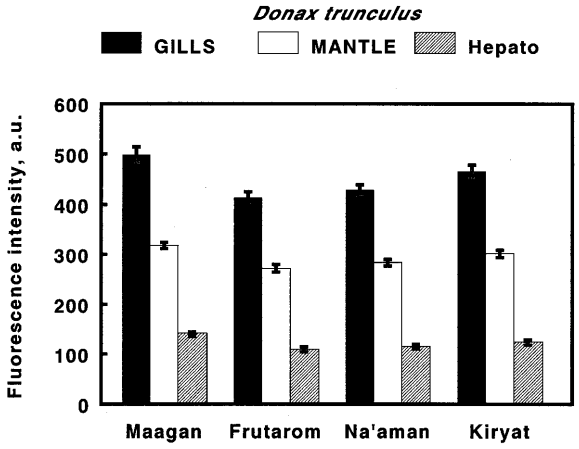

Fig. 9 Non-specific esterase activity in gill, mantle and hepatopancreas epithelium of *D. trunculus* from different sampling sites along the Israeli Mediterranean shore. Number of specimens: Maagan Michael 80; Frutarom 80; Na'aman River estuary 80; Kiryat Yam 40

tissues of molluscs from the polluted sites, e.g. Frutarom, Na'aman River and Ardag (Fig. 9). NSE activities in these molluscs showed no significant seasonal variability.

Catecholamines in nerve ganglia

The intensity of formaldehyde-induced catecholamine fluorescence in nerve ganglia of *D. trunculus, C. florida* and *D. histrio* from different stations exhibited a significant decrease in specimens from the polluted sites (Fig. 10). Seasonal variability was not detected.

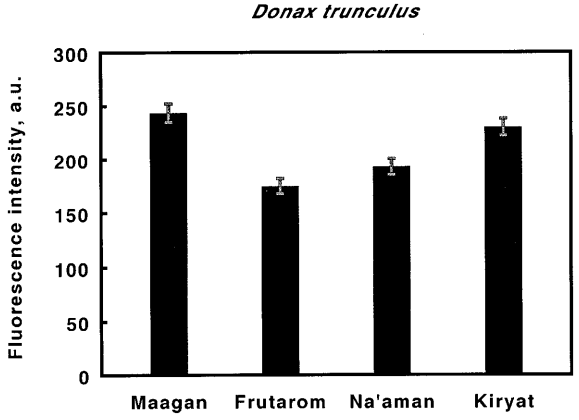

Fig. 10 Formaldehyde-induced catecholamine fluorescence in nerve ganglia of *D. trunculus* from Maagan Michael, Frutarom, Na'aman River estuary and Kiryat Yam. (10 specimens from each site)

Table 5 Activity of cholinesterase in different tissues of *Mytilus edulis* and *Platichthys flesus* in nmol min^{-1} mg^{-1} protein (means±SD). Reference sites in *italics*; *significantly different to reference site (P<0.001)

Species and tissue	Site of collection	Sep 95	Jan 96	Apr 96	Jul 96	Apr 97	Sep 97
M. edulis	Cuxhaven	–	10±2	5±1	7±2	7±2	9±2*
Gills	Eider	–	–	8±1	8±2	11±2	8±2*
	Tiefe Rinne	–	14±10	4±1	9±3	10±3	9±2*
	Spiekeroog	–	–	–	–	–	21±5
M. edulis	Cuxhaven	–	65±33	–	35±14	102±59	121±42
Hemolymph	Eider	–	–	–	50±16	–	43±11
	Dune	–	33±16	–	56±21	90±37.4	49±12
P. flesus	Cuxhaven	121±25	141±14	180±21	138±16	70±8	54±8*
Brain	Eider	126±30	136±16*	169±20	107±18	88±11	86±24
	Tiefe Rinne	143±12	196±24	156±20	138±16	78±9	40±5
	Spiekeroog	–	–	–	–	95.6±16	80±19
P. flesus	Cuxhaven	72±29	132±43	–	52±19*	104±37	80±19
Muscle	Eider	65±28	110±44	–	29±4	119±49	65±24
	Tiefe Rinne	46±18	113±55	–	82±32	106±39	63±25
	Spiekeroog	–	–	–	–	77±26	82±23

Table 6 GST activity in gills of *Mytilus edulis* and hepatic cytosol fraction of *Platichthys flesus* in nmol min^{-1} mg^{-1} protein (means±SD). Reference sites in *italics*; *significantly different to reference sites (P<0.001)

Species and tissue	Site of collection	Jan 96	Apr 96	Jul 96	Apr 97
M. edulis	Cuxhaven	–	–	17.6±4	56±25*
Gills	Eider	–	–	27±3	13±5
	Dune	–	–	30±8	18±15
P. flesus	Cuxhaven	36±13	90±21	41±16	42±11
Liver	Eider	37±12	72±30	46±11	52±23
	Tiefe Rinne	47±24	72±28	66±20	52±19
	Spiekeroog	–	–	–	38±8

Table 7 DNA fragmentation (–log F values) in gills of *Mytilus edulis* and liver of *Platichthys flesus*. Reference sites in *italics*; *significantly different to reference sites

Species and tissue	Site of collection	Jul 96	Oct 96	Apr 97	Sep 97
M. edulis	Cuxhaven	0.24±0.05	0.39±0.04	–	0.39±0.07
Gills	Eider	–	0.49±0.09	–	0.55±0.05*
	Dune	0.39±0.14	0.41±0.12	–	0.32±0.05
	Spiekeroog	–	–	–	0.37±0.08
P. flesus	Cuxhaven	0.43±0.12	0.47±0.09*	0.44±0.07	0.52±0.12
Liver	Eider	0.36±0.05	0.58±0.12*	0.67±0.05*	0.55±0.12*
	Tiefe Rinne	0.44±0.09	0.28±0.07	0.57±0.07*	0.36±0.07*
	Spiekeroog	–	–	0.45±0.08	0.45±0.08

Glutathione-S-transferase activity

The mean GST activity in gills of *M. edulis* was measured as 26±17 nmol·min^{-1}·mg^{-1} protein (n=62 mussels). GST activity exhibited marked seasonal variability (Table 6). In gills of *M. edulis* from Cuxhaven in April 1997, GST activity was significantly higher as compared to those in molluscs from the reference site, Helgoland/Dune (Table 6).

Alkaline DNA-unwinding assay

The alkaline DNA-unwinding assay showed a damage in DNA isolated from soft tissues of *D. trunculus* expressed as negative log F values, which were significantly higher in specimens from Frutarom as compared to specimens from Maagan Michael (Fig. 11). Negative log F for DNA from gills of *M. edulis* demonstrates significant differences between Eider and both reference sites only in samples from September 1997 (Table 7).

In situ acidic DNA-unwinding test

Differential staining of single- and double-stranded DNA by AO demonstrated a significant increase in one-stranded DNA breaks in the gill nuclei of *D. trunculus, C. florida* and *D. histrio* from the polluted sites as compared to those of specimens from the clean sites (Fig. 12a). This test also showed that the relative frequency of the one-stranded DNA breaks was significantly higher in the gill nuclei of *M. edulis* from Eider and Elbe-Cuxhaven than in the nuclei of the same molluscs from Helgoland (Fig. 12b).

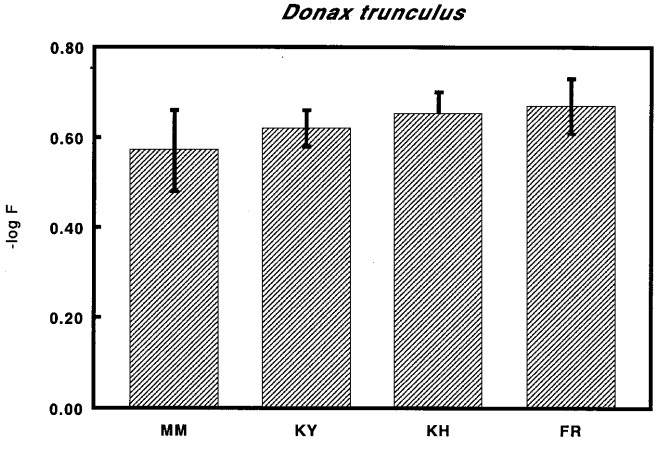

Fig. 11 DNA damage unmasked by alkaline unwinding in *D. trunculus* from different stations along the Israeli Mediterranean shore. Data presented as –log F values, mean±SD, 10 specimens from each site. *MM* Maagan Michael; *KY* Kiryat Yam; *KH* Kiryat Haim; *FR* Frutarom

Micronucleus test

The micronucleus test showed that the frequency of hemocytes containing micronuclei was lowest in *D. trunculus, C. florida* and *D. histrio* from the clean sites and highest in the same species from the polluted sites. The significant differences for *D. trunculus* are shown in Fig. 13a. The frequency of micronucleated hemocytes in *M. edulis* was lowest in specimens from Helgoland and highest in the molluscs from Elbe-Cuxhaven (Fig. 13b). There was a strong significant negative correlation between this frequency of micronucleated cells and MXRtr activities in the gills of the same specimens. A slope of regression line was higher for molluscs from the polluted sites.

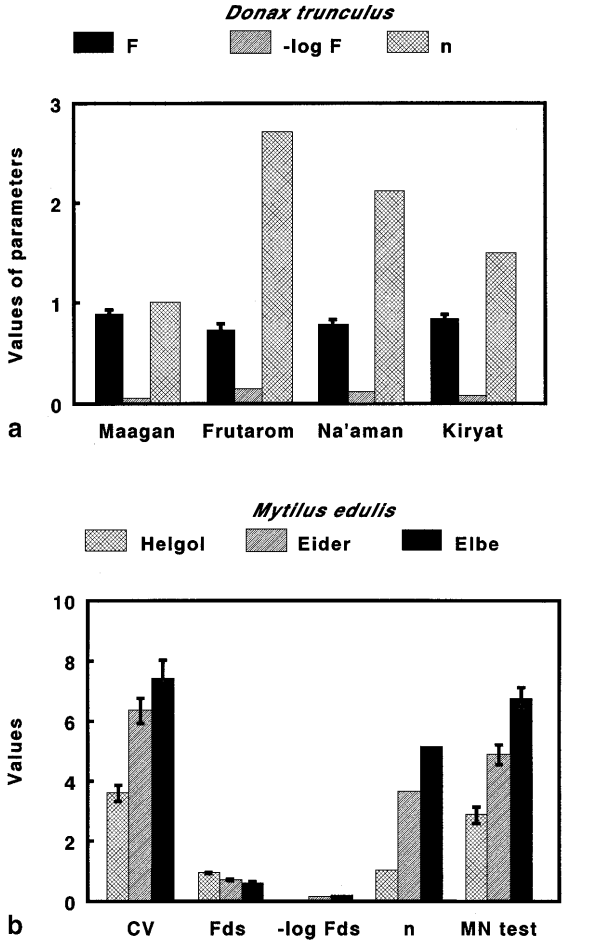

Fig. 12 Fraction of double-stranded DNA (*F* in **a**, *Fds* in **b**), relative frequency of single-stranded DNA breaks (–log *F* in **a**, –log *FdS* in **b**) and mean number of DNA lesions per unwinding units (*n*=–log Fds polluted/-log Fds clean) in gill nuclei of *D. trunculus* (**a**) from Maagan Michael (20 specimens), Frutarom (30 specimens), Na'aman River estuary (25 specimens) and Kiryat Yam (20 specimens) and *M. edulis* (**b**) from Helgoland, Eider and Elbe-Cuxhaven (5 molluscs from each station and 300 nuclei from each mollusc). *CV* of variation; *MN* micronucleus

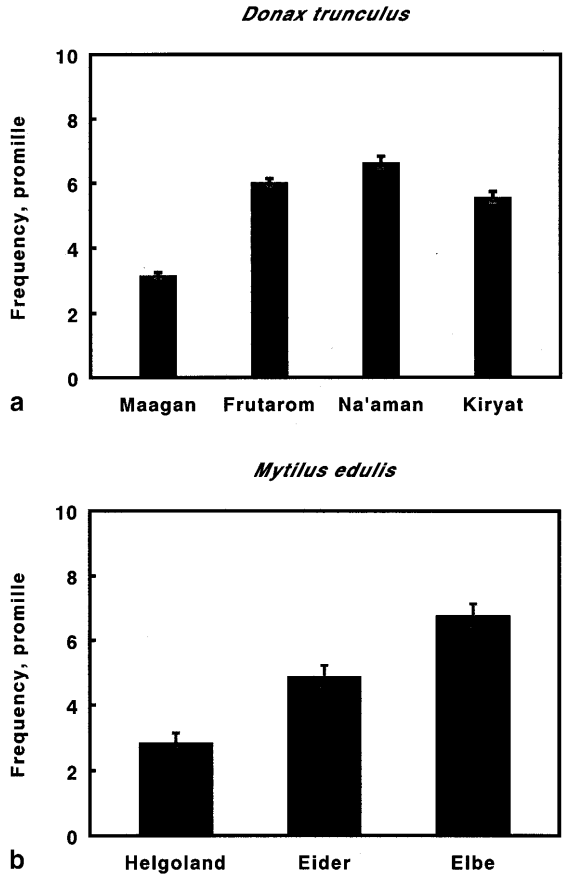

Fig. 13 Frequency of micronucleated hemocytes in *D. trunculus* (**a**) from Maagan Michael (80 specimens), Frutarom (120 specimens), Na'aman River estuary (80 specimens), and Kiryat Yam (40 specimens) and *M. edulis* (**b**) from Helgoland (73 molluscs), Eider (35 molluscs) and Elbe-Cuxhaven (109 molluscs). Three thousand nuclei from each molluscs were examined

Cyto- and histopathological alterations

All quantified cyto- and histopathological alterations in different organs of *D. trunculus*, *C. florida*, *D. histrio* and *M. edulis* from different stations are presented on Fig. 14. A strong negative correlation was detected between the epidemiological characteristic and MXRtr or SATOA activity in the same animal. Pathological alterations detected in different organs of these bivalves are presented below (Fig. 15–19).

Gills Loss of cilia, swelling, hydropic dystrophy, enlargement of lysosomes, formation of autophagosomes, small foci of epithelial cells disintegration and desquamation was expressed in the gills of all molluscs from the polluted sites. Marked necrotic and apoptotic alterations of gill nuclei, edema and swelling of gill filaments and their focal hemocytic infiltration were detected in molluscs from very polluted sites like Frutarom, Ardag (Israel) and Cuxhaven and Eider (Germany) and are exemplified in Figs. 15–17.

Mantle and siphons Mean thickness of mucus layer on the surface of the mantle increased in specimens from the polluted sites. Typical pathological alterations in mantle epithelium were cell swelling, enlargement of lysosomes, formation of autophagosomes and hydropic dystrophy. Small foci of epithelial cells desegregation and desquamation were detected in specimens from the polluted sites, especially Frutarom, Ardag-Cages, Cuxhaven and Eider. Foci of anomalous permeability to FLU in frontier epithelia coincided with small foci of epithelial cell desquamation.

Hepatopancreas Marked cytoplasm swelling and vacuolization including enlargement of lysosomes containing lipofuscin, numerous autophagic vacuoles and small stromal foci of hemocytic infiltration, were found in specimens from the polluted sites, especially Frutarom, Ardag-Cages, Cuxhaven and Eider, as shown in Figs. 18 a, b.

Kidney Marked swelling and hydropic degeneration of epithelial cells, enlargement of their lysosomes, autophagic vacuoles formation and desquamation of some cells

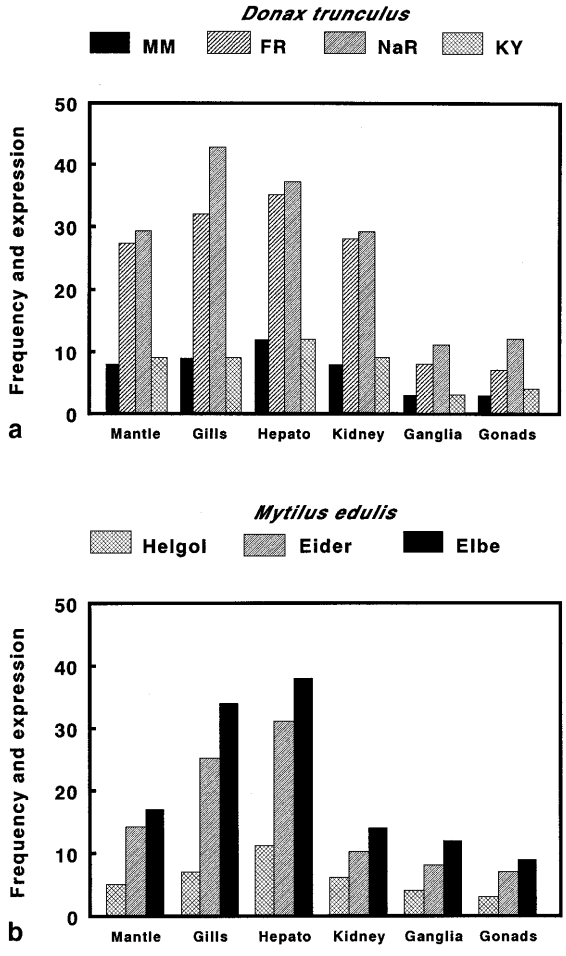

Fig. 14 Expression and frequency characteristics of pathological alteration in organs of *D. trunculus* (**a**) from Maagan Michael (*MM*), Frutarom (*FR*), Na'aman River estuary (*NaR*) and Kiryat Yam (*KY*) (100 specimens from each station) and *M. edulis* (**b**) from Helgoland (73 molluscs), Eider (35 molluscs) and Elbe-Cuxhaven (109 molluscs). Characteristic are expressed as percent of animals with pathological alteration in given organ x coefficient of expression. This coefficient was 1.0 for maximal expression, 0.75 for marked expression and 0.5 for weak expression

were detected in tubular epithelium in *D. trunculus, C. florida* and *D. histrio* specimens from polluted sites in the Mediterranean and Red Sea.

Nerve ganglia Swelling and hydropic dystrophy of some neurons were detected only in specimens from the polluted sites, particularly Frutarom, Ardag-Cages and Cuxhaven.

Phagocytic activity

The results of phagocytic activity tests in mussel hemolymph are summarized in Table 8. The numbers of hemocytes in all mussels collected from piers and rocks of the harbour and estuary sites were approximately 500000 cells/ml. Mussels dredged from the sea bottom at Kiel Harbour (Baltic Sea) also showed a distinct decrease in hemocytes number, most probably due to high pollution of the sediment. Relative phagocytosis activity

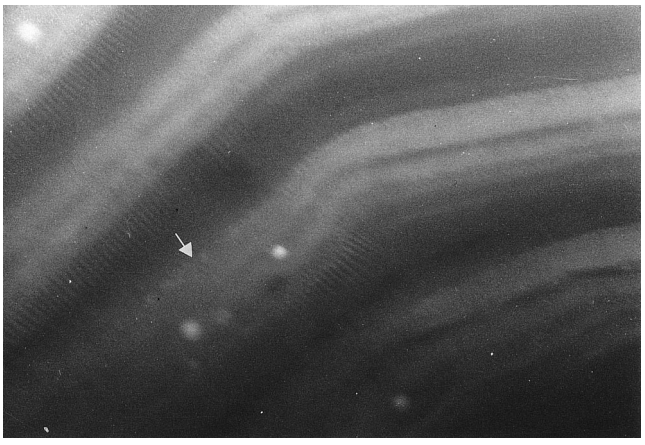

Fig. 15 Contact fluorescent microscopy of gills of living *D. trunculus* from Frutarom after incubation with acridine orange. Loss of cilia in some cells (*arrow*). 600×

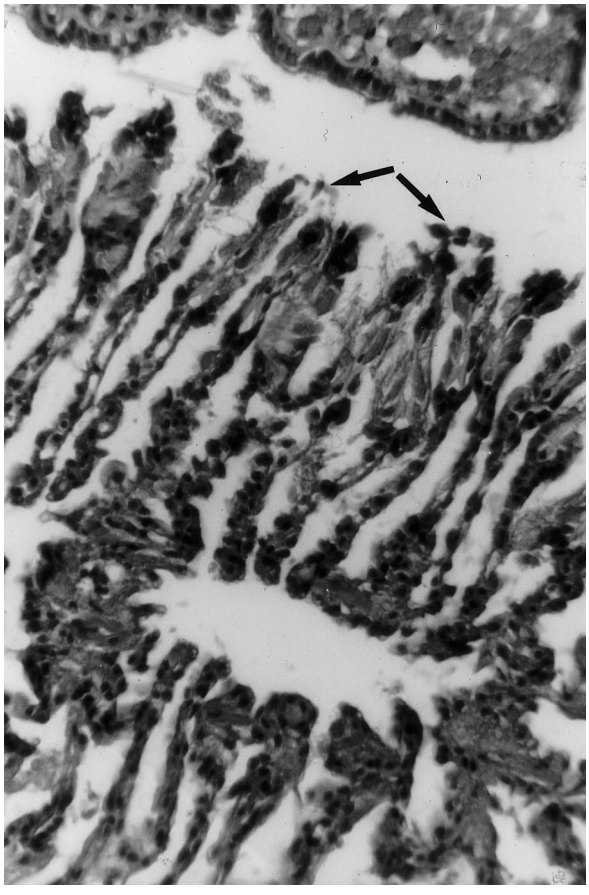

Fig. 16 Focal desquamation of gill epithelial cells (*arrows*) in *D. trunculus* from Frutarom. Conventional slide stained with hematoxylin and eosin. 300×

per cell, expressed as phagocytosis index, resulted in no significant differences (approximately 250 rfu/500000 hemocytes) between samples collected from pier and rocks of the estuaries. A significant increase in the phagocytosis index was found in mussels sampled from the mud beds of the Wadden Sea.

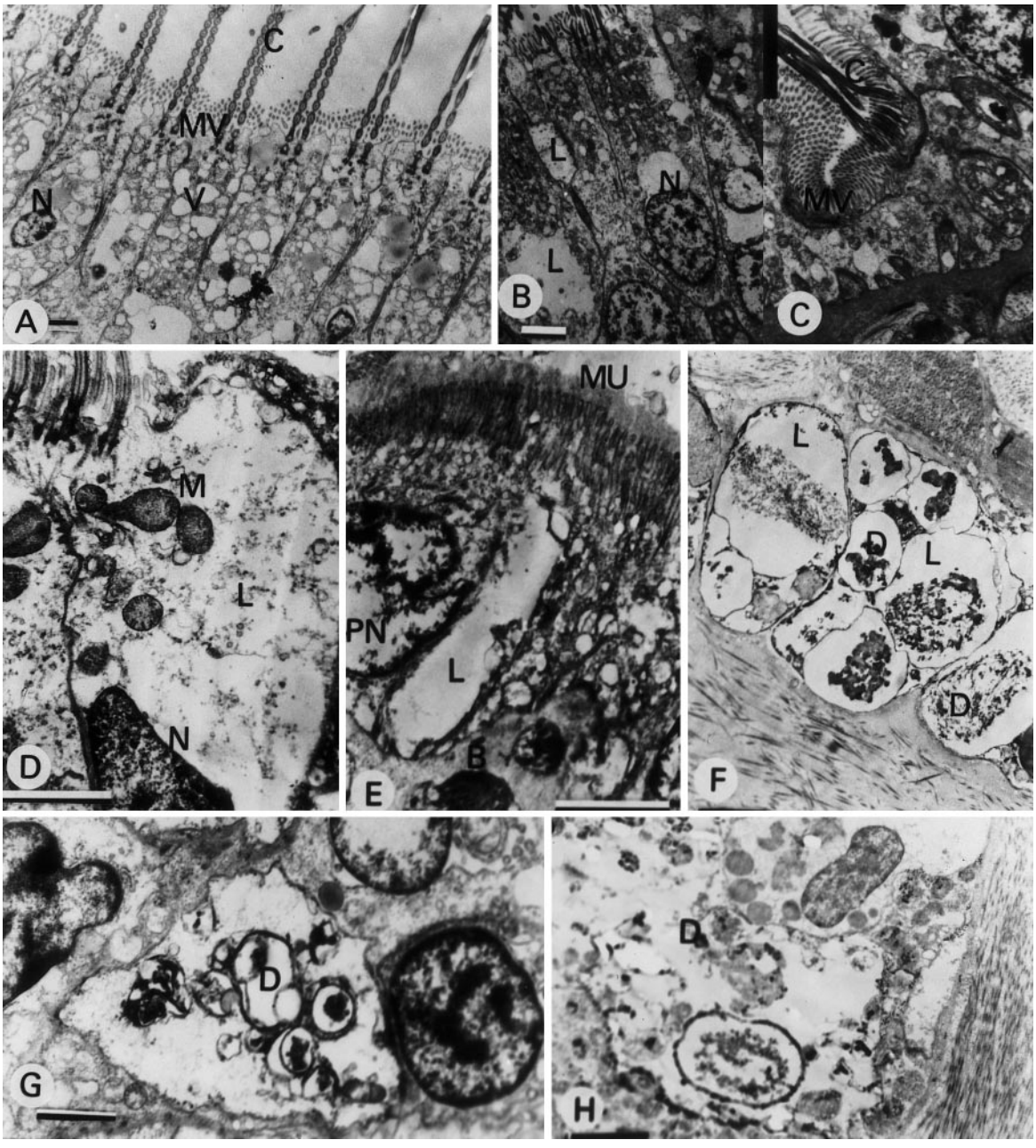

Fig. 17 Transmitted electron microscopy of gills of *D. trunculus* from Maagan Michael (**A**) and Frutarom (**B**–**H**). **B** Swelling of epithelial cells and large vacuole; **C**- degeneration of cilia base; **D**–**H** large autophagic vacuoles contained different cell organoids. Bars 2µm

Table 8 Phagocytic activity in hemolymph of *M. edulis* from different estuary sites (April 1998)

	Cuxhaven	*Eider*	Kiel (piers)	Kiel (sea bottom)
Hemocytes/ml	$3 \cdot 10^5$	$4 \cdot 10^5$	$6 \cdot 10^5$	$8 \cdot 10^3$
Phagocytosis index, rfu/5×10^5 hemocytes	282±185	287±217	230±275	12000±6100
Median	228	245	144	9600

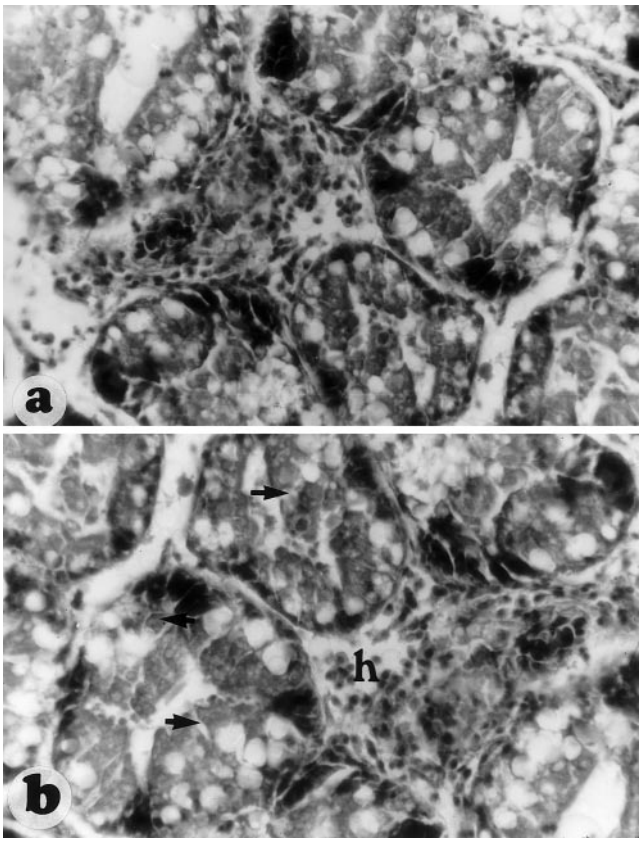

Fig. 18 a, b Marked swelling and vacuolization of glandular epithelial cells (*arrows*) in hepatopancreas (*h*) of *D. trunculus* from Frutarom. **a** Contact epimicroscopy, optical section through fixed tissue block stained with hematoxylin and eosin. **b** Conventional slide from same site stained with hematoxylin and eosin. Both 300×

Mediterranean and Red Sea gastropods:
Patella coerulea and *Cellana rota*

MXRtr and SATOA activity in surface epithelia

MXRtr and SATOA activities were detected in all surface epithelia of adult *Patella coerulea* and its cotaxon *Cellana rota*, and were highest in the pseudogill and lowest in foot epithelia. Mean maximal MXRtr and SATOA activity were highest in specimens from the polluted sites, Shemen Beach and Ardag, and lowest in specimens from the clean sites, Maagan Michael, Achziv and MBL (Fig. 20).

Paracellular permeability of surface epithelia

The permeability to FLU in all the studied specimens was highest in specimens from the polluted sites (Fig. 21).

Intralysosomal NR accumulation

NR accumulation was the highest in *P. coerulea* and *C. rota* from the clean sites and lowest in specimens from the polluted sites (Fig. 22).

ChE activities

In *C. rota* ChE activities were measured separately in soluble fraction from foot muscle and other soft tissues. ChE activities in foot muscle were higher than in soft tissues. Although gastropods from the clean site (LT) had the lowest ChE activity compared to specimens from the most polluted site (fish cages in Ardag), however, the differences between stations were not significant (Fig. 23).

Non-specific esterase activity

In epithelial cells of pseudogills, mantle and hepatopancreas of *P. coerulea* and *C. rota*, NSE activities were higher in specimens from the clean sites, while activity decreased significantly in the tissues of molluscs from the polluted sites (Fig. 24). No seasonal variations were detected.

Catecholamines in nerve ganglia

Intensity of formaldehyde-induced catecholamine fluorescence of nerve fibres and neurones in nerve ganglia of *P. coerulea* and *C. rota* exhibited a decrease in specimens from the polluted sites (Fig. 25). No seasonal variations were detected.

Alkaline and acidic DNA unwinding tests

Damage to DNA isolated from foot tissues of *C. rota* was the lowest in specimens from the clean site (LT) and the highest in molluscs from the polluted site (Ardag-Cages) (Fig. 26). In situ acidic DNA-unwinding test with AO also demonstrated a significant increase in DNA breaks in the pseudogills nuclei of *P. coerulea* and *C. rota* from the polluted sites as compared to those from the clean sites (Fig. 27). No seasonal variations were detected.

Micronucleus test

The micronucleus test showed that the frequency of hemocytes containing micronuclei was the lowest in *P. coerulea* and *C. rota* from clean sites and the highest in populations of the same species from the polluted sites (Fig. 28). No seasonal variations were detected. A significant negative correlation was found between the frequency of micronucleated cells in *P. coerulea* and *C. rota* from all stations and maximal MXRtr activity in the gills of the same specimens. The slope of regression line was higher for molluscs from the polluted sites.

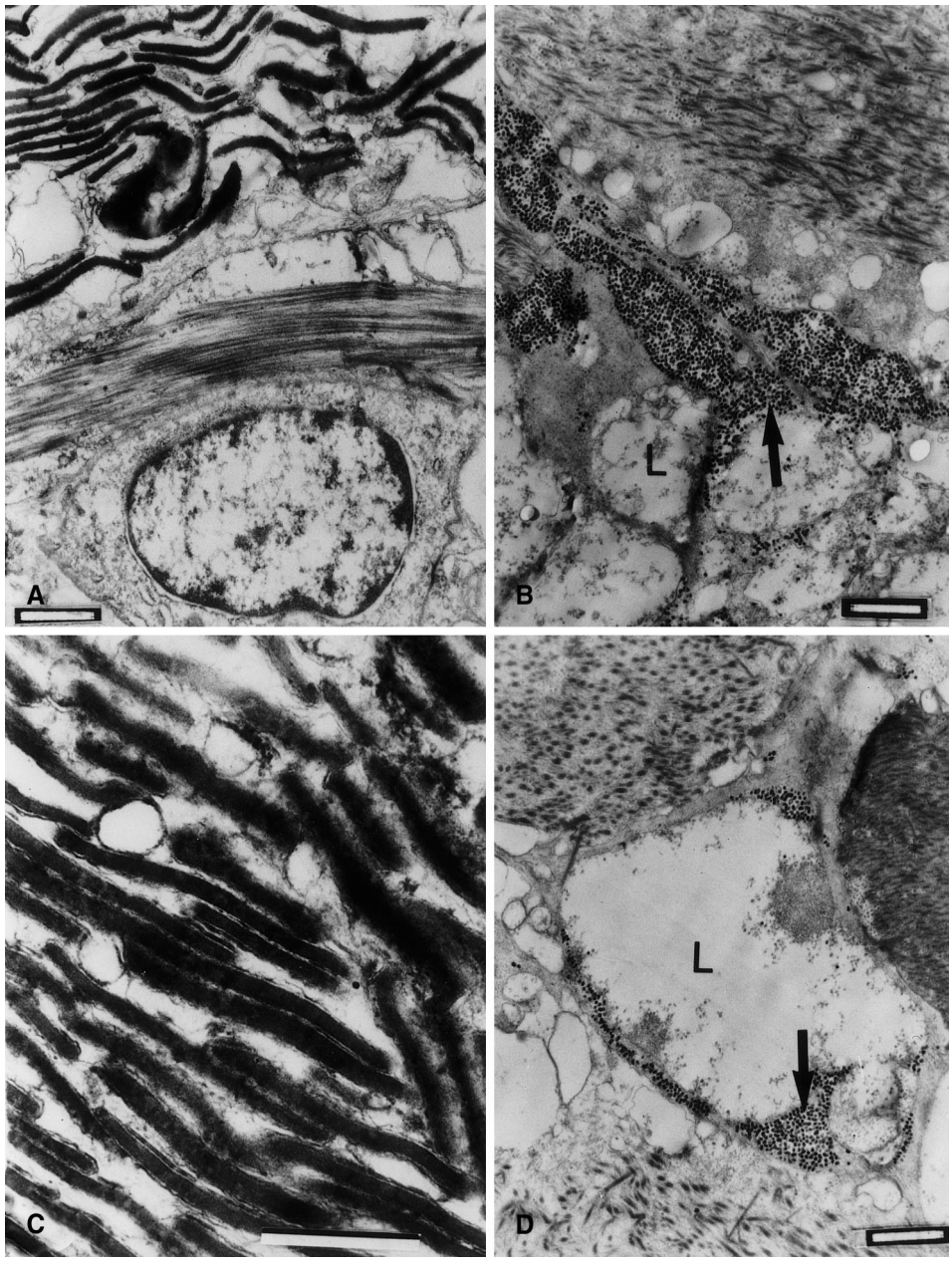

Fig. 19 A–D Transmitted electron microscopy of siphon of *D. trunculus* from Frutarom. **A** Basal part of cell with nucleus. Chromatin condensation along the nuclear envelope. **B** Large vacuole (*L*) and numerous small pigment granules (*arrow*). **C** Hypertrophic endoplasmic reticulum. **D** Large vacuole (*L*) containing pigment granules (*arrow*) and dense material. *Bar* 2 μm

Cyto- and histopathological alterations

Quantified epidemiological characteristics of populations of *P. coerulea* and *C. rota* from different clean and polluted sites are presented on Fig. 29. A strong negative correlation was detected between the epidemiological characteristic of specimens and MXRtr and SATOA activity in the same animal. Common main pathological alterations detected in different organs of *P. coerulea* and *C. rota* were similar to those detected in the study by us of bivalve molluscs. Alterations in the pseudogills epithelium were similar to those in the mantle epithelium.

Fish from the North Sea:
Limanda limanda and *Platichthys flesus*

MXRtr and SATOA activity

During one of the “Walther Herwig III” cruises we investigated “initial” MXRtr activity in the gill epithelium, kidney (renal proximal tubules), liver (bile canaliculi of liver trabecules), and SATOA activity in the kidney and liver of *Limanda limanda* from five different station. The MXRtr activity in the basolateral membrane of renal proximal tubules was highest in specimens from station 15 and lowest in fish from station 42. The MXRtr and SATOA activity in the gills, kidney and liver was also significantly different in specimens from different sampling stations (Fig. 30).

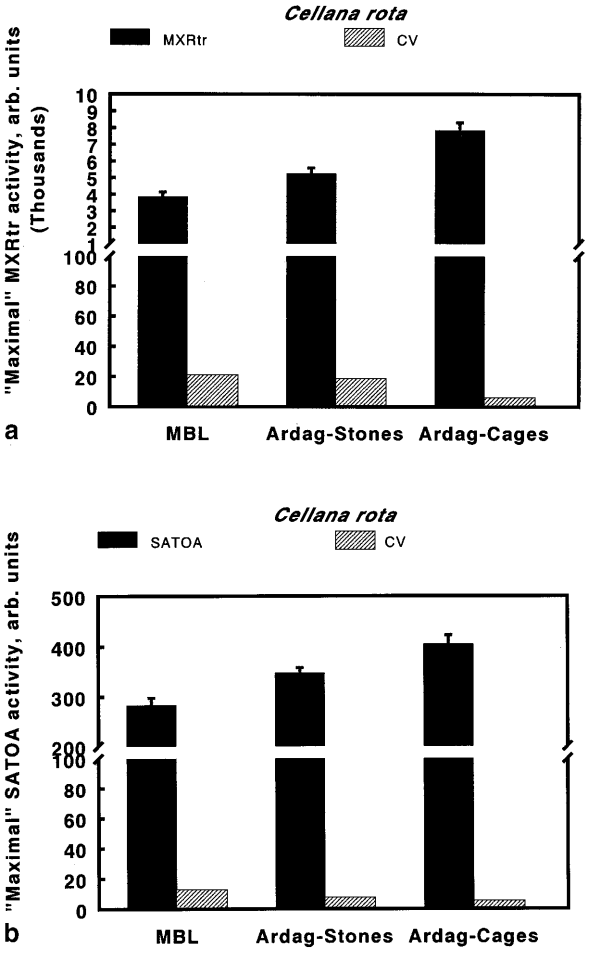

Fig. 20 Mean "maximal" MXRtr (**a**) and SATOA (**b**) activities in pseudogills of *C. rota* from different sampling sites in the Gulf of Aqaba: *MBL* (25 specimens), *Ardag-Stones* (25 specimens) and *Ardag-Cages* (7 specimens). *CV* Coeffizient of variation

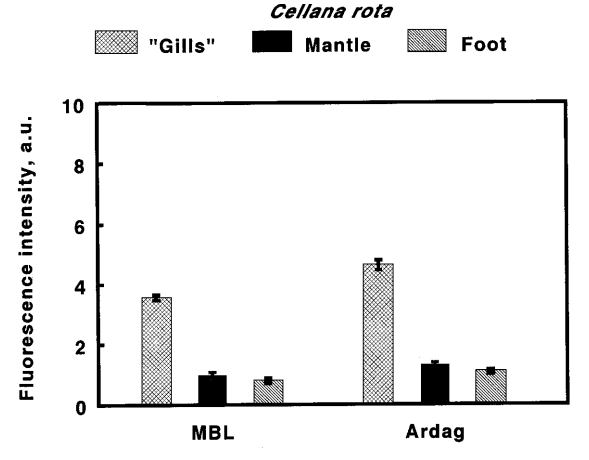

Fig. 21 Paracellular permeability of surface epithelia of *C. rota* from *MBL* (23 specimens) and *Ardag* (22 specimens)

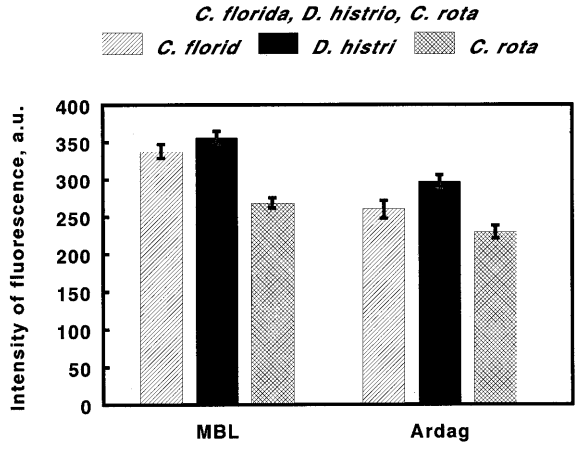

Fig. 22 Intralysosomal neutral red accumulation in gill epithelium of Red Sea molluscs (*C. florida, D. histio and C. rota*) from MBL and Ardag. Achziv (10 specimens for each species and site)

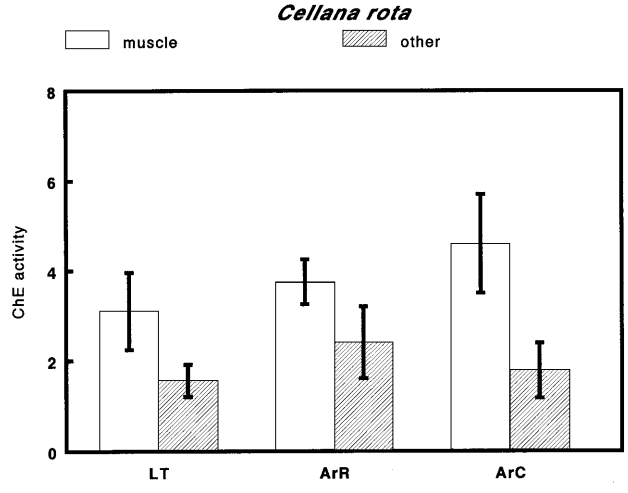

Fig. 23 Cholinesterase activity in muscle tissue and other soft tissues of *C. rota* from different sampling sites in the Gulf of Aqaba. Activity presented as mean±SD, nmol·min^{-1}·mg^{-1} protein, 5 specimens from each station. *LT* Light tower near *MBL*; *ArR* rocks along North Beach (Ardag); *ArC* fish cages in Ardad

GST activity

Mean GST activity in *P. flesus* from all sampling sites ranged from 36 to 90 nmol·min^{-1}·mg^{-1} protein and showed marked seasonal variability but no significant differences between the sampling sites (Table 6). In all samples the highest GST activity was recorded in April 1996. At Spiekeroog, the collected fish showed lowest GST activity, with a mean value of 38±8 nmol·min^{-1}mg^{-1} protein.

ChE activity

ChE activity of flounder from the North Sea reflected differences between tissues as well as temporal and site-

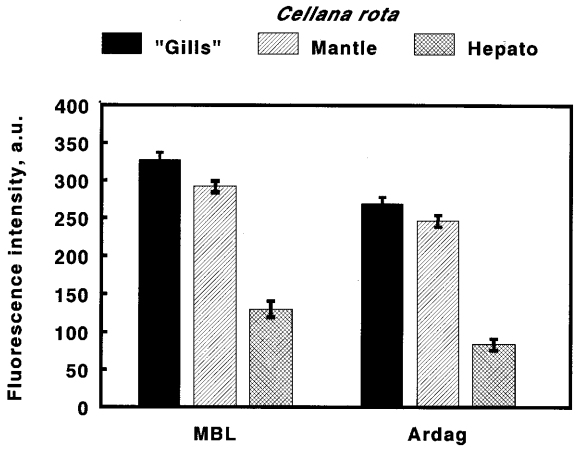

Fig. 24 Non-specific esterase activities in pseudogill, mantle and hepatopancreas epithelial cells of *C. rota* from different sampling sites in the Gulf of Aqaba: *MBL* and *Ardag* Stones (25 specimens from each station)

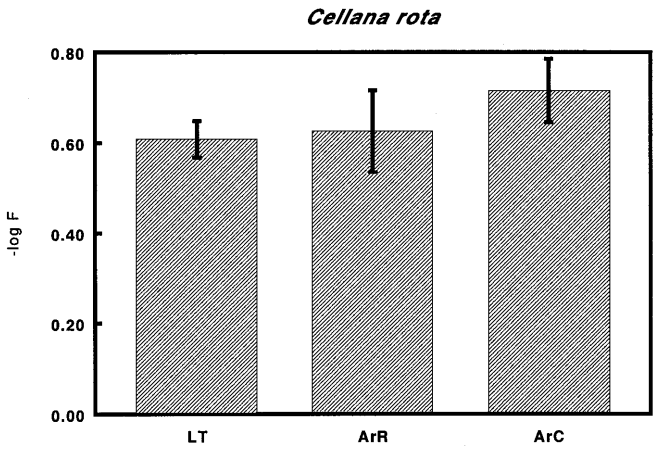

Fig. 26 DNA damage detected by alkaline unwinding of DNA isolated from foot tissue of *C. rota* from different sampling sites in the Gulf of Aqaba. Data presented as –log F values, mean±SD, 10 specimens from each site. *LT* Light tower near MBL; *ArR* rocks along North Beach (Ardag); *ArC* fish cages in Ardag

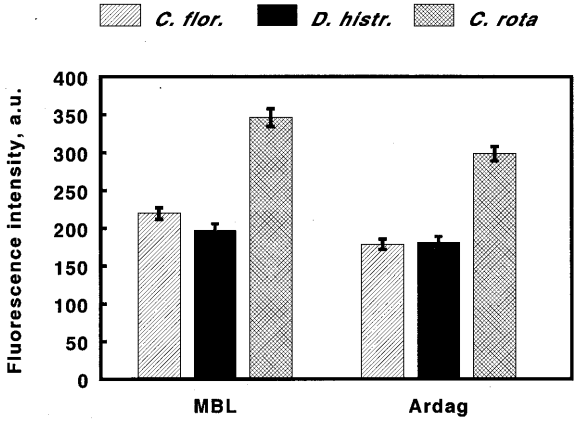

Fig. 25 Formaldehyde-induced catecholamine fluorescence in nerve ganglia from different sampling sites in the Gulf of Aqaba: *MBL* and *Ardag*-Stones (5 specimens from each station)

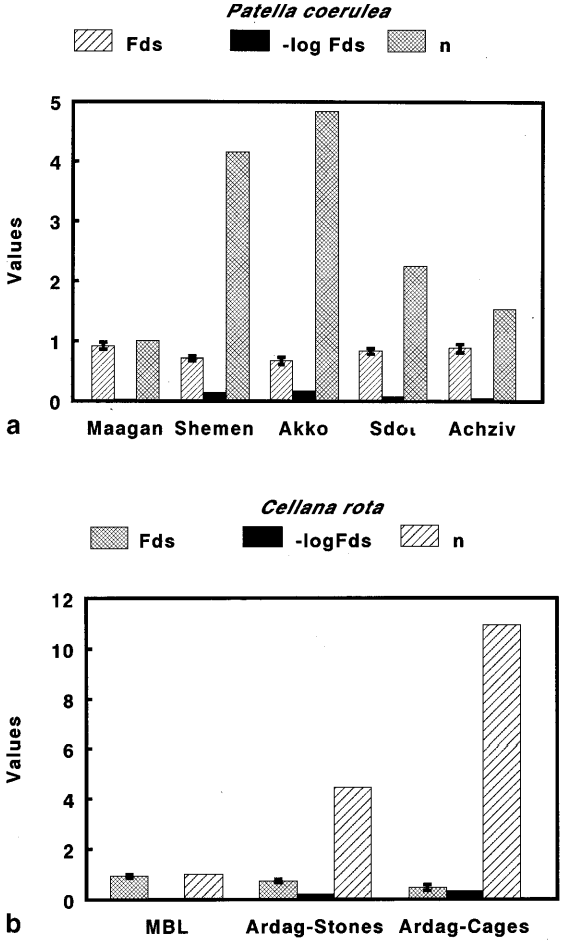

Fig. 27 Fraction of double-stranded DNA (*Fds*), relative frequency of single-stranded DNA breaks (*–log Fds*) and mean number of DNA lesions per unwinding units (*n*=–log Fds pol/–log Fds clean) in pseudogill nuclei of *P. coerulea* (**a**) from Maagan Michael, Shemen Beach, Akko, Sdot Yam and Achziv (10 specimens from each station) and *C. rota* (**b**) from *MBL*, *Ardag-Stones* and *C. rota* (**b**) from *MBL*, *Ardag-Stones* and *Ardag-Cages* (5 specimens from each station)

dependent patterns (Table 5). Brain tissue of *P. flesus* from all sampling sites had the highest ChE activity, with a mean of 118±56 nmol·min^{-1}·mg protein^{-1} (*n*=327), followed by muscle tissue of 86±44 nmol·min^{-1}·mg^{-1} protein (*n*=264). Brain ChE activity in *P. flesus* collected from all sites in 1997 was remarkably lower than that in fish collected during 1996 (Table 5). Significant differences between the clean (reference) and polluted sampling sites were detected only in January 1996 and September 1997. The highest mean ChE activity in flounders from the reference site Helgoland/Tiefe Rinne was about 196±24 nmol·min^{-1}·mg protein^{-1} when sampled in January 1996, and significantly higher than in samples from the estuary site the River Eider. In contrast, fish samples from the Tiefe Rinne in September 1997 had the lowest ChE activity throughout the entire sampling period, with a mean value of 40±5 nmol·min^{-1}·mg protein. Together with the Elbe site, the ChE activity of fish brain in the Tiefe Rinne in September 1997 was significantly

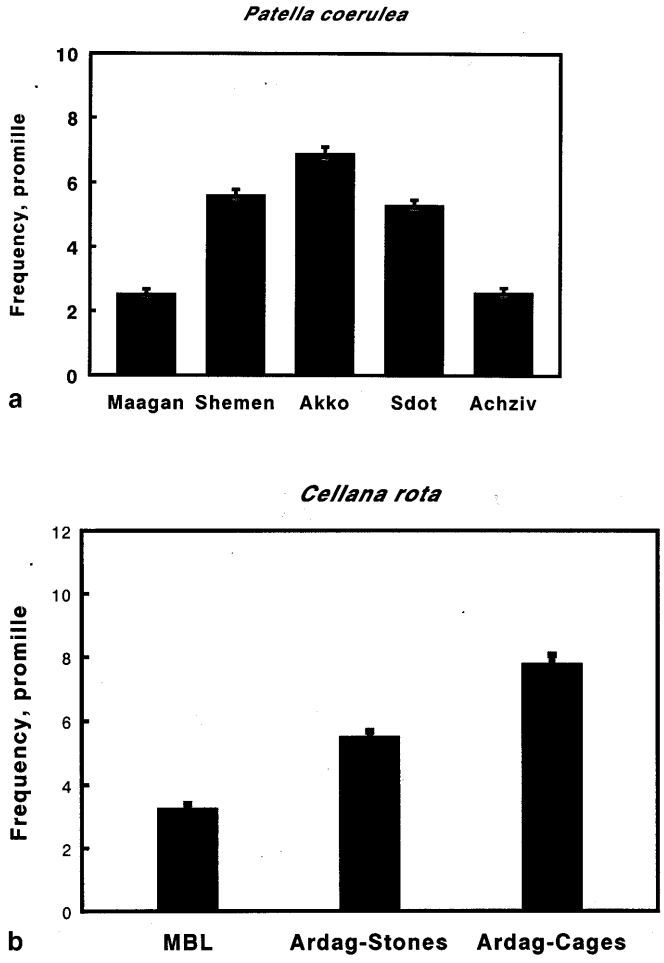

Fig. 28 Frequency of micronucleated hemocytes in *P. coerulea* (**a**) from Maagan Michael (25 specimens), Shemen Beach (45 specimens), Akko (35 specimens), Sdot Yam (25 specimens) and Achziv (20 specimens) and *C. rota* (**b**) from MBL and Ardag-Stones (10 specimens from each station) Ardag-Cages) (7 specimens)

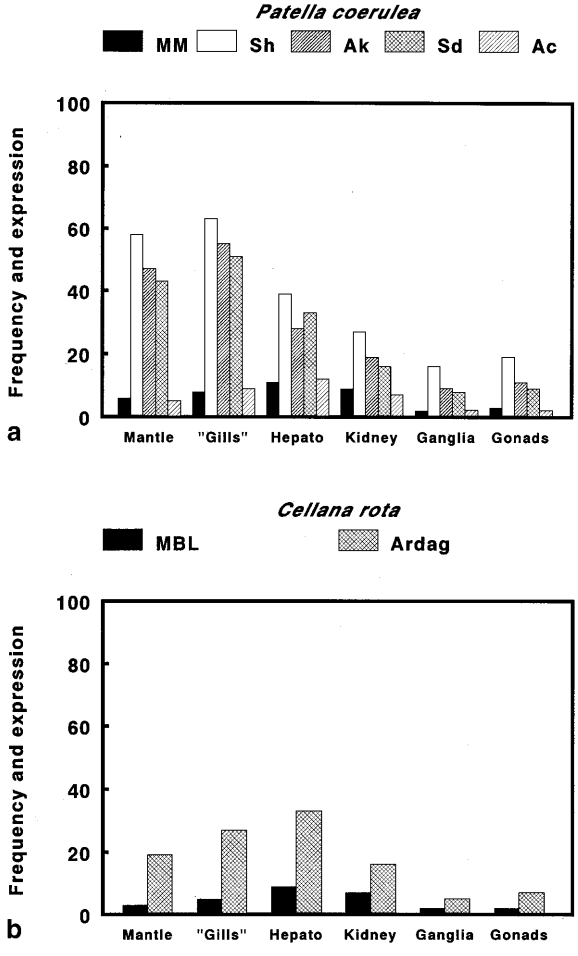

Fig. 29 Expression and frequency characteristics of pathological alteration in organs of *P. coerulea* (**a**) from Maagan Michael (*MM*), Shemen Beach (*Sh*), Akko (*Ak*), Sdot Yam (*Sd*) and Achziv (*Ac*) (50 specimens from each station) and *C. rota* (**b**) from *MBL* and *Ardag* (50 specimens from each station) Characteristics are expressed as percent of animals with pathological alteration in given organ × coefficient of expression. This coefficient was 1.0 for maximal expression, 0.75 for marked expression and 0.5 for weak expression

lower than at Spiekeroog. Muscle tissue of flounder from all sites indicated an increased ChE activity in July 1996 and decreased activity in January 1996 and April 1997. In July 1996 only was a significant decrease in ChE activity in flounder muscle from the Elbe and Eider estuaries ($P<0.001$).

Alkaline DNA unwinding

The damage to DNA isolated from liver of the flounder, *P. flesus*, expressed as negative log F values, showed some differences among stations. Negative log F values of between 0.28 and 0.67 were recorded for all samples from all stations (Table 7). In October 1996 fish samples from Cuxhaven and Eider sites had significantly enhanced negative log F values compared to the site Helgoland/Tiefe Rinne. In April and September 1997 the negative log F values were significantly higher in flounders from the Eider estuary than samples from Spiekeroog.

In situ acidic DNA unwinding

Differential AO staining allowed us to determine total DNA amount and two-stranded DNA fraction (F) in individual erythrocyte nuclei of *P. flesus* from Helgoland, Eider and the mouth of the Elbe (Elbe-Cuxhaven). The majority of nuclei (71.2%) in fish from Helgoland formed the major peak of total DNA content on the histogram (Fig. 31a), with extremities comprising only 1.47% of the nuclei. The peak of total DNA amount in fish from Eider included 67.2% of the nuclei and extremities comprised 2.5% (Fig. 31b). Finally, the peak of the histogram of fish from Elbe-Cuxhaven comprised 55.1% while extremities comprised 4.9% of all examined nuclei (Fig. 31c). Thus, DNA content per nucleus in fish from

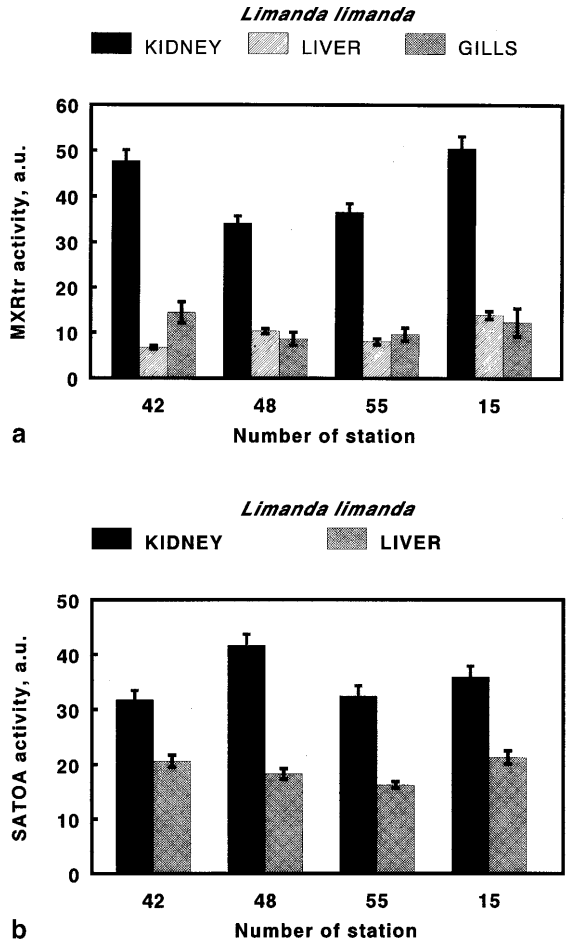

Fig. 30 "Initial" MXRtr (**a**) and SATOA (**b**) activities in organs of dab *Lurianda limanda* from different stations in the North Sea

Eider, and especially from Elbe-Cuxhaven, was significantly more variable than that in fish from Helgoland (Fig. 32). After acidic unwinding the number of DNA breaks was significantly higher in the erythrocyte nuclei of fish from Eider and Elbe-Cuxhaven than in those of fish from Helgoland (Fig. 32).

Micronucleus test

Frequency of micronuclei-containing erythrocytes was significantly higher in fish from Eider and especially Elbe-Cuxhaven than in fish from Helgoland (Fig. 32).

Cyto- and histopathological alterations

The main pathological alterations detected in different organs of *L. limanda* and *P. flesus* were as follows (see Fig. 33). Hyperproduction of mucus by glandular cells in skin of some fish from many stations, as well as edema and swelling of epithelial layer, and broadening of intracellular spaces. Focal desquamation of epithelial cells and small subepithelial lymphocytic infiltrates were de-

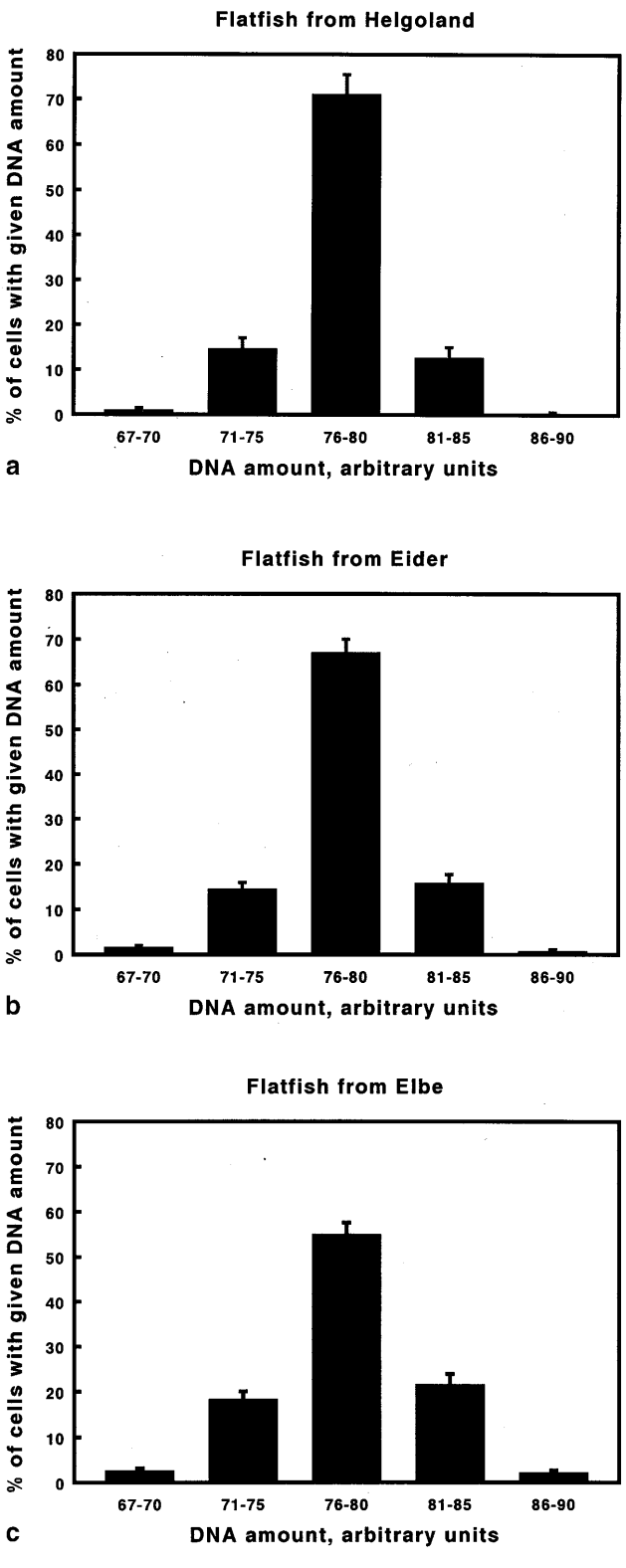

Fig. 31 Histograms of DNA content in erythrocyte nuclei of flatfish *P. flesus* from Helgoland (**a**), Eider (**b**) and Elbe-Cuxhaven (15 fish for each station)

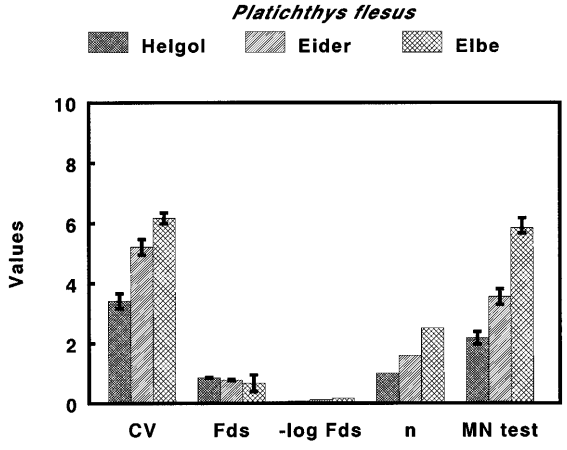

Fig. 32 Frequency of genotoxic and clastogenic lesions in individual erythrocyte nuclei of flatfish *P. flesus* from Helgoland, Eider and Elbe-Cuxhaven (15 fish for each station). Coefficient of variation *(CV)* for DNA content per nucleus; fraction of double-stranded DNA (*Fds*); negtive log of Fds (ll*og Fds*); number of DNA lesions per unwinding units (*n*); frequency of micronucleus containing erythrocytes (*MN*)

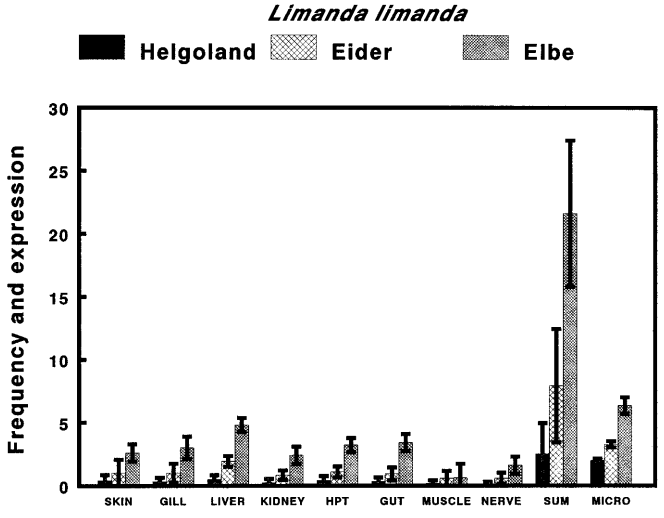

Fig. 33 Expression and frequency characteristics of cyto- and histopathological alterations in organs of dab, *L. limanda*, from Helgoland, Eider and Elbe-Cuxhaven (25 fish for each station). Characteristics are expressed as percent of animals with pathological alteration in given organ × coefficient of expression. This coefficient was 1.0 for maximal expression, 0.75 for marked expression and 0.5 for weak expression

tected in fish from Elbe-Cuxhaven. Similarly, edema and swelling of gill filaments, swelling and hydropic alterations of gill epithelial cells, focal breaks of intercellular junction, desquamation of epithelial cells and small erosia were detected in particular in fish from Elbe-Cuxhaven, Eider and station 15. Foci of anomalous permeability in the gills coincided with small foci of epithelial desquamation in all *L. limanda* examined during the "Walther Herwig III" cruise. Regarding internal organs, marked nuclear polymorphism, focal fatty degeneration and hyaline areas (hypertrophy of smooth endoplasmic reticulum) were observed in the liver of fish from Eider and station 15. Severe fatty degeneration, megalocytosis of hepatocytes, apoptotic bodies, small necrotic foci and macrophage aggregates in liver were detected in the liver of fish from station 15 and especially Elbe-Cuxhaven. Pathological alterations such as swelling and hydropic degeneration were detected in epithelial cells of some proximal tubules of trunk kidney in fish from Eider. Severe hydropic and hyaline degeneration, hyaline cylinders in the lumen of some proximal tubules, and stromal focal lymphocytic infiltration were also found in the kidney of fish from Elbe-Cuxhaven and station 15. The head, kidney and spleen of fish from Elbe-Cuxhaven and station 15 also revealed numerous macrophage aggregates and an enhanced level of apoptosis in hemopoietic cells. In fish from the same stations we also found pathological alterations in gut and skeletal muscles.

The pathological characteristics of the populations of *L. limanda* and *P. flesus* from different sites are shown in Fig. 33. During the "Walther Herwig III" cruise, a strong negative correlation was detected between frequency and expression of the pathology and MXRtr or SATOA activity in the same animals.

Total Biomarker Index

The total biomarker index (TBI) indicating the toxic potential of different sampling sites for our test organisms, mussels and fish, reflects the problem of real "reference" sites in the North Sea. Generally, there was no difference between the TBI of fish and mussels at the different sampling sites. Only in July 1996 was the TBI in Helgoland the lowest and thus able to serve as a reference. In April 1996 the Helgoland site had actually shown the highest TBI. Spiekeroog thus has to be the preferred reference site, owing to its lowest TBI in both April and September 1997.

Discussion

The results of our studies demonstrated that populations (clean) of bivalves, gastropods and fishes from the reference sites along the Israeli and German shore were characterized by:

1. the lowest frequency and expression of histopathological alterations in their organs (data of complete histopathological examination);
2. the lowest frequency of chromosome aberrations and breaks (data of micronucleus test);
3. the lowest level of DNA damage (data of both alkaline DNA unwinding in vitro and acidic DNA unwinding in situ);
4. the highest paracellular impermeability external epithelial layers to water-soluble organic compounds (data of test with fluorescein);

5. the highest ability of lysosomes to accumulate cationic compounds (data of test with neutral red);
6. the highest activity of non-specific esterase and cholinesterase (data of cytochemical and biochemical measurements);
7. the highest content of catecholamines in molluscan brain (data of microfluorimetric determinations).

On the contrary, all or the major part of these parameters in many animals from polluted sampling sites were negatively altered:

1. the frequency and expression of histopathological alterations in their organs increased (data of complete histopathological examination);
2. the frequency of chromosome aberrations and breaks increased (data of micronucleus test);
3. the level of DNA damage increased (data of both alkaline DNA unwinding in vitro and acidic DNA unwinding in situ);
4. the paracellular permeability of external epithelial layers to water-soluble organic compounds increased (data of test with fluorescein);
5. the ability of lysosomes to accumulate cationic compounds decreased (data of test with neutral red);
6. the activity of nonspecific esterase and cholinesterase decreased (data of cytochemical and biochemical measurements);
7. the content of catecholamines in molluscan brain decreased (data of microfluorimetric determinations).

These characteristics permitted us to assess the animals from the reference sites as healthy specimens. Populations consisting of healthy specimens are healthy populations. Animals from polluted sampling sites exhibited negatively altered characteristics of health and might be objectively assessed as sick. The populations with numerous sick specimens might be considered as stress-injured sick populations. Frequency of sick specimens in the population and expression of pathological alterations permit us to assess intensity and nature of environmental pathology as well as biological activity (genotoxic, clastogenic or pathogenic) of the stressors. Thus, the proposed set of cytophysiological, cytochemical, biochemical, cytogenetic and histopathological methods in fact permitted us to examine and assess objectively environmental health of specimens, population and species from different sites along the Israeli Mediterranean and Red Sea shore and the German North Sea.

Data from literature and results of analyses carried out by the chemical group of the MARS1 project, testify that reference sites are cleaner than all other sites, which were considered as polluted. Therefore, alterations in the health of animals and populations collected from these polluted sites can be considered the result of the pollutant action. However, the proposed approach can distinguish biologically safe and dangerous environmental conditions independently from results of chemical analyses.

Examination of the characteristics of environmental health in specimens, population and species from the clean and polluted sampling sites with different pollution history shows also the high sensitivity of antixenobiotic defence mechanisms, particularly external barriers, intracellular depot (lysosomes) and export pumps, as well as their possible special role in health protection. The results of our investigations demonstrate that all the studied species possess numerous antixenobiotic defence mechanisms, particularly export pumps. These two pumps, multixenobiotic resistance-mediated transporter (MXRtr) and the system of active transport of organic anions (SATOA), are located in surface epithelia (gill, mantle and foot epithelia of molluscs and gill epithelium of fish) and in epithelia of excretory organs (renal proximal tubules and liver bile canalicules of fish). Previously we detected similar distribution of MXRtr and SATOA activity in organs of the guppy (Schreiber et al. 1997). Data from literature demonstrate the presence of antixenobiotic defence mechanisms, particularly export pumps, in specimens from different taxa, beginning with protozoa to mammals (Bresler et al. 1990, 1998a; Higgins 1992; Kurelec 1992, 1995, 1997; Gottesman and Pastan 1993; Pritchard and Miller 1993; Bresler and Fishelson 1994; Bresler and Yanko 1995a; Sweet et al. 1997). However, the ecological significance of such export pumps, their regulation and interactions with other antixenobiotic defence mechanisms have been overlooked.

The results of our study showed that MXRtr activities in surface epithelia, especially the gills, of both bivalves and gastropods from polluted sites along the Mediterranean and Red Sea, increased after surviving for 3 days in clean seawater. Kinetic data suggest that gill MXRtr activity in polluted sites is inhibited competitively as compared to the activity in native seawater. The results unmask the competition between marker xenobiotic and certain xenobiotics from polluted environment. It was also shown that various anthropogenic and natural environmental xenobiotics can modify the MXR activity (Williams and Jacobs 1993; Klopman et al. 1997; Smital and Kurelec, 1997). Therefore, comparative kinetic analysis of export pump activities in freshly collected animals and animals surviving in clean water can detect the presence of several xenobiotics in the environment, character of their interactions with transporters (competitive, non-competitive or mixed inhibition), and the possible level. As this is an early and primary response to pollutants, it could offer one of the most sensitive methods for early warning ecological monitoring.

We also showed that the mean activities of MXRtr and SATOA in different populations changed simultaneously so that the activity of both pumps was higher in populations of molluscs from the polluted sites along the Mediterranean and Red Sea as compared to those from the clean sites. Recently, we showed that the frequency polygons of gill MXRtr and SATOA activity in *Donax trunculus* from the clean site were markedly variable, with specimens from the polluted sites demonstrating significantly lower variability as well as a significant shift of polygons to higher values (Bresler et al. 1998a). Such alterations in the frequency polygons may reflect

the process of "industrial selection" of certain MXRtr and SATOA phenotypes. Thus, such studies enable us to disclose of early cryptic alterations in the population structure produced by pollution, and predict the potential fate of the studied species.

It was shown that tissues that bear export pumps also express conjugating enzymes, particularly glutathione-S-transferase (GST). Multiple GST isozymes occur in different tissues of different organisms and mediate both unspecific and specific modulation of reactive xenobiotics (Leaver and George 1996). The GST conjugates with glutathione of both metabolites of MFO/phase I and several non-metabolized xenobiotics. GST activities are species and tissue specific. Our results also show that hepatic GST activity in *P. flesus* was significantly higher than GST activity in gills of *M. edulis.* Increased GST activity occurred in fish liver exposed to a broad spectrum of xenobiotics, including heavy metals, hydrocarbons, pesticides, and pulp mill effluents (Nishimoto et al. 1995; Petrivalsky et al. 1997). Contrary to these findings, our data show the lower GST activity in fish and mussels from the polluted sites in the North Sea. Only in one instance was GST activity enhanced in the liver of flounder from the Eider estuary as compared to the reference sites Helgoland and Spiekeroog. GST activity was also significantly high in gills of mussels from the Cuxhaven. These results probably reflect different steps of adaptation to pollution.

It should be noted that GST, which transforms xenobiotics and their metabolites to the more hydrophilic substrate of SATOA and similar export pumps (GS-X pumps), works congruently with these pumps (Poot et al. 1996; Jakob et al. 1998; Homma et al. 1999). This joint function of MXRtr, SATOA and GST is required to eliminate the lipid-soluble xenobiotics, which are penetrated in the gills by simple diffusion across the lipid bilayer of the plasma membrane.

The study of cholinesterase (ChE) activity was performed in order to monitor neurotoxicity of several environmental pollutants such as organosphosphate and carbamate pesticides, heavy metals and surfactants (Day and Scott 1990; Escartin and Porte 1997). High ChE activities were observed in gills of mussels (*Mytilus galloprovincialis, M. edulis*) and in muscle tissue of fish from the Mediterranean and Baltic Sea (Zinckel et al. 1987; Bocquene et al. 1990; Quadri et al. 1994; Burgeot et al. 1996; Hansen et al. 1998). In our study, the highest ChE activity was observed in the brain of *Platichthys flesus* and in the hemolymph of *M. edulis.* The highest variability of ChE activity, which was found in muscle tissue of the flounder *P. flesus,* was determined probably by cross-activity of acetylcholinesterase and different pseudocholinesterases, particularly non-specific esterases (NSE) occurring in all tissues of fish and molluscs (Galgani et al. 1992; Silva de Assis 1998; Sturm et al. 1998). The ChE activity can show no differences among the sites of collection because pollutants interact with NSE. The level of catecholamines in molluscan brain was a more sensitive and reliable parameter to detect neurotoxicity.

Molluscs and fish from the polluted sites in the Mediterranean, Red Sea and North Sea exhibited marked signs of genotoxicity and clastogenicity. The relatively high DNA damage (high negative log F values) detected by alkaline DNA unwinding was observed in all test organisms: *P. flesus* and *M. edulis* from the North Sea, *D. trunculus* from the Mediterranean Sea and *C. rota* from the Red Sea. This points to a vast genotoxic potential at the polluted sites, especially in the Eider and Elbe estuaries and the coastal area near Frutarom and Ardag. Acidic DNA unwinding in situ exhibited similarly an increase in single-stranded DNA breaks in *M. edulis* and flounders from the Eider and Elbe estuaries, *D. trunculus* from Frutarom and the Na'aman River estuary, *P. coerulea* from Shemen Beach and Akko, and *C. florida, D. histrio* and *C. rota* from Ardag as compared to animals from the corresponding reference sites. Thus, the results of alkaline DNA unwinding in vitro and acidic DNA unwinding in situ, which were made on different specimens collected from the same stations, showed that both these assays revealed a similar enhanced level of DNA breaks in different molluscan species and in the flounder. Therefore, a combination of alkaline DNA unwinding and acidic DNA unwinding in situ guarantees reliable detection of genotoxic effects in molluscs and fish.

Increased breaks of DNA strands were observed recently in *M. edulis* and fish samples collected from a coastal area of the Baltic and the Mediterranean Seas (Nacci and Nelson 1992; Hansen et al. 1998). Due to high pollution of the Baltic Sea, DNA fragmentation in gills of *M. edulis* was about 1.6 times higher than that of *M. galloprovincialis* from the Mediterranean Sea. (Perowska and Protasowicki 1996; Baumard 1997; Hansen et al. 1998). Increased frequency of DNA strand breaks was also detected in freshwater tadpoles, turtles and fish from polluted sites (Meyers-Schone et al. 1993; Sugg et al. 1995; Ralph and Petras 1997).

Increased frequency of micronucleated hemocytes detected in *M. edulis* from Eider and especially Cuxhaven, in *D. trunculus* from Frutarom and the Na'aman River estuary, *P. coerulea* from Shemen Beach, Akko and Sdot Yam, *C. florida, D. histrio* and *C. rota* from Ardag as well as micronucleated erythrocytes in fish from the Eider and Elbe estuaries shows, that pollutants in these sites also have a marked clastogenic activity. High levels of the clastogenicity usually coincide with enhanced levels of genotoxicity determined by DNA unwinding.

Conclusion

The results of this study provide evidences that the novel methodology employed permits us to study the main health parameters, assess the state of health of populations and species, and thus forms a reliable monitoring mechanism for marine coastal areas. It should be noted that this methodology exposes the presence and danger of pollutants by their biological effects independently to data of chemical analysis. For example, alkaline DNA

unwinding in vitro, the fluorescent cytochemical acidic DNA-unwinding assay in situ and the micronucleus test reveal significant signs of environmental genotoxicity and clastogenicity in common marine molluscs or bottom-dwelling fish from certain stations. The environment of these stations contains in fact compounds with genotoxic and clastogenic activity. In such situations chemical analyses show in many cases enhanced levels of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and pesticides. However, dangerous bioactivity of unknown pollutants can also be detected. For example, the northern part of the Gulf of Eilat (Ardag area) is polluted by hotel developments, urban effluents and cage-based fish farming (Angel et al., 1995). At this site cryptic alterations in cell cycle (hormesis), enhanced frequency of DNA break, and high frequency of micronucleated and apoptotic cells were recently detected in the coral *Acropora hyacinthus* (Fishelson et al. 1996a). All these results attest to the presence of certain genotoxic and clastogenic pollutants in the studied environment, probably nitrosoamines.

Our novel methodology also allows us to select reliable common species for ecological monitoring. In general, marine molluscs and other long-living sedentary organisms can also be used for other biochemical, fluorescent cytochemical and cytopathological assays as powerful screening tools for the objective assessment of pollution effects in marine sites.

It should be noted that the application of the biomarker index after Narbonne et al. (1998) to the presented data was made in order to test the efficacy of this method for pollution monitoring in our study. However, the results are only preliminary and further investigation is required in this direction. The comparison between indices of different sampling dates is only possible if a continuous study of the material and all parameters is provided. Additionally, the usefulness of some neurotoxic and genotoxic parameters should be discussed when assessing the effect of pollution on populations.

Finally, we wish to note that our methodology is flexible and compatible with various methods of molecular biology, biochemistry, cytochemistry, physiology, cytogenetic and pathology, which makes it a good tool for complex interdisciplinary investigations in ecotoxicology, ecology and marine biology.

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