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ORIGINAL ARTICLE

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Marine molluscs in environmental monitoring

I. Cellular and molecular responses

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Abstract The study reported here is part of an ongoing effort to establish sensitive and reliable biomonitoring markers for probing the coastal marine environment. Here, we report comparative measurements of a range of histological, cellular and sub-cellular parameters in molluscs sampled in polluted and reference sites along the Mediterranean coast of Israel and in the northern tip of the Gulf of Aqaba, Red Sea. Available species enabled an examination of conditions in two environmental 'compartments': benthic (*Donax trunculus*) and intertidal (*Brachidontes pharaonis*, *Patella caerulea*) in the Mediterranean; pelagic (*Pteria aegyptia*) and intertidal (*Cellana rota*) in the Red Sea. The methodology used provides rapid results by combining specialized fluorescent probes and contact microscopy, by which all parameters are measured in unprocessed animal tissue. The research focused on three interconnected levels. First, antixenobiotic defence mechanisms aimed at keeping hazardous agents outside the cell. Paracellular permeability was 70–100% higher in polluted sites, and membrane pumps (MXRtr and SATOA) activity was up to 65% higher in polluted compared to reference sites. Second, intracellular defence mechanisms that act to minimize potential damage by agents having penetrated the first line of defence. Metallothionein expression and EROD activity were 160–520% higher in polluted sites, and lysosomal functional activity (as measured by neutral red accumulation) was 25–50% lower. Third, damage caused by agents not sufficiently eliminated by the above mechanisms (e.g. single-stranded DNA breaks, chromosome damage and other pathological alterations). At this level, the most striking differences were observed in the rate of micronuclei formation and DNA breaks (up to 150% and 400% higher in polluted sites, respectively). The different mollusc species used feature very similar trends between polluted and reference sites in all measured parameters. Concentrating on relatively basic levels of biological organization—the molecular and cellular level—the parameters measured may have the capacity not only for biomonitoring environmental quality, but also for early warning.

Keywords Antichemical defence mechanisms · Biomonitoring · Environmental health · Marine molluscs · Microfluorometry

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Introduction

The Mediterranean Sea and the northern part of the Gulf of Aqaba (Red Sea) serve as examples of over-exploited seas disturbed by heavy effluent loads (Fishelson 1973; Herut et al. 1993, 1994, 1995), intense maritime transportation and diverse human activities along the coasts, all of which pose a continuing threat to the marine environment, and in some cases also to public health (e.g. Baudart et al. 2000). Recent studies indicate that these regions are being exposed to serious disturbances and are experiencing significant changes (e.g. Bresler and Fishelson 1994; Fishelson 1995; Bresler et al. 1998). While the concentrations of some types of pollutants seem to have declined [for recent measurements of several organic pollutants and metals see Herut et al. (1999); Kress et al. (1999)], the deterioration of the environment and its biota persists (Fishelson et al. 2002). Moreover, a screening for organic pollutants and trace elements conducted within the framework of the MARS 2 project indicates enrichment of a number of regions along the Mediterranean coast of Israel (Feldstein et al. 2003). In order to take preventive or remediation measures towards the conser-

vation of the deteriorating habitats and the species inhabiting them, sensitive and reliable systems must be established and calibrated for monitoring marine pollution.

While it is possible to utilize direct chemical analyses of water and sediment which are usually very sensitive and accurate, monitoring systems which employ direct analysis do not necessarily reflect the true ecological state, for several reasons [for a detailed discussion see Phillips and Rainbow (1994)]. First, the number and range of pollutants known to be discharged to the environment far exceeds the capability of available chemical tests, not to mention unknown pollutants. Second, pollutants may act synergistically, which limits the value of direct measurements dramatically. Finally, the true threat to the environment and its inhabitants also depends on the biological availability of suspected agents, which cannot be accounted for by chemical means. Biomonitoring—the use of biological responses of selected organisms for environmental monitoring—overcomes these problems. Biomonitoring can be applied at various levels of biological integration, from the ecosystem and community at the top of the hierarchy, down to the cellular and molecular levels. The number of responses that can potentially serve for biomonitoring is very large, corresponding to the wide range of biological responses elicited by environmental pollution (ranging from preventive responses, through stress responses to eventual mortality; see Phillips and Rainbow 1994). Nevertheless, considerable attention has been focused on mechanisms directly involved in avoiding or processing environmental pollutants.

An important factor to be considered when establishing a biomonitoring system is the organism to be used. Criteria for selecting biomonitors, including spatial and temporal abundance, ease of sampling and range of detectable biological responses, have been proposed by several investigators (summarized by Phillips and Rainbow 1994). The range of marine organisms used by different researchers is wide (including algae, invertebrates and vertebrates). Bivalve and gastropod molluscs are among the most useful organisms for environmental biomonitoring (Phillips and Rainbow 1994; Boening 1999). In addition to fitting the above criteria, these organisms are relatively site specific, being either attached to the substrate or very restricted in their motility.

Among the species used in this study (see Methods), *Brachidontes pharaonis* (Mediterranean Sea) and *Pteria aegyptia* (Red Sea) are physically attached to the substrate. The two gastropods (*Patella caerulea*, Mediterranean; *Cellana rota*, Red Sea), although motile, always remain on a very restricted area of solid substrates, and do not venture across the water to other areas. *Donax trunculus* is rolled back and forth by wave action, but its ability for very rapid burrowing into the sand provides for very restricted motion and subsequent site specificity. All these organisms are easy to maintain in laboratory aquaria for the duration of the analytical procedures.

During 1996–1998, within the framework of the MARS 1 project, we used fluorescent microscopy to examine cellular- and molecular-level health parameters and antixenobiotic defence mechanisms of mollusc populations along the Red Sea, Mediterranean Sea and North Sea (Bresler et al. 1999). The results obtained in that study demonstrated significant differences, at two levels, between organisms sampled in polluted and reference sites. First, the activity of physiological mechanisms aimed at isolating the organism from its environment was higher in polluted sites. The necessity for higher activity was shown to be due not only to elevated levels of pollutants, but also to damage caused to epithelial layers as shown by higher paracellular permeability (PP) in the polluted sites. Second, molecular, cellular and histological damage was more severe in the polluted sites.

The objectives of the MARS 2 project (1999–2001), reported here, were two fold. First, to explore additional methodologies and their potential for biomonitoring at the molecular and cellular levels. Specifically, we addressed another, intermediate level of cellular antixenobiotic defence—that of mechanisms aimed at minimizing effects of pollutants that have penetrated the cell. Second, to use the methodological basis provided by MARS 1 in order to continue the monitoring over a longer period.

Results of experimental exposure of molluscs to pollutants and chemical analyses of sediments and animal tissue from the sampling sites are also reported elsewhere in this issue (Bresler et al. 2003; Feldstein et al. 2003).

Methods

Species studied and collection sites

Table 1 lists the molluscan species studied and the collection sites in the Mediterranean and Red Sea (Fig. 1). This list is similar, but not identical, to that used by Bresler et al. (1999). Changes in the list of species were in part due to drastic changes in population size. For example, populations of *Donax trunculus*, featuring densities of

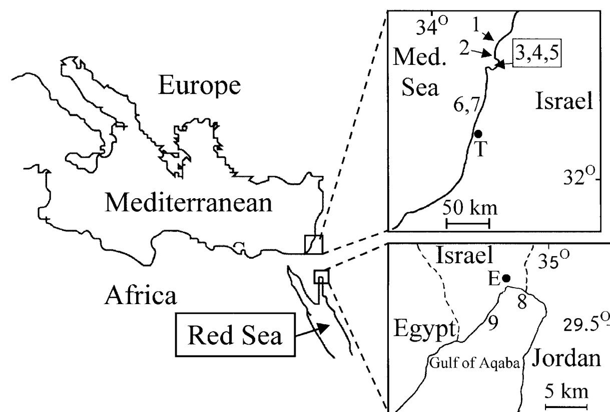

Fig. 1 Sampling sites along the Mediterranean Sea and Red Sea shores of Israel. The sites are numbered from north (*1*-Rosh Haniqra) to south (*9*-MBL) in accordance with Table 1. The city of Tel Aviv (*T*) and the town of Elat (*E*) are marked for orientation

Table 1 Mollusc species used in this study and their collection sites. Map codes match the numbers in Fig. 1. *MBL*=The H. Steinitz marine biology laboratory, Elat

Species	Region	Polluted sites (map code)	Reference sites (map code)	Comments
Donax trunculus	Mediterranean	Na'aman (3) Frutarom (4)	Ma'agan Mikha'el (6)	Bivalve; sandy-bottom; mobile; filter feeder
Brachidontes pharaonis	Mediterranean	Shemen (5) Akko (2)	Caesarea (7)	Bivalve; intertidal hard substrates; attached; filter feeder
Patella caerulea	Mediterranean	Shemen (5)	Rosh Haniqra (1) Caesarea (7)	Gastropod; intertidal hard substrates; mobile; grazer
Pteria aegyptia	Red Sea	Ardag (8)	MBL (9)	Bivalve; submerged hard substrates; attached; filter feeder
Cellana rota	Red Sea	Ardag (8)	MBL (9)	Gastropod; intertidal hard substrates; mobile; grazer

up to 2,000/m^2 in some sites in the early 1990s, have decreased recently for reasons yet unknown. Nevertheless, *D. trunculus* seems to have stabilized, enabling the continuation of prudent sampling. Other species, such as *Mactra corallina*, can no longer be used for biomonitoring as there is no guarantee that specimens will be found when needed.

Especially in the Mediterranean, species were selected for calibration as biomonitors, to enable monitoring of different compartments of the environment. While *D. trunculus* is a sediment dweller, *Brachidontes pharaonis* and *Patella caerulea* inhabit intertidal rocks. In the Red Sea, *Cellana rota* inhabits intertidal substrates while *Pteria aegyptia* attaches to substrates submerged a few metres under the water.

Within the framework of the MARS 2 project, we determined the concentration of trace elements and organic pollutants in the sediments and in the tissue of the studied molluscs. The detailed findings are reported by Feldstein et al. (2003). Briefly, the polluted sites in the Mediterranean Sea (Na'aman, Frutarom, Shemen and Akko) were enriched both in organic and trace element contaminants relative to the reference stations. In the Red Sea, differences between the polluted and reference sites were less pronounced.

Sample treatment

As described previously, the collected live molluscs were transported to the Institute for Nature Conservation Research (Tel Aviv University) and kept in aerated aquaria with artificial seawater for further experiments (Bresler et al. 1999). Molluscs were thus acclimatized for 24–48 h, following which the analytical procedures were done within 2 days (i.e. a total of up to 4 days in the laboratory). The animals were used for vital biophysical, cytophysiological and cytochemical examinations and then fixed in cold absolute methanol and in 5% formaldehyde solution in seawater for three assays: examination of single-stranded DNA breaks, micronucleus test and histopathological examination. With the exception of these three assays, all observations were done on living animals in situ. This is in contrast to methodologies necessitating isolation of physiological components, such as the one often used for the analysis of lysosomal stability in isolated haemocytes (e.g. Matozzo et al. 2001). In addition to the considerable methodological complication, the procedures involved in such isolations may affect the observed results.

Analytical methods

The analytical procedures associated with some of the examined health parameters were previously detailed (Bresler et al. 1999, 2001), and are thus only briefly described here. The hydrophilic fluorescent dye fluorescein was used to examine PP of external epithelia. Damaged epithelial layers show increased permeability through tight junctions, which can be quantified by microfluorometry. The viability of external epithelia was assessed by measuring intralysosomal neutral red (NR) accumulation. The microfluorometrical determination of accumulated NR in situ enhanced markedly the sensitivity and precision of this conventional test of viability. The activity of the multi-xenobiotic resistance transporter (MXRtr) was studied using rhodamine B as a fluorescent marker and a prototypical substrate of this pump. Using verapamil, a specific MXRtr inhibitor, enabled the determination of the net transport rate of rhodamine. Activity of the system of active transport of organic anions (SATOA) was examined using the permeable fluorogenic probe, fluorescein diacetate, which is very rapidly converted to the fluorescent fluorescein by intracellular esterase activity, and the specific SATOA inhibitor, probenecid. For both transport systems (MXRtr and SATOA), one half of each mollusc was incubated with the inhibitor and the other without inhibitor.

The frequency of micronuclei formation (number of cells containing micronuclei per thousand; micronucleus test, MNT) was studied by staining methanol fixed specimens stained with acridine orange. An in situ acidic DNA-unwinding assay with acridine orange served to estimate the frequency of single-stranded DNA breaks as measured by the fraction of double stranded DNA (Fds) out of the total DNA (double stranded + single stranded).

The frequency and expression of cyto- and histopathological alterations were studied using fluorescent contact microscopy and epimicroscopy. Pathological alterations were identified according to conventional classification of general pathological processes (Cotran et al. 1989). The level of expression of these processes was categorized as follows: (1) weak dystrophic alterations—including cell swelling, granular structures within cytoplasm and only few cytoplasmic vacuoles; (2) marked dystrophic alterations—including the above, but with numerous cytoplasmic vacuoles and small lipid droplets; (3) marked dystrophic alterations accompanied by necrobiosis and necrosis—including also signs of cell death like karyopycnosis, karyorrhexis, karyolysis or loss of nucleus.

Additional methods, introduced in the course of the current study, are described below.

Fluorescamine test

This test detects the amount and condition of plasma-membrane surface proteins and other amino-group containing compounds in living cells (Parry et al. 1982; Rawyler et al. 1984; Gareau et al. 1991; Chung 1997; Huijbregts et al. 1998). Live molluscs were incubated in artificial seawater with 20 mM fluorescamine (added from 100 mM stock solution in anhydrous dimethylsulfoxide; Molecular Probes, Eugene, Ore., USA) for 30 s. The reaction was stopped by washing in seawater with 250 mM ethanolamine and fluorescence of external epithelia was measured by contact

microfluorometry (excitation filter 380–420 nm, dichroic mirror 430 nm, barrier filter >450 + supplementary filter 470±10 nm).

Determination of cholinesterase activity in situ

Cholinesterase (ChE) is widely used to estimate neurotoxic impacts of pollutants, especially organophosphates, carbamates, several heavy metals and surfactants. In a previous study, Bresler et al. (1999) used a biochemical method of determination, which exhibited highly variable results. In the current study we used a non-destructive, vital microfluorometrical method based on the ability of N-[4(7-diethylamino-4-methylcoumarin-3-yl)-phenyl]maleimide (CPM), to form a highly fluorescent product with thiocholine (Parviari et al. 1983; Haugland 1999). Living molluscs were incubated in seawater with 0.1 mM acethylthiocholine, 0.2 mM CPM (Molecular Probes) and 0.1 ml Triton X-100 (0.1%) for 30 min. The tissue was then washed, and the amount of fluorescent product was determined by contact microfluorometry (excitation filter 380–420 nm, dichroic mirror 430 nm, barrier filter >450 + supplementary filter 470±10 nm). Preliminary examination demonstrated that this fluorescence was localized at synapses, especially on smooth muscle fibres and was inhibited by eserine. Non-specific background diffuse fluorescence was relatively low.

Determination of ethoxyresorufin-O-deethylase activity in situ

Ethoxyresorufin-*O*-deethylase (EROD) is one of the most often studied cytochrome P450 isoenzymes that is usually studied in isolated microsomal membrane fractions (Kennedy and Jones 1994). However, EROD activity can also be determined in situ, in isolated cells, tissue slides, fish eggs and embryos (Lacy et al. 1992; Stauber et al. 1995; Heinonen et al. 1996; Nacci et al. 1998). We incubated living molluscs in seawater with 0.1 ml Triton X-100 (0.1%) and ethoxyresorufin (final concentration 20 μg/l) for 15 min, washed them, and determined red fluorescence of resorufin by contact microfluorometry (excitation filter 510–550 nm, dichroic mirror 575 nm and barrier filter >590 nm).

Determination of Cu-metallothionein in situ

This assay is based on the strong orange autofluorescence typical of the complex of metallothionein with copper (Cu-MT) when excited by ultraviolet light (Suzuki-Kurasaki et al. 1997; Kurasaki et al. 1998; Paris-Palacios et al. 2000). Living molluscs were incubated in artificial seawater with 150 µg/l copper and the fluorescence intensity of Cu-MT was measured by contact microfluorometry (excitation filter 250±10 nm, dichroic mirror 290 nm, barrier filter >330 nm + supplementary filter 540± 7 nm).

Determination of redox state of the mitochondrial respiratory chain

The metabolic state of the mitochondrial respiratory chain is one of the most significant and sensitive health parameters and its non-invasive, vital microfluorometric examination is widely used in biology and medicine (Chance 1964; Ghosh and Chance 1964; Bresler and Yanko 1995a, 1995b; Chance et al. 1998; Shiino et al. 1999). Because reduced nicotinamide-adenosine dinucleotide (NADH) has marked blue autofluorescence and oxidized flavoprotein (FLAVO) has green autofluorescence, it is possible to monitor in vivo the redox state of different organisms and tissues. The ratio between green and blue fluorescence (metabolic ratio; Mrat) is indicative of the metabolic state of the organism and reflects metabolic alterations. Contact vital microfluorometry was used to examine Mrat in external epithelia of living molluscs. NADH autofluorescence was determined with excitation filter 330–380 nm, dichroic mirror 400 nm and barrier filter >420 ± supplementary filter 450±10 nm. FLAVO autofluorescence was determined with excitation filter 450–490 nm, dichroic mirror 505 nm and barrier filter >520 nm ± supplementary filter 530±10 nm.

Statistical analysis

A preliminary two-way analysis of variance was performed separately for each parameter measured in each species, using Statistica 5.0 for Windows (Stat Soft Ltd), to examine differences between different sampling sites and different seasons. No significant differences were found between seasons within any site (analysis not shown). The data presented below were, therefore, pooled over all sampling campaigns, for each species in each site.

Results

Mediterranean molluscs

Results obtained for the three species of molluscs examined along the Mediterranean coast exhibited remarkably similar trends when polluted sites were compared to reference sites, although the differences were in some cases more obvious than in others. As a general pattern, molluscs from Frutarom and Shemen displayed sharper differences relative to the reference sites than did the ones from Na'aman and Akko. The only exception is the MNT for *Donax trunculus.*

As Fig. 2a, d, g shows, PP to fluorescein and the results of the fluorescamine test (FT) were significantly higher in the gill epithelia of specimens from the polluted sites than in specimens from the reference site. In *D. trunculus* from Frutarom and *Brachidontes pharaonis* and *Patella caerulea* from Shemen the increase in PP was in the narrow range of 70–76%. The signal obtained by FT was weaker and more variable, at 24–36%. Intralysosomal accumulation of NR and ChE activity were significantly lower in gill epithelia of specimens from the polluted sites than in specimens from the reference sites. The results for ChE were more uniform among the different molluscs (a decrease of 32–35% in Frutarom and Shemen relative to reference sites) than the results obtained for NR accumulation (25–48%).

Activities of the antixenobiotic defence mechanisms examined, MXRtr, SATOA and EROD, as well as MT content were significantly higher in specimens from the polluted sites (Fig. 2b, e, h). However, the signals obtained by examining these parameters varied dramatically in strength. MXRtr and SATOA activities were up to 63% and 34% (respectively) higher in polluted sites relative to reference sites. EROD activity was 160–520% higher in the polluted sites, and MT expression increased by 200–350%.

Despite the enhanced activity of these antixenobiotic defence mechanisms, the frequencies of micronuclei-containing haemocytes, as well as the frequencies of single-stranded DNA breaks, were significantly higher in specimens from the polluted sites. MNT values were higher by 100–150%, and the relative number of single-stranded DNA breaks (n) per unwinding unit was higher by 160–400% in polluted sites relative to reference sites (Fig. 2).

Fig. 2 Health parameters measured in the Mediterranean molluscs *Donax trunculus* (**a–c**), *Brachidontes pharaonis* (**d–f**) and *Patella caerulea* (**g–i**). Values shown are means and 95% confidence intervals. The numbers above the bars are sample sizes for each test. Assay acronyms: *PP* paracellular permeability, *FT* fluorescamine test, *NR* accumulation of neutral red, *ChE* cholinesterase activity, *MXRtr* multixenobiotic resistance transporter, *SATOA* system of active transport of organic anions, *EROD* ethoxyresorufin-O-deethylase activity, *MT* copper binding by metallothionein, *Fds* frequency of single-stranded DNA breaks expressed as the fraction of double stranded DNA; *−log Fds*;*n* the ratio between −log Fds of each site to that of the smallest −log Fds; *MNT* micronucleus test. The legend in each right hand chart refers to an entire row of three charts. Names of polluted sites are underlined

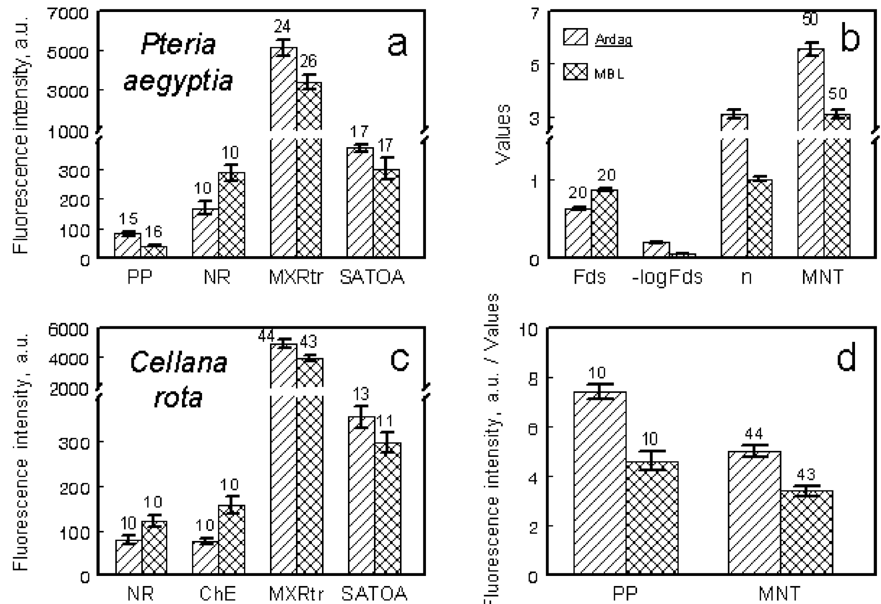

Fig. 3 Health parameters measured in the Red Sea molluscs *Pteria aegyptia* (**a, b**) and *Cellana rota* (**c, d**). Values shown are means and 95% confidence intervals. The numbers above the bars are sample sizes for each test. For explanation of acronyms of the different assays (*X* axis) see Fig. 2. The numbers along the *Y* axis of **d** represent fluorescence intensity for PP and plain values for MNT. The legend in the right hand top chart refers to all four charts. Name of the polluted site (Ardag) is underlined

Table 2 Number and percentage (in parentheses) of molluscs displaying various pathological alterations. Type/severity codes are as follows—gills and mantle: *I*=weak dystrophic alterations,*II*=marked dystrophic alterations, *III*=marked dystrophic alterations and necrobiotic alterations; hepato-pancreas:*I*=few vacuoles with brown pigment, *II*=numerous vacuoles with brown pigment, *III*=focal necrobiotic alterations and hemocytic infiltrates in the stroma; nerve ganglia:*I*=weak dystrophic alterations, *II*=marked dystrophic alterations, *III*=necrobiotic alterations of some nerve cells. The number of individuals examined (*n*) is shown for each mollusc in each site

Organ/tissue	Gills			Mantle			Hepato-pancreas			Nerve ganglia		
Type/severity	I	II	III	I	II	III	I	II	III	I	II	III
D. trunculus												
Frutarom (*n*=120)	27 (23)	15 (13)	9 (8)	21 (18)	13 (11)	5 (4)	39 (33)	27 (23)	11 (9)	6 (5)	3 (3)	1 (1)
Na'aman (*n*=60)	14 (23)	8 (13)	5 (8)	11 (18)	5 (9)	3 (5)	11 (18)	9 (15)	3 (5)	2 (3)	1 (2)	0
Ma'agan Mikha'el (*n*=103)	9 (9)	0	0	6 (6)	0	0	5 (5)	2 (2)	0	0	0	0
B. pharaonis												
Shemen (*n*=60)	23 (38)	14 (23)	8 (13)	16 (27)	11 (18)	7 (12)	13 (22)	19 (32)	17 (28)	8 (13)	3 (5)	3 (5)
Caesarea (*n*=40)	5 (13)	0	0	6 (15)	0	0	9 (23)	2 (5)	0	0	0	0
Patella caerulea												
Shemen (*n*=120)	33 (28)	38 (32)	13 (11)	26 (22)	31 (26)	8 (7)	33 (28)	39 (33)	16 (13)	13 (11)	7 (6)	6 (5)
Rosh Haniqra (*n*=20)	3 (15)	0	0	2 (10)	0	0	2 (10)	1 (5)	0	0	0	0
Caesarea (*n*=60)	7 (12)	0	0	3 (5)	0	0	3 (5)	1 (2)	0	0	0	0
Pteria aegyptia												
Ardag (*n*=50)	13 (26)	19 (38)	16 (32)	11 (22)	12 (24)	6 (12)	14 (28)	21 (42)	11 (22)	4 (8)	1 (2)	0
MBL (*n*=50)	4 (8)	2 (4)	0	3 (6)	0	0	7 (14)	2 (4)	0	0	0	0
C. rota												
Ardag (*n*=40)	9 (23)	18 (45)	11 (28)	7 (18)	15 (38)	6 (15)	14 (35)	19 (48)	7 (18)	5 (13)	2 (5)	0
MBL (*n*=40)	3 (8)	0	0	2 (5)	0	0	6 (15)	2 (5)	0	0	0	0

Cyto- and histopathological examinations revealed much higher frequencies of pathological alterations in specimens from the polluted sites than the reference sites (Table 2). These alterations were observed in the gills, mantle, hepatopancreas, kidney and nerve ganglia of *D. trunculus* and *B. pharaonis*, and in the internal organs and shell of *P. caerulea* [for examples of pathological micromorphologies see Bresler et al. (1999)]. In all types of tissue, the observed level of histopathological effects was higher in molluscs from the polluted sites, in two ways. First, the frequency of any dystrophic alteration was higher. Second, the expression of the alterations was more advanced in specimens from the polluted sites.

Finally, Mrat was significantly lower in external epithelia of specimens from the polluted sites compared to the reference sites (Table 3). The reduction in Mrat was in the narrow range of 35–37% in Frutarom and Shemen relative to the reference sites, whereas in Na'aman it was only 24%.

Table 3 Metabolic state of mitochondria in the gills of molluscs from different sampling sites expressed as the ratio of blue autofluorescence of NADH to green autofluorescence of oxidized flavoprotein (Mrat). Values shown are averages and 95% confidence limits

Species	Sampling site (*n*)	Ratio
D. trunculus	Frutarom (50)	0.386±0.012
	Na'aman River (50)	0.466±0.009
	Ma'agan Mikha'el (50)	0.611±0.005
B. pharaonis	Shemen Beach (30)	0.374±0.015
	Caesarea (30)	0.583±0.011
Patella caerulea	Shemen Beach (50)	0.389±0.013
	Rosh-Haniqra (25)	0.594±0.015
	Caesarea (40)	0.607±0.014
Pteria aegyptia	Ardag (20)	0.371±0.016
	MBL (20)	0.557±0.019
C. rota	Ardag (20)	0.392±0.012
	MBL (20)	0.624±0.015

Red Sea molluscs

Red Sea molluscs were examined in less detail than Mediterranean molluscs. Nevertheless, the results obtained demonstrate significant differences between polluted and reference sites. The results shown in Fig. 3 follow the same trends as those shown for Mediterranean molluscs in Fig. 2. Notably, PP of the gill epithelia in *Pteria aegyptia* specimens was the highest among all investigated mollusc species, displaying an increase of nearly 100% in the polluted site. By contrast, *Cellana rota* showed an increase of only 60% in PP, which is slightly lower than that shown by the Mediterranean molluscs.

Most other parameters measured showed results similar to those obtained for the Mediterranean molluscs, although consistently in the lower range. This includes NR accumulation, ChE activity, MXRtr and SATOA pumps, and DNA breaks. The difference between polluted and reference site shown by the MNT in *P. aegyptia* was lower than that obtained for any Mediterranean mollusc, being only 80% higher in the polluted site.

Discussion

Despite the desirable qualities of an environmental health profile depicted by as many parameters as possible, it should be noted that some of the parameters examined in this study provide sharper signals than others. Thus, there is a trade-off that must be considered when establishing routine biomonitoring programmes. On the one hand, parameters featuring sharper contrast in this study are more likely to be useful for distinguishing between environments typified by milder levels of pollution than the sites in Haifa Bay. They may, therefore, be more useful for early warning, signalling the oncoming of hazardous conditions ahead of time. This potential was demonstrated in the current study, in cases involving more than one polluted site (*Donax trunculus*, Fig. 2a–c) or more than one reference site (*Patella caerulea*, Fig. 2g–i, and to a lesser extent *Brachidontes pharaonis*, Fig. 2d–f) per species. On the other hand, the different parameters studied are of completely different natures, and are probably triggered by different environmental agents. Therefore, it is probably too risky to disqualify any of the parameters in favour of another. Since the nature of future stress agents is not always predictable, it would be prudent to establish comprehensive biomonitoring programmes, using at least the variety of parameters examined in this study. Indeed, it would probably be worth adding markers based on mRNA level alterations in gene expression, which are even more sensitive than protein levels and activities, as suggested by earlier studies within the framework of the MARS 2 project (Mokady and Sultan 1998; Sultan et al. 2000).

Four of the measured parameters provide signals distinguishing polluted sites from reference sites which are far sharper than the other parameters. These are EROD activity, MT expression, DNA breaks and micronuclei formation. The highest relative increase in MT expression observed was ca. ×3.5, in Frutarom (Fig. 2b). By comparison, Ringwood et al. (1999) observed an increase of up to ×5–×8 in some of their industrially polluted sites relative to their reference site. This may rate the most polluted sites along the Mediterranean coast of Israel as moderately polluted. The results obtained in the current study with respect to lysosomal stability (NR) are more similar to those obtained by Ringwood et al. (1999). Whereas we observed a decrease of lysosomal stability of up to 50% (Shemen, Fig. 2d, g), they observed a decrease of ca. 60%. Thus, with respect to pollutants causing intracellular damage, the Israeli coast seems to offer distressingly similar conditions to the ones prevailing in the estuaries in South Carolina, where the other study was done. Finally, with respect to the activity of MXRtr, we observed an increase of more than 60% (Shemen), whereas Ringwood et al. (1999) observed a maximum of ca. 50%.

The above pattern of differences between the results obtained in the two different studies may be caused by a number of factors. First, the examined species were not the same [Ringwood et al. (1999) used *Crassostrea virginica*]. This problem is expected when different areas of the world are involved. Second, the tests used were not the same, even when addressing the same biological mechanisms. Thus, for example, Ringwood et al. (1999) used cellular suspensions for examining NR retention, and isolated MTs in order to measure the amount of bound copper or cadmium, while the current study examined both parameters in living animals in situ. Third, different compositions of either organic pollutants or toxic metals, two of the most widespread classes of pollutants, may affect biological systems differently.

Fluorescent microscopy and micofluorometry, as employed in this study, feature equal or (in most cases) higher detection sensitivity, while being much simpler to execute than other methods. For example, biochemical tests of ChE level and activity are widely used to estimate neurotoxic impacts of pollutants, especially organophosphates, carbamates, several heavy metals and surfactants, on the tissues of marine organisms (Galgani et al. 1992; Escartin and Porte 1997; Silva de Assis 1998). Our data demonstrate the utility of a much simpler fluorometric assay for achieving the same target. However, the suite of methods employed here offer additional advantages, directly relevant to biomonitoring. These techniques allow detection in diverse 'compartments', including environmental samples, biological fluids and internal structures of animals. They allow 'non-invasive', in situ examination of enzyme activity and kinetics in single living cells or tissues, particularly marker enzymes or detoxifying enzymes (Haugland 1999; Bresler and Yanko-Hombach 2000; Bresler et al. 2001). Using the same hardware (i.e. microscope, photomultiplier, etc.), one could utilize both the fluorescence of applied markers and inherent fluorescence of specific cellular components (Bresler and Yanko 1995a, 1995b; Chance et al. 1998; Shiino et al. 1999). Finally, these methods have proved equally useful in organisms ranging from bacteria to human (Bresler et al. 1990, 1998, 1999, 2001; Higgins 1992; Kurelec 1992, 1995, 1997; Pritchard and Miller 1993; Bresler and Fishelson 1994; Bresler and Yanko-Hombach 2000; Eufemia and Epel 2000; Smital et al. 2000).

The flourescamine test is an example of harnessing a well-known biochemical phenomenon to produce a useful biomonitoring marker. In this case it is the interaction of fluorescamine with amino groups of proteins and some phospholipids, in particular phosphatidylethanolamine, on external surfaces of cell membranes (Gareau et al. 1991; Chung 1997; Huijbregts et al. 1998). In our study, fluorescamine-derived fluorescence of external epithelia was always higher in the molluscs from polluted sites (Fig. 2a, d, g). For example, in samples of *B. pharaonis* from the polluted Shemen Beach it was 410.9±10.4 a.u. (fluorescence arbitrary units), whereas in the reference site 330.5±16.5 a.u. Similarly, in *D. trunculus* from the sandy beach of Frutaron it was 493.6±6.5 a.u., whereas in the Caesarea reference site the mean value was 363.5±26.3 a.u.. These data reflect higher amounts of proteins and phosphatidylethanolamine in the plasma

membranes of molluscs, induced by pollution. Concomitantly, our studies revealed an increased activity of protein pumps (MXRtr and SATOA) in the membranes of molluscs from polluted sites.

Another biochemical parameter adopted for ecotoxicological use is the Mrat. Originating in basic biochemistry, this parameter has served for quite some time in the field of medical diagnosis and is also used in other fields (Chance 1964; Ghosh and Chance 1964; Bresler and Yanko 1995a, 1995b; Chance et al. 1998; Shiino et al. 1999; Bresler and Yanko-Hombach 2000). Ecotoxicological use, however, is not yet as widely accepted for Mrat (Bresler and Yanko 1995a, 1995b). Our results (Table 3) demonstrate the utility of this parameter for environmental biomonitoring. High values of Mrat (like those detected in reference sites) are typical of mitochondria in a ’rest’ state, when steady state is maintained between production and use of ATP. Lower values like those detected in polluted sites are typical of mitochondria in an ’active’ state, when the rate of ATP use is higher than ATP production. Thus, molluscs in the polluted sites show signs of a permanent ATP deficit (the main source of metabolic energy) and activated mitochondrial oxidative phosphorylation.

The enhanced activities of antixenobiotic defence mechanisms allow organisms to survive in highly polluted environments, enabling them to withstand environmental stressors in their habitats (Fishelson et al. 2002). It may be argued, therefore, that the environmental stressors initiate a novel selection in favour of the phenotypes that are most adapted to the new, human-mediated conditions (Nevo and Laevi 1989). The general applicability of this statement can be exemplified by considering all five molluscan species studied here together. The results show that, despite the taxonomic distances, the biochemistry and physiology of the molluscs sampled from polluted sites are much closer to each other than to their conspecifics from reference sites.

The present study is an extension of a previous study performed along the Israeli Mediterranean and Red Sea during 1996–1998 (Bresler et al. 1999). Parts of the above discussion are therefore based on 6, rather than 3, years of observations and analyses (from 1996 to 2001). Throughout this research, the studied bivalves and gastropods from the polluted sites exhibited marked alterations in all the examined health parameters, as compared to the molluscs from the reference sites. Applying the methods of this study to various species may enable us to map ‘hotspots’ of pathological alterations and stress responses.

As opposed to direct chemical monitoring, biomonitoring calls our attention to biologically relevant problems, integrates conditions over time and reflects synergistic effects reliably (Phillips and Rainbow 1994). Focusing on biological ‘signals’, indications based on biomonitoring ‘devices’ are essential for the implementation of sound countermeasures to health problems arising from environmental pollution.

Taken together, the various parameters studied unravel complex biological responses to environmental pollution, at the cellular and molecular levels. The types and levels of responses studied here may be viewed as the raw material for future, refined studies, by which specific diagnostic kits may be ‘tailored’ to meet the needs of specific coastlines.

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