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ORIGINAL ARTICLE

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Marine molluscs in environmental monitoring

II. Experimental exposure to selected pollutants

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Abstract In an effort to establish biomonitoring programmes for routine and emergency monitoring of littoral marine habitats, organismal responses are examined in two ways: firstly, in controlled, laboratory studies, where the response may be accurately characterized; secondly, in field-collected specimens, with the hope of obtaining evidence regarding disturbances such as the ones caused by anthropogenic pollution. In many cases, there is a gap between the two types of studies, and different species and experimental and/or analytical procedures are used. In a series of recent studies, we have examined responses of field-collected molluscs, and interpreted our findings with respect to pollution. Here, we report a complementary study, in which molluscs collected from reference and polluted sites were exposed to cadmium or DDT under controlled laboratory conditions. Using fluorescent probes and microfluorometry, we examined the effect of these pollutants on paracellular permeability, lysosomal stability and metabolic status of mitochondria. Our findings indicate that molluscs from polluted sites are less affected, showing significantly smaller alterations in all examined parameters. These findings are in line with previous results showing higher levels of activity of cellular defence mechanisms in molluscs collected from polluted sites. Taken together, the results may be used to establish a reliable biomonitoring system. The sensitivity of the suggested methodology is also expected to qualify such a system for early warning.

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Introduction

In recent decades, coastal zones worldwide have experienced accelerated urban and industrial development leading to increasingly heavy effluent loads on littoral marine ecosystems. One of the prominent symptoms of ecosystem deterioration is a decline or disappearance of populations of sensitive species. However, other species in the same habitat sometimes display a remarkable resistance, which may be attributed to a range of preventive responses, enabling them to withstand environmental stresses (Bresler et al. 1999). The concept of biomonitoring, the use of such organismal responses to monitor the environment (e.g. Lavie and Nevo 1986; Fishelson et al. 1993; Bresler et al. 2003), has developed over the past 40 years. Starting with Folsom et al. (1963), various organismal responses have been studied as potential monitoring tools, attesting to the existence of mal conditions in the environment.

At present, there are numerous studies that illustrate various stress effects of heavy metals and organic pollutants on marine organisms. However, in many cases the evidence linking a specific stressor and response is circumstantial, and the absence of such links posts impediments in the way of sound measures against pollution events and chronic ‘hotspots.’

Studies related to environmental pollutants and their effects on marine biota can be divided into two distinct categories. The first category includes bioassay studies that examine the responses of organisms exposed to specific contaminants under controlled conditions, in vitro or in vivo. For example, cellular responses to various chemicals are widely used in toxicology and ecotoxicology for rapid assessment of dose-response relationships, biochemical and molecular mechanisms of toxicity, target cell and tissue structures, as well as sensitivity of different specimens and species to the selected toxicant (McQueen

1989; Paasivirta 1991; Peterle 1991; Huggett et al. 1992; Malins and Ostrander 1993; Klaassen 1996; Le Pennec and Le Pennec 2001).

The second category includes studies which examine stress responses in field-collected specimens and relate these responses to pollutants found in suspiciously high concentrations at the studied sites. However, in most instances, the type of response, the experimental setups and the studied species are not the same in studies of the two categories. An important question, therefore, is whether the bioassay results from studies in the first category can be compared to the responses observed in second-category studies. In other words, when different species, experimental procedures and organismal responses are used, the results of the first-category studies do not provide a causal explanation for the biological responses observed in the second-category studies.

In a recent series of analyses, using a set of fluorescent microscopic methods, we compared a range of biological responses in selected molluscs collected at various sites along the Mediterranean and Red Sea coasts of Israel (Bresler et al. 1999, 2003). In these 'second-category' studies, we exposed expressions of stress (e.g. increased cell membrane permeability), and preventive (e.g. increased activity of the MXRtr defence mechanism) responses in polluted-site populations compared to conspecific populations from reference (relatively clean) sites.

In a recent research effort within the framework of the MARS 2 project, we performed analyses of heavy metals and organic pollutants in water, sediment and animal tissue from the different sites (Feldstein et al. 2003). As indicated above, however, a question remained whether the organismal responses can be attributed to the pollutants effects.

To examine this question, we conducted a complementary 'first-category' study, in which we exposed molluscs to selected pollutants under controlled laboratory conditions, and examined the same responses using the same species and experimental and analytical procedures as in the field-oriented, second-category study. We examined, in vitro, early responses to acute action from two types of pollutants: water-soluble toxic metal ions (i.e. cadmium), and lipid-soluble pesticides (i.e. DDT). Cadmium (Cd), which is released from fossil fuels and during the production of agricultural fertilizers, paints, alloys, batteries and plastics, was selected because of its dominance in many coastal polluted sites (Paasivirta 1991; Peterle 1991; Maruham 1994; Waalkes and Misra 1996). DDT (dichloro diphenyl trichloroethane) was selected since it is one of the most common and stable contaminants in contaminated coastal waters. It should be noted that both substances were detected at elevated levels in some sites along the Mediterranean coast of Israel, especially in Haifa Bay, which is represented by the Shemen beach site (Pines et al. 1987; Herut et al. 1999; Kress et al. 1999).

Methods

Collection sites and studied species

Continuing with the programme outlined during MARS 2 (see Bresler et al. 2003), we focused our work on the same species of molluscs which we used for biomonitoring and which are common along the Israeli shores of the Mediterranean Sea and Red Sea. In the Mediterranean, the studied species were the clams *Donax trunculus* and *Mactra corallina*, and the limpet *Patella caerulea*. In the Red Sea, the species were the clams *Pteria aegyptia* and *Callista florida*, and the limpet *Cellana rota*. For sampling site locations and characteristics, see Bresler et al. (2003 Fig. 1 and Table 1). *M. corallina* and *Callista florida* were added for this study, and were collected from the same sites as the other clams. 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).

In vitro exposure experiments

For the in vitro experiments with Cd and DDT, we used stock solutions (1 mg/ml) of cadmium acetate (Merck, Germany) in distilled water, and DDT in acetone. Cd is one of the most toxic metals that accumulates in marine food chains (Romeo et al. 1995; Devi et al. 1996) and causes uncoupling of respiration and oxidative phosphorylation in mitochondria, alteration of cellular signalling and gene regulation, DNA damage, alterations of nuclear integrity and apoptosis (McQueen 1989; Paasivirta 1991; Peterle 1991 Huggett et al. 1992; Malins and Ostrander 1993; Devi et al. 1996; Klaassen 1996; Beyersmann and Hechtenberg 1997; Pruski and Dixon 2002). DDT is known to act as an uncoupler of respiration and oxidative phosphorylation within mitochondria; likewise, it is known as an inhibitor of several enzymes including Na, K-ATPase, endocrine disruptor, mutagen and clastogen (McQueen 1989; Paasivirta 1991; Peterle 1991; Worthing and Hance 1991; Huggett et al. 1992; Malins and Ostrander 1993; Maruham 1994; Klaassen 1996).

The shells of live bivalve molluscs (the clams) were opened by cutting the adductor muscles. The two valves, each with intact gills and mantle and half of the foot, were carefully separated. One half (the valve with its soft tissues) was incubated for 2 h at 25°C in aerated artificial seawater containing 1 mg/l Cd or 1 mg/l DDT. The other half was used as control and incubated for 2 h in artificial seawater with 1 ml/l corresponding solvent. Gastropods (the limpets) were incubated in toto in either Cd or DDT.

The paracellular permeability of epithelial layers was measured using the fluorescent anionic marker fluorescein, and intralysosomal accumulation of the cationic fluorescent marker neutral red. These are perhaps the most sensitive and reliable methods for quantitative and non-destructive assessment of cell viability (Bresler et al. 1999; Haugland 1999; Le Pennec and Le Pennec 2001).

The effect of Cd and DDT on the metabolic state of mitochondria was measured by the inherent blue fluorescence of reduced NADH and green fluorescence of oxidized flavoprotein (Chance 1964; Chance et al. 1998; Shiino et al. 1999; Bresler et al. 2003). The ratio between the blue and the green fluorescence, referred to as the metabolic ratio (M_{rat}), was calculated before and after incubation with Cd or DDT. The tissue was then incubated with neutral red or fluorescein to test the effect of the pollutants on lysosomal stability and paracellular permeability, respectively (Bresler et al. 1999).

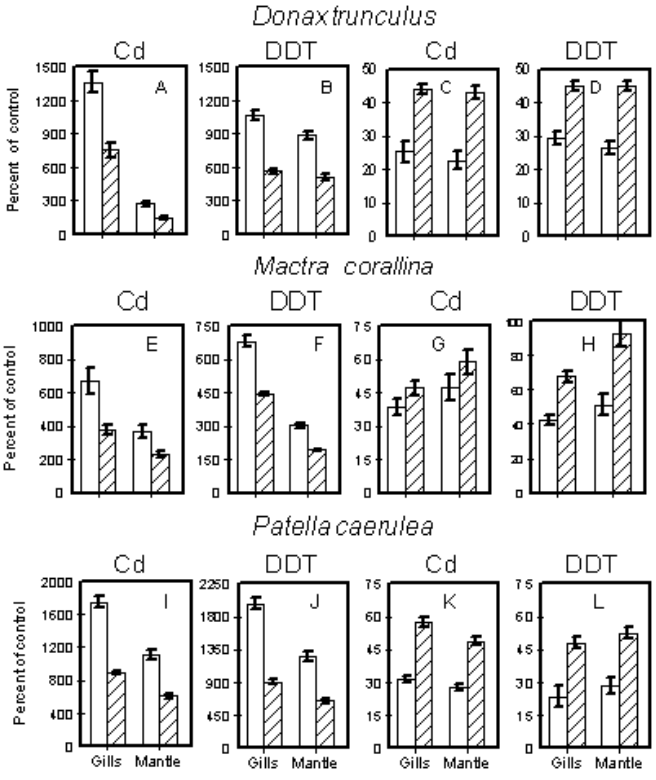

Fig. 1 Paracellular permeability and lysosomal stability in the gills and mantle tissue of the Mediterranean molluscs *Donax trunculus* (**A–D**), *Mactra corallina* (**E–H**) and *Patella caerulea* (**I–L**). The left column charts (**A**, **E**, **I**) and the charts next to them (**B**, **F**, **J**) depict changes in paracellular permeability following incubation with Cd and DDT, respectively. The right column charts (**D**, **H**, **L**) and the charts next to them (**C**, **G**, **K**) depict changes in intralysosomal accumulation of neutral red following incubation with DDT and Cd, respectively. Plotted values are averages and 95% confidence limits. *Open* and *hatched* bars represent reference and polluted sites, respectively. Sample sizes (*n*) are as indicated in Table 1

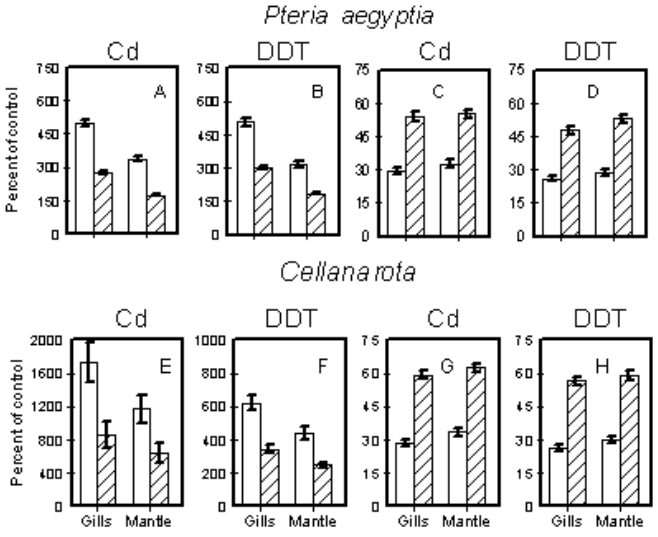

Fig. 2 Paracellular permeability and lysosomal stability in the gills and mantle tissue of the Red Sea molluscs *Pteria aegyptia* (**A–D**), and *Cellana rota* (**E–H**). The left column charts (**A**, **E**) and the charts next to them (**B**, **F**) depict changes in paracellular permeability following incubation with Cd and DDT, respectively. The right column charts (**D**, **H**) and the charts next to them (**C**, **G**) depict changes in intralysosomal accumulation of neutral red following incubation with DDT and Cd, respectively. Plotted values are averages and 95% confidence limits. *Open* and *hatched* bars represent reference and polluted sites, respectively. Sample sizes (*n*) are as indicated in Table 1

Results

The three studied stress symptoms, i.e. paracellular permeability, lysosomal stability and M_{rat}, showed similar trends in molluscs from the Mediterranean Sea and Red Sea.

The paracellular permeability of external epithelia increased dramatically after incubation with Cd or DDT, especially in the gills (Figs. 1, 2), i.e. there was a general difference in the trend of the responses of gill versus mantle tissue for both Cd and DDT. Gill tissue consistently showed sharper responses (Fig. 1). This shift was much more pronounced in animals from the reference sites than in those from the polluted sites (1.7–2.2 times larger).

Intralysosomal accumulation of neutral red decreased after incubation with Cd or DDT (Figs. 1, 2). Once again, animals from the reference sites were more severely affected by the Cd and DDT. In this case, the effect on reference-site molluscs was 1.3–1.5 times stronger. However, there was a difference between gills and mantle in lysosomal stability in only a few cases, as opposed to the difference observed between the tissues for paracellular permeability.

The observed effects were not equally intense in the various molluscs examined. The rocky intertidal gastro-

Table 1 Metabolic state of mitochondria (M_{rat}) in the gills of molluscs before (control) and after incubation with Cd or DDT

Species	Conditions	Site of collection	
		Maagan	Frutarom
Donax trunculus	Control	0.618±0.009	0.406±0.011
	Cd	0.217±0.014	0.254±0.013
	DDT	0.196±0.015	0.225±0.012
Mactra corallina	Control	0.587±0.007	0.381±0.017
	Cd	0.191±0.012	0.246±0.009
	DDT	0.186±0.015	0.236±0.012
		Caesarea	Shemen
Patella coerulea	Control	0.598±0.017	0.377±0.014
	Cd	0.213±0.015	0.244±0.011
	DDT	0.197±0.013	0.239±0.009
		MBL	Ardag
Pteria aegypta	Control	0.588±0.010	0.369±0.012
	Cd	0.181±0.016	0.209±0.011
	DDT	0.177±0.013	0.215±0.009
Cellana rota	Control	0.621±0.008	0.402±0.006
	Cd	0.211±0.015	0.263±0.011
	DDT	0.199±0.013	0.239±0.011
Callista florida	Control	0.592±0.019	0.387±0.150
	DDT	0.187±0.014	0.248±0.017

pods, *Patella caerulea* and *Cellana rota*, exhibited the highest shifts in measured values, followed by the shallow-water clam *D. trunculus*. Bivalves inhabiting deeper water (*M. corallina* and *Pteria aegyptia*) showed the least pronounced alterations. This indicates that deeper habitats are less perturbed.

The M_{rat} (Table 1) was higher in animals from the reference sites than in those from the polluted sites, as also reported by Bresler et al. (2003). The effects of Cd and DDT on the M_{rat} followed the same trend in all mollusc species examined, decreasing dramatically after incubation with either pollutant. As a rule, DDT incubation caused a slightly greater decrease in the M_{rat} than Cd, but this difference was, in most cases, not significant. This decrease was always significantly larger in animals from the reference sites than in those from the polluted sites.

Discussion

The exposure experiments clearly show that both Cd and DDT act as stress agents on the studied species from the Mediterranean Sea and the Red Sea, in accordance with a long list of studies that show the vulnerability of marine organisms to these pollutants. In our study, the stress is reflected in parameters documenting a decline in the activity of antichemical defence systems, at three different levels.

Firstly, as evident from the increase in paracellular permeability, we witnessed deterioration of the barriers restricting the access of xenobiotic substances into the organism's tissues. The magnitude of the effect was, in some cases, more than ten-fold, compared with control animals (Figs. 1 and 2). Secondly, a central cellular mechanism for dealing with xenobiotics that have already permeated—the lysosomes—was observed to deteriorate following exposure to the pollutants. Finally, the physiology of the organism was strongly affected, as evident from the adverse effects on the state of the mitochondrial respiratory chain. These negative alterations can be related to each other. The decrease in the M_{rat} signifies uncoupling between respiration and oxidative phosphorylation that is typical for acute action of Cd and DDT (Devi et al. 1996; Klaassen 1996; Chance et al. 1998). Increased paracellular permeability and decreased intralysosomal NR (neutral red) accumulation are typical cellular alterations produced by the uncoupling and ATP depletion (Trump and Ginn 1969; Cotran et al. 1989).

The pattern of change in M_{rat} is worth closer examination. While molluscs collected from reference sites had a higher M_{rat} than molluscs from polluted sites, they were also more severely affected by the pollutants (Table 1). While the M_{rat} decreased less than two-fold in animals from the polluted sites, it decreased nearly three-fold in molluscs from the reference sites. One way to explain this difference is that animals from the polluted site activate some type of response that was not measured which acts to protect their metabolic pathways. This is in agreement with the results obtained for paracellular permeability and intralysosomal neutral red accumulation. In all cases, the animals from polluted sites are better protected from the effect of xenobiotic, toxic chemicals. Their paracellular permeability increased much less than that of reference site molluscs, their lysosomes deteriorated to a lesser extent, and it is, thus, reasonable that their mitochondria were less affected.

Our previous studies demonstrated a number of defence mechanisms, the levels of which may be responsible for the differences observed between molluscs from the reference and polluted sites. These include export pumps, detoxifying enzymes, and metallothionein (Bresler et al. 1999, 2001, 2003). Metallothionein and reduced glutathione were shown to play an important role in metal detoxification in bivalve molluscs, particularly Cd (Zaroogian and Jackim 2000). MXRtr, an export pump for lipophilic xenobiotics elimination, can decrease DDT penetration into cells. Detoxifying enzymes and glutathione-s-transferase can transform DDT into metabolites that can be eliminated by the SATOA pump (Sheehan et al. 1995; Bresler et al. 1999, 2001, 2003).

An important point that must be addressed prior to using a species as a biomonitor is the genetic homogeneity between reference and polluted sites. In the context of this study, the question is whether the observed differences in levels of anti-chemical defence reflect phenotypic plasticity or speciation. The potential pitfall is that anthropogenic environmental stress (i.e. pollution) may select for better protected phenotypes/genotypes, causing speciation in mollusc populations (Nevo 2001; Fishelson et al. 2003). Differential, site-specific survival would make comparative biomonitoring less efficient. If, indeed, selection has taken place, then any difference observed in biological responses between reference and polluted site animals may combine genetic capabilities with responses. It may sometimes be difficult to differentiate between the purely phenotypic components which are effective for monitoring environmental conditions, and other components which are not. In another study conducted by our research group (Barak et al., in preparation), we carried out a series of transplantation experiments in the field, wherein *Patella caerulea* individuals collected in a polluted site were transferred for a month to a reference site, and vice versa. We then examined the activity of the MXRtr anti-chemical defence mechanism (see Bresler et al. 1999, 2003 for explanations) in the transferred and in control animals. A preliminary examination of the results reassures us that at least this mechanism in *P. caerulea* features phenotypic plasticity, qualifying it as a useful biomonitoring tool.

The three responses of molluscs to pollutants measured in this study are among the parameters suggested for the establishment of a routine biomonitoring programme along the Mediterranean and Red Sea coastal areas (Bresler et al. 2003). The findings reported here may aid refining such a programme. For example, the choice of tissues to be examined may be facilitated by the

knowledge of which type of tissue displays a sharper response to the presence of pollutants.

Searching for direct links between every single pollutant and a wide array of stress responses is an endless task. Moreover, it is reasonable to assume that synergistic effects of various combinations of chemical 'species' are likely to play a very important role in triggering organismal responses. Thus, advocating studies similar to the current one, which will examine all known and suspected pollutants, is unreasonable. Nevertheless, we do advocate making complementary, 'second-category' studies a routine, as it is very important to verify that a biotic response suggested for biomonitoring pollution is in some way related to pollution.

By way of a reasonable compromise, one may wish to proceed with using representative, 'test chemicals' in order to examine the biological responses in the same animals used for biomonitoring. Whether the test chemical used in laboratory experiments is exactly the one found in situ, or just has a similar effect, may sometimes be very difficult to determine. In the current study, Cd and DDT should, therefore, be viewed as representing larger groups of toxic metals and organic compounds, respectively.

The basic assumption during the MARS project was that the elevated stress responses observed in specimens from the polluted sites are related to the effects of pollutants prevailing in the water and sediments. However, so far no experiments have been conducted to establish this link. The reported exposure experiments enable us to relate the specific stress responses examined in the MARS project to specific pollutants that may exist in some of the polluted sites along the Mediterranean coasts (e.g. Shemen Beach). This does not exclude the possibility that additional pollution agents and synergistic effects prevailing in the field participate in eliciting the examined responses.

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