TOXICITY OF FLUORIDE TO THE FRESHWATER MOLLUSC *DREISSENA POLYMORPHA*: EFFECTS ON SURVIVAL, HISTOLOGY, AND ANTIOXIDANT ENZYME ACTIVITY

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SUMMARY: The sensitivity of the freshwater non-indigenous mollusc *Dreissena polymorpha* towards fluoride (F) toxicity was examined in short-term (96-hr LC50; lethal concentration fifty) and long-term (18 days) laboratory experiments. The results indicate that the species is very resistant to F in water, much more than other aquatic invertebrates. In addition, they indicate a concentration- and time-dependent induction of oxidative stress by F exposure and a consequent alteration of the activities of antioxidant enzymes such as superoxide dismutase and catalase. The activity of both of these enzymes progressively decreased with increase of the exposure period when the F concentration was kept constant. Moreover, the appearance of pyknotic nuclei, as well as positivity to TUNEL analysis (Terminal deoxynucleotidyl transferase dUTP nick end labelling) suggests activation of the apoptotic machinery in tissues of animals exposed to high F concentration. This result appears to be related to a sustained condition of oxidative stress.

Keywords: Antioxidant enzymes; Apoptosis; *Dreissena polymorpha;* Fluoride toxicity to molluscs; Freshwater molluscs; Oxidative stress;

INTRODUCTION

The concentration of fluoride ions (F⁻) is significantly increasing in aquatic ecosystems as a consequence of many industrial activities.[1,2] In unpolluted freshwaters, it ranges from 0.1 to 0.3 mg/L, but it can increase more than 100-fold in drainage waters containing phosphate fertilizers, pesticides, and pollution from brick, ceramics, and glass manufacturing.[3] There are many studies regarding the safe concentration of F ions for the algae and aquatic plants, invertebrates, and vertebrates in fresh, brackish, and salt waters.[4-8] These studies have found that aquatic organisms living in soft waters are more adversely affected by F pollution than those living in hard or seawater, since the bioavailability of F ions is reduced with increasing water hardness.

F toxicity to aquatic invertebrates increases with increasing concentration, exposure time, and water temperature, and decreases with increasing individual body size. These results have also revealed that the sensitivity to F can be very different among the species studied. Safe concentrations are greatly variable within classes, families, and genera.[3] In addition, research on the molecular mechanisms of F toxicity indicates that certain xenobiotics can induce excessive production of free radicals and affect the antioxidant defense system.[9,10] Thus, F-induced changes in biochemical parameters in liver, muscle, and testis tissues of freshwater catfish *Clarias batrachus* have been reported,[11] along with altered enzyme activity in muscle and liver of the fish *Channa* punctatus.[12]

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Study aim: The freshwater mollusc, *Dreissena polymorpha* (zebra mussel) is known to be highly resistant to many kinds of pollutants and is frequently employed in biomonitoring.[13-20] In our study of the toxicity of F to this species, we performed short and long-term exposure experiments. In addition, we investigated the histological and biochemical effects of xenobiotics on treated specimens. As fluoride is reported to alter intracellular redox homeostasis and induce oxidative stress,[21] we measured the activity of two antioxidant enzymes, i.e., superoxide dismutase (SOD and catalase, in exposed animals as compared to untreated animals. Moreover, since persistent oxidative stress can induce cell death by apoptosis,[22] we also examined the morphology of the cells in the tissues of exposed and control animals.

MATERIALS AND METHODS

Organisms: Specimens of *Dreissena polymorpha* were obtained from Lake Garda (northern Italy); they were collected at a depth of 2–3 m, their length varying between 1.5 and 2 cm. They were reared in aquaria filled with original lake water (its principal chemical characteristics are listed in Table 1) and acclimated to the laboratory conditions at a temperature of 17±0.5ºC for two weeks before their use. During this phase, the animals were fed daily with liquid food for filter-feeding invertebrates. No mortality occurred during acclimatization.

Table 1. Test conditions for 96-hr and 18-day toxicity bioassays on *Dreissena polymorpha* and chemical-physical characteristics of Lake Garda

	96-hr test	18-day test
Acclimatisation period	14 days	14 days
Water renewal	none	every other day
Feeding	none	liquid food for filter feeding
Temperature	17± 0.5ºC	17± 0.5ºC
Test solution volume	0.6 L	0.6 L
Aeration	yes	yes
Test concentrations (mg F/L)	90, 180, 360, 720, 1440	120, 160, 200, 240
Number of replicates	3	3
Number of mussels for replicates	8	8
Chemical characteristics of Lake Garda (used for the bioassays)	Dissolved oxygen saturation (DO sat) 80% ± 5 Total hardness 106 ± 14 mg CaCO$_3$/L pH 8 ± 0.1 Conductivity 208.5 ± 10 µS/cm [F] < 0.2 mg/L	Dissolved oxygen saturation (DO sat) 80% ± 5 Total hardness 106 ± 14 mg CaCO$_3$/L pH 8 ± 0.1 Conductivity 208.5 ± 10 µS/cm [F] < 0.2 mg/L

Experimental procedures of toxicity bioassays: Various toxicity bioassays were carried out in a thermostated (17±0.5ºC) room using small glass aquaria (0.6 L) aerated to maintain 80±5% oxygen saturation. The test conditions are listed in Table 1. Sodium fluoride (NaF, Merck) working concentrations were checked with a F probe according to the standardized colorimetric method.[23]

In the first short-term (96 hr) bioassay (without water renewal and animal feeding) eight mussels were exposed in triplicate to a range of nominal F^- concentrations of 90, 180, 360, 720, and 1440 mg F^-/L. In the control, the F^- concentration was less than 0.2 mg F^-/L.

Specimens were considered dead when they did not react to any mechanical stimulus. LC50 values at 24, 48, 72, and 96 hr and their respective 95% confidence limits were calculated from mortality data, exposure time, and toxicant concentration, using the multifactor probit analysis (MPA; software SAS ver. 9.1.3).[24] The dependent variable is the probit of the proportion of animals responding to each concentration, and the independent variables were exposure time and toxicant concentration.

For the long-term experiments, 18 days of testing were conducted using the same methods as in the acute tests except that mussels were fed, and water and F^- concentrations were renewed every other day. Eight specimens in triplicate were exposed to a range of nominal F^- concentrations of 120, 160, 200, and 240 mg F^-/ L, based on the results of the short term bioassay.

Since fluoride are taken up directly from water by aquatic animals, the F^- concentration in water was determined after 96 hr and after 11, 15, and 18 days with a F^- ion-specific electrode WTW (Wissenschaftlich-Technische Werkstätten Gmbh); measuring range: 0.02 mg F^-/L up to saturation, with TISAB III to adjust pH to ca. 5.2. Final F^- ion concentrations were then compared with initial concentrations.

Histological and biochemical analysis: Specimens exposed for 24 hr to 120, 240, 360, 720, and 1440 mg/L concentrations of F^- were fixed in 4% paraformaldehyde in 0.4 M cacodylate buffer, dehydrated, and embedded in Paraplast Xtra (Oxford Labware, St. Louis, Mo.). Sections (7 µm) were cut with a Leitz 1212 microtome, mounted on glass slides, deparaffined, and stained for 5 min with Mayer's haematoxylin solution. Slides were then washed, dehydrated, mounted in Eukitt (Fluka), and observed under light microscopy. Pyknotic nuclei appeared as intensely stained spots.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay: To reveal DNA fragmentation, dewaxed and rehydrated sections were incubated in the terminal dUTP nick-end labelling (TUNEL) reaction mixture (*in situ* cell death detection kit, Roche) for 60 min at 37ºC, according to the manufacturer's instructions. Subsequently, they were incubated with a peroxidase-conjugated anti-fluorescein-isothiocyanate (FITC) antibody, stained with 0.63 mM DAB in phosphate buffered saline (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M Kh_2PO_4, 0.065 M Na_2HPO_4) containing 4% hydrogen peroxide, washed in distilled water,

counterstained with haematoxylin, dehydrated, mounted with Eukitt (Fluka) and observed under light microscopy. The presence of fragmented DNA was revealed by dark brown staining.

Superoxide dismutase activity: Total SOD activity was measured in homogenates from specimens exposed for 96 hr at various fluoride concentrations ranging from 0, 240, 360, 720, and 1440 mg/L. At 1440 mg F⁻/L the molluscs did not survive for more than 1 day, and at 360 or 720 mg F⁻/L total mortality was observed in 2–4 days. Animal soft tissues were homogenised in an equal volume of PBS with an Ultraturrax homogeniser. The xanthine oxidase/cytochrome c method, of Crapo et al.,[25] was followed. The cytochrome c reduction by superoxide anions generated by the xanthine oxidase/hypoxanthine reaction was detected at 550 nm at room temperature. SOD enzyme activity was expressed as U/mg of proteins, one unit of SOD being defined as the amount of sample producing 50% inhibition in the assay conditions. The reaction mixture contained 10 µL of sample and 990 µL of a solution containing 46.5 µM KH_2PO_4/K_2HPO_4 buffer (pH 8.6), 0.1 mM ethylenediaminetretraacetic acid sodium salt (EDTA), 195 µM hypoxanthine, 16 µM cytochrome c, and 2.5 µU xanthine oxidase.

Catalase activity: Catalase activity was measured, in the same foregoing samples, following the method of Aebi (1984).[26] Decreases in absorbance of a 1 mL reaction mixture containing 990 µL 50 mM H_2O_2 (ε –0.0436 mM⁻¹cm⁻¹) solution in 50 mM phosphate buffer (pH 7.8) and 10 µL of sample were continuously recorded at 240 nm at 10 s intervals for 1 min. Results are expressed in U/mg of proteins, one unit of catalase being defined as the amount of enzyme that catalyzes the dismutation of 1 µmol of H_2O_2/min.

RESULTS

Short-term experiment: In the short-term bioassays, the mortality of the zebra mussels or mollusks rose with the increase of both F concentration and exposure time. No mortality occurred in the first 24 hr at F concentrations up to 360 mg/L; in the same period, 60% of animals exposed to the 720 mg F⁻/L died. After 96 hr, mortality also occurred at 180 and 360 mg F⁻/L (6.7% and 40%, respectively). Complete mortality occurred at a concentration of 720 mg F⁻/L, and complete survival occurred at a concentration of 90 mg F⁻/L No mortality was observed in controls during the test. Estimated LC50 values for this toxicity bioassay are reported for different exposure times in Table 2. These values exhibited a trend of decreasing survival with increasing exposure times.

**Table 2.** LC$_{50}$, NOEC, and LOEC values for *D. polymorpha*

Time	Water hardness (mg CaCO$_3$/L)	LC50 (mg F$^-$/L) 95% confidence limits are in parenthesis	LOEC (mg F$^-$/L)	NOEC (mg F$^-$/L)
48 hr	106 ± 14	469.41 (364.07–653.97)		
72 hr	106 ± 14	430.15 (331.94–588.3)		
98 hr	106 ± 14	360.88 (292.75–454.33)		
352 hr	106 ± 14		120	90

Long-term experiment: Concentrations that were not toxic in the 96-hr short-term experiments induced mortality when the exposure time was increased up to 18 days. Concentrations below 180 mg F$^-$/L killed no mussels after 96 hr, but at 18 days they had a very clear negative effect on survival: 30% of the exposed animals died at 120 mg F$^-$/L, 47% at 160 mg F$^-$/L, and 80% at 200 mg F$^-$/L with complete mortality at 240 mg F$^-$/L (Figure 1).
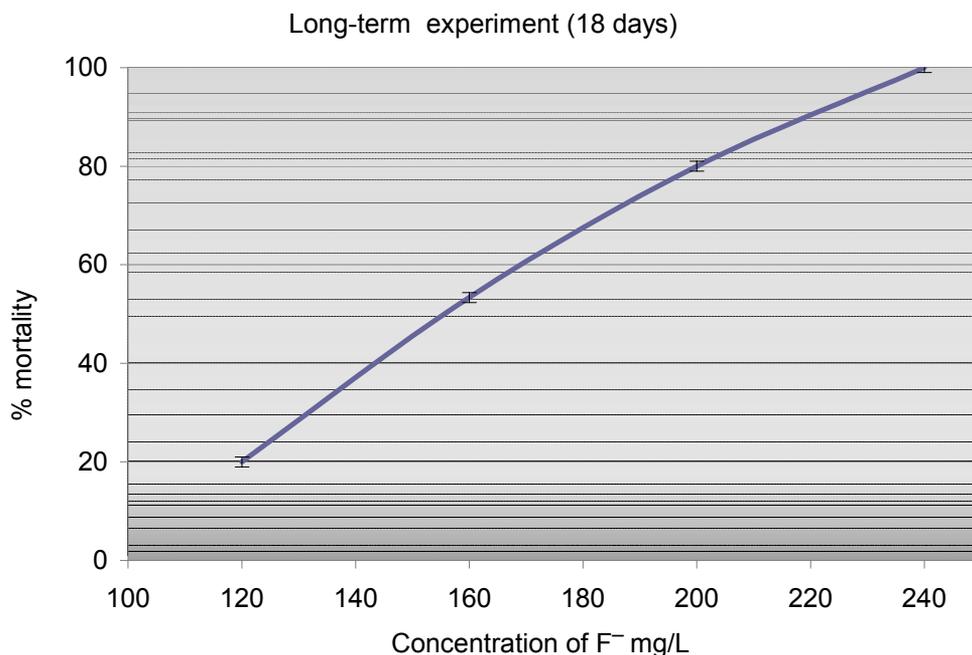
Figure 1. Mortality of *Dreissena polymorpha* exposed to different fluoride concentrations at the end of the log term exposition (18 days).

The observed no-effect concentration (NOEC) and lowest effect concentration (LOEC) were 90 and 120 mg F$^-$/L, respectively (Table 2). It should also be noted that the water F concentration decreased during the bioassays. At the end of the experiment the final reduction ranged between 65 and 70% of the various original concentrations (Figure 2).
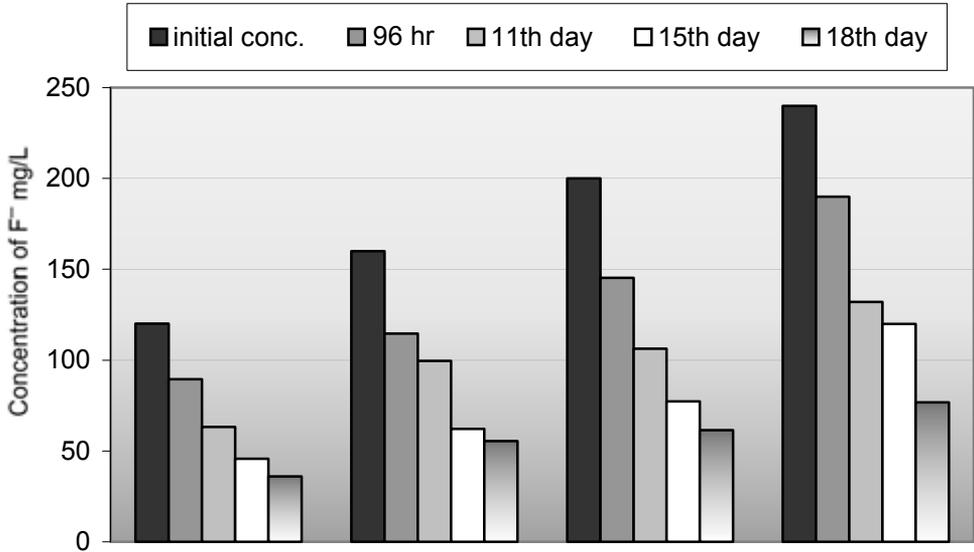
Figure 2. Decrease of fluoride concentration in water during the long-term experiment.

Histological analysis: When the tissues from samples exposed for 24 hr to F were observed under the microscope, no morphological cell alterations were observed at concentrations lower than 240 mg F⁻/L (Figure 3a).

Figure 3a. Histology of *Dreissena* gills. Control section of gills from unexposed animal. Scale bar: 20 μm.
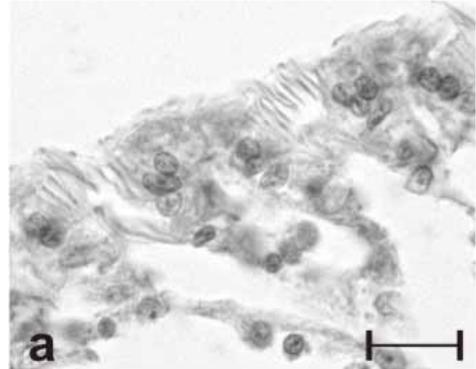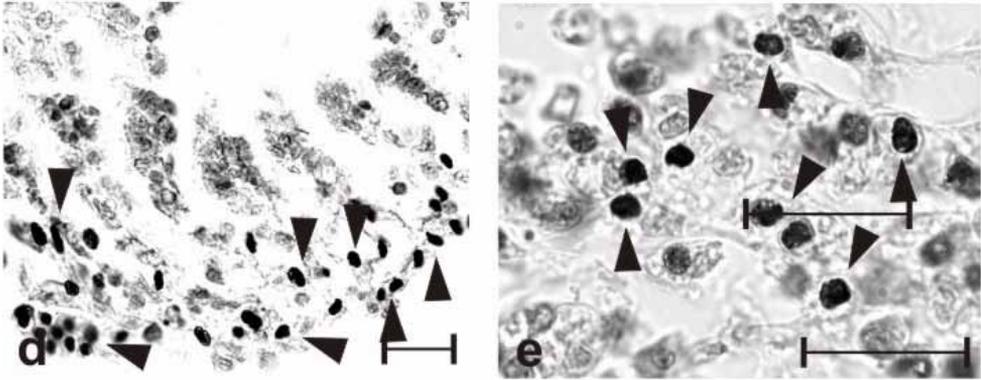

At 360 mg/L, scattered pyknotic nuclei were observed in the branchial epithelium (Figures 3b and 3c).
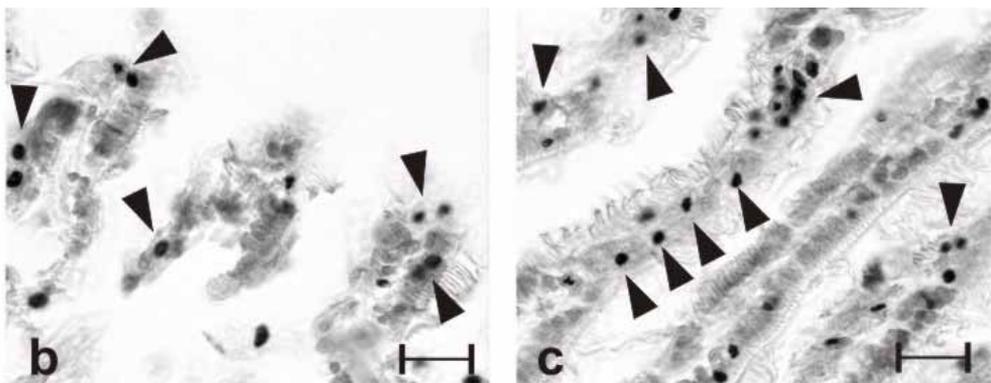
Figures 3b and 3c. Histology of *Dreissena* gills. Gills were taken from specimens exposed for 24 hr to 360 mg F⁻/L. Scale bar: 20 μm.

At 720 mg F⁻/L, the frequency of cells with condensed nuclei in the branchial epithelium increased, but no changes in the cell shape were observed (Figures 3d and 3e).
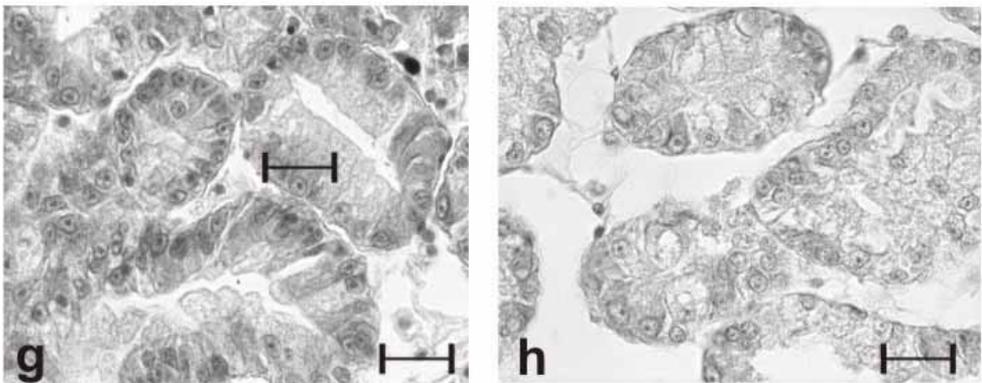
Figures 3d and 3e. Histology of *Dreissena* gills. Gills were taken from specimens exposed for 24 hr to 720 mg F⁻/L. Scale bar: 20 µm.

At 1440 mg F⁻/L, pyknotic nuclei were abundant in the branchial epithelium (Figure 3f).

Figure 3f. Histology of *Dreissena* gills. Gills were taken from specimen exposed for 24 hr to 1440 mg F⁻/L. Scale bar: 20 µm.
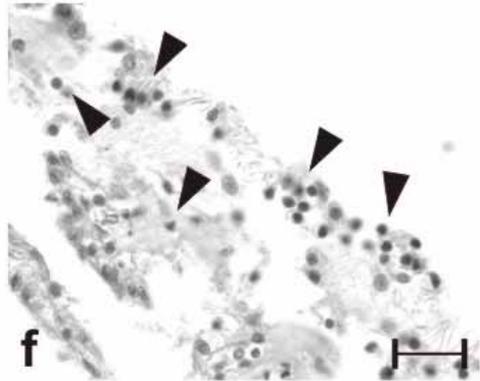
At 720 mg F⁻/L, no alterations were present in cells of the hepatopancreas (Figures 3g and 3h).

Figures 3g and 3h. Histology of *Dreissena* hepatopancreas. 3g: Control section of hepatopancreas from unexposed animal. 3h: Specimen from animal exposed for 24 hr to 720 mg F⁻/L. Scale bar: 20 µm.

At 1440 mg F⁻/L, pyknotic nuclei were abundant in the hepatopancreas (Figure 3i).

Figure 3i. Histology of *Dreissena* hepatopancreas. Specimen from animal exposed for 24 hr to 1440 mg F⁻/L. Scale bar: 20 µm.
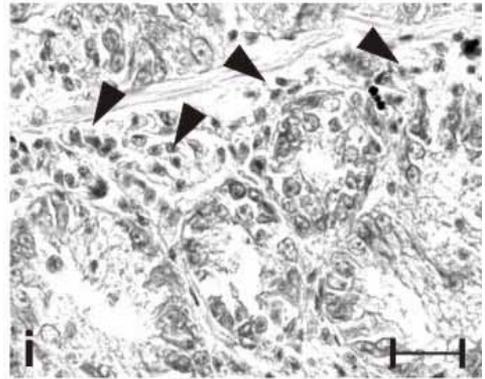

With both the brachial epithelium and the hepatopancreas at 1440 mg F⁻/L, the tissues showed altered morphology of the cells, which appeared shrunk and with abnormal shape (Figure 3i). The TUNEL assay clearly indicated that pyknosis was related to DNA fragmentation, since all the condensed nuclei were positively stained (Figure 4).
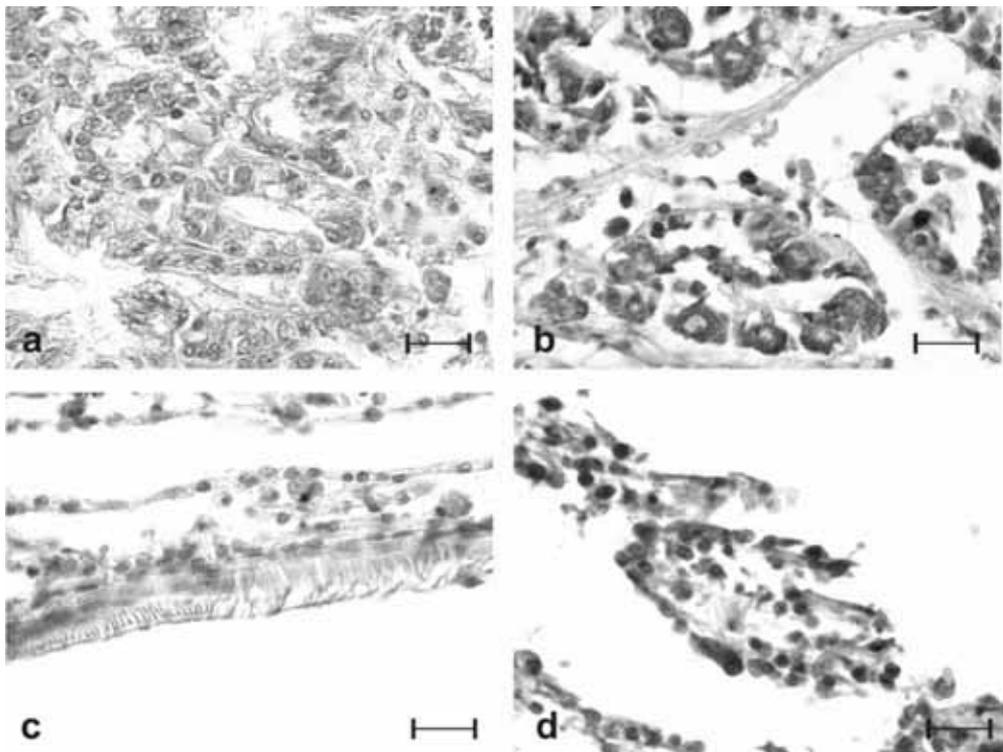
Figure 4a–4d. TUNEL reaction on sections of hepatopancreas (4a and 4b) and gills (4 c and 4d). 4a and 4c: Unexposed animals (control sections). 4b and 4d: Sections from animals exposed for 24 hr to 1440 mg F⁻/L. The presence of fragmented DNA, either inside nuclei or in the cytoplasm, is marked by a dark brown staining; healthy nuclei are stained blue. Scale bars:

SOD and catalase activity: The activity of SOD from animals exposed to F for two days, was significantly different ($p<0.05$) from the control at all the concentrations assayed, with the exception of 1440 mg F⁻/L. The activity was significantly ($p<0.05$) lower with respect to control at 240 and 720 mg F⁻/L,

whereas at 360 mg F⁻/L, a nearly five-fold ($p<0.001$) increase in the activity was observed (Figure 5a). In the case of catalase, the enzyme activity decreased significantly at 360, 720, and 1440 mg F⁻/L (Figure 5c). When the homogenates from samples exposed for various days at the same concentration of 360 mg F⁻/L were compared, the SOD and catalase activities progressively decreased with exposure time. In the case of SOD, the activities were significantly reduced ($p<0.05$) at 3 and 4 days (Figure 5b) compared to one-day exposure, whereas, for catalase, the decreases differed significantly ($p<0.01$) from one-day-exposed animals compared to all the exposure times (Figure 5d).
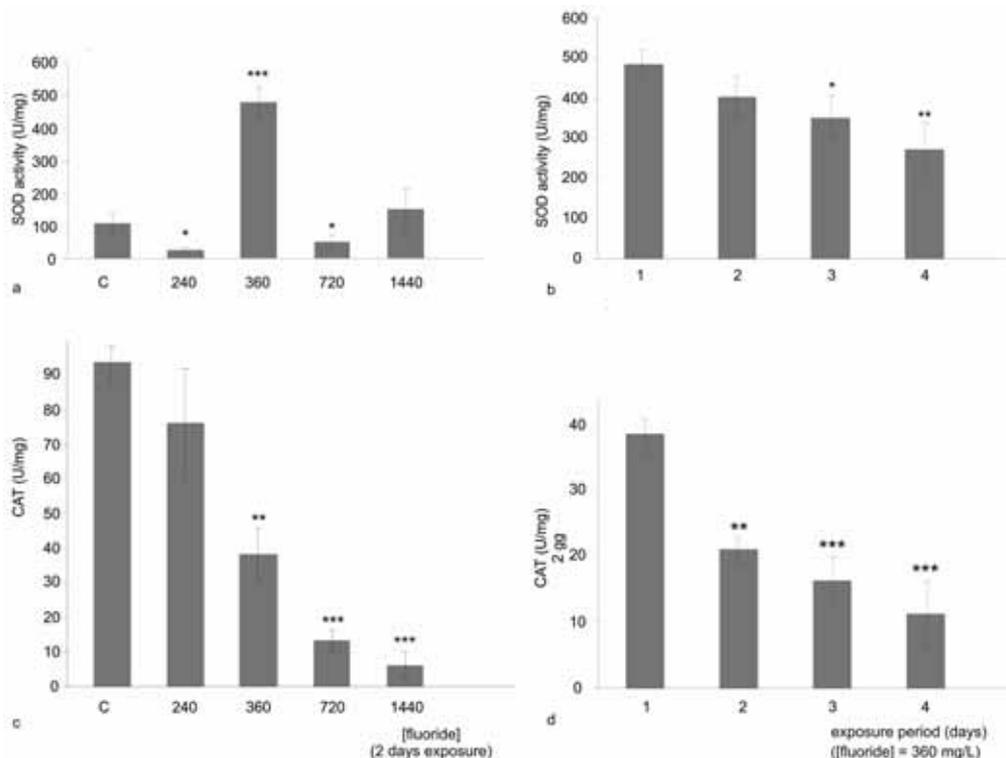
Figure 5a–5d. SOD (5a and 5b) and catalase (5c and 5 d) activity of homogenates from animals exposed for various times to various fluoride concentrations. 5a and 5c: SOD (5a) and catalase (5c) activity of homogenates from animals exposed for 48 hr at various fluoride concentrations. 5b and 5d: SOD (5b) and catalase (5d) activity of homogenates from specimens exposed to 360 mg F⁻/L for 1-4 days. Significant differences with respect to untreated animals (5a and 5c) or 1 day-exposed animals (5b and 5d) are marked by asterisks: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

DISCUSSION

From the results of the short and long-term experiments, *Dreissena polymorpha* appeared to be very resistant to F toxicity, in accordance with its known resistance towards many kind of pollutants. A comparison of our toxicity data with those of other aquatic animals reveals that this species is among the least sensitive to F among the freshwater invertebrates so far studied, being more resistant than *Daphnia magna,* one of the most tolerant species among invertebrates[27] and much more than *Dikerogammarus villosus*, a non-indigenous, widespread species original from the same Ponto-Caspian area.[28]

Concentrations of F up to 120 mg/L appeared to be ineffective in terms of cell morphology during short-term exposure (24 hr). No direct effect was observed at F concentrations lower than 240 mg/L, which fits with the observed resistance of the species to F exposure. Only exposure to higher F levels led to alteration in cells of the branchial and hepatopancreas tissue, which consisted of a dose-dependent induction of nuclear pyknosis and, at 1440 mg/L, the appearance of cell shrinkage.

An increasing number of reports have indicate that one of the causes of F toxicity is the induction of oxidative stress, i.e., the excessive production of reactive oxygen species overwhelming the antioxidant potential of the cells represented by cytoplasmic thiols (e.g., glutathione) and antioxidant enzymes such as SOD, catalase, and glutathione peroxidise.[21,29-32] As persistent oxidative stress causes apoptosis,[22] exposure to F frequently results in the induction of cell death, both *in vivo* and *in vitro*.[21,32-34]

The observed induction of nuclear condensation and cell shrinkage by high F concentrations, events that are typically related to the induction of cell death by apoptosis.[35] Moreover, the TUNEL positivity of nuclei in tissues of animals exposed to high F concentrations agree with the hypothesis of cell death induction in tissues of specimens exposed to high F concentrations. They also stress, once more, the high capability of *Dreissena* to counteract F pollution as morphological signs of unhealthy cells that are visible only at F concentrations higher than 240 mg/L. At these higher F concentrations, both SOD and catalase activities in homogenates from exposed animals were altered in a concentration- and time-dependent manner. At 240 mg F⁻/L, SOD activity was greatly reduced, with respect to untreated *Dreissena* specimens after 48-hr exposure; it significantly increased at 360 mg F⁻/L and decreased again at 720 mg F⁻/L. At 1440 mg F⁻/L, the SOD activity was comparable to that of controls after 24 hr-exposure.

This behaviour is consistent with the repeated induction of SOD gene(s) expression leading to new protein synthesis (for instance, after exposure to 360 mg/L F) upon the inhibition of the antioxidant enzymes by F[21] and oxidative stress conditions. Indeed, most of the studied SOD genes contain antioxidant responsive elements, able to trigger gene expression, in their promoter.[36] Conversely, catalase activity progressively decreased as the F concentration increased, suggesting the absence of gene expression induction and the irreversible inhibition of the enzyme activity by F.[21,29] The activity of both enzymes progressively decreased with the increase of the exposure period when the F concentration was kept constant. Again, this result fits the reported ability of F to inhibit antioxidant enzyme activity.[21] In these circumstances we found no evidence of induction of gene expression, probably because prolonged exposure to F cases to a general alteration of cell metabolism leading, ultimately, to cell death.[32,34]

Further studies on the regulation of gene expression in F-exposed molluscs and the analysis of the transcriptome in various experimental situations will no doubt contribute to a better understanding of the molecular basis of the resistance of *Dreissena polymorpha* to toxic effects of polluted water.

ACKNOWLEDGEMENTS

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