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RESEARCH REPORT

Immunotoxic effects of fluoride on the hemocytes of *Venerupis philippinarum*

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Abstract

The increasing levels of fluoride in the aquatic environments is a matter of concern for freshwater and marine ecosystems. Although data on fluoride toxicity to aquatic organisms are increasing in the scientific literature, few data are available regarding the effects, at cellular level, on freshwater or marine invertebrates. In the present paper, we present data on the effects of *in vitro* exposure to sodium fluoride (NaF) on the hemocytes of the Manila clam *Venerupis philippinarum*. Results indicate that NaF reduces cell spreading and phagocytosis in a dose-dependent way, through the alteration of the actin cytoskeleton. In addition, the compound decreases the stability of the lysosomal membranes, as revealed by the Neutral Red assay. In addition, the observed increase in hemocyte mortality and the parallel rise in DNA fragmentation inside their nuclei, as revealed by the TUNEL assay, suggest a NaF-dependent induction of apoptosis, in accordance with the capability of NaF to induce oxidative stress, a known cause of apoptosis, As hemocytes represent the major defence weapon against foreign, potentially pathogenic microbes, the above results indicate a negative effect of NaF on the immune status of *V. philippinarum*.

Key Words: *Venerupis philippinarum*; bivalve molluscs; sodium fluoride; hemocytes; immunotoxicity

Introduction

Fluorine belongs to the VIIA group of the periodic table and is widely distributed in the environment, mainly as fluorite, a calcium salt of the ionic form fluoride. Fluoride was considered essential for tooth and bone development in mammals, although, today, a lack of consensus is emerging on its possible role in human nutrition, development and growth (Nielsen, 2009). High fluoride concentrations can induce detrimental effects on cells and organisms as they can act as general inhibitors of various enzymes (Barbier *et al.,* 2010). Recent results indicate that, when present at high levels, the ion causes cell toxicity, mainly related to the induction of oxidative stress and the activation of the apoptotic pathway (Barbier *et al*., 2010; Gutowska *et al.,* 2010; Jacinto-Alemán, 2010; Wang *et al.,* 2010; Liu *et al.,* 2011). Fluoride, in fact, can increase the release of cytochrome c from mitochondria, inhibit antioxidant enzyme activity, rise the production of reactive oxygen species and activate MAP-kinase-dependent signaling pathways (Barbier *et al.,* 2010; Inkielewicz-Stepniak and Czarnowski, 2010; Basha *et al.*, 2011; Chen *et al*., 2011; Izquierdo-Vega et al., 2011). In addition, fluoride can interfere with fertilization (Izquierdo-Vega *et al.,* 2008; Izquierdo-Vega *et al.,* 2011), mammalian development (Zhu *et al*., 2013) and amphibian metamorphosis (Zhao *et al*., 2013).

As regards the aquatic environment, fluoride is present in unpolluted waters at concentrations ranging from 0.01 to 0.3 mg/l, however its level can increase more than 100 times as a consequence of human activities (Camargo, 2003). Fluoride can accumulate in the exoskeleton of arthropods and in vertebrate bones, as well as in the soft tissues of some invertebrates such as mussels and barnacles (Barbaro *et al.,* 1981a). As for the toxic effects, fluoride can lead to reduced male and female fecundity, growth impairment and mortality in aquatic organisms (Casellato *et al*., 2013). Seawater or estuarine organisms are usually less affected by high fluoride levels than those living in freshwater (Camargo, 2003): this probably explains the paucity of data available for marine animals.

Bivalve molluscs are filter-feeding organisms widely used in the assessment of the quality of the aquatic environment and fluoride toxicity has been reported in the freshwater mussels *Alasmidonta raveneliana* (Keller and Augspurger, 2005) and

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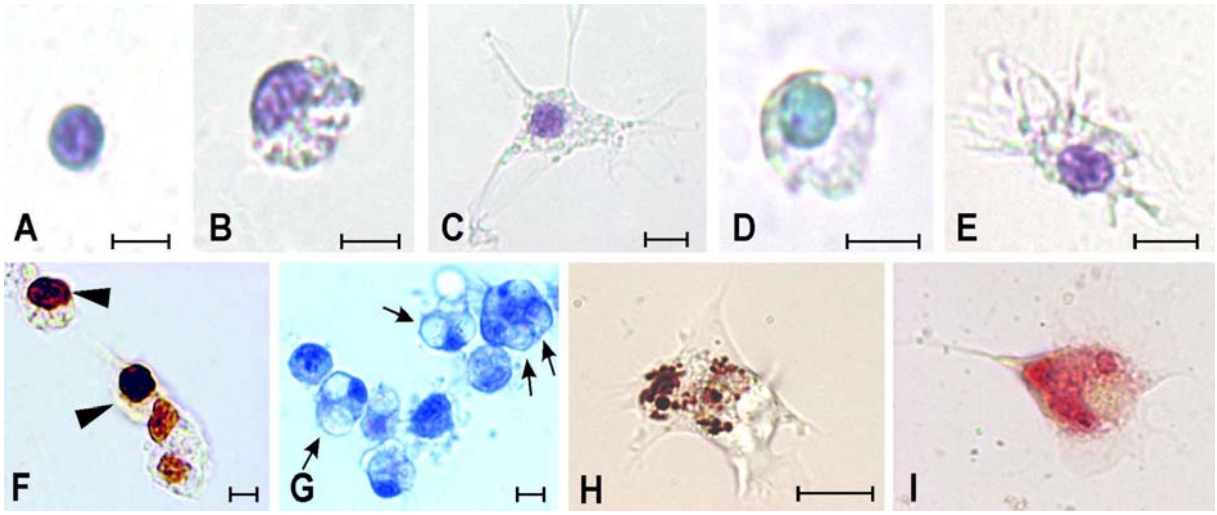

Fig. 1 A-E: fixed hemocytes stained with Giemsa's solution. A: undifferentiated cell; B-C: granulocyte in the round (B) and spreading (C) morphology; D-E: hyalinocyte in the round (D) and spreading (E) morphology. F: TUNEL assay for DNA fragmentation; arrowheads indicates positive nuclei with fragmented DNA. G: Giemsa-stained hemocytes with ingested yeast cells (arrows). H-I: Neutral red assay on control (H) and NaF-exposed (I) cell. Bar = 5 μm.

Dreissena polymorpha (Casellato *et al*., 2012; Del Piero *et al*., 2012).

As invertebrates, bivalves rely only on innate immunity to prevent foreign, potentially pathogenic cells or microbes from entering the organism. Circulating hemocytes represent their major defense weapon and a loss of hemocyte functionality can be deleterious for the whole organism.

At least three hemocyte types have been described in the clam *Venerupis* (previously *Ruditapes* = *Tapes*) *philippinarum*: phagocytes (including granulocytes and hyalinocytes), hemoblasts or undifferentiated cells and serous cells (Cima *et al.,* 2000). This organism is emerging as a reliable model species for studying the effects of xenobiotic at various levels and variations in various parameters, including those relate to immune responses, have been reported in this species as a consequence of the exposure to pollutants (Matozzo *et al*., 2001, 2002, 2012; Matozzo and Marin, 2005; Aguirre-Martínez *et al*., 2013).

In the present work we studied, in *V. philippinarum*, the effects of *in vitro* exposure to fluoride on hemocyte functionality, with particular attention to phagocytes in order to get information on their mechanisms of action. We used NaF concentration ranging from 10 to 250 μg/ml, the lower values resembling the concentration values reported for polluted waters in the lagoon of Venice (Barbaro *et al.,* 1981b) and the higher values being similar to those used for *in vitro* experiments with mammalian cultured cell lines (Barbier *et al.,* 2010). Results indicate detrimental effects on phagocytosis, probably related to cytoskeletal alterations, lysosomal stability, and cell survival, due to the induction of apoptosis.

Materials and Methods

Animals

Venerupis philippinarum from the lagoon of Venice were kept in tanks containing filtered sea water (FSW) and a layer of fine calcareous sand to allow burrowing, in thermostatic rooms, at the temperature of 17 °C. The animals were fed with Liquifry Marine (Liquifry Co, Dorking, UK) for at least 5 days before experiments were undertaken.

Fluoride solution

A storage solution was prepared by dissolving sodium fluoride (NaF) in filtered seawater (FSW) at the nominal concentration of 1 mg/ml. This solution was subsequently diluted in FSW to obtain the working solutions of 10, 50 and 250 μg/ml. FSW was used in controls.

Hemocyte collection

Hemolymph was collected from the anterior and posterior adductor muscle with a plastic syringe and diluted 1:1 with a solution of 0.38 % Na citrate in FSW, pH 7.5, to avoid hemocyte agglutination. Hemolymph was centrifuged at 780xg for 10 min and pellets were resuspended in FSW to give a final concentration of 10^6 cells/ml. Sixty μl of hemocyte suspension were placed in the center of culture chambers, prepared as described elsewhere (Cima, 2010) and left to adhere to coverslips for 30 min at room temperature. For each experiment, pools of hemocytes from various animals were used.

Hemocyte viability assay

To estimate the effects of NaF on hemocyte viability, cell monolayers were exposed to 10, 50 or

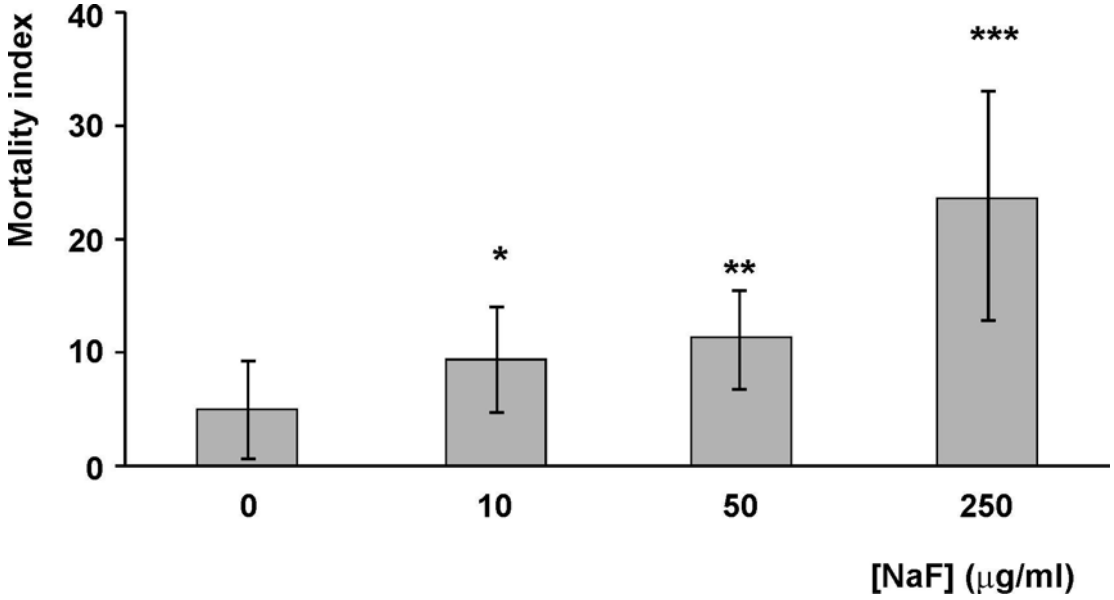

Fig. 2 Mortality index of hemocytes exposed to various NaF concentrations. Significant differences with respect to controls (FSW) are marked by asterisks. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

250 µg/ml of NaF in FSW for 60 min, then incubated with 0.25 % Trypan Blue in FSW for 5 min at room temperature and observed *in vivo* under a Leitz Dialux 22 light microscope (LM) at 1250×. The frequency of blue (= dead) cells was finally evaluated and expressed as the mortality index.

Cell spreading assay

After adhesion to coverslips, hemocytes were incubated for 60 min with NaF at the concentrations reported above; FSW was used in controls. Cells were then fixed in 1 % glutaraldehyde and 1 % sucrose in FSW, stained with 10 % Giemsa solution in FSW and mounted as already described. The morphology of hemocytes was observed under the LM, at the magnification of 1250× (at least 300 hemocytes per slide). Computer-assisted image analysis (Casting Image NT) was performed on fixed hemocyte monolayers to evaluate the phagocyte shape factor, defined as in Ottaviani *et al.* (1997). One hundred cells were considered in each experimental situation. Lower shape factors indicate larger perimeters with respect to the areas and, therefore, an increased amoeboid shape.

Phagocytosis assay

After adhesion of hemocytes to coverslips, cells were incubated for 60 min at room temperature with 60 µl of a suspension of yeast (*Saccharomyces cerevisiae*) cells (yeast:hemocyte ratio = 10:1) in FSW containing NaF at the concentrations reported above. Hemocyte monolayers were then gently washed several times in FSW to eliminate uningested yeast, fixed as reported above, washed 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, pH 7.5), stained with 10 % Giemsa and mounted on glass slides as previously described. Slides were observed under the LM at 1250× and the cells in 10 optic fields (about 200 cells) per slide were counted. The percentage of cells with ingested yeast was evaluated.

Neutral Red retention assay

Lysosomal membrane stability was assessed using a modification of the Neutral Red (NR) retention assay (Lowe *et al.,* 1995), as previously described (Matozzo *et al*., 2001). A stock solution of 0.4 % NR in FSW was prepared. Working solution was obtained by diluting 10 µl of stock solution in 5 ml of FSW. After exposure of hemocytes to NaF for 5, 15, 30 and 60 min, NaF-containing FSW was discarded from culture chambers and 60 µl of NR working solution were added. Ten minutes later, the slides were observed under the LM at 1250×. The percentage of hemocytes showing dye loss from lysosomes into the cytosol which, consequently, appears reddish-pink was expressed as the membrane stability index.

Assay for apoptosis

To reveal DNA fragmentation, cells were exposed for 60 min to NaF, fixed and incubated in the terminal dUTP nick-end labelling (TUNEL) reaction mixture (*in situ* cell death detection kit, Roche) for 60 min at 37 ºC, according to the manufacturer's instructions. Subsequently, they were incubated with a peroxidase-conjugated anti-fluorescein isothiocyanate (FITC) antibody, stained with 0.63 mM 3-3'-diaminobenzidine (DAB) in PBS containing 4 % hydrogen peroxide, washed in distilled water, mounted with Acquovitrex (Carlo

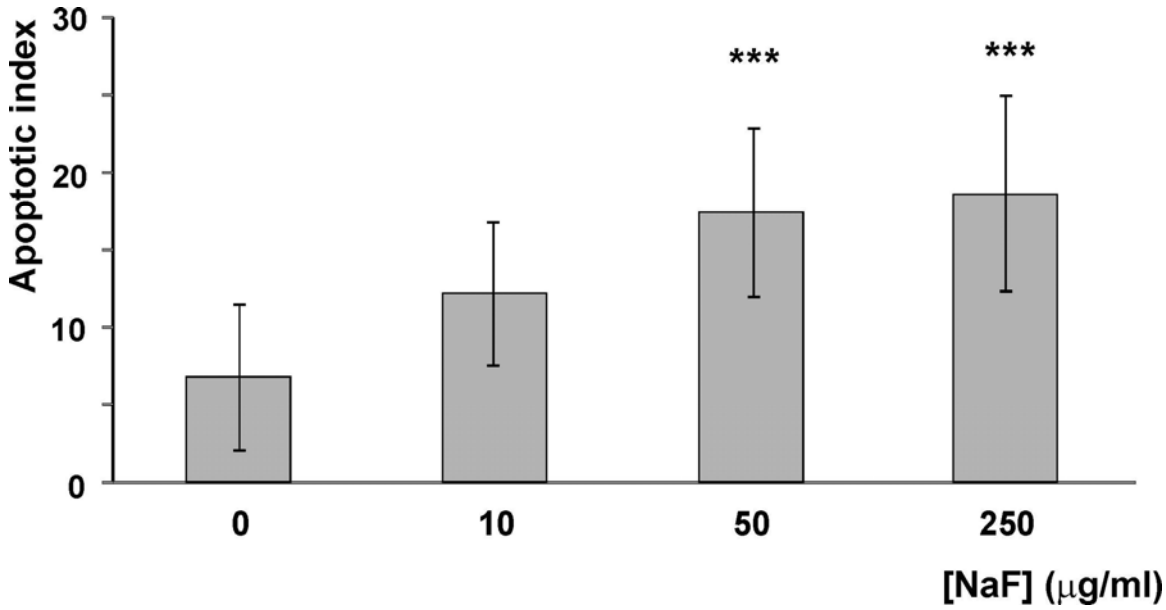

Fig. 3 Apoptotic index of NaF-exposed hemocytes. Asterisks: significant differences with respect to controls (FSW). ***: $p < 0.001$

Erba) and observed under the LM. The presence of fragmented DNA was revealed by dark brown staining and the percentage of positive cells was expressed as the apoptotic index.

Statistical analysis

Each experiment was replicated at least three times (n = 3) with three independent hemolymph pools; data are expressed as mean ± SD. At least 300 cells in 10 optical fields at 1250× were counted under the LM for each experiment. Frequencies were compared with the non-parametric χ^2 test. In the case of the shape factor, data were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variances (Bartlett's test). As ANOVA assumptions were not fulfilled, the non-parametric Mann-Whitney U-test was used for comparison.

Results

The hemocytes of V. philippinarum

Three hemocyte types have been described in *V. philippinarum*: undifferentiated cells (Fig. 1A), phagocytes, and serous cells. Phagocytes represent the majority of the circulating cells, amounting to about 80 % of the total cell number (Cima et al., 2000), and include granulocytes (Figs 1B - C) and hyalinocytes (Figs 1D - E): both the cell types can assume a round (Figs 1B, D) or a spreading (Figs 1C, E) morphology.

NaF alters hemocyte viability and induces apoptotic cell death

Under control conditions, about 3 % of the hemocytes stained with Trypan Blue. This fraction significantly increased to values around 10 % upon the exposure to 10 and 50 μg/ml NaF ($p < 0.05$ and $p < 0.01$, respectively). At 250 μg/ml NaF, the cell mortality rose to about 23 % ($p < 0.001$ with respect to the control; Fig. 2). A significant ($p < 0.001$) increase in the fraction of hemocytes positive to the TUNEL assay (Fig. 1F) with respect to controls was reported for hemocytes treated with 50 and 250 μg/ml of fluoride (about 15 and 20 %, respectively; Fig. 3).

NaF inhibits phagocytosis by clam hemocytes

About 18 % of hemocytes could ingest yeast cells in controls (Figs 1G, 4). When hemocytes were incubated with yeast cells in the presence of fluoride, a significant ($p < 0.01$) reduction in the fraction of phagocytosing cells was obtained for all the used concentrations (Fig. 4).

Exposed hemocytes show changes in cell shape and internal membrane stability

In control slides, more than 50 % of hemocytes had a spreading morphology, whereas most of the cells exposed to 250 μg/ml of NaF, acquired a round morphology. The shape factor of control hemocytes (0.55 ± 0.15) rose significantly (0.61 ± 0.15; $p < 0.001$) when hemocytes were exposed at the highest NaF concentration.

In control hemocytes, the fraction of cells with NR-stained cytoplasm never exceeded 3 % and the dye accumulated inside cytoplasmic granules, probably lysosomes, of phagocytes (Fig. 1H). Under stress conditions, the dye leaked into the cytoplasm which assumed a pinkish color due to membrane alteration. In hemocytes exposed to 250 μg/ml NaF, the percentage of cells with red cytoplasm (Fig.

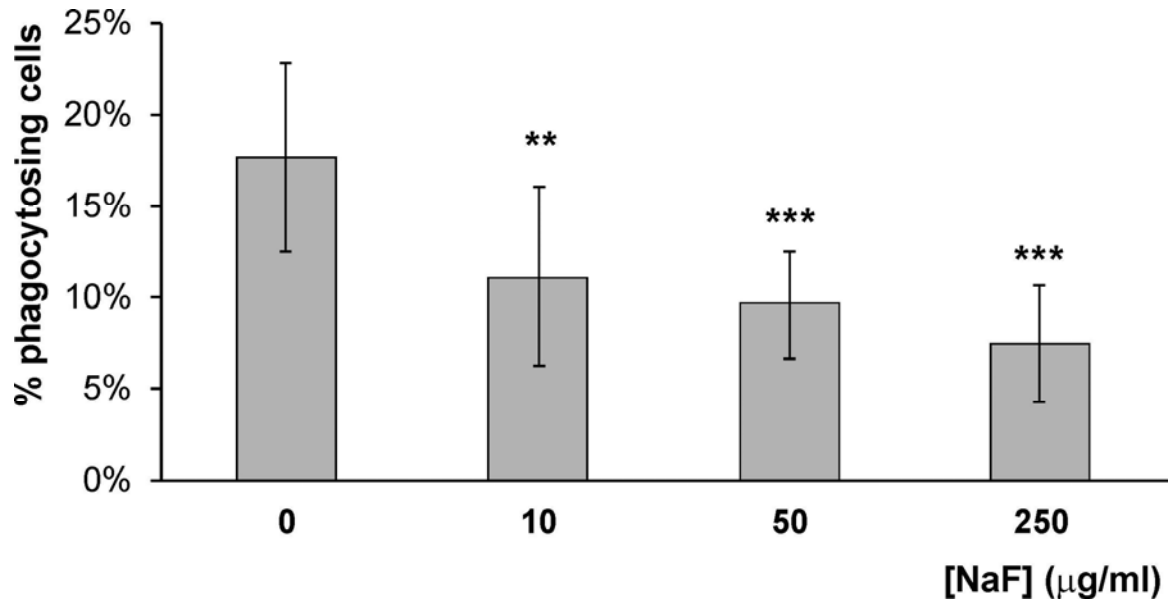

Fig. 4 Percentage of phagocytosing cells in the presence of various NaF concentrations. Significant differences with respect to controls (FSW) are marked by asterisks. **: $p < 0.01$; ***: $p < 0.001$.

1) was significantly ($p < 0.01$) increased after 5 min of exposure and reached the value of 7 % after 60 min of incubation (Fig. 5).

Discussion

Fluoride has been recognized as a cause of toxicity for many aquatic organisms ranging from algae and aquatic plants to invertebrates and fishes (Camargo, 2003). At the cellular level, it acts as an inhibitor of various enzyme activities, and is able to induce oxidative stress and, as consequence, stress responses through the activation of MAPK-related transduction pathways and cell death by apoptosis (Barbier *et al.,* 2010).

In the present paper, we analyzed the effects of *in vitro* NaF exposure on the hemocytes of the clam *V. philippinarum*, a bivalve mollusc widely distributed along the west coast of the Adriatic Sea. As invertebrates, clams rely on innate immunity for their defense and hemocytes play a key role in the response towards potentially pathogenic microbe having entered the organism. Moreover, hemocytes represent a selected cell population to investigate, at the cellular level, the acute effects of exposure to xenobiotics.

The NaF concentration used in our experiments ranged from 10 to 250 μg/ml: the lower values are close to the concentration values reported for polluted waters in the lagoon of Venice (Barbaro *et al.,* 1981b) whereas the higher values, although below those reported from highly polluted waters close to aluminum smelters (Ares *et al*., 1983; Camargo, 2003), are similar to those used for *in vitro* experiments with cultured cell lines (Barbier *et al*., 2010).

As reported before, most of the *V. philippinarum* hemocytes are phagocytes and they can assume a spreading or a round morphology. According to previous hypothesis (Cima *et al.,* 2000), spreading and round cells probably represent different phagocyte differentiation stages, with spreading cells representing actively wandering cells which, once ingested foreign material, withdraw their cytoplasmic extensions and acquire a round morphology. In control hemocytes, the frequency of spreading cells amounts to about 50 % of the total circulating cells: this value is severely decreased in the presence of pollutants which can alter the cytoskeletal organization (Cima *et al*., 1999). The alteration of the hemocyte cytoskeletal organization in the presence of pollutants has been reported also for other marine invertebrate species (Fagotti *et al.,* 1996; Cima *et al*., 1998; Olabarrieta *et al.,* 2001; Gómez-Mendikute and Cajaraville, 2003; Menin *et al.,* 2008; Franchi *et al*., 2013). As spreading and phagocytosis involve the actin component of the cytoskeleton, the observation that fluoride hampers both the processes suggests that, NaF can interfere with actin polymerization. Similar conclusions were reached in studies on mammalian cells in culture (Barbier *et al.,* 2010). The negative effect on actin cytoskeleton can be the consequence of either a direct interaction with microfilaments or an alteration of Ca^{2+} homeostasis: previous studies have reported that fluoride can act at both the levels within the cell (Barbier *et al.,* 2010), although further research is required to better define the relationships between fluoride and cytoskeleton. The observed reduction in phagocytic activity and spreading morphology may also be the consequence of cell death by apoptosis, which affects about 20 %

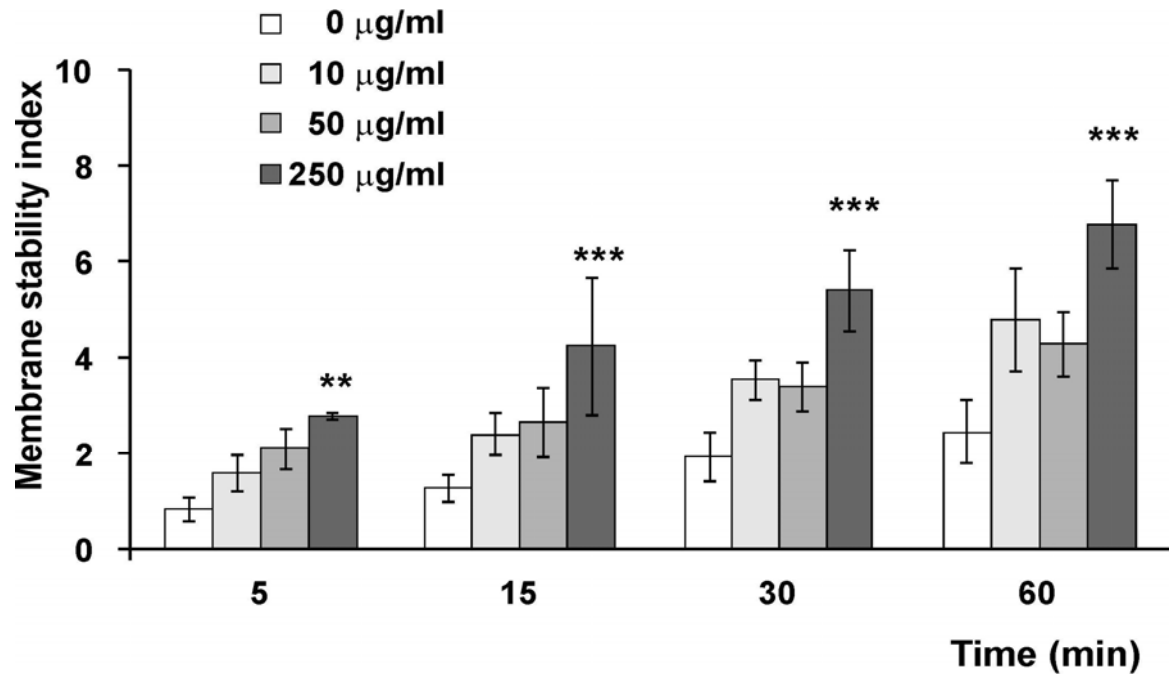

Fig. 5 Membrane stability index of hemocytes exposed to various NaF concentrations for different periods of time. Asterisks: significant differences with respect to the respective controls (FSW). **: $p < 0.01$; ***: $p < 0.001$

of the hemocytes at fluoride concentrations higher than 10 μg/ml. Moreover, the similarity between the values of the mortality and the apoptotic index at the various concentrations, suggests that most of the observed mortality is due to apoptotic cell death. An increase of apoptosis consequent to the exposure to fluoride has been reported in various mammalian cells (Gutowska *et al.,* 2010; Jacinto-Alemán *et al.,* 2010; Wang *et al.*, 2010) where, the down regulation of Bcl-2, the increase in cytochrome *c* release from mitochondria and the activation of both the intrinsic and the extrinsic pathway of cell death have been reported (Barbier *et al.,* 2010). The observed occurrence of apoptosis is likely related to the induction of oxidative stress: this hypothesis fits previous observations demonstrating that one of the main effects of fluoride on cells is the inhibition of antioxidant enzymes and the increase of NADPH oxidase activity with the consequent production of reactive oxygen species (Barbier *et al.,* 2010; Izquierdo-Vega *et al.,* 2011; Liu *et al.,* 2011).

The Trypan Blue exclusion assay evidences the alteration of the cell plasma membrane permeability, a final step in the progression of apoptosis; conversely, the NR assay gives an indication of the stability of the membrane of acid cytoplasmic compartments, abundant in phagocytes in the form of lysosomes, where the dye usually concentrates. The alteration of the internal membranes leads to the leakage of lysosomal content in the cytosol, an additional factor negatively influencing phagocyte functionality, which was clearly observed after 5 min of incubation at 250 μg/ml NaF.

In conclusion, our results show that fluoride affects the functionality of *V. philippinarum* phagocytes and, since the reduction of immune functions can severely compromise the organismal survival, it can be deleterious for the whole biocoenosis. In addition, they stress the need for further studies in order to better understand the molecular pathways and the molecular machine set up by marine invertebrates to protect themselves from the negative effect of pollutants.

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References

Aguirre-Martínez GV, Buratti S, Fabbri E, DelValls AT, Martín-Díaz ML. Using lysosomal membrane stability of haemocytes in *Ruditapes philippinarum* as a biomarker of cellular stress to assess contamination by caffeine, ibuprofen, carbamazepine and novobiocin. J. Environ. Sci. 25: 1408-1418, 2013.

Ares JO, Villa A, Gayosot AM. Chemical and biological indicators of fluoride input in the marine environment near an industrial source (Argentina). Arch. Environ. Contam. Toxicol. 12: 589-602, 1983.

Barbaro A, Francescon A, Polo B. Fluoride accumulation in aquatic organisms in the Lagoon of Venice. Fluoride 14: 102-107, 1981a.

Barbaro A, Francescon A, Polo B, Antonini GF. Fluoride levels in waters of the industrial area of Portomarghera, Lagoon of Venice (Italy). Mem. Biol. Mar. Oceanogr. 11: 91-98, 1981b.

Barbier O, Arreola-Mendoza L, Del Razo LM. Molecular mechanisms of fluoride toxicity. Chem. Biol. Interac. 188: 319-333, 2010.

Basha PM, Rai P, Begum S. Evaluation of fluoride-induced oxidative stress in rat brain: a multigeneration study. Biol. Trace Elem. Res. 142: 623-637, 2011.

Camargo JA. Fluoride toxicity to aquatic organisms: a review. Chemosphere 50: 251-264, 2003.

Casellato S, Masiero L, Ballarin L. Toxicity of fluoride to the freshwater mollusk *Dreissena polymorpha*: effects on survival, histology, and antioxidant enzyme activity. Fluoride 45: 35-46, 2012.

Casellato S, Del Piero S, Masiero L, Covre V. Fluoride toxicity and its effects on gametogenesis in the aquatic oligochaete *Branchiura sowerbyi* Beddard. Fluoride 46: 7-18, 2013.

Chen T, Cui H, Cui Y, Bai C, Gong T. Decreased antioxidase activities and oxidative stress in the spleen of chickens fed on high-fluorine diets. Hum. Exp. Toxicol. 30: 1282-1286, 2011.

Cima F. Microscopy methods for morpho-functional characterisation of marine invertebrate haemocytes. In: Méndez-Vilas A, Álvarez JD (eds), Microscopy: science, technology, applications and education, microscopy book series, Vol. 2, n. 4, Badajoz: Formatex Research Center, pp 1100-1107, 2010.

Cima F, Ballarin L, Bressa G, Burighel P. Cytoskeleton alterations by tributyltin (TBT) in tunicate phagocytes. Ecotoxicol. Environ. Saf. 40: 160-165, 1998.

Cima F, Marin MG, Matozzo V, Da Ros L, Ballarin L. Biomarkers for TBT immunotoxicity studies on the cultivated clam *Tapes philippinarum* (Adams and Reeve, 1850). Mar. Poll. Bull. 39: 112-115, 1999.

Cima F, Matozzo V, Marin MG, Ballarin L. Haemocytes of the clam *Tapes philippinarum*: morphofunctional characterisation. Fish Shellfish Immunol. 10: 677-693, 2000.

Del Piero S, Masiero L, Casellato S. Influence of temperature on fluoride toxicity and bioaccumulation in the nonindigenous freshwater mollusk *Dreissena polymorpha* Pallas, 1769. Environ. Toxicol. Chem. 31: 2567-2571, 2012.

Fagotti A, Di Rosa I, Simoncelli F, Pipe RK, Panara F, Pascolini R. The effects of copper on actin and fibronectin organization in *Mytilus galloprovincialis* haemocytes. Dev. Comp. Immunol. 20: 383-391, 1996.

Franchi N, Ballarin L. Influence of cadmium on the morphology and functionality of haemocytes in the compound ascidian *Botryllus schlosseri*. Comp. Biochem. Physiol. 158C: 29-35, 2013.

Gómez-Mendikute A, Cajaraville MP. Comparative effects of cadmium, copper, paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. Toxicol. In Vitro 17: 539-546, 2003.

Gutowska I, Baranowska-Bosiacka I, Baśkiewicz M, Milo B, Siennicka A, Marchlewicz M, *et al.* Fluoride as a pro-inflammatory factor and inhibitor of ATP bioavailability in differentiated human THP1 monocytic cells. Toxicol. Lett. 196: 74-79, 2010.

Inkielewicz-Stepniak I, Czarnowski W. Oxidative stress parameters in rats exposed to fluoride and caffeine. Food Chem. Toxicol. 48: 1607-1611, 2010.

Izquierdo-Vega JA, Sánchez-Gutiérrez M, Del Razo LM. Decreased *in vitro* fertility in male rats exposed to fluoride-induced oxidative stress damage and mitochondrial transmembrane potential loss. Toxicol. Appl. Pharmacol. 230: 352-357, 2008.

Izquierdo-Vega JA, Sánchez-Gutiérrez M, Del Razo LM. NADPH oxidase participates in the oxidative damage caused by fluoride in rat spermatozoa. Protective role of α-tocopherol. J. Appl. Toxicol. 31: 579-588, 2011.

Jacinto-Alemán LF, Hernández-Guerrero JC, Trejo-Solís C, Jiménez-Farfán MD, Fernández-Presas AM. *In vitro* effect of sodium fluoride on antioxidative enzymes and apoptosis during murine odontogenesis. J. Oral Pathol. Med. 39: 709-714, 2010.

Keller AE, Augspurger T. Toxicity of fluoride to the endangered unionid mussel, *Alasmidonta raveneliana*, and surrogate species. Bull. Environ. Contam. Toxicol. 74: 242-249, 2005.

Liu H, Sun JC, Zhao ZT, Zhang JM, Xu H, Li GS. Fluoride-induced oxidative stress in three-dimensional culture of OS732 cells and rats. Biol. Trace Elem. Res. 143: 446-456, 2011.

Lowe DM, Fossato VU, Depledge MH. Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice lagoon: an in vitro study. Mar. Ecol. Prog. Ser. 129: 189-96, 1995.

Matozzo V, Marin MG. 4-Nonylphenol induces immunomodulation and apoptotic events in the clam *Tapes philippinarum*. Mar. Ecol. Prog. Ser. 285: 97-106, 2005.

Matozzo V, Ballarin L, Pampanin DM, Marin MG. Effects of copper and cadmium exposure on functional responses of hemocytes in the clam, *Tapes philippinarum*. Arch. Environ. Contam. Toxicol. 41: 163-170, 2001.

Matozzo V, Ballarin L, Marin MG. *In vitro* effects of tributyltin on functional responses of haemocytes in the clam *Tapes philippinarum*. Appl. Organomet. Chem. 16: 169-74, 2002.

Matozzo V, Costa Devoti A, Marin MG. Immunotoxic effects of triclosan in the clam *Ruditapes philippinarum*. Ecotoxicology 21: 66-74, 2012.

Menin A, Ballarin L, Bragadin M, Cima F. Immunotoxicity in ascidians: antifouling compounds alternative to organotins - II. The case of Diuron and TCMS pyridine. J. Environ. Sci. Health 43B: 644-654, 2008.

Nielsen FH. Micronutrients in parenteral nutrition: boron, silicon, and fluoride. Gastroenterology 137: S55-S60, 2009.

Olabarrieta I, L'Azou B, Yuric S, Cambar J, Cajaraville MP. *In vitro* effects of cadmium on two different animal cell models. Toxicol. In Vitro 15: 511-517, 2001.

Ottaviani E, Sassi D, Kletsas D. PDGF- and TGF-β-induced changes in cell shape of invertebrate immunocytes: effect of calcium entry blockers. Eur. J. Cell Biol. 74: 336-341, 1997.

Wang ZH, Li XL, Yang ZQ, Xu M. Fluorine-induced apoptosis and lipid peroxidation in human hair follicles *in vitro*. Biol. Trace Elem. Res.137: 280-288, 2010.

Zhao H, Chai L, Wang H. Effects of fluoride on metamorphosis, thyroid and skeletal development in *Bufo gargarizans* tadpoles. Ecotoxicology 22: 1123-1132, 2013.

Zhu JQ, Si YJ, Cheng LY, Xu BZ, Wang QW, Zhang X, *et al*. Sodium fluoride disrupts DNA methylation of H19 and Peg3 imprinted genes during the early development of mouse embryo. Arch. Toxicol. 2013 [in press].