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# Histological changes and Micronucleus induction in the Zebra mussel *Dreissena polymorpha* after Paraquat exposure

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**Summary.** The herbicide paraquat (PQ), still widely used in developing countries, represents a serious risk factor for human and environmental health. To test the sublethal effects of PQ on the freshwater bivalve *Dreissena polymorpha*, mussels were exposed to 0.125, 0.250, 0.500 mg/L for 7 and 14 days and histologically screened. PQ's genotoxic potential was also determined in haemocytes by the micronucleus, MN, assay. At concentrations ≥ 0.250mg/L, severe lesions, such as cellular vacuolation, lysis and thinness of the germinative epithelia were observed in the digestive gland and testis. A positive trend between the number of granulocytes and all PQ concentrations was observed in both gonads and digestive glands, addressing the inflammatory capacity of this herbicide on these tissues. Mussels exposed to PQ also exhibited a significant MN induction. The spontaneous MN frequencies ranged from 2.75 to 4.25‰, while PQ-induced MN rates in treated mussels were between 3.50 and 12.50‰. The histopathological effects on the digestive and reproductive systems, as well as the MN induction in the haemocytes, confirmed the cytotoxic and genotoxic effects of PQ also in *D. polymorpha*.

**Key words:** *Dreissena polymorpha*, Histopathology, Genotoxicity

## Introduction

The dipyridyl compound Paraquat (PQ, 1-1'-dimethyl-4-4'-bypiridylium dichloride) is a non-selective contact herbicide, widely used for the control and management of weeds. PQ is a fast-acting compound with a broad spectrum of activity which destroys all green plant tissues (Dodge, 1971). It is used for plantation crops (banana, cocoa-palm, coffee, oil-palm, rubber, etc.), and for citrus fruits, apples, plums, vines, and tea. On certain crops (potato, pineapple, sugar-cane, sunflower), PQ is also used as a desiccant, while in cotton plantations, at roadsides, along railways and around buildings and homes in rural areas of developing countries, it is employed as a defoliant (Wesseling et al., 2001). This herbicide can reach water bodies as run-off from agricultural farm lands as well as from direct applications in static or slow-moving waters, where it is commonly used at low (0.1-2 mg/L) concentrations (Calderbank, 1972), even if sometimes higher concentrations are required. PQ usually quickly dissipates from natural water systems and its disappearance is due to its adsorption by sediment and suspended material, and by absorption and uptake by aquatic plants and algae (Summers, 1980). PQ is quite toxic and its toxicity to animals and humans has been exhaustively described (Summers, 1980). However, bibliographic data report little information about the effects of this compound on invertebrates which represent highly sensitive levels of trophic chains, and which can provide relevant indications in environmental risk assessment monitoring programs. In this field molluscs, and in particular bivalves, are considered suitable organisms for monitoring purposes on account of their sessile status and filter feeding behaviour (Widdows and Donkin, 1992). This kind of monitoring was first applied in a marine environment using the blue mussel *Mytilus edulis* (Goldberg et al., 1978), but later also in the freshwater environment with the zebra mussel *Dreissena polymorpha* (Bias and Karbe, 1985; De Kock and Bowmer, 1993).

Although a good deal of data is available regarding the lethal effect of PQ on molluscs (PANNA, 2004), little is known about its histopathological effect on them. Moreover, several data reported PQ as a powerful genotoxic agent since it was able to induce DNA

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damage in several living systems (Salam et al., 1993; Martínez-Tabche et al., 2004) e.g. increasing the frequency of micronuclei (D'Souza et al., 2005). The micronucleus assay (MN) has proven most suitable for assessing genotoxic effects of environmental contaminants in effluents and in polluted water bodies (Mersch and Beauvais, 1997) and it has been already applied on molluscs, mainly in the marine and estuarine environments (see Mersch et al., 1996). In freshwater environments the MN test has been successfully applied with the mussel *Anodonta cygnea* (Scarpato et al., 1990), the green-lipped mussel *Perna viridis* (Siu et al., 2004), and also with the zebra mussel *D. polymorpha* (Mersch and Beauvais, 1997; Pavlica et al., 2000) which resulted highly sensitive, thus a good test species.

By exposing mussels to sublethal and realistic environmental PQ concentrations of 0.125, 0.250, 0.500 mg/L, we focused our investigation on the histopathological effects of this herbicide simultaneously examining its MN induction in the haemocytes of *D. polymorpha*. This paper represents the first step of an in-depth study regarding PQ toxicity on the zebra mussel and its goal is to give preliminary data towards understanding the cyto- and genotoxic effects of PQ on the zebra mussel, whose role as a biomonitoring organism and ecotoxicological test species is well known. The results are discussed with respect to the implications for the use of these techniques in environmental monitoring studies.

## Materials and methods

### *Field samplings*

*D. polymorpha* was collected from a relatively unpolluted site in Lake Como, near the town of Abbadia Lariana (Province of Lecco, N. Italy). Scuba dives were made to bring up rocks covered with mussels from 2-3 m depth. Colonies were initially rinsed in the field. Once on shore, mussels were first placed in shock-proof plastic sheets, then stored in thermic bags without water and transported to the laboratory in less than 2 hr, with care not to damage any specimens.

### *Maintenance of stock mussels*

Once in the laboratory, all the mussels were rinsed again and left on the substrates to which they were attached. Damaged or dying mussels were discarded, while the living were randomly placed in two 70L aerated aquaria with about 500 specimens each, and acclimatized at 20 ± 0.5°C. Twice a day they were fed with frozen samples of ALGAMAC-2000® (Aquafauna Bio-Marine Inc., CA). The feeding samples were prepared by dissolving 5g/L of ALGAMAC-2000® in de-ionized water and then freezing the final suspension in amounts of 12.5 ml each. The aquaria were cleaned every other day and the dead mussels were removed. The mussels were maintained in the above conditions for ten days before the start of treatment.

### *Water and chemicals used*

The water for storage and subsequent experiments was made using de-ionized water with 1% seawater added. PQ with over 98% purity, 4',6-diamidino-2-phenylindole (DAPI) and 1,4-Diazabicyclo[2.2.2] octane (DABCO) were supplied by Sigma Chemical Co. (St. Louis, MO).

### *Experiment plan*

Healthy mussels, greater than 15 mm in shell length, were randomly detached from the rocks by cutting their byssus threads, divided into 4 groups with each group placed in an aerated glass aquarium with glass cover. The volume of water in each aquarium was 15 L and the mussels 100. Mussels were cleaned by removing detritus and any epibiota on the shells when necessary. Preliminary toxicity data generated in our laboratory led us to work with three nominal PQ concentrations: 0.125, 0.250 and 0.500 mg/L and one control group. These concentrations were within the range of PQ concentrations recommended for aquatic weed control (Calderbank, 1972). Histological and genotoxic effects were evaluated on the 7$^{th}$ and 14$^{th}$ day of exposure. Each day, the solutions were renewed and the dead mussels removed from the aquaria. The mussels were fed every day. The experiment run only one time, thus no statistical comparisons were made between control and PQ-exposed groups.

### *Histopathological analyses*

Twenty-five mussels from each PQ experimental group and 25 from the control were randomly sampled 7 days after the start of exposure and fixed in Bouin's fluid for successive histopathological analysis. For the remaining mussels, the treatment continued for one more week and all the survivors were fixed on the 14th day. From these mussels, 30 from each PQ group and 30 from the control were randomly selected for histopathological study. The samples were dehydrated in an ascending alcohol series and embedded in Bio-Plast (melting point 57°C). Using a rotary microtome, samples were cut in 7µm transverse sections at the proximal, central and distal levels of the visceral sack. Ten serial sections from each portion were placed on microscope slides and left to dry overnight at 37°C. Slides were then stained with Mayer's Haemalum and Eosin mounted in Eukitt (Kindler GmbH, Freiburg) and observed under a Leica DMRA2 light microscope. Additional histochemical stain (Periodic Acid Schiff, PAS) was used when appropriate to further investigate specific lesions. Microphotographs were taken with a Leica DC320F digital camera. All mussels were observed and analyzed microscopically for the occurrence of diseases and inflammatory response. The number of granulocytes

was determined in the most severely affected field of digestive diverticula and gonads, in an area of 90,000 $\mu m^2$.

Histopathological changes in the digestive gland (DG), were scored according to a four step semi-quantitative evaluation as reported in Figure 1. The scoring for histopathologic alterations considered the number and severity of lesion detected in the DG of the mussels. For example, specimens with no observable lesions in the tissues were rated as 0. Those with small number and slight tissue lesions were considered to be in mild condition and were given a score of 1. Those with moderate number and moderate lesions were scored as 2, and those specimens with a high number of extensive lesions were considered as severe and were scored as 3. To evaluate the general DG condition, a Digestive Gland Degeneration Index (DGDI), was calculated as the mean of the cytological score of all specimens of each experimental group. This type of index has been used by a number of authors in recent years (de Kock and Bowmer, 1993) and it only summarizes the health of the specimens in a very general way according to the criteria in Fig. 1

### *Micronucleus assay*

Eight mussels from each experimental group were sampled for the micronucleus assay (MN) after 7 and 14 days of exposure. The upper edge of the mussel shells was gently broken and about 100 µl haemolymph were drawn up from the sinus near the posterior adductor muscle by a hypodermic syringe. The haemolymph aliquots were spread over electrostatically charged slides and cells were allow to settle in a humidified chamber at

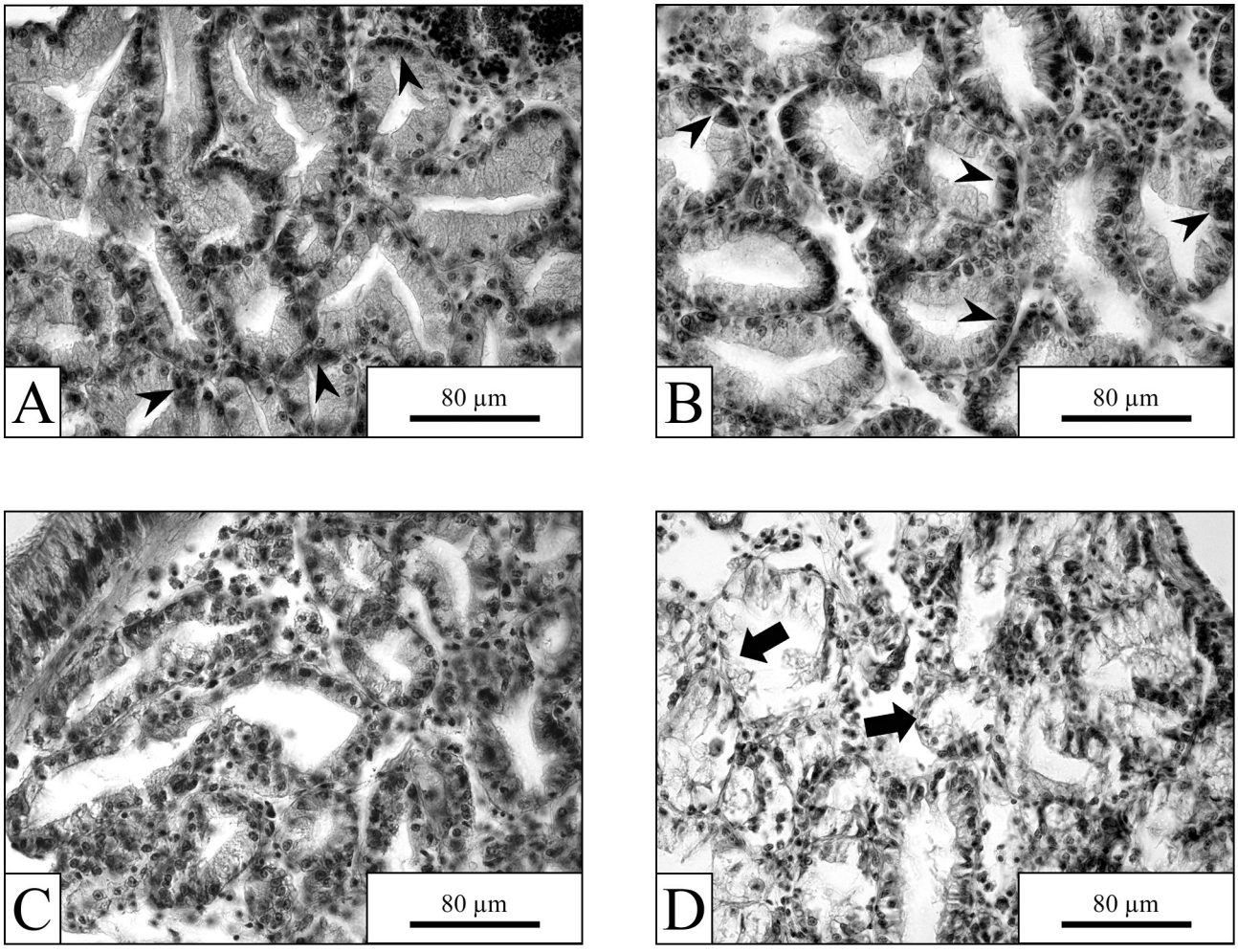


**Fig. 1.** Histopathological pictures of the *D. polymorpha* Digestive Gland at different severeness levels (SL). SL0 = Normal condition (**A**); SL1 = Mild alterations **(B)**; SL2 = Moderate alterations **(C)** and SL3 = Severe alterations **(D)**. See text for the cytological description of the SL. Arrowheads: BPC; Arrows: Necrotic cells

room temperature for about 20 min. Cells were then fixed for 15 min in 10% neutral buffered formalin, repeatedly rinsed with distilled water and air dried. After being washed in PBS 0.1M, pH 7.2, cells were permeabilized with 0.4% Triton X-100 in PBS, abundantly rinsed in PBS and stained with 20 µg/ml DAPI for 20 min, then washed in PBS, mounted in a 2% glycerol-based medium (DABCO, Sigma Chemical Co., St. Louis, MO), stored at 4°C in the dark and processed within one week. The slides were scored under a Leica DMRA2 fluorescent microscope equipped with a Hg 100W ultra high pressure mercury lamp and a Leica filter cube A for UV, with excitation filter BP 340-380 nm, dichromatic mirror 400 nm and suppression filter LP 425 nm. On each slide, 500 intact and well-individualized cells were examined at 1000x magnification. MN were identified according to Mersch and Beauvais (1997).

### *Statistical analysis*

Ninety-five percent comparison intervals were calculated for the mean granulocyte number and for the DGDI. The non parametric Mann Whitney U-test was used to compare the number of granulocytes and micronuclei among the experimental groups both at 7 and 14 days (Siegel and Castellan, 1992). Statistical comparisons were considered to be significant at the 5% level ($p<0.05$).

## Results

No mortality was observed in the experimental groups, except for one specimen in the 0.250 mg/L PQ group, after 14 days.

### *Histopathological study*

Digestive gland (DG)

According to the cytological score of the DG (Fig. 1), SL0 (SL=Severeness Level) indicates the optimal tissutal condition, while SL1, SL2 and SL3 correspond to increasing pathological pictures. In the normal condition, the DG shows intact digestive tubules with regular epithelium surrounded by well-defined basal membranes. The digestive cells (DC) and the basophilic

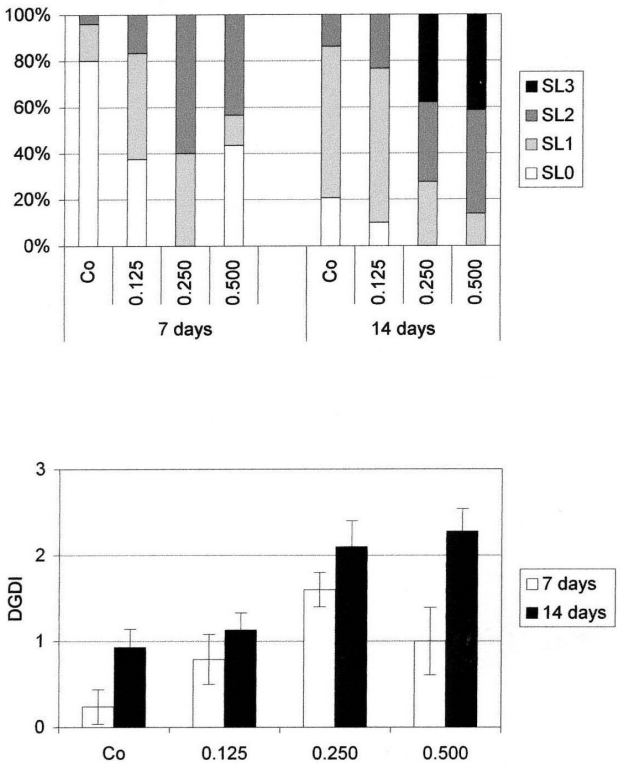


**Fig. 2.** Histopathological conditions of the *D. polymorpha* Digestive Gland. Upper panel - Histograms with relative frequencies of SL in Controls (Co), and PQ groups, at 7 and 14 days. Lower panel - Digestive Gland Degeneration Index (DGDI). Vertical bars: 95% confidence intervals. Concentration: mg/L

**Table 1.** Number and frequencies of mussels with histological lesions

| | Co | | PQ mg/L 0.125 | | 0.250 | | 0.500 | |
|---|---|---|---|---|---|---|---|---|
| | 7th | 14th | 7th | 14th | 7th | 14th | 7th | 14th |
| Ovary (n) | 2 | 3 | 3 | 2 | 5 | 8 | 6 | 4 |
| Ovary (%) | 18.2 | 16.7 | 27.3 | 11.1 | 33.3 | 44.4 | 50.0 | 30.8 |
| Testis (n) | 0 | 1 | 0 | 0 | 0 | 6 | 5 | 13 |
| Testis (%) | - | 8.3 | - | - | - | 50.0 | 38.5 | 76.5 |
| Digestive Gland (n) | 1 | 4 | 4 | 7 | 15 | 21 | 10 | 25 |
| Digestive Gland (%) | 4.0 | 13.3 | 16.0 | 23.3 | 60.0 | 70.0 | 40.0 | 83.3 |
| Gut (n) | 0 | 1 | 1 | 0 | 0 | 5 | 0 | 5 |
| Gut (%) | - | 3.3 | 4.0 | - | - | 16.7 | - | 16.7 |

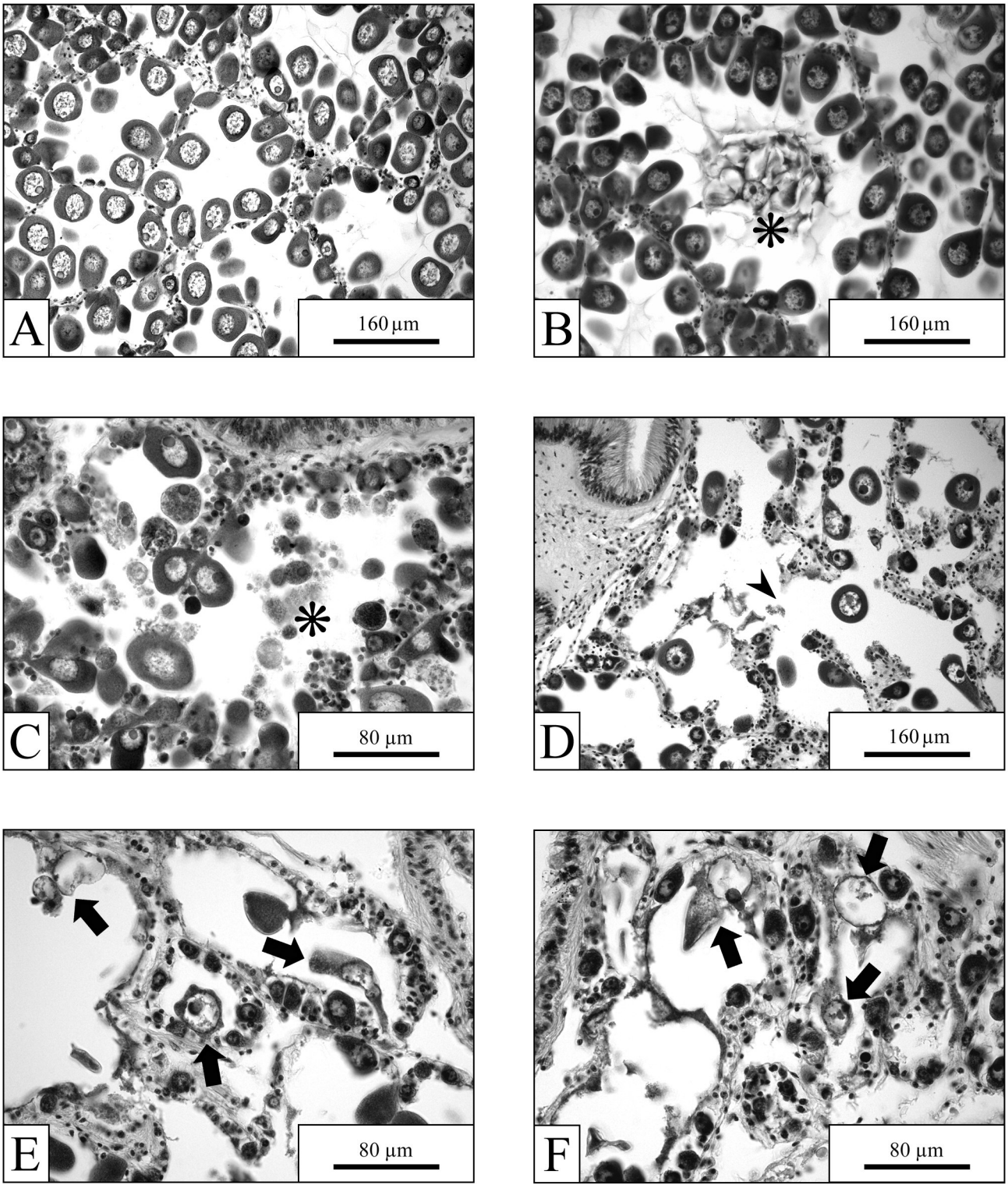


**Fig. 3.** Histological sections of ovaries. **A:** Control; **B:** 0.250 mg/L PQ at 7 days; **C:** 0.500 mg/L PQ at 7 days; **D-F:** 0.500 mg/L PQ at 14 days. Asterisk: Cellular debris; arrowheads: Breakdown of the acinar wall; arrows: Degenerating oocytes.

pyramidal cells (BPC) are clearly distinguishable, the former appearing well nourished, the latter, assembled to form crypts (Fig. 1A). The PAS positive brush border of the DC regularly lines the narrow lumen of the tubules and their dense eosinophilic cytoplasms present numerous positive globules. In SL1, the DG shows increasing vacuolations of the DC, poor PAS positive granulations in the cytoplasms, partially disrupted brush borders and thinness of the digestive epithelia, often accompanied by irregular and dilated lumina (Fig. 1B). In SL2 the histological condition of the DG shows most tubules in disintegrating phases with the epithelia showing degenerating DC with condensed or vacuolated cytoplasms. The structural integrity of the epithelium is not preserved and cellular debris accumulates in the enlarged lumen. At this severeness level, the DC generally reveal nuclear alteration and sometimes they appear indistinguishable from the BPC (Fig. 1C). The increased necrosis of the DC determines an increased phagocitic activity in the interstitial tissue, as demonstrated by the granulocytic infiltration often observed. The worst cellular condition corresponds to SL3 in which the degenerating phenomena involve almost all the digestive tubules and many necrotic cells are present in the epithelia. The digestive epithelium appears completely disorganized, having lost its shape (Fig. 1D). The BPC also degenerate and become indistinguishable from the DC and granulocyte infiltration increases further.

Different percentages of mussels with DG scored as SL0, SL1 and SL2 were observed after 7 and 14 days, but the heaviest condition (SL3) was observed only after 14 days in 0.250 and 0.500 mg/L PQ groups (Fig. 2). This fact reflects on DGDI, whose values are also reported in Figure 2. After 7 days, the DGDI of the PQ treated mussels were higher than that of the controls and

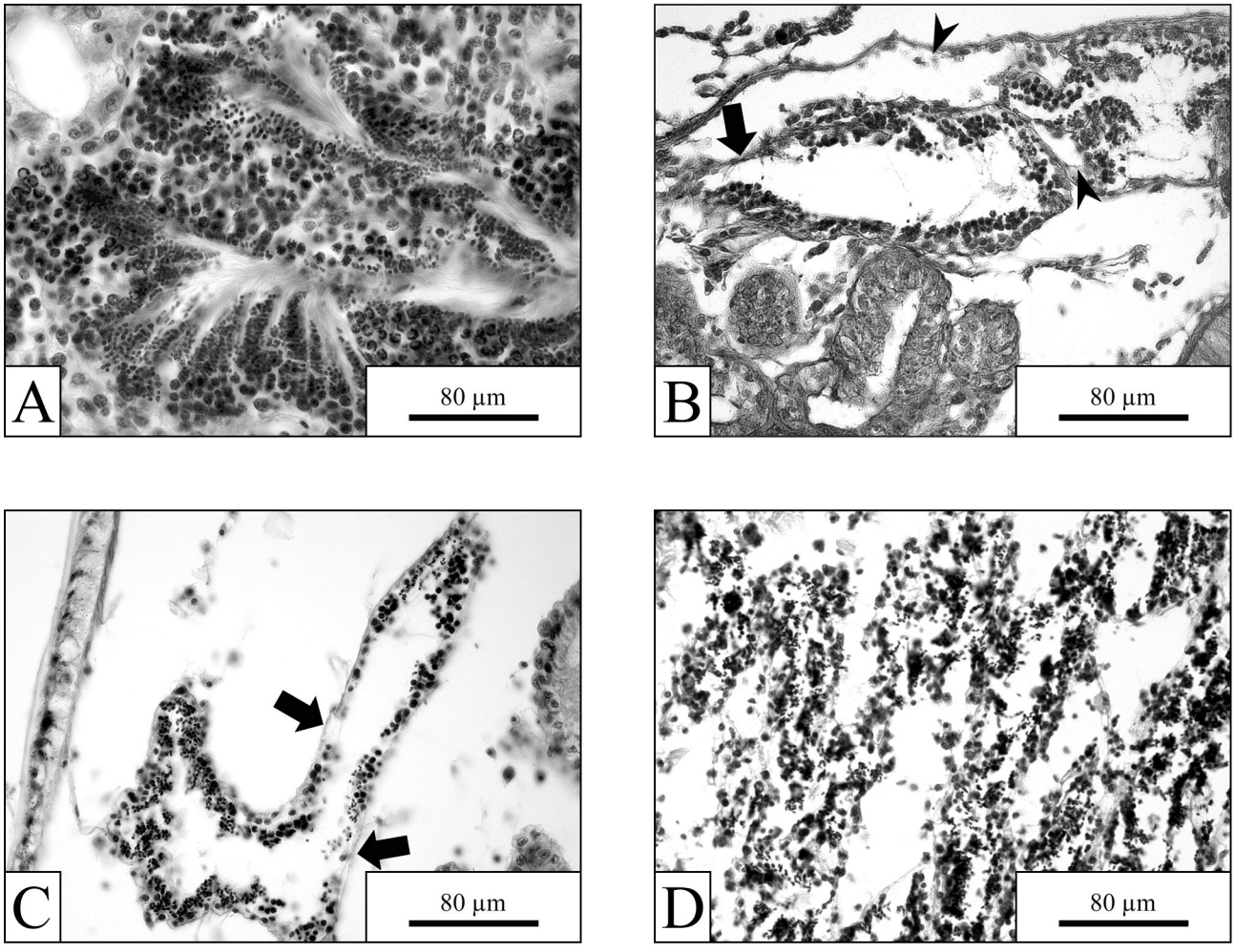


**Fig. 4.** Histological sections of testes. **A:** Control; **B:** 0.500 mg/L PQ at 14 days; **C:** 0.250 mg/L PQ at 14 days; **D:** 0.500 mg/L PQ at 14 days. Arrowheads: Heavy vacuolations inside the basal membrane; arrows: Thinness of the germinative layer and atrophy of the acini.

the highest value was observed in 0.250 mg/L PQ group with a score of 1.6. At this time, no mussels were ever observed as SL3, while 60% of the mussels at 0.250 mg/L PQ and 43.5% at 0.500 mg/L PQ were scored as SL2. After 14 days, the DGDI values were always higher than those after 7 days, with a clear trend with PQ concentrations (Fig. 2). Many treated mussels, in particular 72.4% at 0.250 mg/L and 86.2% at 0.500 mg/L, showed severe alterations of the DG with some specimens scored as SL3 (Fig. 2). At these concentrations, the DGDI values exceeded 2, indicating high PQ toxicity on the DG at concentrations higher than 0.125 mg/L.

The PAS-activity showed a progressive reduction from the control to the 7$^{th}$ and 14$^{th}$ day groups, the latter being the most affected. In the digestive cells of these mussels, the PAS-reaction was greatly reduced and restricted only to the brush border, when present. The PAS-positive globules, which were clearly evident and widely distributed in controls, disappeared in the 0.500 mg/L PQ group, where the digestive cells had heavily vacuolated cytoplasm.

Ovary

The non-affected ovaries showed intact acini with developing and mature oocytes (Fig. 3A). However, some mussels from all the experimental groups, controls included, were observed with slight alterations of the germinative epithelium (Table 1). These pathological pictures mainly consisted of lyses of the apical portion of the oocytes, determining the presence of cellular debris in the acinar lumen (Fig. 3B). The histological condition of the mussels exposed to PQ worsened with increasing concentration and exposure time, but their number did not. After 7 days of exposure to the highest PQ concentration, 50% of female mussels showed ovarian lesions (Table 1). Most of the oocytes were degenerating and large amounts of cytoplasms and condensed nuclei and nucleoli were often detectable in the lumina. (Fig. 3C). After 14 days the heaviest ovarian lesions were detected. The acinar walls sometimes broke (Fig. 3D), the oocytes shrank and their nuclei became vacuolated (Fig. 3E, F). In a few case some oocytes entirely disintegrate while other showed pyknotic nuclei.

Testis

Testicular lesions mainly consisted of acinar atrophy, thinness of the germinative layer with only few developing spermatogonia and lack of mitotic figures. Diffuse vacuolations were noted inside both the germinative epithelium and the basal membrane. These pathologies were observed after 7 days of exposure only in the 0.500 mg/L group (38%), while after 14 days at 0.250 mg/L (50%) and at 0.500 mg/L (76%) (Table 1). In the most affected testes the irregular basal membranes, evidenced by the PAS reaction, testified the disintegration of the germinative epithelia. At these levels many granular haemocytes were detected indicating the heavy macrophagic activity associated with tissue degeneration. Sometimes the wall of the acini was broken, with consequent release of spermatocytes and spermatids in the haemocoel. Histological pictures of affected testes are shown in Figure 4.

Gut

At 7 days, no pathological pictures were observed, except for one specimen showing slight and localized vacuolation at 0.125 mg/L PQ. After 14 days 5 mussels at 0.250 and as many at 0.500 mg/L had diffuse epithelial vacuolations with cells that, in the most affected fields, appeared completely detached from the

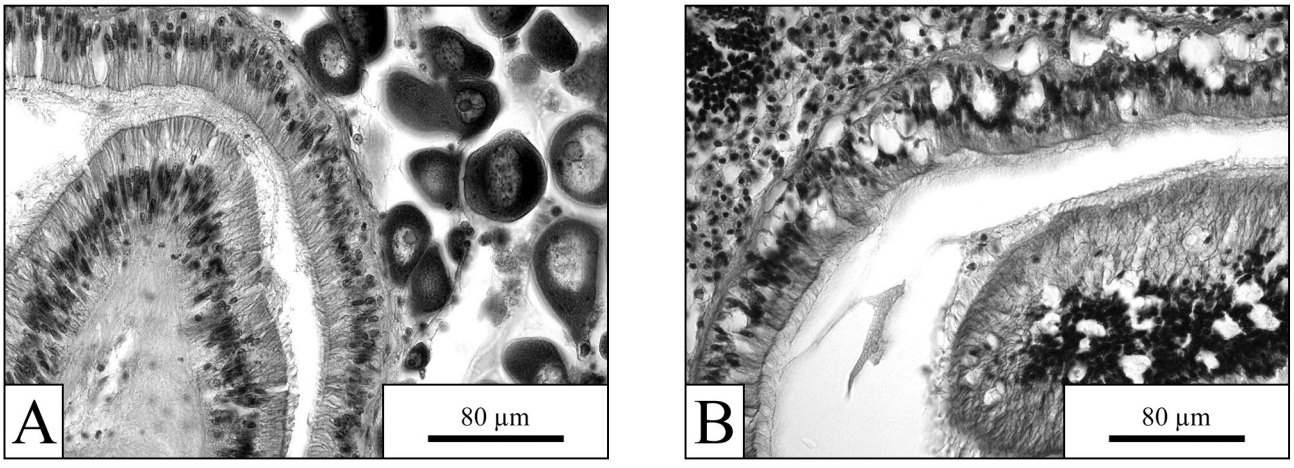


**Fig. 5.** Histological sections of gut epithelium. **A:** Control; **B:** 0.500 mg/L PQ at 14 days.

basal membrane (Fig. 5).

Level of tissue inflammation

As reported in Figure 6 a clear trend among PQ concentrations and the number of granulocytes was observed in the gonad and the DG. After 7 days, significant levels were reached at 0.250 and 0.500 mg/L PQ in both DG ($p<0.05$; $p<0.01$, respectively) and gonad ($p<0.01$; $p<0.05$, respectively). At 14 days in DG and gonad, the level of tissue inflammation in treated mussels was always higher than controls, even if significant differences were reached only at 0.500 mg/L ($p<0.05$).

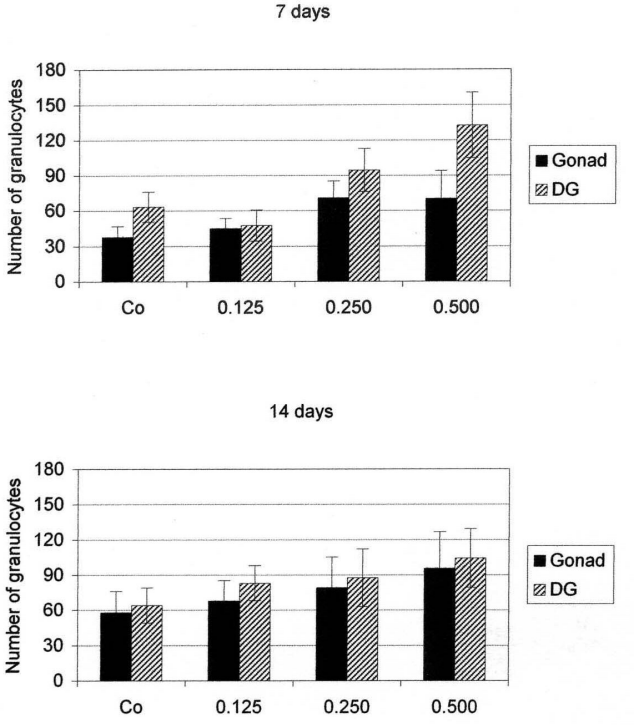


**Fig. 6.** Mean granulocyte number in the gonad and Digestive Gland (DG) of *D. polymorpha*, after 7 (upper panel) and 14 (lower panel) days of exposure to three different concentrations of PQ. Co = Controls. Vertical bars = 95% confidence intervals. Concentration = mg/L

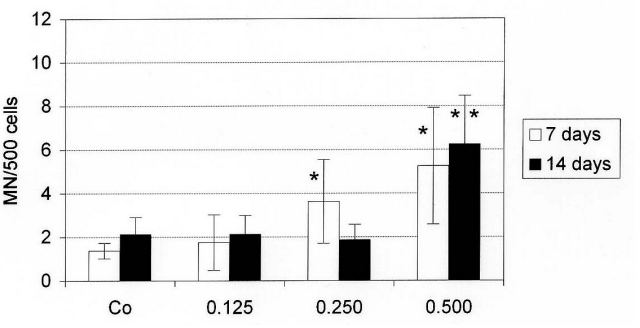


**Fig. 7.** Mean micronucleus (MN) number in Controls (Co) and PQ groups measured at 7 and 14 days. Vertical bars: 95% confidence intervals. *: $p<0.05$ vs control; **: $p<0.01$ vs control. Concentration: mg/L

*MN Assay*

In control mussels the spontaneous level of MN frequencies ranged from 2.75 and 4.25 ‰ (Fig. 7). After 7 days of exposure, MN frequencies progressively increased with PQ concentrations, reaching significantly high values at 0.250 and 0.500 mg/L ($p<0.05$). After 14 days the number of MN did not result concentration-dependent and significant differences from the control were registered only at 0.500 mg/L ($p<0.01$). In this experimental group the highest MN rate of 6.25/500 cells was scored (Fig. 7).

## Discussion

The absence of mortality observed in *D. polymorpha* even after 14 days of exposure to 0.500mg/L PQ suggests that zebra mussels can tolerate this herbicide and that its environmentally available concentrations do not affect the viability of these bivalves. On the contrary, the severe histopathological damage detected in the male and female reproductive tissues clearly endanger not only the reproductive success of the affected mussels, but indicated a PQ-associated risk for the whole mussel population. From our results, only mussels exposed to 0.125 mg/L PQ had little or no pathological stresses, but this concentration is close to the lowest recommended limit of concentration for aquatic weed control (Calderbank, 1972).

In the literature there are many references to PQ toxicity in aquatic animals. With regards to fish, for example, the 96 hr $LC_{50}$ ranges from 11.84 mg/L in *Oreochromis niloticus* (Babatunde et al., 2001), to 67.40 in *Cnesterodon decemmaculatus* (Di Marzio et al., 1998). The amphibian embryos are much more sensitive than fishes showing a 96 hr $LC_{50}$ of 1.3 mg/L in *Rana pipiens* (Linder et al., 1990) and a 120 hr $LC_{50}$ of 0.138 mg/L in *Xenopus laevis* (Vismara et al., 2000). Regarding molluscs, data comes from studies on different species, but all agree that PQ may be considered as slightly or moderately toxic (PANNA, 2004). Nemcsóc et al. (1997) while reporting PQ to be lethal only at high concentrations in *Anodonta cygnea*, also found that it was rapidly incorporated into different organs, causing tissue necrosis. In previous work with the gastropod *Physa fontinalis* exposed to the same PQ concentrations tested in the present study (Bacchetta et al., 2002), no differences in mortality were observed in the experimental groups, but a time- and concentration-dependent alteration of ovipository activity and a clear

trend between PQ concentrations and degenerating oocyte percentages. The histological analyses of the present work showed diffuse tissutal damages in the gonads (Fig. 3, 4), particularly the testis, digestive gland (DG) and gut (Fig. 1, 5), together with high levels of tissue inflammation in both gonads and DG (Fig. 6). This is consistent with the observations of Streit (1979) and Nemcsóc et al. (1997), who reported that PQ is a non-lipophilic compound which is known to accumulate equally in all organs.

This compound also seems to be responsible for increased inflammation in mussel tissues. In fact the trend we observed between PQ concentrations and the number of granulocytes in the gonads and the DG faithfully reflected the level of exposure and the degree of tissue degeneration. In particular, the number of granulocytes increased with increasing PQ concentrations, except in the DG at day 7 in the 0.125 mg/L group (Fig. 6). The use of this marker is based on the observation that granular haemocytes in molluscs are involved in detoxification processes, due to their batteries of hydrolytic enzymes, which also work in intracellular digestion (Giamberini et al., 1996). Although the level of tissue inflammation does not represent a specific histological marker, this has already been successfully used in biomonitoring programs with *Mytilus edulis* (Wedderburn et al., 2000), as well as with *D. polymorpha* (Regoli et al., 2001; Binelli et al., 2004).

The atrophy of the acini in testes, the thinness of their germinative layers and the lack of mitotic figures we observed are in agreement with: (i) the results of Rios et al. (1995) who reported that PQ induced a significant decrease of the mitotic index in treated mice, and (ii) the results obtained by Lutz-Ostertag and Henou (1974) who observed a great reduction in the number of gonocytes in bird embryos exposed to PQ. In invertebrates, a reduction in the number of mitosis subsequent to PQ exposure was already observed in the testes of the oligochaetes (Bacchetta et al., 2000), confirming that PQ presents cytotoxic properties on the male germ line. The number of pathological ovaries in all PQ groups indicates that this herbicide has a cytotoxic effect on oocytes too (Table 1; Fig. 3). Similar effects were already reported in the gastropod *P. fontinalis* exposed to PQ (Bacchetta et al., 2002), as well as in other molluscs exposed to different xenobiotics (Rasmussen et al., 1983; Lowe and Pipe, 1986).

In bivalves, besides digestion, the DG has a significant role in general metabolism and detoxification processes, and it represents the most sensitive organ to contaminants. While the effects of pollution on the DG have been widely studied in *Mytilus*, even at the molecular level (Lowe and Fossato, 2000; Domouhtsidou and Dimitriadis, 2001), data referring to its freshwater counterpart, *D. polymorpha*, are relatively scanty. Histopathological effects on the DG of zebra mussels in starving conditions were investigated (Bielefeld, 1991), as well as in pollution effect monitoring studies (Janssen et al., 1992), and in laboratory test (Zupan and Kalafatiç, 2003). As reported in Table 1, several mussels showed frequent lesions of the DG, ranging from cell degeneration to that of entire branches. Contrary to a previous work where no histological damage was found on the hepatopancreas of the snail *P. fontinalis* even after day 14 of exposure to 0.500 mg/L PQ (Bacchetta et al., 2002), in *D. polymorpha* heavy effects on DG were also seen at lower concentrations. This suggests the high sensitivity of the *D. polymorpha* DG to chemical pollutants, and the key role of this species in freshwater monitoring programs. Besides, vacuolation of bivalve DG and digestive epithelial thinning have been previously demonstrated after pollutant exposure (Kela and Bowen, 1995; Wedderburn et al., 2000).

The zebra mussel was also successfully used for genotoxicity monitoring by the micronucleus (MN) induction in the haemocytes. According to Mersch and Beauvais (1997) a minimum sample size of 500 cells from each of 4 individuals should provide representative information about the induction of MN; the results presented here should be reliable, because a sample of 500 cells was taken from each of 8 mussels, increasing intrasamples variability, but with the benefit to increase also their representativity. Mersch et al. (1996) have demonstrated that in *D. polymorpha* the exposure to reference clastogens under laboratory conditions gave positive results for MN induction and the haemolymph to be the more appropriate test tissue for environmental genotoxicity assessment. This paper reported a baseline MN frequency of 0-3‰ in haemocytes, but these authors also recorded spontaneous MN rates of 3.4‰ in another control zebra mussel sample (Mersch and Beauvais, 1997). Generally, MN frequency in bivalve haemocytes was found to be close to 1‰ (Wrisberg et al., 1992; Burgeot et al., 1995; Mersch et al., 1996), but levels as high as 4.6‰ have been scored in *Mytilus galloprovincialis* haemocytes from unpolluted sites (Burgeot et al., 1996). In agreement with these data, our results showed in control mussels MN frequencies of 2.75‰ and 4.25‰ after 7 and 14 days. The MN induction by PQ progressively increased with increasing concentration at 7 days reaching significant levels at 0.250 and 0.500 mg/L ($p<0.05$), while at 14 days it significantly differed from the controls only at 0.500 mg/L (Fig. 7). In the shorter exposure time the MN response was concentration dependent, but this trend vanished at longer time, in particular the similar MN frequencies found in control, 0.125 and 0.250 groups at 14 days was probably due to DNA repair mechanisms stimulated by the mutagen. Such a response was already observed by Martínez-Tabche et al. (2004) who highlighted PQ genotoxic potential in the rainbow trout *Onchorhynchus mikiss*. Considering the great damage we found at tissue level, it is rather surprising the limited MN inducibility in the haemocytes. This was observed and discussed also by other authors (Wrisberg et al., 1992; Mersch et al., 1996), who suggested the exposition to toxicants to increase mussels' stress, leading to a

reduced mitotic activity. It is likely that the two possible hypothesis, that is, diminished mitotic activity and DNA repair mechanisms, work together contributing to keep the MN number low. It should be noted that since the haemocytes do not divide in the haemolymph (Cheng, 1981), the formation of MN happened elsewhere, perhaps in the connective tissue (Dopp et al., 1996). The spontaneous MN rate of 2.75‰ and 4.25‰ after 7 and 14 days in the controls must be evaluated considering that mussels from these groups are equally stressed by the treatment procedure, like those exposed to PQ. This appears to be a cost in the application of the MN assay in laboratory tests, which on the contrary did not reflect non-specificity of different forms of environmental factors.

PQ has been used to study cellular responses to oxidative stress, because of its capacity to produce Reactive Oxygen Species (ROS). These are highly reactive transient chemical species, formed during normal aerobic cellular metabolism, that are able to initiate damage to the various intracellular components including nucleic acids, lipids and proteins. The principal ROS of physiological relevance are the hydroxyl and superoxyde radicals which are implicated in the pathology of a diverse range of disorders (Grune et al., 1995). In solution PQ is almost completely dissociated into positive and negative ions. The toxic effect is promoted by the metabolic activities of the cells, which reduce the positive ion to form a relatively stable free radical, via mitochondria (Wang et al., 1992) or accept an electron from NADPH, via cytochrome P-450 in microsomes (Bus et al., 1976). In the presence of oxygen, the PQ free radical is oxidized to reform the original ion, but gives rise to hydrogen peroxide, and, consequently, to hydroxyl radicals causing lipid peroxidation, which lead to cell death (Dodge, 1971).

Petushok et al. (2002) reported a decrease in cytochrome P-450 levels in unionid mussels after 10mg/L PQ treatment and attributed this effect to the destruction of the endoplasmic reticulum or mitochondria membrane, where cytochromes P-450 are bound. This data is in agreement with the damage we observed in the DG of PQ-treated mussels, where the digestive epithelium was widely vacuolated and/or with degenerating cells. Also considering that the pathological enlargement and membrane destabilization of the lysosomes in the digestive cells of molluscs derive from exposure to pollutants (Lowe et al., 1981; Domouhtsidou and Dimitriadis, 2001), we can argue that membrane peroxidation induced by PQ-derived oxidative stress endangered the xenobiotic detoxification system associated both with smooth endoplasmic reticulum and mitochondria, and destabilize the lysosomal system, causing cellular lysis. Also, PQ genotoxicity was linked to the capability of this herbicide to induce oxidative stress by free radicals production (Melchiorri et al., 1998; Ortiz et al., 2000; Martínez-Tabche et al., 2004), considering the well documented free radicals relationship with DNA strand breakage and deoxyribose degradation (Salam et al., 1993). Moreover, Melchiorri et al. (1998) demonstrated melatonin, an efficient free radical scavenger, to reduce PQ-induced genotoxicity in mice. In particular, these authors observed that melatonin administration greatly reduced the increase in PQ-induced MN formation at both 48 and 72h following PQ administration, linking without any doubt the PQ toxicity to the free radical generation. The good responses given by the haemocytes in the present work indicate that they are sensitive targets for PQ and that this cellular type is suitable for the study of the oxidative mediated damage to DNA also in the zebra mussel. In conclusion, our results demonstrated the heavy cytotoxic and genotoxic properties of PQ on *D. polymorpha*, suggesting also a larger use of this species in laboratory toxicity tests to better understand the physiological and morphological responses to stressors of a species which is commonly employed in many field biomonitoring programs.

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