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NOVEL ANTIFOULING AGENT ZINC PYRITHIONE: DETERMINATION, ACUTE TOXICITY, AND BIOACCUMULATION IN MARINE MUSSELS (*MYTILUS GALLOPROVINCIALIS*)

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Abstract—Antifouling biocide zinc pyrithione (ZnPT) and its biological fate have received little attention because this compound was assumed not to be persistent in marine ecosystems. An analytical procedure was developed that has proved to be efficient and very sensitive in extracting ZnPT and its main secondary products, Zn and ionized pyrithione (PT^-), from both seawater and biological samples, namely in the gills and digestive gland of the bioindicator species *Mytilus galloprovincialis*. Short-term experiments were carried out to investigate ZnPT toxicity and bioaccumulation. The effects on survival and tissue bioaccumulation of ZnPT and its secondary products were studied on adult mussels from a natural population, collected in the harbor area of Porto Santo Stefano (Italy) and exposed to sublethal doses of the biocide for up to 7 d. Zinc pyrithione was shown to be persistent in the experimental seawater in the short term. A basal level of ZnPT and ionized PT^- was detected in the mussels, indicating that ZnPT availability in the sampling site is already high enough to induce a detectable accumulation in individuals of the native population. Zinc pyrithione rapidly accumulated in the tissues of the exposed mussels, proportionately to both exposure concentration and time, identifying the gills and digestive gland as important targets in the biological pathway of the contaminants. Even though the 7-d median lethal concentration (LC50) = 8.27 μM established here appears high with respect to reported ZnPT environmental concentrations, the results indicate that this biocide could represent a threat for marine organisms in coastal environments and that further investigations on its biological effects at sublethal doses are needed. Environ. Toxicol. Chem. 2010;29:2583–2592. © 2010 SETAC

Keywords—Zinc pyrithione Mussels Median lethal concentration Analytical determination Bioaccumulation

INTRODUCTION

The recent ban on tributyltin (TBT) in antifouling paints established by the International Maritime Organization has resulted in the use of substitute booster biocides [1]. In particular, the use of zinc pyrithione (ZnPT) is of increasing significance. Though its employment as an organic booster biocide is relatively new, ZnPT-based antifouling products are widely applied on yachts and vessels in Europe, with a constant increase in production over recent years [2–4].

Biofouling on ship hulls is caused by a large diversity of aquatic organisms (e.g., algae, barnacles, and mussel embryos). High toxicity to a broad spectrum of species is a feature of antifouling biocides desired by the user. Thus, it is not unexpected that pyrithiones have been found to induce a variety of toxic effects in different test species. In particular, data regarding ZnPT acute toxicity on mussel and copepod embryos and on crustaceans have been reported [5–10]. However, many species in coastal marine ecosystems may become unintended targets of the biocide released by antifouling treatments.

Zinc pyrithione was assumed to be environmentally neutral in the long term, because it could easily photodegrade to less toxic compounds [11,12]. Zinc pyrithione decomposes rapidly in direct sunlight, due to so-called photolysis, with a reported half-life of 8.3 ± 0.9 min in artificial seawater [13]. However, the radiation that is effective for photolysis is of significance only close to the water surface. Zinc pyrithione is suggested to persist in the marine environment where the influence of light is limited, such as in waters and sediments shaded under parking vessels in marinas and harbors [13,14]. In fact, great uncertainty still exists concerning the actual persistence of the biocide in the marine water. Upon degradation of ZnPT, Zn^{2+} is released and several decomposition products are formed. Among these, ionized pyrithione (PT^-) is also known as a broad-spectrum biocide and as a common additive in marine antifouling paints in the form of sodium pyrithione (NaPT) [15].

Different methods have been proposed for the determination of ZnPT in water samples. In particular, ZnPT analyses were performed using a bioassay with radioactive reagent [13] or by high-performance liquid chromatography (HPLC) coupled with atmospheric pressure chemical ionization mass spectrometry [4,16,17], but, to the best of our knowledge, no studies have been carried out on the biological fate and potential bioaccumulation of the biocide in marine organisms.

The Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) is widespread on hard bottoms of confined habitats such as harbors and marinas, where ZnPT may be released as a result of using antifouling paints and may also become a source of Zn contamination. Because of its biological and ecological characteristics (sessile lifestyle, abundance and ubiquity, tolerance to environmental stress, and high accumulation capacity of a wide range of contaminants) [18], this marine mussel is usually used as a sentinel organism.

The aims of this research were to develop a reliable analytical procedure for the extraction and measurement of zinc pyrithione in both seawater and biological samples, and to investigate the biological effects of ZnPT, in terms of acute toxicity and bioaccumulation in adult *M. galloprovincialis*, experimentally exposed to the biocide for up to 7 d. Bioaccumulation responses were highlighted by quantifying both ZnPT

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and its main secondary products ionic zinc (Zn^{2+}) and PT^{-}, to gather data on the biological fate of the biocide. In addition, preliminary experiments were carried out to study the effect of light and oxygenation on ZnPT degradation in artificial seawater, to evaluate ZnPT actual availability in the same experimental conditions as used in the exposure tests with mussels.

MATERIALS AND METHODS

Chemicals and experimental conditions

Zinc pyrithione (48% aqueous dispersion ZnPT; Rutgers Chemicals AG), ionic zinc ($ZnCl_2$; Panreac Quimica), 2-mercaptopyridine-1-oxide sodium salt (NaPT; Fluka), copper nitrate hemi (pentahydrate; Sigma-Aldrich), propyl 4-hydroxybenzoate (Fluka), sodium bicarbonate (Sigma-Aldrich), and nitric acid (J.T. Baker) were of analytical grade. Dichloromethane (DCM; Sigma-Aldrich) and methanol (Carlo Erba) were HPLC grade. The HPLC grade water was obtained by means of a Milli-Q Water System (Millipore). The solvents were filtered through a 0.45 μm Millipore membrane filter and degassed before and during HPLC.

Stock solutions were freshly prepared by dissolving ZnPT in the nontoxic organic dissolvent dimethylsulfoxide (DMSO; Carlo Erba), approximately 1 h before the start of experiments or the renewal of experimental solutions. The experimental solutions were obtained by serial dilution of the stock solution in artificial seawater (Reef Crystals™, 38 practical salinity units, 20°C, pH 8.1, total zinc 0.04 μg/L), and were kept under dark conditions until their use, to avoid photolysis. All treatment and control solutions contained the same amount of DMSO (~0.1‰). The experimental solutions were renewed every 48 h (static renewal experiments) and the quality parameters (pH, temperature, and dissolved oxygen) were measured daily. The experiments were carried out in 5-L or 30-L glass aquaria placed in an environmentally controlled room (constant temperature 20°C, light:dark cycle 12:12 h). Lighting was provided by lamps (390–800 nm) placed approximately 1.5 m above the aquaria, and by indirect sunlight through the windows (with all the aquaria equally distant from the windows). During the exposure experiments, the animals were placed on plastic grids suspended 15 cm below the water surface.

Preliminary experiment: ZnPT degradation

To assess the persistence of ZnPT in the laboratory conditions, evaluating the effect of light and oxygenation on the degradation of the biocide and on the formation of secondary products, Zn^{2+} and PT^{-}, a short-term (48 h) fate experiment was performed. Three 30-L aquaria filled with artificial seawater with the addition of ZnPT up to a nominal concentration of 1.5 μM (0.48 μg/L), were exposed to different conditions: The first was kept in light conditions (12:12 h light:dark cycle) with no oxygenation, the second was maintained in light conditions (12:12 h light:dark cycle) with oxygenation (5.5–6.0 mg O_2/L), and the third in dark conditions with oxygenation (5.5–6.0 mg O_2/L). Three samples (10 ml) were collected from each aquarium at time 0 and after 0.5, 1, 6, 12, 24, and 48 h, and stored at 4°C in dark conditions until sample processing and analysis.

Biological material

Adult mussels (shell length >5 cm) were collected throughout November 2007 from a natural population inhabiting a quay in the busy tourist harbor of Porto Santo Stefano, Monte Argentario, Italy (superficial water 38 PSU, 15°C, 5.6 mg O_2/L), to perform the exposure experiments. Mussels were also sampled from an unpolluted spot outside the harbor area (superficial water 38 PSU, 14°C, 5.9 mg O_2/L) to record the basal levels of bioaccumulation of the studied compounds in a presumably nonimpacted population (reference group).

All animals were transported to the laboratory in portable ice boxes and maintained in aquaria with running natural seawater (38 PSU, 20°C, 5.5–6.0 mg O_2/L, constant conditions) for one week to acclimatize. The animals were not fed during acclimatization or during the exposure experiments. After acclimatization, the animals sampled inside the harbor area were used for the exposure experiments (acute toxicity tests and biological fate experiments).

On the contrary, the animals from the reference group did not undergo exposure experiments and were analyzed for basal levels of ZnPT, PT^{-}, and zinc: Five samples of three specimens were randomly collected, the individuals from each sample were dissected, their gills and digestive glands were pooled separately and stored at −80°C until analysis. Animals still in a visible reproductive status were also discarded and replaced.

Exposure experiments

ZnPT acute toxicity tests. Preliminary range-finding experiments were performed to determine the appropriate dose ranges for the tested chemical (data not shown). Next, short-term tests were carried out to establish the acute lethal toxicity of zinc pyrithione on *M. galloprovincialis* and to identify a range of sublethal concentrations of the contaminant that could be used in the subsequent exposure experiments. The specimens were exposed to 0 (control), 1, 2, 4, 8, 16, 32, 64, and 128 μM ZnPT (respectively, 0, 0.32, 0.64, 1.3, 2.5, 5.1, 10.2, 20.4, and 40.8 μg ZnPT/ml) for up to 7 d, using five 5-L aquaria each containing five animals for every exposure concentration. The aquaria were checked daily for mortalities. Mussels were considered dead when the valves gaped and did not close when manipulated. The number of dead specimens was recorded to calculate the 48-h, 96-h, and 7-d LC50s (median lethal concentrations for 50% of sampled animals). Nominal concentrations were used throughout the present study. The acute toxicity tests were conducted in accordance with American Society for Testing and Materials E729-96 [19].

Biological fate experiments. Two different 7-d experiments were performed. In experiment 1, the mussels were exposed to a sublethal ZnPT concentration (1.5 μM ZnPT, 7-d mortality <20%), to the molar equivalent concentration of ionic Zn (1.5 μM $ZnCl_2$), and to a ZnPT/$ZnCl_2$ mixture (0.75 μM ZnPT + 0.75 μM $ZnCl_2$). In experiment 2, nonlethal ZnPT doses (7-d mortality <5%) were used: Mussels were exposed to 0.4 μM ZnPT, 0.4 μM $ZnCl_2$, and to a ZnPT/$ZnCl_2$ mixture (0.2 μM ZnPT + 0.2 μM $ZnCl_2$).

The experiment was designed to evaluate the contribution of the Zn fraction generated by ZnPT degradation in the biocide toxicity to mussels. In each experiment, the mussels were exposed to a dose of ZnPT, the mixture of ZnPT and ionic Zn that would derive from a partial degradation (50%) of the biocide, and the concentration of ionic Zn that would originate from complete degradation.

Moreover, since the nominal concentration of Zn is the same for all the treatment groups (ZnPT, mixture, and ionic zinc), the experimental design allows us to evaluate, in the three different situations, in which form Zn is accumulated in mussel tissues (ZnPT, ionic Zn, non-ZnPT organic Zn) and whether ionic Zn could interfere with ZnPT bioaccumulation. Zinc, in other

words, was used as a tracer for the bioaccumulation and biological fate of the biocide.

A control group of mussels kept in a contaminant-free medium was set up for each experiment (control). For each experimental group (control, ZnPT, mixture, and ionic zinc), 90 individuals were used, divided into three 30-L aquaria, each containing 30 mussels. Mortality was assessed after 2 and 7 d of exposure. For each experimental group, at time 0 and after 2 and 7 d of exposure, specimens were randomly collected from the three aquaria, and five experimental samples of three randomly chosen mussels of similar size (difference in size within each group $\leq$0.5 cm) were created. The individuals from each sample were dissected, and the gills and digestive glands were pooled separately and stored at -80°C until bioaccumulation analysis. Animals still in visible reproductive status were discarded and replaced.

Sample processing

Artificial seawater samples (10 ml) were extracted twice with dichloromethane (2×3.0 ml). The gills and digestive gland pools were weighed, homogenized with dichloromethane (1.0 ml), and centrifuged to extract pyrithione chelates. The extraction procedure was repeated twice and the supernatants were combined. Aliquots of the extracts (500 μl) from both tissue and water samples, diluted when necessary, were treated with copper nitrate aqueous solution (300 μl; 0.5 M) and mixed thoroughly (vortexed for 5 min) to obtain the quantitative transchelation of the pyrithione chelates into the stable complex, copper pyrithione (CuPT), as suggested in other studies [3,4,20].

The aqueous layer was then removed and the obtained extracts were subjected to reversed phase–high-performance liquid chromatography (RP-HPLC) analysis. To verify whether the pyrithione chelate extracted from the samples and subsequently transformed into CuPT was in fact ZnPT, aliquots of the DCM extracts were evaporated to dryness with nitrogen, digested with 69% nitric acid to release all present metals in their ionic form, and analyzed by atomic absorption spectroscopy (AAS) to check the identity of the metals in the organic extract. The analysis proved that no detectable amount of FePT nor CuPT was present in the organic extracts.

To investigate the presence of ionized pyrithione, an aliquot of the residues of the samples (both water and tissues) obtained from the aforementioned extraction, was treated with copper nitrate aqueous solution (300 μl; 0.5 M) and mixed thoroughly (vortexed for 4 min) to transform any ionized pyrithione present into CuPT. The samples were then extracted in duplicate with dichloromethane (2×1.0 ml) and the extracts analyzed by RP-HPLC.

A further aliquot of the residues was analyzed by AAS to determine the ionic Zn content, and another was analyzed to quantify the total Zn (except Zn as ZnPT) after complete bomb digestion in the microwave (Milestone ETHOS TC Labstation) with 69% nitric acid and then analysis by AAS. The organic Zn non-ZnPT-bound concentration was obtained by subtracting the ionic zinc from the total Zn concentration.

Chromatographic analysis

The CuPT levels in the samples were determined by HPLC coupled to a ultraviolet detector (Jasco). Analyses were performed at 35°C on a LiChroCART Purospher RP-18e (125×4 mm; 5.0 μm) column equipped with a Purospher RP-18e (4.0×4.0 mm; 5.0 μm) guard column (Merck kGaA). The mobile phase consisted of methanol and water under the following gradient conditions: initial, 55% methanol; 0 to 20 min, from 55 to 75% methanol; 20 to 25 min, from 75 to 100% methanol; 25 to 35 min isocratic at 100% methanol. Manual injection was performed by a Rheodyne 7725i injection valve equipped with a 6.0 μl loop. The column eluates were monitored at 240 nm. Retention time for CuPT: 8.40 min. Under these chromatographic conditions, no interfering peak due to the biological matrix was observed, eluting with a retention time corresponding to that of copper pyrithione.

Copper pyrithione calibration curve (RP-HPLC)

The copper chelate (CuPT) was prepared at room temperature by treating 2-mercaptopyridine-1-oxide sodium salt in aqueous solution (0.5 M; 4 ml) with an excess of copper nitrate aqueous solution (0.5 M; 10 ml) under magnetic stirring. The precipitate obtained was filtered, washed with water, and dried under vacuum. The identity of the synthesized derivative was confirmed by ultraviolet spectrophotometric analyses. A stock solution was prepared by dissolving the appropriate quantity of CuPT in dichloromethane to yield a concentration of 1.30 mM; working standard solutions in the range of 0.13 to 1.30 mM were prepared by appropriate dilution of the stock solution with methanol, and the resulting samples were processed in triplicate (6.0 μl). Linear regression analysis was performed by the external standard method using propyl-4-hydroxybenzoate (0.40 mM in methanol). The peak-area ratio of CuPT to the external standard was plotted against the analyte concentration and a linear curve was obtained with a correlation coefficient of $r = 0.995$, slope $= 2.699$ (± 0.143), and intercept $= 0.078$ (± 0.026).

RP-HPLC: Detection limit, recovery, reproducibility

Several ($n = 10$) repeated experiments were done by injecting a standard solution of CuPT to verify the reproducibility of the HPLC procedure; the relative standard deviation (RSD)% obtained for retention time and peak area were 1.12 and 3.16%, respectively. By using replicate extractions and a signal-to-noise ratio of 3 and 10, a limit of detection (LOD) of 24 nmol/L and a limit of quantitation (LOQ) of 80 nmol/L were obtained and validated, respectively. Concentrations that were found to be under the detection limit were reported as 0.

To quantify the recovery of ZnPT from the biological tissues, a blank sample (control gills) and another portion of the same blank sample, spiked with a known amount of ZnPT, were homogenized, extracted twice with dichloromethane, and subjected to transchelation with copper nitrate. The peak area ratios of CuPT to the external standard were plotted against the previously calculated calibration curve. The ZnPT content in the spiked sample was calculated by subtracting its level in the nonspiked sample from that found in the spiked sample, and the percentage recovery was found to average 96%.

Metal concentrations (Zn, Cu, Fe) were determined with a flame atomic absorption spectrometer (Varian SPECTRAA10). Working standard solutions for all analyzed metals were prepared from 1.0 g/L standard solutions (Panreac Quimica), to obtain the calibration curves. The accuracy of the analytical method was checked by using Standard Reference Material (Mussel Tissue, National Institute of Standards and Technology 2977) and procedural blanks.

Statistical analyses

The normality of all the data was first assessed using a κ^2-test. The mortalities after 48 h, 96 h, and 7 d were used to estimate the LC50s. The data were analyzed using the trimmed Spearman–Karber (TSK) method [21] using the TSK Program

Version 1.5 (Environmental Monitoring Systems Laboratory). The LC50s at the respective time intervals with corresponding 95% confidence intervals (CI) are reported.

The homogeneity of variance was assessed by means of the Bartlett's test. To highlight the differences in biological conditions (soft tissue weight and shell length) and in the content of contaminants among the experimental groups, as well as among the time intervals, one-way analysis of variance (ANOVA) was performed followed by the Duncan multiple comparison test. When homogeneity of variances could not be established, two alternative versions of the F ratio, namely the Welch F [22] and Brown–Forsythe F [23] ratios were used. These procedures are robust when the assumption of homogeneity of variances is not met [24,25].

The Student's t test was performed to compare levels of contaminants between the reference and control mussels collected after acclimatization at time 0. To assess the relationship between the bioaccumulation levels and exposure concentrations, as well as between bioaccumulation and time of exposure, a linear regression model was applied and the Fisher–Snedecor's F test was performed to assess the hypothesis of linear independence. The β values, along with their CI, were calculated by means of Student's t test, to allow a statistical comparison. The statistical analyses were performed using SPSS software. A statistical level of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Analytical procedure

The procedure that was developed is based on a sequence of extraction and analysis steps, which involved the use of both a liquid chromatographic (RP-HPLC) and an AAS approach. This analytical procedure was conceived and set up to determine not only ZnPT, but also to quantify the levels of its main secondary products, PT^- and Zn, both in ionic and organic non-ZnPT-bound forms, in the same analytical sample.

The tested procedure proved to be efficient in analyzing all the studied fractions in both artificial seawater and biological samples, and allowed the evaluation of ZnPT degradation in artificial seawater as well as its biological fate. In particular, chromatographic analyses permitted sensitive detection of ZnPT and PT^- (LOQ = 80 nM; LOD = 24 nM), with high values of reproducibility and recovery (see *RP-HPLC: Detection limit, recovery, reproducibility* section).

ZnPT degradation in artificial seawater

The ZnPT, PT^-, and ionic zinc concentrations in artificial seawater during the experiments are shown in Figure 1. No organic zinc other than ZnPT was detected. The ZnPT concentration followed a similar pattern when exposed to light and to light + oxygenation, showing a fast initial decrease and then no significant change after the first 24 h (ANOVA). Photolysis is confirmed as playing a significant role in the degradation of the biocide, while oxygenation had no significant effect. However, a considerable portion of ZnPT, approximately 50% of the initial quantity, did not degrade, and persisted in the artificial seawater, even after 48 h of light exposure, allowing the conclusion that after several hours a considerable quantity of biocide was still available for intake, in disagreement with data reported by some authors [12,13,17].

An important consideration is that photolysis rate is strongly dependent on the photon flux density and spectral distribution of the light source. Thus, different results are to be expected whether light is provided by laboratory fluorescent lamps together with indirect solar radiation, as in the current case, or whether the ZnPT solutions are exposed to direct sunlight or xenon arc lamps, as in the case of the previously cited studies.

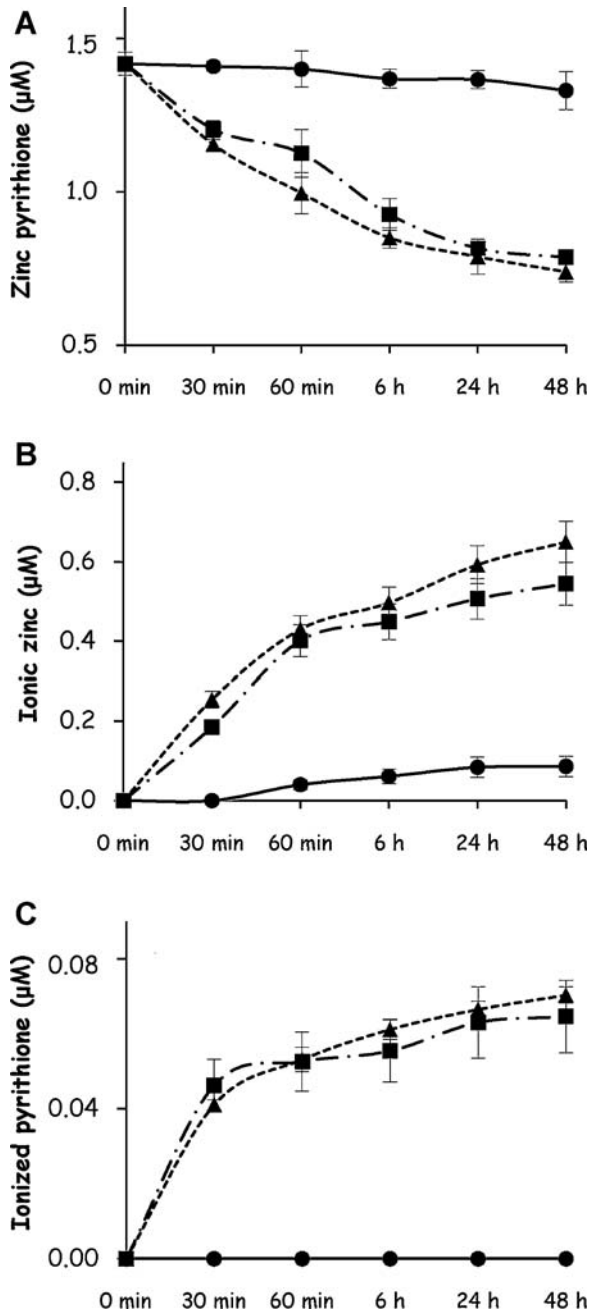

Fig. 1. Time-dependent variation of zinc pyrithione (ZnPT) (**A**), ionic zinc (**B**), and ionized pyrithione (**C**) concentrations (mean ± standard deviation) in artificial seawater containing a nominal initial concentration of 1.5 μM ZnPT. Three aquaria were exposed to different conditions: light conditions with no oxygenation (■), light conditions with oxygenation (▲), and dark conditions with oxygenation (●) ($n = 3$).

No significant degradation could be established under dark conditions throughout the whole experiment time. Under light conditions, as a consequence of the degradation pattern, ionic Zn^{2+} and PT^- concentration in seawater initially increased and then remained unchanged after 24 h (ANOVA). In particular, PT^- concentration was shown to be quite low, slightly above the detection limit, while most of the Zn released by photolysis could be found in the solution in its ionic form.

The preliminary experiment on ZnPT degradation was performed to evaluate the availability of the biocide in the tested conditions. Even though nominal concentrations were used in the subsequent exposure experiments, it can be estimated that the actual concentration of the biocide gradually decreased to 50% of the initial dose, until the renewal of the experimental medium, which was performed every 48 h.

No detectable concentration of CuPT was found in artificial seawater (AAS detected no copper in the DCM extracts, see *Sample processing* section), meaning that Cu levels were low in the experimental medium. In fact, ZnPT transchelation into CuPT was demonstrated to occur only when sufficient Cu was present, and it can become an important factor in stabilizing pyrithiones in the marine environment, and especially in polluted marinas where Cu contamination is often high [3,4,14,20,26]. Moreover, CuPT has been demonstrated to have even higher toxicity to marine organisms than its analogous ZnPT [3,14].

ZnPT effect on survival of M. galloprovincialis

The results of the acute toxicity tests, performed to assess the effect of ZnPT on adult *M. galloprovincialis* survival, are shown in Figure 2 (dose–response curves). Efforts were focused mainly on the toxicity of ZnPT to algae [9] or to larval stages. Bellas et al. [5] calculated 48-h median effective concentration (EC50) values of 7.7 and 8 nM on sea urchin *Paracentrotus lividus* and marine mussel *Mytilus edulis* embryos, respectively, while Karlsson and Eklund [27] reported for three-week-old specimens of the harpacticoid copepod *Nitocra spinipes* a 96-h LC50 of 178 nM. Only a few studies have been conducted to assess the effect of ZnPT on adult marine invertebrates. Hossain et al. [7] reported a 48-h LC50 value of 31 nM for the calanoid copepod *Acartia omorii* under light conditions, while Onduka et al. [28] calculated a 24-h LC50 of 0.88 μM for the crustacean *Tigriopus japonicus* in the dark. Sánchez-Bayo and Goka [10] obtained 48-h LC50 values in the range 0.4 to 1.6 and 0.24 to 0.62 μM ZnPT for ostracod and cladoceran juveniles. Moreover, a 96-h LC50 value of 11.52 ± 3.12 ZnPT nM was obtained in acute toxicity tests on the marine polychaete *Dinophilus gyrociliatus* under light:dark cycle 12:12 h conditions [29].

The acute toxicity values calculated here for *Mytilus galloprovincialis* (48-h LC50 = 45.9 ± 19.0 μM; 96-h LC50 = 15.1 ± 4.2 μM; 7-d LC50 = 8.27 ± 2.52 μM) are quite different from all the other experimental evidence reported up to now for other marine invertebrates. Zinc pyrithione toxicity for adult specimens of *M. galloprovincialis* in the experimented light conditions is one order of magnitude lower than that for other adult organisms studied, and at least two orders of magnitude lower than for embryos of other marine invertebrate species studied. In fact, *M. galloprovincialis* is confirmed to be particularly resistant to contaminants [18]. An important consideration is that ZnPT-based antifoulants were found to be more toxic to marine species than other common antifoulants, i.e., Irgarol®, Diuron®, Zineb®, and Sea-Nine® [6,8,9,30,31].

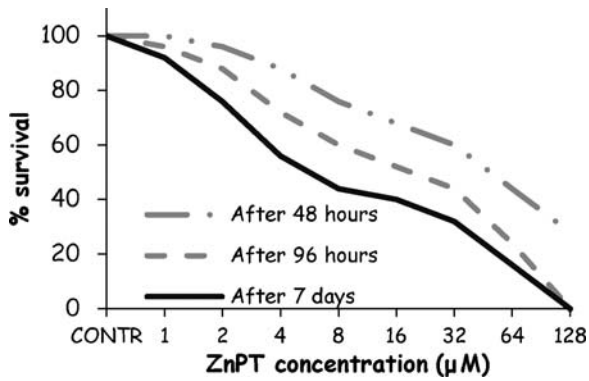

Fig. 2. The effect of zinc pyrithione (ZnPT) on survival of adult *Mytilus galloprovincialis* (% of live specimens) ($n = 25$). A dose–response curve is presented for each observation time.

Biological fate experiments: Mortality data

The mortality data obtained from the biological fate experiments are consistent with the results of the acute ZnPT toxicity tests. After 7 d, 15 and 7% mortality values were detected in mussels exposed to 1.5 μM ZnPT and to the mixture (0.75 μM ZnPT + 0.75 μM $ZnCl_2$) in experiment 1, respectively, while mortality below 5% was noticed in mussels exposed to 0.4 μM ZnPT in experiment 2. On the contrary, no mortality was detected in the MIXTURE group in experiment 2 (0.2 μM ZnPT + 0.2 μM $ZnCl_2$), nor in the experimental groups exposed to ionic Zn alone. Ionic Zn was demonstrated to have no effect on the survival of *M. galloprovincialis*, even at the highest tested concentration (1.5 μM).

It can be assumed that the acute toxicity detected in groups exposed to ZnPT or to the mixture is to be imputed exclusively to the biocide. The presence of ionic Zn in the MIXTURE groups at the tested concentrations did not increase ZnPT toxicity, notwithstanding the fact that this biocide is reported to be a Zn ionophore, and intracellular Zn is considered to be important in the ZnPT mechanism of toxicity [32]. Zinc pyrithione, it seems, explicates its toxicity regardless of the presence of further Zn in the experimental medium.

Since the ionic Zn concentrations used in the exposure experiments caused no mortality, it can be stated that the quantity of Zn released from the degradation of ZnPT at sublethal concentrations appears to have no effect on *M. galloprovincialis* survival. However, such a Zn release in seawater may acquire greater importance, considering the amount of this metal accumulating in the coastal environment, derived from multiple anthropogenic sources other than the widespread usage of paints.

Biological fate experiments: Bioaccumulation

The concentrations of ZnPT, PT^-, ionic and organic zinc in the gills and digestive glands of *M. galloprovincialis* are shown in Figures 3–6. Soft tissue weight and shell length were not considered as covariates in the analysis of the bioaccumulation data, because ANOVA showed no differences in these biological condition parameters among the experimental groups.

ZnPT and PT^- bioaccumulation. No detectable ZnPT nor PT^- basal levels were found in the reference mussels, collected outside the harbor. Data from the exposure experiments are shown in Figures 3 and 4. A basal level of the compounds was detected both in the gills and digestive glands of the specimens sampled inside the port area (time 0). The levels of the contaminants did not change significantly in the control group during the experiment (ANOVA). In the exposed groups, a similar bioaccumulation pattern was shown for both ZnPT and PT^-, with PT^- concentration at a much lower level. The data clearly indicate rapid accumulation in mussels exposed to ZnPT (in both experiments, ZnPT > mixture > ionic zinc = control, after 48 h; ANOVA), the extent of which depends on exposure time and ZnPT concentration.

Bioaccumulation increases during the 7-d exposure with a higher intensity in the digestive gland compared to the gills, as demonstrated by the regression coefficients β, the values of which are significantly higher in the gland in ZnPT-exposed groups (Table 1). The highest slope values were observed in

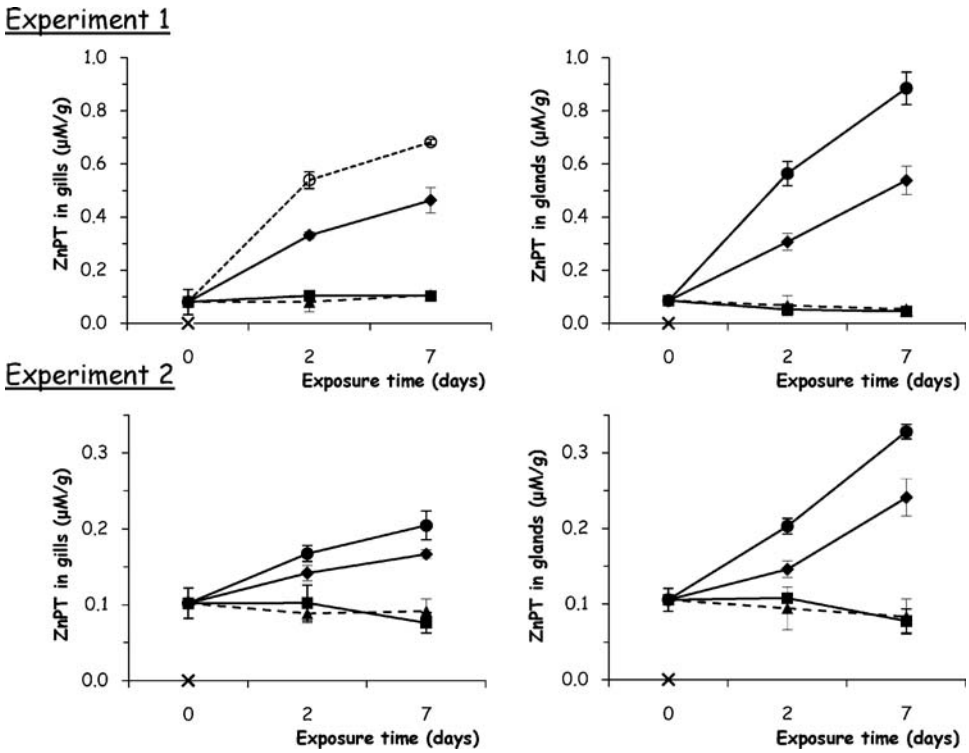

Fig. 3. Zinc pyrithione (ZnPT) concentration (mean ± standard deviation) in gills (left) and digestive gland (right) of *Mytilus galloprovincialis* ($n = 5$). Experiment 1: mussels exposed to 1.5 µM ZnPT (ZnPT ●), 1.5 µM $ZnCl_2$ (ionic zinc ■), 0.75 µM ZnPT + 0.75 µM $ZnCl_2$ (mixture ◆). Experiment 2: mussels exposed to 0.4 µM ZnPT (ZnPT ●), 0.4 µM $ZnCl_2$ (ionic zinc ■), 0.20 µM ZnPT + 0.20 µM $ZnCl_2$ (mixture ◆). For both experiments: control (▲) = mussels kept in contaminant-free artificial seawater; reference (×) = mussels collected outside the harbor area.

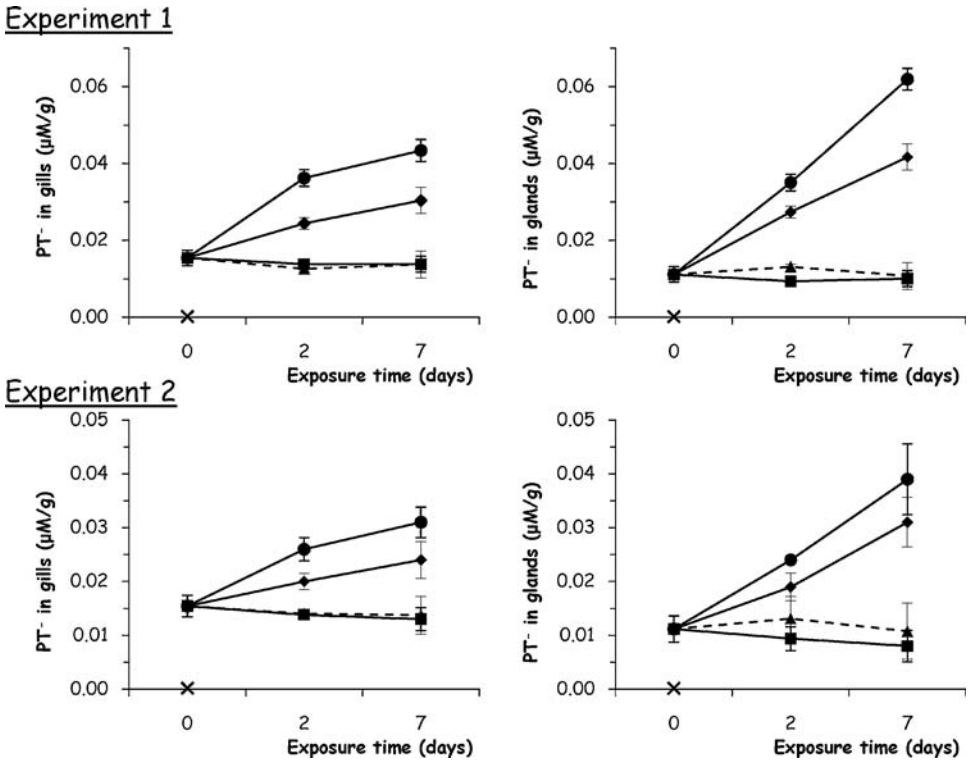

Fig. 4. Ionized pyrithione (PT^-) concentration (mean ± standard deviation) in gills (left) and digestive gland (right) of *Mytilus galloprovincialis* ($n = 5$). ZnPT = zinc pyrithione. Experiment 1: mussels exposed to 1.5 µM ZnPT (ZnPT ●), 1.5 µM $ZnCl_2$ (ionic zinc ■), 0.75 µM ZnPT + 0.75 µM $ZnCl_2$ (mixture ◆). Experiment 2: mussels exposed to 0.4 µM ZnPT (ZnPT ●), 0.4 µM $ZnCl_2$ (ionic zinc ■), 0.20 µM ZnPT + 0.20 µM $ZnCl_2$ (mixture ◆). For both experiments: control (▲) = mussels kept in contaminant-free artificial seawater; reference (×) = mussels collected outside the harbor area.

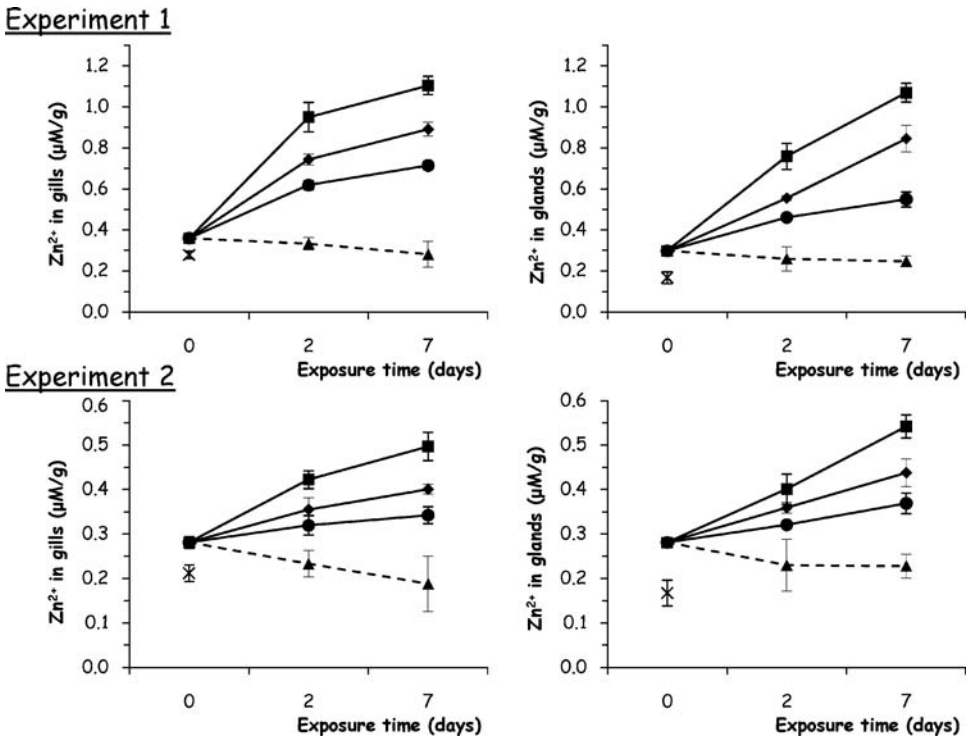

Fig. 5. Ionic zinc concentration (mean ± standard deviation) in gills (left) and digestive gland (right) of *Mytilus galloprovincialis* ($n = 5$). Experiment 1: mussels exposed to 1.5 µ µ µM ZnPT (ZnPT ●), 1.5 µM $ZnCl_2$ (IONIC ZINC ■), 0.75 µM ZnPT + 0.75 µM $ZnCl_2$ (mixture ◆). Experiment 2: mussels exposed to 0.4 µM ZnPT (ZnPT ●), 0.4 µM $ZnCl_2$ (ionic zinc ■), 0.20 µM ZnPT + 0.20 µM $ZnCl_2$ (mixture ◆). ZnPT = zinc pyrithione. For both experiments: control (▲) = mussels kept in contaminant-free artificial seawater; reference (×) = mussels collected outside the harbor area.

mussels exposed to 1.5 µM ZnPT: Bioaccumulation rates of 0.399 µM ZnPT/day and 0.025 µM PT^-/day were calculated for the digestive gland, while values of 0.301 µM ZnPT/day and 0.014 µM PT^-/day were established for the gills.

The data show that bioaccumulation of these compounds proceeded in the gills and in the digestive gland with a similar intensity during the first 2 d, with no difference in concentrations between the two tissues (*t* test, $p < 0.05$). Subsequently,

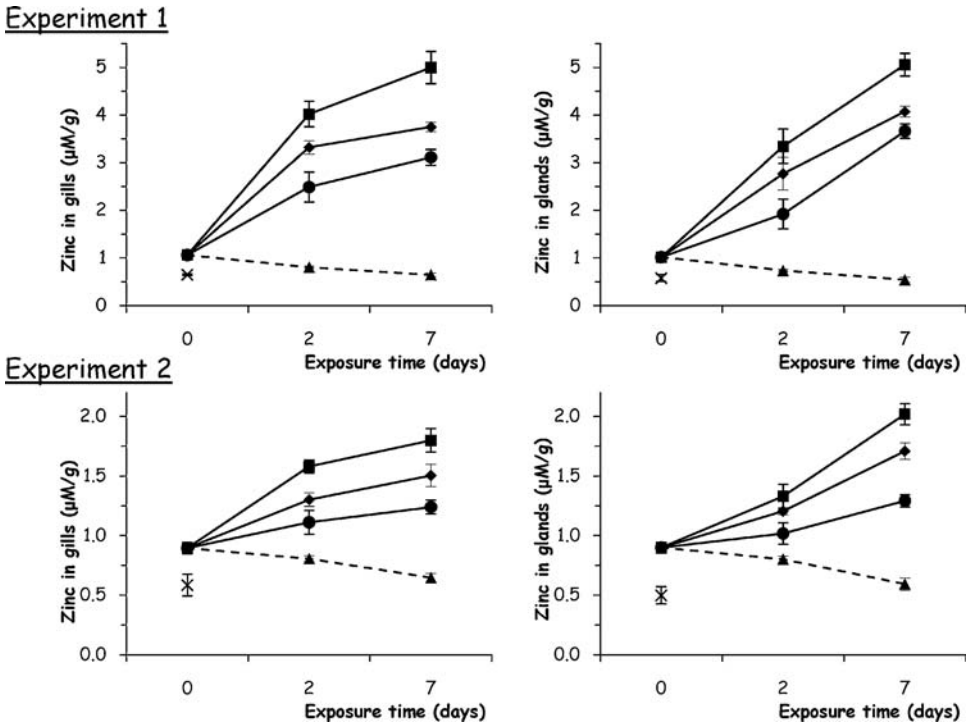

Fig. 6. Non-zinc pyrithione (ZnPT) organic zinc concentration (mean ± standard deviation) in gills (left) and digestive gland (right) of *Mytilus galloprovincialis* ($n = 5$). Experiment 1: mussels exposed to 1.5 µM ZnPT (ZnPT ●), 1.5 µM $ZnCl_2$ (ionic zinc ■), 0.75 µM ZnPT + 0.75 µM $ZnCl_2$ (mixture ◆). Experiment 2: mussels exposed to 0.4 µM ZnPT (ZnPT ●), 0.4 µM $ZnCl_2$ (ionic zinc ■), 0.20 µM ZnPT + 0.20 µM $ZnCl_2$ (mixture ◆). For both experiments: control (▲) = mussels kept in contaminant-free artificial seawater; reference (×) = mussels collected outside the harbor area.

Table 1. Relationship between the bioaccumulation of zinc pyrithione (ZnPT), ionized pyrithione (PT^-), ionic and non-ZnPT organic zinc in *Mytilus galloprovincialis* and exposure time (length of the experiments = 7 d)[a]

	ZnPT		PT^-		Zn^{2+}		Organic Zn	
Experimental group	Gills	Gland	Gills	Gland	Gills	Gland	Gills	Gland
Experiment 1								
ZnPT	0.301	0.399[b]	0.0140	0.0254[b]	0.1372	0.1256	1.026	1.522[b]
Mixture	0.151	0.226[b]	0.0075	0.0153[b]	0.2657	0.2734	1.345	1.728[b]
Ionic zinc	0.008	−0.007	−0.0008	−0.0002	0.3719	0.3857	1.967	2.019
Control	0.013	−0.020	−0.0008	−0.0006	−0.0394	−0.0252	−0.207	−0.237
Experiment 2								
ZnPT	0.051	0.111[b]	0.0078	0.0139[b]	0.0307	0.0440	0.172	0.196
Mixture	0.032	0.067[b]	0.0043	0.0099[b]	0.0699	0.0785	0.205	0.405[b]
Ionic zinc	−0.012	−0.014	−0.0012	−0.0016	0.1179	0.1305	0.452	0.760[b]
Control	−0.005	−0.011	−0.0008	−0.0002	−0.0465	−0.0265	−0.123	−0.153

[a] Linear regression parameters β (μM/day) are shown. The β values in the gills and digestive gland are compared. Experiment 1: The mussels were exposed to 1.5 μM ZnPT (ZnPT); 1.5 μM $ZnCl_2$ (ionic zinc); 0.75 μM ZnPT + 0.75 μM $ZnCl_2$ (mixture). Experiment 2: The mussels were exposed to 0.4 μM ZnPT (ZnPT); 0.4 μM $ZnCl_2$ (ionic zinc); 0.20 μM ZnPT + 0.20 μM $ZnCl_2$ (mixture). Control: The mussels were kept in contaminant-free artificial seawater.
[b] Significant difference in the β value between gills and gland within the same experimental group.

bioaccumulation in the gills increased at a slower rate, while in the digestive gland it continued at a constant rate during the 7 d of the experiment (Figs. 3 and 4). Moreover, ZnPT and PT^- bioaccumulation was shown to increase hand-in-hand with the exposure concentration, both after 2 and 7 d, as shown by r^2 and β values (r^2 always ≥0.9; Table 2).

Zinc bioaccumulation. The data on ionic and organic non-ZnPT zinc levels for all the experimental groups are shown in Figures 5 and 6. The basal concentrations in both tissues at time 0 were significantly higher in mussels collected inside the harbor than in those sampled outside the harbor (reference) (*t* test). In the control group, ionic Zn levels did not vary significantly during the experiment, while a little but significant depuration occurred after 7 d in the case of organic Zn (ANOVA). In the exposed groups, accumulation of Zn, in both its ionic and organic forms, increased with time, depending on the availability of ionic Zn (in both experiments, ionic zinc > mixture > ZnPT; ANOVA). Moreover, there is a significant relationship between Zn bioaccumulation and ZnPT-exposure concentration in ZnPT- or mixture-exposed groups, both after 2 and 7 d, meaning that the ionic Zn fraction derived from ZnPT dissociation in the experimental medium is accumulated in the analyzed tissues, both as ionic and as organic Zn (Table 2).

ZnPT fate in mussels. No evidence exists, at the time of writing, on bioaccumulation, nor on metabolism of ZnPT in mussels. Preliminary speculations can be made based on the results of these experiments, although certainly additional research should be performed to elaborate on these findings.

Given its chemical properties [33], zinc pyrithione is likely to partially dissociate in tissues, as has been demonstrated to occur in artificial seawater, and to release free Zn and ionized pyrithione. On the same basis, it can be speculated that PT^-, as well as ionic Zn, accumulated in tissues should derive not only from the intake from seawater, but also from the internal dissociation of the biocide. That notwithstanding, the current data indicate that ZnPT enters mussel tissues and is rapidly accumulated as such in the gills and digestive glands, which unmistakably have an important role in its uptake and bioaccumulation. Zinc pyrithione is accumulated in mussels proportionally to availability in the experimental medium, apparently with no efficacious internal degradation nor regulation in the short term. In fact, very little quantities of ionized pyrithione are present in tissues after 7 d in comparison to ZnPT, therefore it can be hypothesized that PT^- stored in tissues derives from the direct uptake from the experimental medium, in which it is formed from the dissociation of ZnPT. No enzyme activity seems to occur in the short term to metabolize the biocide into PT^-, which is known to be a less biologically active compound [34], unlike what has been reported for the antifoulant TBT, as an example, which is rapidly metabolized after being accumulated [35].

As previously shown, the intensity of accumulation of both ZnPT and PT^- in the gills is less pronounced after the second day of exposure (see *Biological fate experiments: Bioaccumulation* section), because these compounds are probably in part mobilized and transferred to other tissues via the hemolymph. On the contrary, the digestive gland is shown to accumulate at a constant rate during the 7 d of the experiment, meaning that it is

Table 2. Relationship between bioaccumulation of zinc pyrithione (ZnPT), ionized pyrithione (PT^-), ionic and non-ZnPT organic zinc in *M. galloprovincialis,* and exposure ZnPT concentration (length of the experiments = 7 d)[a]

	ZnPT		PT^-		Zn^{2+}		Organic Zn	
Tissue/exposure time	r^2	β	r^2	β	r^2	β	r^2	β
Gills/2 d	0.90	0.11[b]	0.99	0.005[b]	0.90	0.16[b]	0.93	0.84[b]
Gills/7 d	0.91	0.15[b]	0.92	0.009[b]	0.96	0.23[b]	0.94	1.09[b]
Gland/2 d	0.90	0.12[b]	0.89	0.005[b]	0.94	0.12[b]	0.92	0.68[b]
Gland/7 d	0.95	0.19[b]	0.93	0.011[b]	0.97	0.21[b]	0.96	1.14[b]

[a] Linear regression parameters r^2 and β are shown. The mussels were exposed to different ZnPT concentrations: 0.0 μM ZnPT (control group: mussels kept in contaminant-free artificial seawater); 1.5 μM ZnPT (ZnPT group) and 0.75 μM ZnPT (mixture group) in Experiment 1; 0.4 μM ZnPT (ZnPT group) and 0.20 μM ZnPT (mixture group) in Experiment 2.
[b] Significant linear relationship between bioaccumulation and exposure ZnPT concentration.

probably to be considered a storage organ for both compounds, at least in the short term. In fact, the current data seem to indicate that the accumulation of ZnPT and PT^{-} in mussels proceeds along a similar path in comparison to inorganic zinc. It is well known that, in mussels, metal uptake is through the gut, mantle, and gills. Zinc, as well as other heavy metals, is transported from the gills via the hemolymph, either as a high molecular weight complex or in the granular amoebocytes, to the digestive gland and the kidney. In mussels, the digestive gland constitutes the main center for metabolic regulation, participating in the mechanism of homeostatic regulation, as well as in the process of detoxification of xenobiotics [36,37].

The present results show strong accumulation of Zn in mussels in the short term, proportionally to ionic Zn availability in the experimental medium and, importantly, in both the gills and digestive gland Zn is mostly accumulated as organic Zn. It is well known that in mussel cells, metallothioneins bind Zn, as well as other heavy metals such as Cu and Cd, to provide protection against metal toxicity [38,39].

The presence of ionic Zn in the experimental solutions did not interfere with ZnPT uptake and accumulation in mussels, nor did it affect its toxicity in terms of effects on survival, differently from what was expected (see *Biological fate experiments: Mortality data* section). It seems quite clear that toxic effects on mussels are to be expected in relation to pyrithione accumulation, while the quantity of Zn deriving from ZnPT degradation constitutes a much smaller threat.

Additional research is necessary to verify the bioaccumulation path of ZnPT in the long term, and to evaluate whether mechanisms of regulation and/or excretion of the contaminant take place. Moreover, it is fundamental to evaluate the potential effects of ZnPT bioaccumulation on mussels, especially with regard to those tissues where ZnPT is stored, given the established high biological activity and toxicity of the compound. Given that ZnPT was shown to induce toxic effects such as apoptosis at nanomolar concentrations [32], it can be hypothesized that the rapid bioaccumulation response could couple with disruption of homeostasis, consequently leading to stress or damage responses even at very low doses.

CONCLUSIONS

The procedure developed, based on the differential extraction of ZnPT and its main secondary products, proved to be efficient and highly sensitive in detecting ZnPT and PT^{-} in seawater and mussel samples. This method constitutes an incisive and cost-effective tool that could be well used in monitoring ZnPT in the marine environment.

Zinc pyrithione was shown to be persistent in artificial seawater and to accumulate in mussel tissues. These findings, along with its proven high biological activity and toxicity to marine species, make it clear that this biocide has the potential to cause environmental damage. Although ZnPT concentration in harbors and marinas is probably not yet high enough to have an effect on adult *M. galloprovincialis* survival, as shown by LC50 values calculated here, it could comprise a risk for marine organisms, especially for those that are not as tolerant to contaminants as bivalves.

Importantly, no ZnPT and PT^{-} were detected in the reference mussels collected outside the harbor of Porto Santo Stefano. On the contrary, a basal level of both compounds was detected in mussels from inside the harbor area, indicating that ZnPT availability in the sampling site is already sufficiently high to determine detectable accumulation in *M. galloprovincialis*.

Moreover, the high bioaccumulation rates recorded in the exposure experiments confirm that ZnPT is able to easily enter the biological compartment and could potentially accumulate in the trophic chain. This is even more alarming if one considers that concentrations of the pyrithione ligand of up to 105 nM have already been detected in marinas [40], which is a concentration that is only slightly lower than the sublethal values found here.

In conclusion, these findings indicate the need for monitoring of ZnPT and its secondary products in biota, as well as in seawater and sediment, especially in fishery harbors and marinas where the water exchange is limited and antifouling paints containing pyrithiones, such as Zinc Omadine® and Copper Omadine® (Arch Chemicals), are abundantly applied to fishing and pleasure boats. Further investigations on the ecotoxicological effects of sublethal or nonlethal doses of the biocide are desirable, with the aim of obtaining the necessary information on its sustainability as a safe alternative to TBT in marine antifouling products.

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