Acute Toxicity of a Marine Emerging Pollutant (Promethazine Hydrochloride) on *Artemia* sp

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**ABSTRACT:** Recently, there has been a worrying increase in the pollution of the aquatic ecosystem caused by emerging contaminants (ECs) detected in wastewater effluent discharges. Although traces of ECs in waters have been found in low concentrations, it leads to negative effects for nontarget organisms. Antihistamines are a class of drugs largely used, whose metabolites are widespread in the aquatic ecosystem. The aim of the study was to evaluate the short-term effects of promethazine hydrochloride on nauplii of *Artemia* sp. A high percentage of mortality and morphological alterations were found. The results suggest a possible correlation between exposure to antihistamine and an acceleration of larval development.

**INTRODUCTION**

A large quantity of chemical products necessary for the treatment and prevention of human and animal diseases are annually consumed worldwide. These emerging contaminants such as pharmaceuticals, personal care products, and endocrine disruptor compounds have been detected in wastewater effluent discharges. Consequently, they and their metabolites are present in the environment, and their long-term impact on the environment is not known. They have been included between “marine emerging pollutants,” substances that enter the environment and cause adverse ecological and human health effects but are still largely unregulated and whose potential effects are poorly known. The release of these biologically active compounds in the aquatic environment may pose a serious threat to the aquatic ecosystem. Antihistamines are a class of drugs frequently used to treat the symptoms of allergic reactions. These drugs are absorbed orally and metabolized in the liver; small amounts of the unchanged drug are then excreted in the urine. They act on specific histamine receptors, inhibiting their activity. Histamine interacts with four different receptor subtypes called H1, H2, H3, and H4, variously distributed. According to the different types of receptors, two main classes of compounds are distinguished: antihistamines H1 and H2. Promethazine (PM) (RS) -N, N-dimethyl-1-phenothiazin-10-yl propan-2-amine is a phenothiazine derivative. Its function is to inhibit histamine H1 receptors without blocking their secretion.

Antihistamines are a class of pharmaceutical products widely used, whose active ingredients or metabolites are globally widespread in influent, effluent, and surface waters, particularly in Europe and North America. As regards aquatic organisms, active pharmaceutical ingredients pose an alarming risk to nontarget aquatic organisms, as many taxa have evolutionarily conserved molecular drug targets similar to humans. Some studies have evaluated the toxicity of antihistamines on model organisms such as *Daphnia magna* and *Amphibalanus Anamitrite*. These studies show that the test performed on *D. magna* can detect toxicity effects at low concentrations, while the studies on *Amphibalanus amphitrite* have evaluated the possible use of antihistamines as products for antifouling, as some nontoxic concentrations would still allow the larvae to metamorphose in the adult stage. *Artemia* sp. also known as brine shrimp is a small saltwater crustacean, belonging to the Anostraca order. *Artemia* sp. is a well-known organism in ecotoxicology, it has a great osmoregulatory capacity, and it can tolerate a wide range of salinity. In fact, it is not expected to be very sensitive to the action of several contaminants thanks to its high tolerance to salinity and it is usually correct for the mortality criterion, especially compared to other crustaceans such as *D. magna*, *D. pulex*, and *Thamnocephalus platyurus*.

**MATERIALS AND METHODS**

An artificial sea water (ASPM water) has been used for cysts’ hatching and for the exposure of nauplii during the experiment.

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According to another study performed by Guzzella, this artificial sea water was prepared by adding seven different salts in 1 L of distilled water. The prepared solution was stirred until the salts were completely dissolved and was kept in the dark at 4 ± 1 °C; before its use, the solution was brought to room temperature (20–25 °C) and the pH was verified, the optimal value of which is 7.4. This solution has been prepared every two days to guarantee a complete purity. The use of ASPM water has some advantages compared to natural sea water, such as the lack of microorganisms, the constant maintenance of pH, and the easy reproducibility in the laboratory to obtain standardized experimental conditions.

The solutions containing the antihistamine have been prepared by solubilizing different quantities of promethazine hydrochloride (PM, 99% pure, in powder) (FARMALABOR-Associated Pharmacists) in 99 mL of artificial saltwater (ASPM) and 1 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich); DMSO is an agent that guarantees a complete solubility of the antihistamine in ASPM, without affecting the results of toxicity tests. Starting from literature data, six concentrations of PM were tested (40, 25, 20, 10, 5, and 2.5 μg/mL). All the solutions have been resuspended by vortex before use.

The nauplii used for the toxicological test were obtained from cysts marketed by JBL (JBL GmbH & Co. KG, Germany) and another type of cyst marketed by the HOBBY company (Hobby, Gelsdorf, Germany) was used as a replicate. The hatching of the cysts was obtained in a beaker (1000 mL) with ASPM water and an aerator to keep the microbubble air insufflation constant. Hatching of the cysts occurred between 24 and 48 h, and stage II and III nauplii were transferred using a micropipette to multiwell plates (96-well, BRANDplates). According to Pecoraro et al., the ecotoxicological test has been performed. In each well, we have put a single individual and 300 μL of each solution containing the different concentrations of PM has been inserted. The controls were inoculated only with ASPM water, while positive controls were performed by inoculating ASPM water containing 1% V/V DMSO (Sigma-Aldrich). The specimens were not fed during the tests. A total of 144 nauplii were exposed to each concentration of PM and 144 nauplii for Control (CTRL) and Control plus DMSO (CTRL + DMSO) respectively. Two replicates are performed. The numbers of surviving nauplii in each well were counted under a stereomicroscope (Leica EZA) after 24 and 48 h. Larvae are considered dead when they remain immobile for at least 10 continuous seconds of observation and the test is not considered valid if the number of deaths in the control solution is greater than 10%. The nauplii that appeared completely immobile were considered as dead, and the mortality rates for each treatment and relative control assays were calculated. For each concentration tested and respective controls, some specimens (15 individuals for each test) were randomly collected at 24 and 48 h of exposure. They were observed using an optical microscope (Leica DMLB) equipped with a camera (Leica DFC295), sacrificed, and fixed in formalin (4%, Bio-Optica) to evaluate the development and any morphological alterations of larvae. The presence of morphological alterations as growth inhibition or malformations compared to controls can be considered an important indicator of toxicity.

The data obtained were processed, and the mortality rates between the nauplii exposed to the different concentrations of PM and the control nauplii, both at 24 and 48 h, were compared using the one-way analysis of variance (ANOVA) test followed by the Tukey test (α < 0.05) using the statistical software Past4Project (version Past4.03). Bar chart graphs of mortality rate (24 and 48 h) were realized using GraphPad Prism software (version 9.3.1). The LC50 value at certain PM concentrations was calculated using the AAT Bioquest (Quest Graph LC50 Calculator) at the durations (24 and 48 h) of nauplii exposure.

**RESULTS**

Percentages of mortality of nauplii exposed to different concentrations of PM at 24-48 h are reported in the following figures. Figure 1 shows that percentages of mortality at 24 h of exposed nauplii were 60% for 40 μg/mL, 31% for 25 μg/mL, and 28% for 20 μg/mL. Lower percentages of mortality, respectively, for 10 μg/mL (11%), 5 μg/mL (10%), and 2.5 μg/mL (4%) are reported. Conversely, controls (CTRL) and positive control groups (CTRL + DMSO) show a percentage of mortality of 3%. Under the highest concentrations of PM exposure, the percentages of mortality of nauplii were statistically different from the controls by the one way ANOVA test followed by Tukey’s test. A statistically significant difference (p < 0.05) for the three highest concentrations of PM has been found (Figure 1, *).

Figure 2 shows notable percentages of nauplii’s mortality at 48 h: 100% of mortality of exposed nauplii to 40 μg/mL, 97% of mortality for 25 μg/mL, and 93% of mortality for 20 μg/mL of PM. Lower percentages of nauplii’s mortality, respectively, 56% for 10 μg/mL, 31% for 5 μg/mL, and 18% for 2.5 μg/mL have been obtained. Controls (CTRL) and positive control groups (CTRL + DMSO) show a percentage of mortality of 6 and 7%, respectively. Under the highest concentrations of PM at 48 h exposure, the percentages of mortality of nauplii were statistically different from the controls by the one-way ANOVA test followed by Tukey’s test. Statistically significant differences

![Figure 1](https://example.com刽)
(p < 0.05) have been obtained for all the concentrations tested compared to controls (Figure 2, *).

Moreover, the percentages of mortality of nauplii exposed to different concentrations of PM at 24-48 h have been used to calculate statistically effective concentration which causes a lethal effect on 50% of exposed nauplii (LC$_{50}$). In this study, the value of LC$_{50}$ is 18.10.

The nauplii seemed to have abundantly passed the third stage of development already at 24 h (Figure 3), compared to the controls, suggesting the existence of a possible correlation between exposure to antihistamine tested and an acceleration of larval development as described for D. magna. These data are supported by a marked vesiculation and an onset of detachment of the cuticle probably attributable to an early molt in exposed subjects (Figures 345).

Moreover, it is also possible to find morphological changes in the digestive tract (Figure 5A,B) which has some constriction in different parts, more visible at 48 h (Figure 5C,D). A possible explanation for this condition could be attributed to a sort of vesiculation that tends to put pressure on the digestive tract. Our results showed high percentages of mortality at 24 h for only the highest concentrations (40, 25, and 20 μg/mL), while at 48 h percentages of mortality were significant statistically compared to controls for each concentration of PM.

**DISCUSSION**

The presence of antihistamines, including promethazine, and their metabolites in the aquatic ecosystem poses serious risks to many nontarget organisms. Some studies have investigated the toxic effects of antihistamines on several model organisms, but only a few have evaluated growth, survival, or LC$_{50}$. For this reason, a robust sensitivity distribution of species exposed to antihistamines cannot be estimated because of the current scarcity of data. Currently, in the literature there are not studies that used Artemia sp. for the evaluation of toxic effects because of exposure to the antihistamine PM. Another study in the literature showed that no lethal effect has been observed for tested concentrations of PM (2.5–20 μg/mL) on another crustacean species (Amphibalanus amphitrite). The authors have demonstrated that larvae were alive and able to move without disturbance during the entire periods of experiments.
(24–96 h) for all the concentrations tested. Furthermore, larvae were able to regularly metamorphosis from the juvenile to adult stage at the end of the treatment, except for those exposed to the highest concentration (20 μg/mL). Another study, in contrast, conducted on *D. magna*, little freshwater crustacean belonging to Cladocera order, has shown a lower value of LC$_{50}$ (LC$_{50}$ = 1.6) than that one obtained in our research on *Artemia* sp. nauplii (LC$_{50}$ = 18.10). However, the authors have shown how the exposure to PM led to an acceleration of the development of the larva at the concentrations tested (0.12–9.4 μg/mL). In *Artemia* sp. nauplii from the II instar, successive cell divisions are at the basis of the formation of transverse lines in the antero-ventral region of the thorax; these lines form those that will later give rise to the gills of the thoracopods in the central-most region. During the development of II to IV instar, the formation of these lines is increasingly evident.$^{16,29}$ In the present study, as shown from the figures, the exposed nauplii showed more developed lines in the antero-ventral regions than those usually present in III instar nauplii already at 24 h compared to controls. This evidence suggests a possible correlation between exposure to PM and an acceleration of larval development of *Artemia* sp. nauplii instars, reported for *D. magna*. Furthermore, the morphological alterations showed sublethal effects on the exposed nauplii compared to the controls, visible even at the lowest concentrations. This study adds important data relating to the toxicity of antihistamines and in particular highlights the toxic effects caused on brine shrimp nauplii, an organism that could become a new model species in this field. In conclusion, *Artemia* sp. appears to be a good candidate for toxicity evaluation in relation to the presence of antihistamines in the aquatic environment, appearing responsive through developmental and morphological alterations. However, further studies are needed to better assess the various effects on different levels, such as behavioral, physiological, and reproductive parameters, because of the exposition to a such pollutant. Finally, to standardize data and propose a valid organism model, it is necessary to define the species of *Artemia* used in the experiments, because different species may respond differently to the same chemical stressor.

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Conceptualization, S.I., R.P., and M.V.B.; methodology, S.I. and S.E.B.; investigation, S.I., R.P., S.E.B., and M.C.; data curation, S.I.; writing-original draft preparation, S.I., R.P., and M.V.B.; writing-review and editing, E.M.S., G.F., and A.S.; supervision, R.P. and M.V.B.; funding acquisition, M.V.B. All authors have read and agreed to the published version of the manuscript. S.I. and R.P. have contributed equally to this work and share first authorship.

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**REFERENCES**

(1) Grassi, M.; Rizzo, L.; Farina, A. Endocrine disruptors compounds, pharmaceuticals and personal care products in urban wastewater: implications for agricultural reuse and their removal by adsorption process. *Environ. Sci. Pollut. Res.* 2013, 20, 3616–3628.

(2) Zhang, H.; Ibara, M.; Hanamoto, S.; Nakada, N.; Jurgens, M. D.; Johnson, A. C.; Tanaka, H. Quantification of pharmaceutical related biological activity in effluents from wastewater treatment plants in UK and Japan. *Environ. Sci. Technol.* 2018, 52, 11848–11856.

(3) Cheng, N.; Wang, B.; Wu, P.; Lee, X.; Xing, Y.; Chen, M.; Gao, B. Adsorption of emerging contaminants from water and wastewater by modified biochar: A review. *Environ. Pollut.* 2021, 273, No. 116448.

(4) Jones, O. A. H.; Voulvoulis, N.; Lester, J. N. Potential ecological and human health risks associated with the presence of pharmaceutically active compounds in the aquatic environment. *Crit. Rev. Toxicol.* 2004, 34, 335–350.

(5) Tornero Alvarez, M.; Hanke, G. Potential chemical contaminants in the marine environment: An overview of main contaminant lists. EUR 28925 EN ; Publications Office of the European Union: Luxembourg, 2017.

(6) Oaks, J. L.; Gilbert, M.; Virani, M. Z.; Watson, R. T.; Meteyer, C. U.; Rideout, B. A.; Shivaprasad, H. L.; Ahmed, S.; Chaudhry, M. J.; Arshad, M.; Mahmood, S.; Ali, A.; Khan, A. A. Diclofenac residues as a cause of population decline of white-backed vultures in Pakistan. *Nature* 2004, 427, 630–633.

(7) Oetken, M.; Nentwig, G.; Löffler, D.; Ternes, T.; Oehlmann, J. Effects of pharmaceuticals on aquatic invertebrates. Part I. The
antileptic drug carbamazepine. Arch. Environ. Contam. Toxicol. 2005, 49, 353–361.

(8) Kidd, A. K.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U. S. A. 2007, 104, 8897–8901.

(9) Keller, G. A.; Di Girolamo, G. Antihistamines: past answers and present questions. Curr. Drug Saf. 2010, 5, 58–64.

(10) Kristofco, L. A.; Brooks, B. W. Global scanning of antihistamines in the environment: Analysis of occurrence and hazards in aquatic systems. Sci. Total Environ. 2017, 592, 477–487.

(11) Kostich, M. S.; Batt, A. L.; Lazorchak, J. M. Concentrations of prioritized pharmaceuticals in effluents from 50 large wastewater treatment plants in the US and implications for risk estimation. Environ. Pollut. 2014, 184, 354–359.

(12) Berninger, J. P.; du, B.; Connors, K. A.; Eytcheson, S. A.; Kolkmeier, M. A.; Prosser, K. N.; Valenti T., Jr.; Chambliss, K.; Brooks, B. W. Effects of the antihistamine diphenhydramine on selected aquatic organisms. Environ. Toxicol. Chem. 2011, 30, 2065–2072.

(13) Lutel, K.; Dhalwai, R. S.; van Raai, T.; Heyland, A. Sea urchin histamine receptor 1 regulates programmed cell death in larval Strongylocentrotus purpuratus. Sci. Rep. 2018, 8, 4002.

(14) Furuhagen, S.; Fuchs, A.; Lundström Belleza, E.; Breitholtz, M.; Gorokhova, E. Are pharmaceuticals with evolutionary conserved molecular drug targets more potent to cause toxic effects in non-target organisms? PLoS One 2014, 9, No. e105028.

(15) Jin, C.; Qiu, J.; Xiao, L.; Feng, K.; Zhou, X. Antifouling activities of anti-histamine compounds against the barnacle Amphibalanus (=Balanus) amphitrite. J. Exp. Mar. Biol. Ecol. 2014, 452, 47–53.

(16) Gajardo, G. M.; Beardmore, J. A. The Brine Shrimp Artemia: Adapting to Critical Life Conditions. Front Physiol. 2012, 3, 185.

(17) Vanhaecke, P.; Persoone, G.; Claus, C.; Sorgeloos, P. Research on the development of a short term standard toxicity test with Artemia nauplii. In The Brine shrimp Artemia, Persoone, G., Sorgeloos, P., Roels, O., Jaspers, E., Eds.; Vol. I: Morphology, genetics, radio-biology, toxicology; Universa Press: Wetteren, Belgium, 1980; p. 345.

(18) Liberato, G.; Prato, E.; Migliore, L.; Cicerò, A. M.; Manfra, L. A review of toxicity testing protocols and endpoints with Artemia spp. Ecol. Indic. 2016, 69, 35–49.

(19) Cantarella, M.; Gorras, G.; di Mauro, A.; Scuderi, M.; Nicotra, G.; Fiorenza, R.; Sciré, S.; Scalisi, E. M.; Brundo, M. V.; Privitera, V.; Impellizzeri, G. Mechanical milling: A sustainable route to induce structural transformations in MoS2 for applications in the treatment of contaminated water. Sci. Rep. 2019, 9, 974.

(20) Fiorenza, R.; Balsamo, S. A.; D’Urso, L.; Sciré, S.; Brundo, M. V.; Pecoraro, R.; Scalisi, E. M.; Privitera, V.; Impellizzeri, G. CeO2 for water remediation: comparison of various advanced oxidation processes. Catalysts 2020, 10, 446.

(21) Balsamo, S. A.; Fiorenza, R.; Condorelli, M.; Pecoraro, R.; Brundo, M. V.; Lo Presti, F.; Sciré, S. One-Pot Synthesis of TiO2-rGO Photocatalysts for the Degradation of Groundwater Pollutants. Materials 2021, 14, 5938.

(22) Guerra, R. Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluents. Chemosphere 2001, 44, 1737–1747.

(23) Nunes, B. S.; Carvalho, F. D.; Guilhermino, L. M.; Van Stappen, G. Use of the genus Artemia in ecotoxicity testing. Environ. Pollut. 2006, 144, 453–462.

(24) Manzini, P., Azzoni, R., Sansoni, G., Spaggiari, R., Baldaccini, G.N., Fochetti, R., Mancini, L. Saggio di tossicità acuta con Artemia sp. In Biologia ambiente, Centro Italiano Studi di Biologia Ambientale (C. I. S. B. A.) Bolletino, Ed.; Bolletino CISBA n. 1/ 1997: Milano, Italy, 1997; Vol. 1, pp. 4–9.

(25) A.P.A.T. (Agenzia per la Protezione dell’Ambiente e per i Servizi Tecnici) & IRSA-CNR (Istituto di Ricerca sulle Acque del Consiglio Nazionale delle Ricerche). Manuale e linee guida 29/2003; APAT Rapporti: Rome, Italy, 2003; Vol. 3(8000), pp. 63–67.