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Environmentally realistic concentrations of the antibiotic Trimethoprim affect haemocyte parameters but not antioxidant enzyme activities in the clam *Ruditapes philippinarum*

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ABSTRACT

Several biomarkers were measured to evaluate the effects of Trimethoprim (TMP; 300, 600 and 900 ng/L) in the clam *Ruditapes philippinarum* after exposure for 1, 3 and 7 days. The actual TMP concentrations were also measured in the experimental tanks. The total haemocyte count significantly increased in 7 day-exposed clams, whereas alterations in haemocyte volume were observed after 1 and 3 days of exposure. Haemocyte proliferation was increased significantly in animals exposed for 1 and 7 days, whereas haemocyte lysate lysozyme activity decreased significantly after 1 and 3 days. In addition, TMP significantly increased haemolymph lactate dehydrogenase activity after 3 and 7 days. Regarding antioxidant enzymes, only a significant time-dependent effect on CAT activity was recorded. This study demonstrated that environmentally realistic concentrations of TMP affect haemocyte parameters in clams, suggesting that haemocytes are a useful cellular model for the assessment of the impact of TMP on bivalves.

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1. Introduction

The presence of pharmaceuticals in aquatic ecosystems is a matter of concern due to the potential toxicological risk to non-target species (Fabbri, 2015). Numerous studies have demonstrated that these compounds continuously enter aquatic ecosystems as non-metabolised substances or as metabolites (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003; Bringolf et al., 2010). Both pharmaceutical consumption and the careless disposal of unused or expired medications contribute considerably to the environmental introduction of pharmaceuticals. Exhaustive descriptions of the sources and fate of environmental pharmaceuticals have been provided by Santos et al. (2010) and Matozzo (2014).

It is well known that antibiotics are used extensively (several tons per year) worldwide in both human and veterinary medicine as well as in aquaculture to prevent or to treat microbial infections and to enhance the growth and feed efficiency of livestock. Due to their considerable use, detectable concentrations (in the ng/L–μg/L range) of antibiotics have been measured in various aquatic ecosystems (Sarmah et al., 2006; Kümmerer, 2009; Zheng et al., 2012; Manzetti and Ghisi, 2014).

The antibiotic Trimethoprim (TMP) is an inhibitor of dihydrofolate reductase and is widely used in both human and veterinary medicine to treat gastrointestinal and respiratory tract infections. After administration, TMP is rapidly absorbed by the gastrointestinal tract, but approximately 60% is excreted unaltered (Sweetman, 2005). As a consequence of its excessive use in human and veterinary medicine and aquaculture, TMP enters aquatic environments (Boxall et al., 2003; Kümmerer, 2009; Santos et al., 2010; Zhang et al., 2013). In aquaculture, TMP is used along with sulfadiazine to treat bacterial fish diseases (Rigos and Troisi, 2005; Sapkota et al., 2008; Kümmerer, 2009). The prophylactic and therapeutic use of TMP in aquaculture can lead to elevated antibiotic residues in both biotic and abiotic matrices, such as ponds, marine sediments, aquaculture products, wild fish, and other natural aquatic environments that are impacted by aquaculture facilities (Sapkota et al., 2008). For example, in Vietnam, TMP was found at a concentration of 1.04 mg/L in water samples from shrimp aquaculture ponds (Le and Munekage, 2004).

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TMP poses a potential environmental risk because its removal rate by activated sludge in sewage treatment plants (STPs) can be low (less than 50%) (de Souza et al., 2009). Relatively high concentrations of TMP (35–2550 ng/L, with a mean value of 781 ng/L) were detected in raw municipal wastewater from the Western Balkan region (Bosnia and Herzegovina, Croatia and Serbia) (Terzić et al., 2008). TMP was detected in 94% of water samples from 139 US streams, with a maximum concentration of 710 ng/L (Kolpin et al., 2002). TMP was detected in 93% of the STP effluent samples (the median level of detection varied from 27 to 89 ng/L, depending on the flow conditions), whereas in the surface waters of the Han River (Korea), TMP was detected in 73% of samples, with a concentration up to 546 ng/L in the mainstream (Choi et al., 2008). Hirsch et al. (1999) detected TMP in STP effluents and surface water at concentrations of 660 ng/L and 200 ng/L, respectively. Lacey et al. (2012) reported the presence of TMP in STP effluents at concentrations up to 600 ng/L. In a final aeration lagoon for the treatment of domestic and hospital wastewater (Canada), Gagné et al. (2012) reported a TMP concentration of approximately 200 ng/L.

Regarding coastal areas, Zhang et al. (2012) measured a maximum TMP concentration of 330 ng/L in Laizhou Bay in the Bohai Sea (China), whereas Minh et al. (2009) reported a maximum concentration of 216 ng/L in Victoria Harbour, Hong Kong. Relatively high concentrations of TMP were recently detected in STP effluent samples (up to 1200 ng/L) and marine surface waters (up to 570 ng/L) collected from May 2011 to April 2012 and in STP effluent (up to 1170 ng/L) and marine surface waters (up to 870 ng/L) collected from May 2011 to August 2011 from different sites around the Irish coastline (McEneff et al., 2014).

Despite evidence that TMP is present in aquatic ecosystems, few studies have investigated the effects of TMP on non-target organisms (Gagné et al., 2006; Binelli et al., 2009a, 2009b; Papis et al., 2011; De Liguoro et al., 2012; Carlsson et al., 2013). This study is the first to evaluate the effects of TMP on haemocyte parameters and antioxidant enzyme activities in the clam *Ruditapes philippinarum*. The hypotheses we tested were that environmentally realistic concentrations of TMP affect cellular and biochemical parameters in clams and that TMP effects vary depending on both the exposure concentrations and duration. To test these hypotheses, clams were exposed for 1, 3 and 7 days to 300, 600 and 900 ng TMP/L, and several biomarkers indicative of cytotoxic (immunomarkers), as well as of oxidative stress (antioxidant enzyme activities), were measured in haemocytes and in gills and digestive gland, respectively. Haemocytes were chosen to assess TMP toxicity in clams as they are involved in immune responses, whereas gills (a tissue in immediate contact with waterborne pollutants) and digestive gland (involved in accumulation and metabolism of organic contaminants) were selected as they are target tissues for several contaminants. Lastly, the actual concentrations of TMP in the exposure medium were also measured.

2. Materials and methods

2.1. Clams and exposure to TMP

Specimens of *R. philippinarum* (3.5–3.8 cm shell length) were collected from a reference site located inside an area licensed for clam culture in the southern basin of the Lagoon of Venice (Italy) and were acclimatised in the laboratory for 5 days before exposure to TMP. Clams were maintained in large aquaria that had a sandy bottom and aerated seawater (salinity of 35 ± 1 psu, temperature of 17 ± 0.5 °C) and were fed daily with microalgae (*Isochrysis galbana*). A stock solution (100 mg/L) of TMP (CAS: 738-70-5; molecular weight: 290.32; p*K*a: 6.60; log K_{ow}: 0.65) (Sigma–Aldrich, Milano, Italy) was prepared in ethanol (EtOH), whereas working solutions were prepared daily by diluting the stock solution in seawater.

The exposure was performed in winter, far from the sexual maturity period of the clams; this non-reproductive condition avoided spawning and reduced possible additional stress during the experiments. Clams (40 per concentration) were exposed for 1, 3 and 7 days to 0 (control), EtOH or 300, 600 or 900 ng/L TMP. The nominal concentrations were chosen based on data on TMP concentrations in aquatic ecosystems (see references in the Introduction section). For the solvent control, EtOH was added at the highest concentration (10 μL/L) used in the TMP treatments. Clams were maintained in 35 L glass aquaria (without sediment) containing aerated seawater under the same thermohaline conditions that were used during the acclimatisation period. Every day, the water was changed, and TMP and microalgae were added.

2.2. Chemical analysis (UHPLC-Q-TOF-MS)

Actual TMP concentrations were measured in water samples collected from the exposure tanks approximately 15 min after the first application (t = 0) and after 24 h (before the water was renewed and before the second application). Seawater samples (50 mL per tank) were collected and stored at −20 °C until analysis. HPLC-grade methanol was supplied by Romil. Water was purified using a Milli-Qplus system from Millipore (Milford, MA, USA). Chromatography was performed using an Agilent 1290 UHPLC system. The chromatographic column was Zorbax Eclipse Plus C18 – Rapid Resolution HD (2.1 × 50 mm, 1.8 μm) with a column temperature of 30 °C. The mobile phase consisted of 0.1% formic acid (solvent A) and 0.1% formic acid in methanol (solvent B). The following solvent gradient was used: 0–0.5 min, 97% A; 3 min, 5% A; 10 min, 5% A. The flow rate was kept at 0.2 mL/min, and the injected volume was 20 μL. Mass spectrometry was performed on a Q-TOF system (Xevo G2-SQTof – Waters); the system was operated using MassLynx software (Waters). The data were collected between m/z 50 and 1200 Da. The ESI capillary was set to 2.0 kV. The desolvation temperature was adjusted to 450 °C, and the source temperature was set at 100 °C. The desolvation flow (nitrogen) was 800 L/h, and the cone gas flow was 20 L/h. The cone voltage was set to 40 V.

The linearity of the method was evaluated using seven standard solutions calibrated over the range of 0.2–10 μg/L. A calibration curve was established by plotting the peak area ratio of the calibration solutions *vs* the nominal concentrations of TMP. Linearity was determined via linear regression analysis. The limit of detection was defined as the lowest concentration resulting in a peak area of three times the baseline noise (S/N > 3) in solvent. The limit of quantification was defined as the lowest concentration level resulting in a peak area of ten times the baseline noise (S/N > 10) in solvent. Measurements of the intra- and inter-day variability were utilised to determine the reproducibility of the method. TMP standard solutions were analysed to determine the intra-day repeatability (examined in one day) and inter-day repeatability (determined on 3 different days). The relative standard deviation (RSD) was calculated as a measurement of method reproducibility (n = 3) (Table 1). Recovery experiments were performed at three different levels of standard and recovery was in the range 106–113%.

2.3. Haemolymph and tissue collection

Haemolymph was collected from the anterior adductor muscle using a 1-mL plastic syringe, placed in Eppendorf tubes and stored at 4 °C. At each sampling time, three pools of haemolymph (from four bivalves each) from each experimental condition were prepared (final volume of at least 2.5 mL). Pooling was necessary to

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