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Effects of 2-(4-Methoxyphenyl)-5, 6-trimethylene-4H-1, 3, 2-oxathiaphosphorine-2-sulfide on biomarkers of Mediterranean clams *Ruditapes decussatus*

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ABSTRACT

The effects of exposure to a novel synthetic organophosphorus compound, 2-(4-Methoxyphenyl)-5, 6-trimethylene-4H-1, 3, 2-oxathiaphosphorine-2-sulfide (OMTOS) concentrations (Control=0, *C*1=0.01, *C*2=0.1, *C*3=1 and *C*4=10 μg/L) were investigated in the clam *Ruditapes decussatus*. Vitellogenin (Vg)-like protein levels in haemolymph from males and females were investigated. Concentrations of 1 μg/L and 10 μg/L significantly decreased Vg levels in male haemolymph after 7 days, whereas significant variations were only found in females treated with 10 μg/L. On the other hand, superoxide dismutase (SOD), catalase (CAT) and acetylcholinesterase activities (AChE) in whole soft tissue were measured after 2, 4 and 7 days of exposure to the same series of concentrations. After 2 days of exposure, 0.1, 1, and 10 μg/L of OMTOS increased SOD activity significantly, but this decreased with 10 μg/L after 4 and 7 days. No changes in CAT activity were observed after 2 days compared to controls. OMTOS significantly reduced AChE activity after 4 and 7 days in treated clams with the highest concentration 10 μg/L, but it did not induce significant variations at the other concentrations tested. Our study demonstrates that OMTOS alters biochemical parameters in *R. decussatus*, even at low concentrations, and suggests differing modes of action of the contaminant. Using clams is a powerful tool to provide valuable insights into possible mechanisms of environmental toxicity of novel synthetic organic products both in non-target organisms and the marine ecosystem. Additionally, our results highlight that biomarker responses facilitate elucidation of putative mechanisms of action of OMTOS in non-target species.

1. Introduction

Organic phosphorylated products represent a large group of substances used either by humans for personal health and cosmetic reasons or in agribusiness to enhance growth or health of livestock. Oxathiaphosphorines are an important class of compounds in medicinal chemistry due to their interesting antimicrobial, antifungal and antitumor activities (Khidre and Kamel, 2008; Krstić et al., 2012). Pharmacological evaluation of these compounds has previously been performed by screening against various fungi including *Penicillium brevicompactum*, *Aspergillus niger* and *Aspergillus fumigatus* using a food poisoning technique. Oxathiaphosphorines caused 100% inhibition of spore germination in *A. niger* at 500 mg/mL. When tested against bacterial species including *Bacillus subitilis*, *Bacillus cereus* and *Escherichia coli*, oxathiaphosphorines showed high inhibitory effect against all the tested organisms, possibly attributable to the presence of the phosphorus moiety (Khidre and Kamel, 2008). Significant advances in the controlled synthesis of this class of organic compounds have been made in recent years. Therefore, the contribution to the synthesis of a new series of organophosphates is needed. 2-(4-Methoxyphenyl)-5, 6-trimethylene-4H-1, 3, 2-oxathiaphosphorine-2-sulfide (OMTOS) is a novel oxathiaphosphorine derivative recently synthesised by (Aouani and Touil, 2014). This category of organic compounds has attracted increasing interest in nanotechnology as chemical ligands for the synthesis of nanomaterials aimed at applications in nanomedicine. OMTOS represents an analogue of cyclophosphamide (CP) and ifosfamide (IF) known as the two oldest and most widely prescribed alkylating cytostatic medicines (Česen et al., 2015). These

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drugs were also found in hospital wastewaters (Steger-Hartmann et al., 1997). Therefore, analogues of these compounds such as OMTS can most probably succeed in reaching aquatic ecosystems. In marine ecosystems, the polar nature of most synthetic products makes them directly bioavailable to filter feeding organisms. Bivalves represent an important economic resource for many coastal populations worldwide. These organisms are constantly subjected to different types of environmental stress, due to their sessile condition and filter feeding habits. They have been extensively utilized as bioindicators in environmental toxicology in the past few decades. The clam *Ruditapes decussatus* is of significant economic interest, abundant and resistant to a wide variety of pollutants and environmental stressors, making them ideal sentinel organisms for marine monitoring programs. Field studies have exploited measurement of several biomarkers in bivalves. Indeed, they provide an early signal of significant biological effects in aquatic environmental monitoring, especially for the sub-organism responses (Fang et al., 2009). Vitellogenins (Vgs) are glycophospho-lipo-proteins normally synthesised by mature females in response to endogenous estrogens, released into the bloodstream, and stored in developing oocytes (Wahli et al., 1981). Measurement of Vg levels has been proposed as a useful biomarker to assess the estrogenic effects of various contaminants in aquatic invertebrates (Matozzo et al., 2008). In addition, several enzymes have been selected as biomarkers for specific chemicals (Ortiz-Zarragoitia et al., 2006; Raftopoulou and Dimitriadis, 2010). Environmental stressors can potentially cause oxidative stress in these organisms, since there is a close relationship between environmental stress and the rate of production of cellular reactive oxygen species (ROS). ROS, including the hydroxyl radical (●OH), superoxide anion radical ($O_2\bullet-$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), are potentially highly-toxic to cells if their rate of production exceeds their rate of removal by antioxidant defenses. Oxidative stress leads to oxidation of key cell components such as proteins, DNA and fatty acids. To protect against ROS, cells possess specific antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), amongst others (Soldatov et al., 2007;). Many field studies have demonstrated the usefulness of measurement of acetylcholinesterase (AChE) activity in invertebrates as a biomarker of exposure in coastal waters (Kwong, 2002; Dutta and Arends, 2003; Solé et al., 2010; Gagné et al., 2011).

Despite the wide spectrum of biological activities associated with oxathiaphosphorines, knowledge on their toxicity is limited. In order to study the possible environmental and human health risks posed by these chemicals, information regarding their occurrence in the aquatic environment, particularly in aquatic species, is urgently required. The aim of this study was to test the occurrence and relative effects of OMTOS at different concentrations, on marine clams collected from sites around the Bizerte lagoon, Tunisia.

2. Materials and methods

2.1. Animal sampling

R. decussatus of 2.5 ± 0.5 cm length size were collected from a reference site of the Bizerte Lagoon (37°10′27.36″N, 9°46′ 50.62″E Tunisia) in January 2014. This site has been previously studied by our group (Dellali et al., 2001; Sellami et al., 2014a; Sellami et al., 2014b). Animals were immediately transferred to the laboratory and placed in an aquarium at a constant temperature of 19 °C with natural seawater (dissolved oxygen 6.2 mg/L, pH 7.8). Seawater was changed every day and kept constantly aerated.

After a 7-day acclimation period, two experiments were performed. The first aimed to assess the potential estrogenicity of OMTOS by measuring Vg-like protein levels in male and female clams. To this end, clams were exposed for 7 days during the prespawning phase, when it was possible to distinguish sex by microscopic observation of gonadal smears. Clams were exposed to 0, DMSO, 0.01, 0.1, 1 and 10 µg/L. Due to its low solubility in water, a stock solution of OMTOS was prepared in DMSO and stored at room temperature (18–20 °C) for the duration of the experiments. Working solutions were prepared daily by diluting the stock solution in seawater. In DMSO controls, solvent was added at the highest concentration used in OMTOS treatments (20 µl/L). During exposure, clams were maintained in glass aquaria containing aerated seawater (1 L/animal), in identical thermo-haline conditions used for the acclimation period. Water and OMTOS solutions were changed daily. At the end of exposure but prior to sexing, haemolymph was individually collected from the anterior adductor muscle of both control and OMTOS-exposed clams with a 1-mL plastic syringe and placed in Eppendorf tubes on ice. Haemolymph from males and females was pooled to obtain 5 replicates (2 animals each) per sex from each experimental condition. Pooling was necessary to obtain enough haemolymph protein material for ALP analysis. Haemolymph was centrifuged at 780 g for 10 min to remove the haemocytes. The shells were then opened and sexing (identification of oocytes and spermatozoa) was performed by microscopic observation (400 ×) of smears of gonadal tissue. Cell-free haemolymph samples were frozen and stored at −80 °C until processed.

A second experiment was performed to evaluate OMTOS effects on antioxidant enzymes and AChE activities. Clams (10 animals per concentration) were exposed for 2, 4 and 7 days to the OMTOS concentrations indicated above. SOD, CAT, GST and AChE activities were individually measured in whole soft tissue. After each period of treatment (2, 4 and 7 days), the experiment was concluded, clams were fixed in liquid Nitrogen and then dissected on ice. After homogenization by polytron homogenizer in 10 mM Tris/HCl, pH 7.2, containing 500 mM sucrose, 1 mM EDTA and 1 mM PMSF, the supernatants were collected by centrifugation at 20,000 × *g* (4 °C for 30 min) to be finally stored at −80 °C until required for biochemical analysis.

No mortality was observed under the conditions used and all animals were observed to be feeding normally.

2.2. Chemistry

OMTOS (1) was prepared according to the procedure reported by (Aouani and Touil, 2014) (Fig. 1). The structure of OMTOS was confirmed by infra-red (IR) and nuclear magnetic resonance (NMR) spectroscopies. ^1H, ^{31}P, and ^{13}C NMR spectra were recorded with $CDCl_3$ as the solvent, on a Bruker AC-300 spectrometer operating at 300.1 MHz for 1 H, 121.5 MHz for ^{31}P and 75.5 MHz for ^{13}C. The chemical shifts are reported in ppm relative to tetramethylsilane (internal reference) for ^1H and ^{13}C NMR and relative to 85% H_3PO_4 (external reference) for ^{31}P NMR. The coupling (*J*) constants are reported in Hz. For the ^1H NMR, the multiplicities of signals are indicated by the following abbreviations: s: singlet; d: doublet; t: triplet; q: quartet; quint: quintet; m: multiplet. IR spectra were recorded on a Nicolet IR200 spectrometer (Thermo Electron Scientific Instruments LLC, Madison, WI, USA). The General procedure for the synthesis **of** OMTOS started by adding the Mannich base hydrochloride (0.01 mol) to Lawesson's reagent (0.005 mol) and dry toluene (30 mL). The mixture was then heated under reflux with stirring for 8–48 h. After cooling, the mixture was extracted with water (2 × 30 mL). The organic phase was dried over Na_2SO_4 and concentrated under vacuum. The obtained residue was chromatographed on a silica gel column using a mixture of ether and petroleum ether (1:1) as eluent.

The spectral data for OMTOS were: