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Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus* spp.) and their comparison with standardized toxicity tests

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ABSTRACT

Human pharmaceuticals, like the lipid lowering agent gemfibrozil and the non-steroidal anti-inflammatory drug diclofenac are causing environmental concern. In this study, the marine mussel (*Mytilus* spp.) was exposed by injection to environmentally relevant and elevated (1 μg/L and 1000 μg/L) concentrations of both compounds and biomarker expression was observed. Gemfibrozil exposure induced biomarkers of stress (glutathione *S*-transferase and metallothionein) at both concentrations 24 h and 96 h after exposure, respectively. Biomarkers of damage (lipid peroxidation (LPO) and DNA damage) were significantly affected, as well as the biomarker for reproduction, alkali-labile phosphate assay, indicating the potential oxidative stress and endocrine disrupting effect of gemfibrozil. Diclofenac significantly induced LPO after 96 h indicating tissue damage. Additionally standard toxicity tests using the marine species *Vibrio fischeri*, *Skeletonema costatum* and *Tisbe battagliai* showed differences in sensitivity to both drugs in the mg/L range. Results indicate a suite of tests should be used to give accurate information for regulation.

1. Introduction

The presence of human and veterinary pharmaceuticals in the aquatic environment has become an area of growing concern in recent years. Human pharmaceuticals, including their metabolites and conjugates, mainly enter marine surface waters, rivers and lakes through the release of treated effluents from municipal sewage treatment plants (Daughton and Ternes, 1999; Fent et al., 2006). Due to their continuous release and persistence these novel contaminants can be found in the aquatic environment with concentrations ranging from low μg/L to high ng/L (Daughton and Ternes, 1999; Halling-Sørensen et al., 1998; Metcalfe et al., 2003). Within this diverse and extensive group of human pharmaceuticals, it is known that the lipid lowering agent gemfibrozil (GEM) and the non-steroidal anti-inflammatory drug (NSAID) diclofenac are found in receiving waters throughout the western world, including in the Irish aquatic environment (Halling-Sørensen et al., 1998; Lacey et al., 2008; Metcalfe et al., 2003).

Gemfibrozil belongs to the class of fibric acid derivates, which reduce plasma triglyceride and cholesterol concentrations. Hence it induces the lipoprotein lipolysis, the fatty acid uptake and reduces the hepatic triglyceride production. Additionally it is known to induce peroxisome proliferation by binding to nuclear peroxisome proliferator-activated receptors (PPAR α, β, and γ) (Staels et al., 1998). GEM has been reported to have effects on non-target organisms, like in goldfish, where it was able to bioconcentrate and reduced the plasma testosterone levels in males (Mimeault et al., 2005, 2006).

Among human pharmaceuticals, the non-steroidal anti-inflammatory drug diclofenac is the most commonly found within the aquatic environment (Landsdorp et al., 1990). It is prescribed to humans to treat a variety of acute and chronic pain and inflammatory conditions. It acts by inhibition of prostaglandin synthesis by inhibiting both cyclooxygenase-1 and -2 (COX-1 and COX-2) (Gan, 2010). In marine invertebrates, prostaglandins are important to regulate the ion transport, oogenesis, spermatogenesis and immune defence (Rowley et al., 2005). Diclofenac has known effects in non-target organisms, like fish, where it can bioaccumulate and change cellular reactions in liver, kidney and gills (Hoeger et al., 2005; Schwaiger et al., 2004; Triebskorn et al., 2004).

Currently standard toxicity tests are used to assess the potential risk of contaminants entering the environment. Generally these are based on short-term acute toxicity (Crane et al., 2006; Halling-Sørensen et al., 1998), which can provide valuable information. However, it is agreed in many studies (Crane et al., 2006; Fent et al., 2006; Santos et al., 2010) that there is a need for chronic toxicity tests to predict ecological consequences due to exposure.

Bivalves, such as the blue mussels (*Mytilus edulis*) are commonly used in ecotoxicology and have been extensively used in various monitoring programmes, such as the Mussel Watch Programme (Kimbrough et al., 2008). Due to their wide distribution,

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sentinel, long life cycle and filter feeding behavior, they are a good bio-indicator species for pollution (Viarengo and Canesi, 1991).

The aim of this study was to evaluate the possible occurrence of chronic effects resulting from exposure to selected pharmaceuticals at environmental and elevated concentrations using the marine blue mussels (*Mytilus* spp.) as the model organism. Biomarkers of stress (glutathione *S*-transferase (GST) and metallothionein (MT)), damage (lipid peroxidation and DNA damage (DNA)) and reproduction (vitellin-like proteins) were analyzed. These results were compared with standard acute toxicity tests using the marine species *Vibrio fischeri*, *Skeletonema costatum*, *Tisbe battagliai*.

2. Materials and methods

2.1. Mussel collection and experimental set up

Blue mussels (*Mytilus* spp.) were collected in March 2009 from a pristine site in the west of Ireland (Lettermullan, Co. Galway). The size of the mussels ranged between 4 cm and 5 cm. The mussels were transported back to the lab, cleaned and acclamatized for 3 days in dechlorinated artificial sea water (ASW) at 10 °C (±1 °C). Water temperature (in °C), pH, oxygen and nutrients (ammonia (NH_4^+ in mg/L), nitrate (NO_3 in mg/L) and nitrite (NO_2 in mg/L)) were measured at the study site and daily during exposures. The water in each tank was changed daily. Taking into consideration an approximate hemolymph volume of 1 mL, 10 μL of solution containing 1 μg/L or 1000 μg/L gemfibrozil or diclofenac was injected using a microsyringe and a 22G needle into the posterior adductor muscle of each mussel. Control and solvent control mussels were injected with 10 μL of dechlorinated ASW and dimethyl sulfoxide (DMSO), respectively. Experiments were conducted under 12:12 light: dark regime and were performed in triplicate. After 24 h and 96 h the visceral mass was dissected and frozen at −80 °C for further analysis. To determine the Fulton condition factor ($CF = ((L/W^3) \times 100)$ the shell length (in cm) and the wet weight of the visceral mass (in g) of each mussel were taken.

2.2. Tissue preparation and biochemical analysis

The gonad and digestive gland were dissected over ice and homogenized in a Hepes–NaOH-buffer containing 130 mM sodium chloride (NaCl), 25 mM Hepes–NaOH, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol with a pH of 7.4 at a weight: volume ratio of 1:5. Subsamples of homogenate were frozen at −80 °C for later analysis of LPO, DNA damage and total protein content (TP). The remaining homogenate was centrifuged at 12,000*g* for 10 min at 4 °C, the supernatant (S12) was separated from the pellet and frozen at −80 °C and was later used to determine glutathione *S*-transferase activity, alkali labile phosphate (ALP) and metallothionein. The biomarkers were normalized against total protein content in both the homogenate and supernatant (Bradford, 1976). The sex of each mussel was determined using the squash technique.

2.3. Metallothionein

The evaluation of metallothionein activity was carried out by the method described by Viarengo et al. (1997). A sample of 500 μL supernatant of digestive gland was added to 500 μL 95% ethanol with 8% chloroform. After mixing it was centrifuged at 6000*g* for 10 min at 4 °C. Fifty microlitre RNA, 10 μL hydrogen chloride (HCl) and 1.2 mL of cold ethanol were added to the 500 μL of the supernatant (S6) and frozen for 25 min at −80 °C. Following a second centrifuge step (6000*g* for 1 min at 4 °C) the supernatant was removed and the pellet (precipitate) was re-suspended with 300 μL of 87% ethanol and 1% chloroform. After centrifuging at 6000*g* for 1 min at 4 °C the supernatant was removed and the pellet was re-suspended with 150 μL NaCl, 150 μL HCl containing 4 mM EDTA and 300 μL Ellmans reactive containing 0.4 mM dithionitrobenzoate, 2 M NaCl and 0.2 M potassium dihydrogen phosphate (KH_2PO_4) with a pH 8. The absorbance was read after 5 min at 412 nm using a spectrophotometer. Glutathione buffer containing 1 mM glutathione in 0.1 M HCl was used as a standard and the amount of metallthionein was expressed as nmol/mg protein.

2.4. Glutathione S-transferase activity

The activity of glutathione *S*-transferase was determined using the method by Boryslawskyi et al. (1988). About 200 μL of 1 mM glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene in a buffer of 10 mM Hepes–NaOH containing 125 mM NaCl with a pH of 6.5 were added to a sample of 50 μL of S12 of digestive gland. The absorbance was read after 5 min at 340 nm using a spectrophotometer and data was expressed as μmoles GSH transferase activity/mg protein.

2.5. Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid method adapted from Wills (1987). About 150 μL of digestive gland homogenate was added to 300 μL 10% trichloroacetic acid (TCA) containing 1 mM iron sulfate ($FeSO_4$) and 150 μL 0.67% thiobarbituric acid (TBA). After heating for 10 min the samples were centrifuged for 5 s at 10,000*g* to remove the precipitate. Two hundred microlitre of sample was added to a black 96 well plate and the fluorescence was measured at 515 excitation/545 nm emission. Blanks and standards solutions of tetramethoxypropane were prepared in homogenate buffer. Enzyme activity was expressed as μmoles/mg protein.

2.6. DNA damage (strand breaks)

Adapting the method from Olive (1988) the DNA damage was assessed. About 25 μL of homogenate of digestive gland was added to 200 μL 2% sodium dodecyl sulfate (SDS) containing 10 mM EDTA, 10 mM tris–base and 40 mM sodium hydroxide (NaOH). After 1 min 200 μL of 0.12 M potassium chloride (KCl) was added and the solution was heated for 10 min at 60 °C. Following 30 min cooling at 4 °C the samples were centrifuged at 8000*g* for 5 min at 4 °C. Fifty microlitre homogenate was added to 150 μL of Hoescht (bisBenzimide) containing 0.4 M NaCl, 4 mM sodium cholate and 0.1 M tris–acetate, pH 8.5–9. After 5 min mixing the fluorescence at 360 nm excitation/450 nm emissions were recorded. Homogenate buffer was used as blanks. Salmon sperm DNA standards (Sigma Aldrich) were added to a buffer solution containing 50 mM tris–acetate and 1 mM EDTA, pH 8 and the formation of DNA strand breaks were expressed as μg/mg proteins.

2.7. Vitellin-like proteins

The organic alkali-labile phosphate technique (Blaise et al., 1999) was used to determine the vitellogenin (Vn) content in the supernatant of gonadal tissues. About 180 μL of the S12 samples were added to 97.2 μL 35% acetone, mixed and left for 10 min. After centrifugation (10,000*g* for 5 min) the supernatant was removed. Eighty microlitre of NaOH was added and mixed for 30 min at 60 °C and then added to a 96 well plate. Additionally 5 μL of 100% TCA, 125 μL deionised water, 25 μL of molybdate reactive and 25 μL of 1% ascorbate was added and left on a plate