Marine Environmental Research 127 (2017) 126–136

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Assessing the impact of diclofenac, ibuprofen and sildenafil citrate (Viagra®) on the fertilisation biology of broadcast spawning marine invertebrates

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ARTICLE INFO

Article history:
Received 31 December 2016
Received in revised form
3 April 2017
Accepted 4 April 2017
Available online 8 April 2017

Keywords:
Sperm motility
NSAID
Pharmaceuticals
Drugs
Benthos
Marine invertebrate

ABSTRACT

Exposure to synthetic chemicals is a key environmental challenge faced by aquatic organisms. The time and dose effects of the pharmaceuticals diclofenac, ibuprofen, and sildenafil citrate on sperm motility and successful fertilisation are studied using the echinoderms, *Asterias rubens* and *Psammechinus miliaris*, and the polychaete worm *Arenicola marina*, all important components of the marine benthos. Motility was reduced for all species when exposed to diclofenac concentrations ≥0.1 μg/L. Exposure to ≥1.0 μg/L of ibuprofen affected only *P. miliaris* gametes and fertilisation success of *A. marina*. *A. rubens* and *P. miliaris* sperm increased in both percentage motility and swimming velocity when exposed to sildenafil citrate at concentrations ≥18 and ≥ 50 ng/L, respectively. Pre-incubation of sperm with sildenafil citrate significantly increased fertilisation success in *A. rubens* and *P. miliaris* but not in *A. marina*. Pre-incubated *A. rubens* oocytes fertilised successfully in ibuprofen. According to EU Directive 93/67/EEC, diclofenac is classified as a very toxic substance to gametes of *A. rubens, P. miliaris,* and *A. marina* (EC_{50} = 100–1000 μg/L) while ibuprofen is classified as very toxic to gametes of *P. miliaris* but non-toxic to gametes of *A. marina* (EC_{50} > 10,000 μg/L). The present study indicates that diclofenac exposure may have negative impacts on invertebrate reproductive success, whereas ibuprofen potentially may compromise *P. miliaris* reproduction. This study provides a valuable insight into the mechanisms that allow marine invertebrates to survive and reproduce in contaminated and changing habitats.

1. Introduction

Pharmaceutical compounds are a growing class of environmental contaminants within the broad category of pharmaceutical and personal care products (Dietrich et al., 2002). Many pharmaceuticals and their metabolites are detected in wastewater and sewage treatment plants — implicated as the primary sources of environmental discharge (Daughton and Ternes, 1999). Despite most pharmaceuticals being detected in aquatic environments in the nanogram-per-litre (ng/L) to low microgram-per-litre (μg/L) ranges (Fent et al., 2006; Kasprzyk-Hordern et al., 2008; Triebskorn and Hetzenauer, 2012), owing to their high biological and pharmacokinetic activities, risks to aquatic biota cannot be excluded (Jobling et al., 2003).

Non-steroidal anti-inflammatory drugs (NSAIDs) are used primarily for analgesic and anti-inflammatory purposes, acting as non-selective inhibitors for one or both cyclooxygenase enzyme isoforms (COX-1 and -2) by interfering with the inflammatory mediator (Zou et al., 1999). NSAIDs are the most widely taken oral drugs category and have been detected in significant quantities in municipal effluent (Tixier et al., 2003). Diclofenac (2-[(2,6-dichlorophenyl)amino] phenylacetic acid) and ibuprofen ((+/−)-2-(p-isobutylphenyl) propionic acid) are two commonly used NSAIDs, with seawater concentrations ranging between 0.6-843 and 0.01–2370 ng/L respectively (Ankley et al., 2007; Fent et al., 2006; Gaw et al., 2014). Diclofenac, which has a low removal rate during wastewater treatment, is commonly identified in aquatic ecosystems, with greywater discharge being the principal release pathway (Heberer and Feldmann, 2005). Ibuprofen can remain in the aquatic phase once discharged and is considered fairly persistent, bioaccumulative, of low volatility, and with a low tendency for absorption onto organic matter (Bendz et al., 2005;

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http://dx.doi.org/10.1016/j.marenvres.2017.04.005
0141-1136/

Fent et al., 2006; Johnson et al., 2007; Maurer et al., 2007; Schwaiger et al., 2004).

Diclofenac and ibuprofen exposure can impact aquatic organisms, including bacteria, algae, molluscs, crustaceans, and teleost fish (Cleuvers, 2003; Dietrich and Prietz, 1999; Ericson et al., 2010; Escher et al., 2005; Han et al., 2006; Heckmann et al., 2007; Hoeger et al., 2005; Seigel et al., 2011); although test concentrations are often higher than those measured in the aquatic environment. Until now, little focus has been directed towards the potential effects on animal reproductive success (Hayashi et al., 2008; Memmert et al., 2013); this translates to inadequate risk assessment.

Sildenafil citrate, also known as Viagra®, is a phosphodiesterase type 5- inhibitor (PDE5 blocker) widely used to treat human male erectile dysfunction (Althof et al., 2006; Glenn et al., 2009) and other conditions requiring the management of pulmonary hypertension (Grant and El-Fakahany, 2004). Sildenafil citrate concentrations in wastewater are detected up to 10 ng/L (Fr. Schröder et al., 2010; Nieto et al., 2010). In contrast to the NSAIDs, the environmental impact of the discharge of sildenafil citrate and its metabolites into aquatic environments is essentially unknown. Rocco et al. (2012) concluded that sildenafil citrate can exert genotoxic effects in teleosts when exposed to 26.25 ng/L for up to 35 days, whilst chronic exposure to mutagenic chlorination derivatives slowed invertebrate population growth (Temussi et al., 2013).

The European Council Directive 2001/83/EC (EC, 2011) concluded an environmental risk assessment should be conducted before authorising marketing of a medicinal product for human use. A two-phased (Phase I and Phase II) tiered assessment concept has been proposed (AMEA, 2005) with Phase I to predict environmental concentrations in surface water (PEC_{sw}). If the PEC_{sw} value is below 0.01 μg/L, and exhibits no environmental concern, no further assessments are required. However, if the PEC_{sw} exceeds 0.01 μg/L a Phase II environmental effect analysis is required.

This study assesses the effects of exposure to environmentally realistic concentrations of diclofenac, ibuprofen, and sildenafil citrate over a range of short-term exposure periods on the reproductive success (as measured by sperm motility and fertilisation success) of selected ecologically important benthic marine invertebrates; two echinoderms, *Asterias rubens* (sea star) and *Psammechinus miliaris* (sea urchin), and a polychaete worm *Arenicola marina* (lugworm). The results should allow for a better understanding of the effects of pharmaceutical contaminants on the reproductive success of ecologically important marine invertebrates and potentially permit extrapolations to predict population effects that may inform future risk assessments.

2. Materials and methods

2.1. Collection and maintenance of animals

Asterias rubens collected using fishing creels from the Amble coast, Northumberland, UK (55.32 °N, 1.55 °W) from the end of March to early May 2010–2012, were transported in seawater to the laboratory and held in a flow-through seawater aquarium at 5 °C with constant darkness until required. Animals were spawned within one week. *Psammechinus miliaris* were collected in July from two locations on the west coast of Scotland: the Isle of Cumbrae, UK (55.76 °N, 4.94 °W) in 2010 and Oban, UK (56.41 °N, 5.47 °W) during 2011 and 2012. Urchins were transported to the laboratory in tanks filled with ambient seawater and aerated by a portable electric pump. In the laboratory they were held in flow-through tanks at 10 °C and 12L: 12D photoperiod until required. Animals were spawned within one week. *Arenicola marina* were collected by digging during low tide, using a flat pronged fork from beaches at Alnwick, Northumberland, UK (55.38 °N, 1.60 °W) during late October to late December 2010–2012. Once removed from the sand, they were placed into buckets containing small amounts of seawater and sand, and returned to the laboratory where they were sexed by observation of the gametes present in the coelomic cavity under bright illumination (Pacey and Bentley, 1992). Where this was inconclusive, a small drop of coelomic fluid was removed using a disposable syringe fitted with a 21-g hypodermic needle and examined under a light microscope. Following sexing, the animals were kept individually in plastic containers filled with 0.22 μm filtered seawater (FSW), renewed daily, and kept at 5–6 °C. The animals were left for at least 24 h before spawning to allow gut evacuation.

2.2. Spawning induction, gametes collection and preparation of test solutions

The spawning protocols were followed as published elsewhere; *A. rubens* (Caldwell et al., 2002; Williams and Bentley, 2002); *A. marina* (Pacey and Bentley, 1992); and *P. miliaris* (Caldwell et al., 2004). Gametes were collected in Eppendorf tubes and stored on ice until required.

Ibuprofen (CAS no. 15687-27-1), diclofenac sodium salt (CAS no. 15307-79-6), and sildenafil citrate (CAS no. 171599-83-0) were obtained from Sigma-Aldrich UK, with a chemical purity of 98%. Stock solutions were prepared in high-performance liquid chromatography grade methanol (Sigma-Aldrich, UK). Methanol concentrations did not exceed 0.001% v/v in any experiment. Both ibuprofen and diclofenac were assayed at concentrations ranging from 0.01 μg/L to a maximum of 1 mg/L, whereas sildenafil citrate was assayed within ranges of 2 ng/L to 1 μg/L. The upper concentration was informed by the tolerance of each bioassay species. Exposure durations of up to one hour were used for *A. rubens* and *P. miliaris* and up to two hours for *A. marina*.

2.3. Sperm motility

Sperm motility was measured using computer assisted sperm analysis (Caldwell et al., 2011). Between six to nine replicate sperm suspensions (1000 μl) were prepared for each treatment according to the times and concentrations required and mounted on clean, concave glass slides. Sperm motility for each sample was recorded for five seconds in ten fields of view (50–100 sperm per field) providing a total of 60–90 fields for each treatment and time interval. Curvilinear velocity (VCL; μm/s) was assessed, which represents the time-averaged velocity of the sperm head along the actual trajectories of individual spermatozoa.

2.4. Fertilisation success

To test for the effects of oocyte pre-incubation, two hundred and fifty oocytes were incubated in 1 ml of test medium; either solvent control, diclofenac, ibuprofen or sildenafil citrate at set concentrations and times in Eppendorf tubes at 15 °C. After incubation, the oocytes were washed three times with FSW and transferred into 24-well microplates to which unexposed sperm, pooled from three males, was added to give a final concentration of 2.5×10^6 sperm/ml.

The effects of sperm pre-incubation was tested by incubating sperm pooled from three males at a concentration of 5×10^6 sperm/ml in set concentrations of the solvent control, diclofenac, and ibuprofen, or sildenafil citrate at 15 °C. The exposed sperm were then added to unfertilised oocytes that were not previously exposed to the test chemicals. After each time point, 250 μl of sperm was added to the unfertilised oocytes to give a final concentration of 2.5×10^6 sperm/ml.