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A multibiomarker approach to explore interactive effects of propranolol and fluoxetine in marine mussels

Silvia Franzellitti [a, b, *], Sara Buratti [b], Bowen Du [c], Samuel P. Haddad [c], C. Kevin Chambliss [d], Bryan W. Brooks [c], Elena Fabbri [a, b]

[a] University of Bologna, Department of Biological, Geological, and Environmental Sciences, via Selmi 3, 40100 Bologna, Italy
[b] University of Bologna, Interdepartment Centre for Environmental Science Research, via S. Alberto 163, 48123 Ravenna, Italy
[c] Department of Environmental Science, Baylor University, Waco, TX 76798, USA
[d] Department of Chemistry and Biochemistry, Baylor University, Waco, TX 76798, USA

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ABSTRACT

A multi-biomarker approach, including several lysosomal parameters, activity and mRNA expression of antioxidant enzymes, and DNA damage, was employed to investigate the nominal effects of 0.3 ng/L fluoxetine (FX) and 0.3 ng/L propranolol (PROP) alone or in combination (0.3 ng/L FX + 0.3 ng/L PROP) on Mediterranean mussels after a 7 day treatment. FX co-exposure appears to facilitate PROP bioaccumulation because PROP only accumulated in digestive gland of FX + PROP treated mussels. Lysosomal parameters were significantly impaired by FX + PROP treatment, while no clear antioxidant responses at the catalytic and transcriptional levels were observed. Biomarker responses led to a "medium stress level" diagnosis in FX + PROP treated mussels, according to the Expert System, whereas 0.3 ng/L PROP or FX alone did not induce consistent stress conditions. These findings suggest vulnerability of coastal marine mussels to FX and PROP contamination at environmentally relevant levels.

1. Introduction

Developing an understanding of the biological consequences of pharmaceutical residues in aquatic ecosystems has become a research priority over the last decade, given the increased knowledge on the occurrence and widespread distribution of these contaminants worldwide. Indeed, many ingested pharmaceuticals are minimally metabolized by target organisms and not completely degraded during conventional wastewater treatments; thus, residues are released from final effluent discharges to receiving systems (Daughton and Ternes, 1999; Kolpin et al., 2002). Population growth, urbanization, aging populations, more effective delivery of health services, and climatic changes further contribute to concentrated releases of these substances in urban inland and coastal water bodies (Brooks, 2014). Because pharmaceuticals are inherently designed to have biological activity, and many therapeutic and adverse drug reaction targets are evolutionarily conserved across aquatic organisms (Gunnarsson et al., 2008), identifying inherent chemical properties (Brooks, 2014; McRobb et al., 2014) associated with cellular/molecular interactions resulting in adverse outcomes at environmental concentrations is necessary for protecting aquatic organisms (Franzellitti and Fabbri, 2014; Schmitt et al., 2010).

Compared with freshwaters, studies on pharmaceuticals' fate and effects in marine environments are under-represented in literature (Gaw et al., 2014) presumably assuming that dilution and dispersion processes significantly decrease the risks to marine wildlife. Nevertheless, recent studies reported notable amounts of emerging contaminants being transported to coastal areas via riverine inputs, or due to their use in mariculture, and locally effluents from coastline WTPs are discharged directly in seawater to protect surface waters (Fenet et al., 2014; Jiang et al., 2014). Spot data on pharmaceuticals occurrence in seawaters are reported worldwide (Gaw et al., 2014), with exposure risks being strongly related to their hydrodynamic behaviors in marine ecosystems

* Corresponding author. Department of Biological, Geological, and Environmental Sciences (BIGEA), University of Bologna, Via Sant'Alberto 163, 48123 Ravenna, Italy.
E-mail address: silvia.franzellitti@unibo.it (S. Franzellitti).

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(Bayen et al., 2013; Fenet et al., 2014). In particular, many pharmaceuticals display high affinity for suspended solids and bottom sediments (Bayen et al., 2013), suggesting exposure in sediment-dwelling and filter-feeding marine benthos may be more relevant than for pelagic species.

The present study used the Mediterranean mussel (*Mytilus galloprovincialis*) as a model species to explore potential pharmaceutical responses in marine benthonic invertebrates. Mussels live at the sediment/water interface and filter large volumes of water, suspended materials and colloids (Viarengo et al., 2007). As such, they can accumulate micro-pollutants through the gills (dissolved substances) and the digestive tract (substances adsorbed on particles) (Gómez et al., 2012; Martinez-Bueno et al., 2013, 2014). Our objective was to investigate possible adverse outcomes triggered by a combination of fluoxetine (FX), a selective serotonin reuptake inhibitor used in the treatment of depression and other mood disorders, and propranolol (PROP), a β-adrenergic receptor antagonist used to counteract cardiovascular pathologies (Weir, 2009). These compounds have been detected in coastal environments at concentrations in the range of ≤3–596 ng/L (FX; Vasskog et al., 2008; Benotti and Brownawell, 2007) and 0.3–6329 ng/L (PROP; Wille et al., 2011, 2010; Yang et al., 2011) and also may be effectively bioaccumulated by mussels (Du et al., 2014a; Ericson et al., 2010; Franzellitti et al., 2014).

Interactive studies of PROP and FX in aquatic organisms are lacking (Brausch et al., 2012), but may be of concern for aquatic wildlife. In humans, PROP and FX are contraindicated medications because FX is a potent inhibitor of CYP450-mediated metabolism (Hardman and Limbird, 2001). Whether co-exposure to FX influences toxicokinetics and bioaccumulation of PROP also in aquatic organisms is not understood. However, environmental consequences from pharmaceutical mixtures are identified as the #1 priority research need to understand risks of long term exposure to pharmaceuticals (Boxall et al., 2012; Rudd et al., 2014). Such information may provide support to the ongoing discussion of expanding biological approaches supporting pharmaceutical risk assessment in aquatic ecosystems (Caldwell et al., 2014).

In the present study a multi-biomarker approach reflecting the general health status of mussels and the counteracting responses was employed to further investigate FX and PROP effects on marine mussels. This built on our recent studies, where pharmaceutical adverse modes of action were assessed under single-chemical exposure conditions (Franzellitti et al., 2011, 2014), because interactive effects in the present study were examined under the same exposure scenario used previously (Franzellitti et al., 2013). Though there is undeniable power in the use of biomarkers to elucidate pathways and mechanisms of adverse effects of contaminants, simply examining the simultaneous variation of different biomarkers it is generally difficult to obtain an inter-comparable assessment of the organism health status, so that their practical use for assessing the degree of environmental risk is still not always clear (Viarengo et al., 2000). We attempt to alleviate this through the application of the Mussel Expert System (Dagnino et al., 2007) able to integrate information derived from different biomarkers within a synthetic health status index, thus allowing common criteria in the evaluation of the biological effects of pollutants.

2. Materials and methods

2.1. Experimental animals and holding conditions

Specimens of *M. galloprovincialis* (5–7 cm in length) were collected from the northwestern Adriatic Sea coast by fisherman of the “Cooperativa Copr.al.mo” (Cesenatico, Italy), and transferred to the laboratory in seawater tanks with continuous aeration. Animals (30 per aquarium) were acclimated for 3 days in aquaria containing 60 L of aerated artificial 35 psu seawater at 16 °C, under a natural photoperiod. Mussels were fed once a day with an algal slurry (Koral filtrator, Xaqua, Italy). Fifteen mussels were sampled at zero time to assess parameters at the onset of each experiment.

2.2. Test substances

Mussels were treated with propranolol ((±)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol) hydrochloride (PROP) and with fluoxetine ((±)-N-methyl-γ-[4-(trifluoromethyl)phenoxy] benzenepropanamine) hydrochloride (FX) (Sigma Aldrich, Milan, Italy; purity ≥ 98%). According to the manufacture's datasheets, PROP and FX are water soluble up to 50 mg/mL and to 4 mg/mL, respectively. Therefore, stock solutions were prepared in distilled milliQ-grade water at a 0.1 mg/mL concentration, aliquoted and stored at −20 °C. To achieve treatment levels, 1 aliquot of stock solution was employed and diluted to achieve the suitable volume to be spilled in each vessel. As the vessels used in this study are made up of a plastic material for use with foodstuff, vessels walls should neither absorb nor release chemicals. Although we cannot exclude that some interaction of FX and PROP with plastic could have occurred, our observations in previous studies (Brooks et al., 2003; Stanley et al., 2007) suggest it is minimal, also considering the concentrations used for the treatments. Half-life for the pharmaceuticals in water solutions was about 102 days (FX) and 30 days (PROP) (Kwon and Armbrust, 2006; Yamamoto et al., 2009), whereas in this study pharmaceutical administration was on a daily base along with mussel feeding and after water changes.

2.3. Experimental design

Mussel treatments with FX, PROP, or their combination were carried out as reported by Franzellitti et al. (2013) and Franzellitti and Fabbri (2013). A total of 480 mussels were randomly selected and divided in groups of 20 animals each, and transferred to vessels containing 20 L of water. One liter of seawater *per* mussel is the suitable volume to avoid overloading and prevent the onset of unfavorable health conditions. For each experimental condition, 6 vessels containing a total of 120 mussels represented the 6 replicates. Mussels were treated for 7 days with nominal 0.3 ng/L PROP, 0.3 ng/L FX, or with the combination FX + PROP (0.3 ng/L + 0.3 ng/L). These nominal concentrations fall within the lower range of environmental levels for the compounds and were selected for this study because of the previously induced interactive effects on MOA relevant parameters (Franzellitti et al., 2013). A group of unexposed (control) mussels were maintained in parallel to the treatment groups. Seawater was renewed each day and the chemicals added from stock solutions as described above along with mussel feeding.

At the end of the experiment, haemolymph was taken from the abductor muscle of individual mussels. The gills, digestive gland and mantle/gonad complex were dissected from individuals and used immediately or snap-frozen in liquid nitrogen and stored at −80 °C. There was no mortality during the exposure period. Mussels at zero time were immediately analyzed for biomarkers to assess their initial health status, and results were not significantly different from mussels maintained for 7 days under control conditions (data not shown).

We established independent replicates within each treatment group by considering the 6 vessels as the operative replication level (N = 6). FX and PROP bioaccumulation was assessed on duplicate pooled tissues, each pool consisting of digestive glands, gills or mantle/gonads from 12 animals. Lysosomal membrane stability was measured in haemolymph sampled from 4 individual mussels