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The conceptual imperfection of aquatic risk assessment tests: highlighting the need for tests designed to detect therapeutic effects of pharmaceutical contaminants

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Abstract

Standardized ecotoxicological tests still constitute the fundamental tools when doing risk-assessment of aquatic contaminants. These protocols are managed towards minimal mortality in the controls, which is not representative for natural systems where mortality is often high. This methodological bias, generated from assays where mortality in the control group is systematically disregarded, makes it difficult to measure therapeutic effects of pharmaceutical contaminants leading to lower mortality. This is of concern considering that such effects on exposed organisms still may have substantial ecological consequences. In this paper, we illustrate this conceptual problem by presenting empirical data for how the therapeutic effect of Oxazepam—a common contaminant of surface waters—lower mortality rates among exposed Eurasian perch (Perca fluviatilis) from wild populations, at two different life stages. We found that fry hatched from roe that had been exposed to dilute concentrations (1.1 ± 0.3 µg L⁻¹) of Oxazepam for 24 h 3–6 days prior to hatching showed lower mortality rates and increased activity 30 days after hatching. Similar effects, i.e. increased activity and lower mortality rates were also observed for 2-year old perch exposed to dilute Oxazepam concentrations (1.2 ± 0.4 µg L⁻¹). We conclude that therapeutic effects from pharmaceutical contaminants need to be considered in risk assessment assays to avoid that important ecological effects from aquatic contaminants are systematically missed.

Keywords: risk assessment, therapeutic effects, Eurasian perch, benzodiazepines, contamination, ecotoxicology

Introduction

Traditional ecotoxicological tests (e.g. OECD protocols) are frequently used for risk assessment of pharmaceuticals in the environment (European Medicines Agency 2006), and their results are used for informing the public about potential environmental effects in countries such as Sweden (www.fass.se). However, pharmaceuticals are different than other aquatic contaminants, as their pharmacological effects occur at concentrations much lower than concentrations that may be toxic. This introduce a conceptual dilemma as pharmaceutical contaminants, at least in theory, have the capacity to improve fitness of exposed individuals, but the tests used for risk assessment are designed to measure harmful effects. Indeed, the need for using new approaches that focus on the
pharmaceuticals as substances that pose physical harm to exposed individuals in the environment continue to prevail knowledge this is the first study that present empirical data illustrating that a pharmaceutical contaminant can generate therapeutic effects beneficial for the health of exposed individuals and we discuss how such effect may cascade into different ecosystem effects depending on the environmental settings they occur in.

Materials and methods

Collection of 2-year old (2+) perch and pre-treatment

We retrieved 2-year old Eurasian perch in late May from lake Bjönsjön (18 km southwest of Umeå, Sweden with no inputs of effluence water) using a beach seine net. The perch were placed in laboratory holding tanks (600 l) containing aged tap water, and were fed zooplankton (Daphnia pulex) twice daily for two weeks. Typical water quality parameters of the non-chlorinated tap water are: pH 8.0, ammonium 0.005 mg l\(^{-1}\), nitrite <0.003 mg l\(^{-1}\), iron 0.03 mg l\(^{-1}\) and conductivity 13 mS m\(^{-1}\). Fish (n = 120) were then randomly assigned to an exposure setting with Oxazepam (control, low, or high) and were placed, individually, in oxygenated (using airstones) aquaria (n = 120; 14 cm high × 14 cm wide × 22 cm long) filled with aged tap water to a depth of 12 cm. In the low- and high-concentration treatments, fish were exposed to nominal concentrations of 1 µg l\(^{-1}\) and 1000 µg l\(^{-1}\) Oxazepam (analytical grade Oxazepam from Sigma-Aldrich), respectively. There were no significant differences in length or weight of the perch assigned to different exposure settings (P always >0.6). We did not keep track of the sex of individual perch since there is no non-invasive method of determining sex of 2-year old perch.

Roe collection, pre-treatment and exposure

In the beginning of May, six roe strings were collected from a coastally (brackish water) spawning perch population at Rovågern (16 km northeast of Umeå, Sweden). The collected roe is from a separate population than the 2-year old perch and embryonic development had proceeded for at most four days at the time of collection. Roe from each string was placed in individual, oxygenated (using oxygen cannulas) aquaria (n = 114; 14 cm height × 12 cm width × 20 cm length) with 2.8 l of aged tap water. The aquaria were randomly placed on shelves in a climate-controlled room. Temperature was set to 10 °C, and the light regime to 5:19 h dark:light regime, to mimic natural conditions for this time of the year (May/June).

Each subsection of roe was exposed to one of three different nominal Oxazepam concentrations (n = 12, per concentration) of 0 µg l\(^{-1}\) (control), 1 µg l\(^{-1}\) (low) and 1000 µg l\(^{-1}\) (high) in aquaria (6 cm height × 9 cm width × 9 cm length). All subsections of roe were exposed for a 24 h period during
the first 9 days of embryonic development (n = 108), and subsequently categorized into three exposure groups depending on the day of exposure: roe exposed during day 1–3, 4–6 or 7–9 in embryonic development. Both in the beginning and at the end of the exposure, water samples (15 ml) from each aquarium were collected and frozen for later analyses, to validate constant concentrations. After exposure, the roe was submerged twice in aged tap water to remove possible Oxazepam contamination, before being placed back into their individual home aquaria. Thereafter, the temperature was increased gradually from 10 to 17 °C to accelerate hatching that onset three days after cessation of the exposure. Directly after hatching, the fry were given zooplankton ad libitum, and 60 perch fry were randomly collected and placed together in aquaria (n = 114; 14 cm height × 12 cm width × 20 cm length). Fish mortality under ad libitum food conditions over the first 30 days following hatching was recorded.

**Behavioral assays**

2-year old (2+) Eurasian perch (n = 75) was assayed for three behavioral traits (sociality, boldness and activity) both before and after a seven-day exposure according to previously established tests (Brodin et al 2013) described in detail in the supplementary information, available at stacks.iop.org/ERL/9/084003/mmedia. Only activity of the perch fry (n = 147) was assayed as the boldness and sociality tests was originally developed for more mature individuals. Activity was measured 30-days after exposure. All procedures involving catching and handling of fish were permitted by the Ethical Committee on Animal Experiments in Umeå and comply with current Swedish law.

**Chemical analyses**

Analyses of water samples from the aquaria, before and after exposure, and fish tissue were carried out using a triple quadrupole mass spectrometer connected to a liquid chromatograph (Quantum Ultra EMR, Thermo Fisher Scientific, San Jose, CA, USA). For full information of this method see Brodin et al (2013).

**Statistics**

Effects on activity and sociality of 2-year old perch resulting from the exposures were tested using two-way ANOVAs (data normal distributed), followed by a Tukey-HSD post-hoc test. Pair-wise Kruskal–Wallis tests were used to analyze significant within- and between-group effects on boldness (data not normally distributed). Effects on fry mortality and activity were tested using a non-parametric Kruskal–Wallis analysis (data not normally distributed).

**Results**

For the roe exposure, the analyses of water showed an average Oxazepam concentration of 1.1 μg L⁻¹ ± 0.3 (mean ± 1 SD, n = 20) and 984 ± 157 μg L⁻¹ (n = 20) in the low and high treatments, respectively (table 1). The average water concentration of Oxazepam in the experiments with 2-year old perch was 1.2 ± 0.4 μg L⁻¹ (n = 20) in the low treatment and 965 μg g⁻¹ ± 110 (n = 20) in the high treatment (table 1). Analyses of water samples collected prior to and after the experiments did not indicate any change in concentration over the course of the study (data not shown). In the low and high treatments, average perch muscle concentrations of Oxazepam were 7 μg g⁻¹ ± 1.5 (n = 20) and 6100 ± 1100 μg g⁻¹ (n = 20), respectively, and thus about six times higher than in the water.

As expected, mortality rates were high among hatched fry (figure 1(a)) and relatively high among 2-year old perch (figure 1(b)), but were significantly reduced by Oxazepam exposure in comparison to the control. For the fry, both concentration (H₂ = 10.508, P = 0.005) and day of exposure (H₂ = 11.579, P = 0.009) had significant effects on mortality. Here, fry hatched from roe exposed to the high concentration treatment during day 7–9 of embryonic development showed a significantly lower mortality than the control and those exposed day 1–6 (figure 1(a)). Similarly, the low concentration treatment showed a lower mortality for the fry hatched from roe exposed during day 7–9, but this effect was only close to significant (H₂ = 3.187, P = 0.074). Mortality was lower in the high concentration treatment than in the control and low concentration treatments, where the difference between low and high treatments was most pronounced for fry hatched from roe exposed during day 4–6. In accordance with fry mortality, a significant (H₂ = 12.197, P = 0.002) reduction in mortality induced by Oxazepam exposure was also observed for the 2-year old perch, where mortality was lower in both the low and high treatments in comparison to the control (figure 1(b)).

**Behavioral effects**

Activity of the fry hatched from roe exposed to Oxazepam was significantly affected by day of exposure (H₂ = 6.162, P = 0.046), but not significantly affected by the two concentration treatments (P > 0.4). The significant effect of day of exposure was caused by a higher activity in the fry hatched from roe exposed during day 7–9 in comparison to those exposed during day 1–3 and 4–6 (figure 2). High mortality rates in the control hindered comparisons of activity between this group and the others.

Significant effects on activity, sociality or boldness following Oxazepam exposure were seen for 2-year old perch (figures 3(a)–(c)). There were no differences in behavior between the control, low, and high treatment prior to Oxazepam exposure, and the behavior of the control group did not change significantly from before to after exposure. However, in both the low and the high treatment, significant increases in activity were noted after, compared to before, exposure (figure 3(a)), (F₁,₄₆ = 4.740, P = 0.037 and F₁,₄₆ = 22.010, P < 0.001, respectively), and the activity of the fish exposed to the high concentration was also significantly higher than the control group (F₁,₄₆ = 5.137, P = 0.010). Furthermore,
Table 1. Water concentrations of Oxazepam (mean ± 1 S.D.) used for exposure of perch roe and 2-year old perch. Also shown are the resulting muscle concentration of the 2-year old perch.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>Treatment</th>
<th>Water (µg L⁻¹)</th>
<th>Muscle tissue (µg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roe</td>
<td>20</td>
<td>Control</td>
<td>&lt;LOQ</td>
<td>ND</td>
</tr>
<tr>
<td>Roe</td>
<td>20</td>
<td>Low</td>
<td>1.1 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Roe</td>
<td>20</td>
<td>High</td>
<td>984 ± 157</td>
<td>ND</td>
</tr>
<tr>
<td>2-year old</td>
<td>20</td>
<td>Control</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>2-year old</td>
<td>20</td>
<td>Low</td>
<td>1.2 ± 0.4</td>
<td>7.4 ± 1.5</td>
</tr>
<tr>
<td>2-year old</td>
<td>20</td>
<td>High</td>
<td>965 ± 110</td>
<td>6100 ± 1100</td>
</tr>
</tbody>
</table>

<LOQ = below level of quantification; ND = not determined.

Oxazepam exposure made the 2-year old perch bolder in the high concentration treatment compared to before exposure (P<0.001) and compared to the control group (P<0.001) (figure 3(b)). Also, fish in the high concentration treatment became less social after exposure compared to their pre-treatment performance \((F_{1.46} = 5.156, P = 0.029)\) and to the control group \((F_{1.46} = 6.633, P = 0.003)\) (figure 2(c)).

Discussion

Therapeutic effects versus toxicological effects

Assessment of biological effects is currently ranked as the most important research question by ecotoxicologists working with aquatic contaminants (Boxall et al. 2012), and various approaches for risk assessment tests has been suggested (Ankley et al. 2007, Arnold et al. 2013, Brooks et al. 2009, Christen et al. 2010, Huggett et al. 2003, Rand-Weaver et al. 2013). In the European Medicines Agency guidelines (European Medicines Agency 2006), as well as in other suggested risk assessment approaches, impacts of pharmaceuticals on aquatic organisms are mainly quantified using methods where toxic endpoints constitute key variables and where zero mortality in the control group is a prerequisite; hence, approaches that cannot detect possible positive effects on survival. From our findings it is evident that Oxazepam contamination can generate effects on fish that cannot be measured in such tests, i.e. increased survival (figures 1(a), (b)) and behavioral effects, such as increased activity (figures 2, 3(a)), increased boldness (figure 3(b)) and reduced sociality (figure 3(c)).

In a predator-free environment, as represented by the aquaria used in our assays, the therapeutic effects of an anxiolytic drug such as Oxazepam stimulates feeding (Brodin et al. 2013); hence, increased survival among the exposed individuals in our experiments was likely induced by the anxiolytic effect of the drug improving feeding rates. That Oxazepam contamination generates increased survival and activity in this setting is therefore not surprising given the previous findings. However, the result is of high conceptual importance; here we measured effects that stand in complete contrast to what would be expected from the common conceptual view of a pollutant, i.e. a substance that pose harmful effects on exposed individuals. These effects cannot be measured in risk assessment tests currently used for pharmaceuticals (EMEA 2006) or in tests constituting the current trends in contamination research in general (Blasco and Pico 2009), mainly as tests are designed with the traditional view of an aquatic contaminant in mind and thus biased towards detecting toxic effects. Biases in ecotoxicological test has previously been discussed. For example, biases towards continuous testing of pharmaceuticals that has received large attention in the past, i.e. the ‘Mathew effect’, has been previously highlighted (Daughton 2014). Based on our findings, we argue that there is an additional unrecognized (stealth) bias in risk assessment induced by the repetitive use of exposure test incapable of measuring beneficial health effects. Considering that pharmaceuticals in general are made for curing diseases and improving health, it is somewhat surprising that beneficial therapeutic effects from dilute concentrations of pharmaceuticals are not systematically tested. Intuitively, enhanced individual performance might not seem to pose a problem. Nevertheless, in natural environments, any contaminant-induced alteration of the ecosystem should be considered when doing risk assessments (Brooks et al. 2009), and preferential survival of some exposed organisms may lead to complex cascading ecosystem effects. The prevailing conceptual view of a contaminant, underlying virtually all ecotoxicological tests, likely limits detecting therapeutic effects from pharmaceuticals of ecological importance and that the consequences of pharmacological effects needs more recognition. In particularly since the ecological consequences of a positive effect on a species survival might be as severe as those induced by similar-sized reduction in survival.

Our findings also point towards additional important biases in ecotoxicological assays. Few risk assessment studies include quantitative measures on behavioral effects, and, when used, behavioral effects are predominately measured on experimentally stocked fish, such as zebrafish (Danio rerio) (Oggier et al. 2010) or fathead minnows (Pimephales promelas) (Garcia-Reyero et al. 2011, Painter et al. 2009). Based on our findings, we raise concerns regarding the limited use of behavioral tests in the EMEA guidelines as well as the predominant use of stocked fish when doing risk assessment with pharmaceuticals in general. It is evident from our results that the ecological important behavioral effects may occur at sub-lethal concentrations, and these are missed when focusing on toxic endpoints. Second, we found increased survival when wild fish individuals were exposed to an anxiolytic drug, suggesting that laboratory environments favor survival of stress tolerant individuals. Hence, stocked fish populations, kept for several generations in artificial settings, are likely to be more stress tolerant and display a behavioral repertoire that is unrepresentative for wild populations. As a consequence, behavioral effects, as well as effects on for example survival, could be missed in ecotoxicological tests when using fish from laboratory stocks or fish farms.
The oxazepam effect

Mechanisms behind the benzodiazepine-induced increase in activity are not fully understood and interpretations between studies vary. One hypothesis is that benzodiazepines cause a ‘paradoxical reaction’ where this sedative causes hyperactivity among fish at low concentrations (Oggier et al 2010). Studies showing that hyperactivity among children can be induced by Diazepam (Saias and Gallarda 2008) support this hypothesis. Another plausible hypothesis is that increased activity is caused by exposed individuals experiencing the same therapeutic effect as the drug is designed to mediate, i.e. increased stress release and reduced anxiousness, and thus fish become more willing to take risks resulting in increased activity (Brodin et al 2013). This hypothesis is supported by the positive response in both boldness and activity induced by Oxazepam seen in this study (figures 3(a), (b)) and previously (Brodin et al 2013). Further support for the second hypothesis is also provided by the Oxazepam-induced reduction in mortality (figures 1(a), (b)), as stress release and improved feeding during isolation is the most likely explanation to the lower mortality of exposed individuals compared to control individuals. It seems unlikely that the absence of hyperactivity would cause mortality of the untreated individuals. Therefore, we interpret the observed behavioral change in the 2-year old perch as being caused by reduced anxiousness and less stress of exposed individuals.

In contrast to the effects observed for the 2-year old perch, the mechanisms behind the increased activity of the fry cannot be attributed to the therapeutic anxiolytic effects occurring through the assay, but only to the exposure occurring during the last days prior to hatching. This is because activity was measured 30 days after exposure and, thus, long after the neuronal-based effect had ceased, i.e. the half-life of Oxazepam is less than a day in humans and other mammals (Greenblatt 1981). Here, the higher activity is likely reflecting the better physiological status of the fry hatched from highly exposed roe. This finding indicate that exposure during the last few last days of embryonic development induces behavioral modifications, at that time so beneficial for the embryo or recently hatched fry in the artificial setting, that generate positive effects on survival and health at a later stage, in life. Nevertheless, the mechanism(s) behind the behavioral on the fry cannot be determined with the current dataset. Given that mortality rates vary between the treatments, increased activity could simply be explained by the survival of different individuals or a different physiological status of the fry hatched from exposed roe. Even though we cannot determine the underlying mechanisms behind the Oxazepam induced effects on fry mortality and behavior, it represents thus far unknown effects of Oxazepam exposure that warrants further studies.

Environmental relevance

Average concentrations around 1.2 μg Oxazepam l⁻¹ used in the low treatments was clearly below 1.9 μg Oxazepam l⁻¹.
Figure 3. Results from behavioral assays of 2-year old perch on (a) activity, (b) boldness, and (c) sociality. Behavior prior to exposure is shown as white bars and behavior after exposure is shown as black bars. Significant differences are indicated by * (P<0.05) or *** (P<0.001). Error bars represent ±1 S.E.

which is a concentration reported for treated wastewater effluent (Loos et al 2013). Wastewater effluent constitute a major component of many urban surface waters, where nearly 100% of the annual flow can be derived from treated effluent (Waiser et al 2011). The average Oxazepam concentration around 7 μg kg⁻¹ found in fish tissue from the low-concentration treatment is directly comparable to concentrations found for two out of ten wild fish caught in River Fyris, a mid-sized river in southern Sweden contaminated by effluent water (Brodin et al 2013). The overlap between Oxazepam concentration in wild fish and our experimental fish, despite substantial dilution of wastewater in the river, indicates that wild fish can be exposed to benzodiazepine contamination sufficient for altering their behavior. An attraction to wastewater effluent could be one reason for why wild perch show such high Oxazepam muscle-tissue concentrations, despite a dilution of the wastewater in River Fyris. Wild fish has been suggested being attracted to wastewater effluent for several reasons. Partly because the outlets often represents patches of high food density for fish and also due to more preferential water temperatures during the winter (Hynes 1960).

Importantly, perch used in the experiment were either recently hatched or collected in early spring directly after ice break, i.e. after a long winter with low food availability, and were thus recently exposed to severe environmental stress. Environmental stress is known for amplifying effects from aquatic contaminants in ecotoxicological assays (Knillman et al 2012). The experimental set up with fish exposed to environmental stress likely made the anxiolytic effect of the drug and its impact on mortality stronger than if the individuals would have been in better condition (i.e. summer and autumn fish). Even though we measured mortality rates of the fry that is directly comparable to natural systems where mortality for perch can be about 92% during the first week after hatching (Viljanen and Holopainen 1982), the measured effects on mortality should not be used as representative for natural conditions. In a naturally complex environment including predators the net effect on survival from an anxiolytic behavior could be fatal and result in increased mortality. Although our study was not performed in an environment directly representative for natural conditions, it clearly demonstrate that beneficial effects may be expected for contaminated individuals at environmental relevant concentrations. Commonly used ecotoxicological tests are unable to detect such effect, which makes it impossible to estimate how likely beneficial fitness effects from other pharmaceutical contaminants may be in the environment. Clearly, this is of concern as it implies that the ecotoxicological research community may have systematically missed important effects of ecological relevance.

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References

Ackerman C J and Cline H T 2007 Refining the roles of GABAergic signaling during neural circuit formation Trends Neurosci. 30 382–9


Arnold K E et al 2013 Assessing the exposure risk and impacts of pharmaceuticals in the environment on individuals and ecosystems Biol. Lett. 9 20130492


Boxall A B A et al 2012 Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. 120 1221–9

Huggett D B, Huggett D B and Boxall A B A 2009 Pharmaceuticals and personal care products: research needs for the next decade Environ. Toxicol. Chem. 28 2469–72
Calisto V and Esteves V I 2009 Psychiatric pharmaceuticals in the environment Science 77 1257–74
Caminada D, Escher C and Fent K 2006 Cytotoxicity of pharmaceuticals found in aquatic systems: comparison of PLHC-1 and RTG-2 fish cell lines Aquat. Toxicol. 79 114–23
Christen V, Hickmann S, Rechenberg B and Fent K 2010 Highly active human pharmaceuticals in aquatic systems: a concept for their identification based on their mode of action Aquat. Toxicol. 96 167–81
European Agency for the Evaluation of Medicinal Products (EMEA) 2006 Environmental Risk Assessment of Medicinal Products for Human Use. CPMP/SWP/4447/00 (London: EMEA)
Heberer T 2002 Tracking persistent pharmaceutical residues from municipal sewage to drinking water J. Hydrol. 266 175–89
Hynes H B N 1960 The Biology of Polluted Waters (Liverpool: Liverpool university press)
Loos R et al 2013 EU-wide monitoring survey on waste water treatment plant effluents Water Res. 47 6475–87
Mompatat S, Thomas O and Le Bot B 2011 Contamination levels of human pharmaceutical compounds in French surface and drinking water J. Environ. Monit. 13 2929–39
Saia T and Gallarda T 2008 Paradoxical aggressive reactions to benzodiazepine use: a review Encephale 34 330–6
Ternes T A 1998 Occurrence of drugs in German sewage treatment plants and rivers Water Res. 32 3245–60