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The fate and effect of pharmaceuticals in boreal surface waters

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Dissertation for PhD

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Till Elin

”Jag ska göra några sista kalkyler
Och studera förloppet i ett experiment
Om förändringarna stöder teorin
Så kan vatten förvandlas till vin.”

- Kjell Höglund, 1984

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Sammanfattning

Spår av läkemedel utgör ett vanligt fenomen i sjöar, floder och vattendrag då läkemedelsrester från mänsklig konsumtion tar sig in i akvatiska ekosystem via utsläpp av avloppsvatten. Syftet med denna avhandling var: **i**) att skapa en bättre förståelse för läkemedlets öde i akvatiska ekosystem; och **ii**) att öka vår kunskap om möjlig beteendepåverkan på abborre (Perca fluviatilis) av ett ångestdämpande läkemedel (oxazepam) som är vanligt förekommande i ytvatten. I mina studier använde jag en serie storskaliga fältexperiment för att utvärdera tester gjorda i kontrollerade laboratoriemiljöer. Min avhandling visar att de småskaliga inkubationer som vanligtvis används för att utvärdera läkemedels nedbrytningshastighet i vattenmiljöer fungerade bra för att förutsäga läkemedels öde under den första veckan i naturen. Men dessa experiment och de konceptuella modeller som tillämpas på dem kunde inte förutse att de studerade läkemedlen (trimethoprim, diclofenac, hydroxyzine, diphenhydramine och oxazepam) kan förbli lösta i vattnet i flera månader. Mina resultat visar även att läkemedlen förblir bioaktiva i månader, och att upptaget av olika läkemedel varierade mycket mellan trofiska nivåer. Bottenlevande (bentiska) arter verkade i allmänhet ta upp de studerade läkemedlen i högre utsträckning än arter i högre trofiska nivåer i födoväven så som fisk. Icke desto mindre ackumulerade abborre det studerade ångestdämpande läkemedlet oxazepam, och effekten av oxazepam på abborrars beteende testades i två sjöar, där jag använde akustisk telemetri för att spåra deras in situ-beteende. I dessa två sjöar gjorde jag två huvudsakliga iaktagelser. Först upptäckte jag att abborrarnas beteende i deras naturliga miljö är korrelerat med laboratoriebeteende om jag använde information från flera laborationstester och gjorde flerdimensionella beteendeprofiler över studerade individerna. För det andra hittade jag inga tydliga effekter på abborrebeteendet av oxazepam när detta läkemedel tillsattes i den ena sjön. Noterbart var att jag i den sistnämnda behandlingen använde mycket högre koncentrationer av oxazepam än vad som någonsin uppmätts i förorenade miljöer. Jag drar slutsatsen att förenklade laboratorieexperiment har en viss förmåga att förutsäga läkemedels öde och effekter i komplexa naturliga ekosystem, men att det finns skäl att tro att laboratoriemiljöer kan underskatta persistens av läkemedel i vattenlevande ekosystem samt att de kan generera en beteenderespons för oxazepam som inte är representativ för akvatiska ekosystem. I den sistnämnda miljön verkar sociala drivkrafter vara av yttersta betydelse för fiskbeteende och en förklaring till varför laborationstest gjorda på isolerade individer kan ge missvisande prediktioner för beteende i naturen.

Abstract

Traces of pharmaceuticals are often found in streams, rivers, and lakes as the result of effluent water discharge. This dissertation aims to create a better understanding of the fate of drugs in aquatic ecosystems and how oxazepam, an anxiolytic pharmaceutical commonly detected in surface waters, affects the behavior of perch (Perca fluviatilis). To address these issues, I used a series of large-scale field experiments to evaluate predictions made in controlled laboratory experiments. My dissertation shows that small-scale incubations commonly used to assess the persistence of pharmaceuticals (trimethoprim, diclofenac, hydroxyzine, diphenhydramine and oxazepam) in aquatic environments effectively predicts the fate of dissolved drugs in freshwater during the first week of contamination. However, these experiments and the conceptual models failed to predict that pharmaceuticals can remain dissolved in freshwater for months. In addition, the results suggest that the drugs remain bioactive for months and that the uptake of different drugs varied widely between trophic levels. For example, benthic species generally had a higher affinity to accumulate the studied drugs than species in higher trophic levels; however, the anxiolytic drug oxazepam was found in perch. To test the effect of oxazepam on perch behavior, I used acoustic telemetry to track the perch in situ (i.e., in the ponds). The in situ behavior of perch correlated with laboratory behavior when findings from several trials were merged into multidimensional behavioral profiles of the studied individuals, although oxazepam did not conclusively affect perch behavior in line with earlier theories, when though concentrations were much higher than concentrations measured in any contaminated environments. I conclude that simplified laboratory experiments have some predictive power regarding the fate and effects of pharmaceuticals in complex natural ecosystems, but laboratory environments may underestimate persistence of drugs in aquatic ecosystems and fail to detect important social drivers of animal behavior in natural settings.

Keywords: Aquatic ecosystems, behavioural effects, ecotoxicology, acoustic telemetry, field verification, social network

List of papers

This thesis is based on the following four studies, referred to in the text by their respective roman numerals:

I. **Fahlman, J**., Fick, J., Karlsson, J., Jonsson, M., Brodin, T., & Klaminder, J. (2018). **Using laboratory incubations to predict the fate of pharmaceuticals in aquatic ecosystems.** Environmental Chemistry, 15(8), 463-471.

II. Lagesson, A., **Fahlman, J**., Brodin, T., Fick, J., Jonsson, M., Byström, P., & Klaminder, J. (2016). **Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web - Insights from a field experiment.** Science of the Total Environment, 568, 208-215.

III. **Fahlman, J**., Hellström, G., Jonsson, M., Veenstra, A., & Klaminder, J. (2020). **Six common behavioral tests and their relevance for fish performance in nature.** Science of the Total Environment, 732, 139101.

IV. **Fahlman, J**., Hellström, G., Jonsson, M., Fick, J., Rosvall M. & Klaminder, J. (2020). **Behavior of shoaling fish populations is not responsive to anxiolytics after habituation to lake conditions.** Submitted to Science Advances.

Author contributions

Paper **I**

JoF and **JoK** designed the study. **JoF** collected the data. **JeF** performed the chemical analyses. **JoF** performed the statistical analysis and wrote the paper under supervision from **JoK**. All authors contributed with comments to the paper.

Paper **II**

JoF and **JoK** designed the study. **JoF** collected the field data. **JeF** performed the chemical analyses. **AL** performed the statistical analysis and wrote the paper under supervision from **JoK**. All authors contributed with comments to the paper.

Paper **III**

JoF designed the study with assistance from **JoK**, **GH** and **MJ**. **JoF** and **GH** collected the data. **JoF** performed the statistical analysis and wrote the paper under supervision from **JoK** and **MJ**. All authors contributed with comments to the paper.

Paper **IV**

JoF designed the study with assistance from **JoK**, **GH** and **MJ**. **JoF**, **GH** and **JeF** collected the field data. **JeF** performed the chemical analyses. **JoF** performed the statistical analysis and wrote the paper under supervision from **JoK** and **MJ**. All authors contributed with comments to the paper.

Author abbreviations

JoF: Johan Fahlman, **JK**: Jonatan Klaminder, **AL**: Annelie Lagesson, **JaK**: Jan Karlsson, **TB**: Tomas Brodin, **JeF**: Jerker Fick, **MJ**: Micael Jonsson, **PB**: Per Byström, **GH**: Gustav Hellström, **AV**: Arno Veenstra, **MR**: Martin Rosvall

List of papers not included in this thesis

I. Klaminder, J., Jonsson, M., Leander, J., **Fahlman, J**., Brodin, T., Fick, J., & Hellström, G. (2019). **Less anxious salmon smolt become easy prey during downstream migration.** Science of the total environment, 687, 488-493.

II. Hellström, G., Brodin, T., Jonsson, M., Olsen, H., Leander, J., **Fahlman, J.**, Fick, J. & Klaminder, J. (2020). **Environmentally relevant concentrations of the common anxiolytic pharmaceutical oxazepam do not have acute effect on spawning behavior in mature male Atlantic salmon (Salmo salar) parr.** Journal of Applied Ichthyology, 36(1), 105-112.

III. Lagesson, A., Brodin, T., **Fahlman, J.**, Fick, J., Jonsson, M., Persson, J., Byström, P. & Klaminder, J. (2018). **No evidence of increased growth or mortality in fish exposed to oxazepam in semi-natural ecosystems.** Science of the Total Environment, 615, 608-614.

IV. Klaminder, J., Hellström, G., **Fahlman, J.**, Jonsson, M., Fick, J., Lagesson, A., Bergman, E. & Brodin, T. (2016). **Drug-induced behavioral changes: using laboratory observations to predict field observations.** Frontiers in Environmental Science. 4, 81.

V. Klaminder, J., Brodin, T., Sundelin, A., Anderson, N. J., **Fahlman, J.**, Jonsson, M., & Fick, J. (2015). **Long-term persistence of an anxiolytic drug (oxazepam) in a large freshwater lake.** Environmental Science & Technology. 49(17), 10406-10412.

Abstracts of the papers

I. Environmental Chemistry (2018), 15(8), 463-471.

Using laboratory incubations to predict the fate of pharmaceuticals in aquatic ecosystems.

Johan Fahlman, Jerker Fick, Jan Karlsson, Micael Jonsson, Tomas Brodin and Jonatan Klaminder.

Environmental persistence is a key property when evaluating risks with excreted pharmaceuticals in aquatic ecosystems. Such persistence is typically predicted using small-scale laboratory incubations, but the variation in aquatic environments and scarcity of field studies to verify laboratory-based persistence estimates create uncertainties around the predictive power of these incubations. In this study we: (1) assess the persistence of five pharmaceuticals (diclofenac, diphenhydramine, hydroxyzine, trimethoprim and oxazepam) in laboratory experiments under different environmental conditions; and (2) use a three-month-long field study in an aquatic ecosystem to verify the laboratory-based persistence estimates. In our laboratory assays, we found that water temperature (TEMP), concentrations of organic solutes (TOC), presence of sediment (SED), and solar radiation (SOL) individually affected dissipation rates. Moreover, we identified rarely studied interaction effects between the treatments (i.e. SOL x SED and TEMP x SOL), which affected the persistence of the studied drugs. Half-lives obtained from the laboratory assays largely explained the dissipation rates during the first week of the field study. However, none of the applied models could accurately predict the long-term dissipation rates (month time-scale) from the water column. For example, the studied antibioticum (trimethoprim) and the anti-anxiety drug (oxazepam) remained at detectable levels in the aquatic environment long after (~150 days) our laboratory based models predicted complete dissipation. We conclude that small-scale laboratory incubations seem sufficient to approximate the short-term (i.e. within a week) dissipation rate of drugs in aquatic ecosystems. However, this simplistic approach does not capture interacting environmental processes that preserve a fraction of the dissolved pharmaceuticals for months in natural water bodies.

II. Science of the Total Environment (2016). 568: 208-215.

Bioaccumulation of Five Pharmaceuticals at Multiple Trophic Levels in an Aquatic Food Web – Insights from a Field Experiment

Annelie Lagesson, **Johan Fahlman**, Tomas Brodin, Jerker Fick, Micael Jonsson, Per Byström, and Jonatan Klaminder.

Pharmaceuticals derived from manufacturing and human consumption contaminate surface waters worldwide. To what extent such pharmaceutical contamination accumulates and disperses over time in different compartments of aquatic food webs is not well known. In this study we assess to what extent five pharmaceuticals (diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine) are taken up by fish (European perch) and four aquatic invertebrate taxa (damselfly larvae, may fly larvae, waterlouse, and ramshorn snail), by tracing their bioconcentrations over several months in a semi-natural large-scale (pond) system. The results suggest both significant differences among drugs in their capacity to bioaccumulate and differences among species in uptake. While no support for in situ uptake of diclofenac and trimethoprim was found, oxazepam, diphenhydramine, and hydroxyzine were detected in all analyzed species. Here, the highest bioaccumulation factor (tissue:water ratio) was found for hydroxyzine. In the food web, the highest concentrations were found in the benthic species ramshorn snail and waterlouse, indicating that bottom-living organism at lower trophic positions are the prime receivers of the pharmaceuticals. In general, concentrations in the biota decreased over time in response to decreasing water concentrations. However, two interesting exceptions to this trend were noted. First, may fly larvae (primarily grazers) showed peak concentrations (a fourfold increase) of oxazepam, diphenhydramine, and hydroxyzine about 30 days after initial addition of pharmaceuticals. Second, perch (top-predator) showed an increase in concentrations of oxazepam throughout the study period. Our results show that drugs can remain bioavailable for aquatic organism for long time periods (weeks to months) and even re-enter the food web at a later time. As such, for an understanding of accumulation and dispersion of pharmaceuticals in aquatic food webs, detailed ecological knowledge is required.

III. *Science of the Total Environment (2020). 732, 139101*

Six common behavioral tests and their relevance for fish performance in nature.

Johan Fahlman, Gustav Hellström, Micael Jonsson, Arno Veenstra and Jonatan Klaminder.

Behavioral traits measured in laboratory settings are commonly used when predicting ecological effects and evolutionary outcomes in natural systems. However, uncertainties regarding the relevance of simplified lab-based behavioral tests for complex natural environments have created doubts about the use of these tests within aquatic ecology and ecotoxicology. In this study, we scrutinize the assumption that fish performance in six commonly applied behavioral assays have relevance for *in situ* behavior, by comparing individual behavior tracked in both artificial laboratory settings as well as in two natural lakes. We show that: i) commonly measured behavioral traits of individual fish (*Perca fluviatilis*) have low predictive power for within-lake behaviors if interpreted alone, but that; ii) composite variables synthesized from several (six) behavioral assays explain important *in situ* measures

such as swimming activity, dispersion, home-range size, and habitat preference. While our findings support recent criticisms against the use of single behavior tests for predicting environmental effects, we provide empirical evidences suggesting that fish performances in multiple laboratory assays are highly relevant for fish behavior in nature. As a majority of studies are currently conducted using single tests, we argue for the importance of increasing the number of assays in studies attempting to foresee ecological outcomes of behavioral differences.

IV. Submitted

Behavior of shoaling fish populations is not responsive to anxiolytics after habituation to lake conditions.

Johan Fahlman, Gustav Hellström, Micael Jonsson, Jerker Fick, Martin Rosvall and Jonatan Klaminder.

A current theory in environmental science is that dissolved anxiolytics from wastewater effluent can cause maladaptive anti-predator behaviors in fish with potentially devastating impacts on fish populations. Here, we show that fish behavior in the wild is resilient to anxiolytics as behaviors coupled to shoaling and temperature overrides previously documented impacts on individual fish. Our finding is demonstrated using in situ measurements of European perch (Perca fluviatilis) behavior after manipulation of a whole-lake ecosystem with an anxiolytic (oxazepam). The results suggest that exposure to oxazepam concentrations a magnitude higher than that ever measured in contaminated rivers, had a minute impact on perch anti-predator behaviors being mainly dependent on the surrounding social network structure. We suggest that previous documented effects of anxiolytics on fish behavioral is an artefact of artificial stress in laboratory settings that are not present in nature where habituated fish are an active part of a social context.

Introduction

Pharmaceuticals in the environment

Medicinal plants and various chemical compounds have been used for over a millennia to improve human health – the oldest known pharmaceutical recipe is approximately 5000 years old (Petrovska, 2012). The modern pharmaceutical industry developed in the 18th century, parallel to the scientific revolution. However, the path to achieve human health and longevity has not been straight and our understanding of the possible side effects of medicinal compounds often comes with missteps. For example, although now known to be deadly, mercury has a long history in medicine, treating everything from syphilis (Sartin and Perry, 1995) to dental cavities (Bates, 2006) (Ullrich et al., 2001). Similarly, the Romans used lead as a medicine, a sweetener, and a plumbing material (Retief and Cilliers, 2006), but now even the general public understands that lead is an environmental hazard and compromises health (Nriagu, 1998).

About half a century ago, researchers first uncovered the incomplete removal of used and excreted pharmaceuticals from wastewater treatment plants (WWTP) (Stumm-Zollinger and Fair, 1965). At the time, this discovery did not result in the same public outcry that Rachel Carson's book *Silent Spring* caused just a few years earlier (Carson, 2002). However, concern for pharmaceuticals in the environment has grown and is now a respected research field. Progress in analytical chemistry has made it possible to detect pharmaceutical contaminants at very low concentrations (i.e., ng/l) and studies using these techniques suggest almost every freshwater body receiving wastewater effluent contains drugs in their therapeutic forms (aus der Beek et al., 2016).

Since the first discoveries of pharmaceutical pollution and the analytical breakthroughs in the 1990s (Heberer, 2002; Ternes, 1998), research targeting the fate of various therapeutic substances has increased, with new methods facilitating the detection of an increasing number of pharmaceuticals detected in the environment (Calisto and Esteves, 2009; Daughton and Ternes, 1999; Kosjek et al., 2012; Löffler et al., 2005). More than 4000 pharmaceuticals are on the market (Boxall et al., 2012), and over 600 of these have been detected in surface waters worldwide (aus der Beek et al., 2016). These pharmaceuticals degrade through biotic and abiotic processes, transforming these products into potentially highly bioactive compounds (La Farre et al., 2008). It is now known that pharmaceuticals can enter organisms through many pathways and can result in many unintended ecological impacts (Boxall et al., 2012).

Pharmaceutical pollution may be more relevant than ever as annual global consumption rates are measured in many thousands of tons and the pharmaceutical market is predicted to increase by about 25% between 2018 and 2023 (Aitken et al., 2019). This rapidly growing market in combination with examples where both terrestrial (Oaks et al., 2004) and aquatic ecosystems (Kidd et al., 2007) have been decimated by individual drugs entering the environment has resulted in efforts to perform risk assessment of potential impacts of individual substances entering international markets (EMA, 2006; FASS, 2012). These risk assessments include assessing a drug's environmental persistence, potential for bioaccumulation, and effect on biota. Currently, the Organization for Economic Co-operation and Development (OECD) guidelines recommend that risk assessments be conducted in controlled small-scale laboratory settings. Although highly reproducible and standardized, the environmental relevance

of these tests is unclear (Challis et al., 2014). Therefore, the scientific community is requesting that these test results be verified in the field (Challis et al., 2014; Webster et al., 1998).

Lessons learned from OECD tests

Although the environmental relevance of OECD standardized laboratory tests remains uncertain, these tests have provided valuable insights into the fate and effect of pharmaceuticals. Section 2 of the OECD guidelines for the testing of chemicals – "Effects on Biotic Systems" – includes a battery of recommended laboratory approaches for environmental toxicity screening (OECD, 2020a). Studies using similar approaches have found that pharmaceuticals tend to display low toxicity compared to other pollutants. Indeed, this finding is expected since low toxicity is one of the prerequisites for approving pharmaceutical products, and toxicity tests, which often rely on fish and rodents, are generally used to remove harmful substances during the early stage of the drug development. Toxicity of pharmaceuticals in aquatic environments generally starts becoming visible at concentrations above 1 mg/l (Fent et al., 2006), and most pharmaceuticals are found at concentrations of ng/l (Fick et al., 2017; Petrović et al., 2014), while traditional pollutants, such as metals (Warnick and Bell, 1969) and PCBs (Stalling and Mayer Jr, 1972), can have toxicity thresholds at low µg/l. Similarly, in Section 3 of the OECD guidelines for the testing of chemicals – "Environmental Fate and Behavior" (OECD, 2020b) – pharmaceuticals are generally not as persistent as traditional pollutants, with half-lives ranging between days and weeks in water (Calisto et al., 2011; Lam et al., 2004; Packer et al., 2003). In contrast, other organic pollutants, such as PCBs, are named according to how persistent they are (Persistent Organic Pollutants, POPs) and can remain in the environment for months or even years (Jones and De Voogt, 1999; Wania and Mackay, 1996). However, as mentioned above, there are over 600 pharmaceuticals that have been detected in the environment, all with their own intrinsic chemical properties, and a near constant input of drugs that compensate for rapid dissipation rates have made researchers refer to them as "pseudo-persistent" (Challis et al., 2014; Daughton and Ternes, 1999). As with any other chemical, certain pharmaceuticals are more persistent than others, such as some psychoactive substances (Calisto et al., 2011), antibiotics (Al-Ahmad et al., 1999), and antihistamines (Topp et al., 2012). Furthermore, as pharmaceuticals degrade, other substances (i.e., metabolites) are created, and these can become highly bioactive and compromise health.

Despite the value of standardized laboratory practices, OECD-style tests do not replicate true environmental scenarios as they are designed to minimize external factors so as to isolate the effect of the investigated compound. Various versions of these testing procedures have been questioned regarding lack of both temporal (Davies et al., 2003) and contextual (Kowalczyk et al., 2015) complexity and scale within specific fields. Obviously, laboratory testing cannot completely mimic natural settings. For example, laboratory testing cannot mimic the spatiotemporal scale of a natural system. That is, artificial settings cannot completely replicate seasonality, stochasticity, and biotic and abiotic processes and interactions. As laboratory testing cannot replicate the complexity of natural conditions, field experiments are needed that can validate laboratory-based predictions (Challis et al., 2014). The demand for more realistic environments when assessing the fate of pharmaceuticals has resulted in an increasing number of studies using experimental units more analogous to natural conditions, such as mesocosms (Kunkel and Radke, 2008; Li et al., 2015; Posselt, 2020; Walters et al., 2010), ponds (Klaminder et al., 2015), and whole ecosystems and field studies (Klaminder et al., 2015; Schaper et al.,

2018a; Schaper et al., 2018b). To date, studies that have conducted field experiments in fully functional freshwater ecosystems are limited and more work is required to disentangle how mechanisms such as sedimentation, photodegradation, and microbial processes affect the environmental fate of pharmaceuticals, especially their interaction with one another.

Behavioral modifications as a driver of ecosystem change

Although OECD-style tests have shown that most pharmaceuticals display limited toxicity at low concentrations, a few real-world exceptions exist where non-target organisms have been affected by drug exposure on a population level albeit in very different ways. Perhaps the most (in)famous example is the decline of the white-rumped vulture (*Gyps bengalensis*) in Pakistan due to diclofenac exposure (Oaks et al., 2004). Another recognized example is the collapse of a fathead minnow (*Pimephales promelas*) population due to exposure to low levels of synthetic estrogen affecting gonadal development, causing recruitment failure (Kidd et al., 2007). Kidd et al. noted that that the minnows altered their spawning behavior, but it is unclear whether this behavioral change contributed substantially to the fish population collapse (Kidd, personal communication). Alterations of invertebrate behavior could have played a role in the measured ecosystem's effects caused by the synthetic estrogen (Kidd et al., 2014). However, the role of behavior as a driver of ecosystem change is largely unknown.

Since the onset of modern environmental science, it has been known that measuring how contaminants alter behavior is a more sensitive tool than toxicity tests for detecting the effects of pollutants on organisms (Warner et al., 1966). Over the last 50 years, behavior has been used as a non-lethal endpoint, where effects from chemical stressors have been shown not only for pharmaceutical exposure (Brodin et al., 2013), but also for heavy metal (Sandheinrich and Atchison, 1989), pesticide (Warner et al., 1966), and artificial light (Longcore and Rich, 2004) exposure as well as ocean acidification as the result of carbon dioxide (Munday et al., 2014). However, one major problem with behavioral ecotoxicology is how to interpret subtle behavioral alterations in both the presence and absence of effects (Tanoue et al., 2019) as well as in terms of what the ecological consequences of this changed behavior might be (Brodin et al., 2014). Interpreting sub-lethal behavioral alterations in an ecological context is difficult (Sprague, 1971). However, more recent improvements of behavior trials and identifications of behavioral traits of central importance for animal behavior in the wild have generated a notion that behavioral effects as the result of pharmaceutical exposure can now be interpreted in an ecological context (Brodin et al., 2014).

Over the last two decades, the field of animal behavior has argued that the accuracy of ecological prediction based on behavioral profiles of individuals can be improved if tests are designed to measure five significant traits: boldness, exploration, sociality, activity, and aggression (Reale et al., 2007). This relatively new theoretical framework is based on the assumption that animals, including human beings, show differences along these five main traits that are robust across variable contexts and time – i.e., animal personality (Mittelbach et al., 2014; Reale et al., 2007; Sih et al., 2004b; Wolf and Weissing, 2012). Animal personality theory differs from alternative views that consider individual factors such as hunger and metabolic processes as key drivers of fish activity and risk taking (Metcalfe et al., 2016). Animal personality theory suggests that the individual judged to be the boldest, most

exploratory, and most active in behavior trials will always be more active and risk taking than other individuals. These five traits – boldness, exploration, sociality, activity, and aggression – have been suggested to have fundamental evolutionary (Moretz et al., 2007) and ecological (Sih et al., 2012) consequences as they affect fitness traits such as growth, reproduction, and survival (Reale et al., 2007; Sih et al., 2004a). However, few studies have tested the ecological relevance of laboratory behavioral trials, so the scientific community has requested field verifications of laboratory experiments (Conrad et al., 2011). Further complicating interpretations is the fact that studies on boldness have shown both positive (Biro and Post, 2008) and negative effects (Adriaenssens and Johnsson, 2011) on growth.
The notion that behavioral trials in fish can be used for ecological predictions if they target key traits has attracted ecotoxicologists who want to interpret behavioral changes in response to chemical stressors in ecological contexts. As a result, laboratory trials targeting fish boldness, exploration, sociality, activity, and aggression traits have been used to screen subtle effects of pharmaceutical contaminant (Brandão et al., 2013; Brodin et al., 2013; Brodin et al., 2014; Nunes et al., 2008). However, many of these studies have not included tests of how repeatable behavioral traits are over time or across environmental contexts, a suggested key criterion for the use of the theoretical framework underlying animal personality research (Bell et al., 2009). In fact, the lack of repeatability is shared by about 62% of all behavioral studies using key traits derived from the theory of animal personality (Niemela and Dingemanse, 2018). In other words, although similar terminology of traits is used both within ecotoxicology and animal personality research, their meaning and robustness might differ.

Although the measurements of behavior traits have been advocated to be a powerful tool for screening non-lethal effects of chemicals (Dell'Omo, 2002), skepticism remains regarding the use of non-lethal endpoints in ecotoxicology (Johnson et al., 2020). Other criticisms note the limited consensus regarding how behavioral changes should be interpreted and highlight that expert opinions differ in their interpretation of small non-linear behavior effects (Hanson et al., 2017; Huerta et al., 2016). Recently, more uncertainty regarding the use of behavioral endpoints is derived from the revelation that some pioneer works showing behavior modifications in response to acid stressors (Munday et al., 2014; Nilsson et al., 2012) have been proven very difficult to reproduce (Clark et al., 2020). Hence, there is currently an urgent need to scrutinize to what extent behavioral traits measured when screening for effects of pharmaceuticals can be used for ecological predictions.

Oxazepam as a modifier of fish behavior

Central in the debate about the use of behavioral trials within ecotoxicology is drugs that are designed to alter behavior and have been detected in numerous aquatic ecosystems. Psychoactive pharmaceuticals, such as anxiolytics, have received particular interest due to their ability to change behavior via target receptors also present in many aquatic organisms (Gunnarsson et al., 2008). Benzodiazepines, an anxiolytic group of pharmaceuticals used for the treatment of anxiety and alcohol withdrawal, targets the GABA-a receptor in the central nervous system (Sternbach, 1979). Oxazepam, a benzodiazepine, has been shown to alter fish behavior in trials “borrowed” from animal personality research (Brodin et al., 2013; Brodin et al., 2017; Huerta et al., 2016; Saaristo et al., 2019; Vossen et al., 2019). Fish exposed to oxazepam have shown increased activity, reduced sociality, and bolder behavior (Brodin et al., 2013; Brodin et al., 2017; Saaristo et al., 2019). Tests of bolder or anti-anxiety

behavior are directly designed to measure individuals' ability to explore potentially risky environments, which seems to be explained by the fact oxazepam controls anxiety by binding to GABA-a receptors. In addition, the increased activity and reduced social behavior (increased distance to conspecifics) are behavior changes characteristic of an anti-anxiety like behavior such as reduced activity and shoaling typical for anti-predator behaviors (Sih et al., 2004a). Behavioral effects of oxazepam exposure have been shown for many teleost fish species, such as Eurasian perch (*Perca fluviatilis*) (Brodin et al., 2013), roach (*Rutilus rutilus*) (Brodin et al., 2017), Atlantic salmon (*Salmo salar*) (Hellström et al., 2016), fathead minnow (*Pimephales promelas*) (Huerta et al., 2016), burbot (*Lota lota*) (Sundin et al., 2019), and zebrafish (*Danio rerio*) (Vossen et al., 2019). Interestingly, the sensitivity to oxazepam seems to differ among these species, with wild caught zebrafish being the most sensitive with behavioral alterations at water concentrations of 0.57 µg/l (Vossen et al., 2020). In addition, bioconcentration of oxazepam differs between (Heynen et al., 2016c; Meredith-Williams et al., 2012) and within species (Heynen et al., 2016a), depending on social context. Studies indicate that within-species sensitivity to oxazepam depends on rearing environment – e.g., wild-caught zebrafish are more sensitive than laboratory-reared conspecifics (Vossen et al., 2020). Reversibility (Sundin et al., 2019) and tolerance (Vossen et al., 2019) to the behavioral effects of oxazepam have been observed in burbot and zebrafish, respectively, indicating that observed alterations of behavior are not permanent. Despite proven behavioral alterations in laboratory environments, the first attempts to explore ecological (i.e., field) effects of oxazepam exposure indicate no significant impact on growth and survival (Lagesson et al., 2018). Lagesson et al. (2018) suggest that environmental drivers such as temperature might be more important in controlling fish behavior than oxazepam, a view that was later supported in multi-stressor laboratory trials (Saaristo et al., 2019). However, Lagesson et al.'s study (2018) could not determine whether the behavior of the studied perch aligned with predictions of earlier laboratory studies. In short, the study was unable to resolve whether the lack of effect on mortality rates from oxazepam was due to a situation where the effects of the expected reduced anti-predator behavior were cancelled out by direct negative effects by oxazepam on the predator efficiency or if the result was caused by oxazepam-induced behavioral change. However, there are indications that behavioral effects on fish caused by oxazepam exposure are expressed in natural environments (Klaminder et al., 2016; Klaminder et al., 2019). Because these studies use pre-exposed and human-handled fish, their results reflect the treatment effects on fish subjected to artificial stress rather than natural conditions.

The ecological consequences of oxazepam are directly related to the interaction of the environment, the pharmaceutical industry, and the release of contaminated effluent water into natural freshwaters. However, there is a somewhat unexpected connection between oxazepam-induced behavioral modifications and behavioral changes associated with increased atmospheric CO_2 concentrations in marine systems. In short, increased dissolved CO_2 is suggested to affect ion-regulatory adjustments in blood and tissue, which depolarizes the GABA-a receptor (Nilsson et al., 2012), causing behavioral alterations similar to the therapeutic effect of oxazepam. In addition, behavioral changes due to oxazepam exposure and ocean acidification largely mimic one another as both conditions seem to reduce anti-predator behavior and increase boldness scores in laboratory trials (Brodin et al., 2013; Dixson et al., 2010; Klaminder et al., 2019; Munday et al., 2010). Additionally, both the GABAergic behavioral effects caused by oxazepam (Vossen et al., 2019) and ocean acidification (Hamilton et al., 2014) have been suggested to diminish over a relatively short time (i.e., weeks). Despite the similarities between behavioral effects of oxazepam exposure and ocean acidification, there are some important differences between these two independent environmental issues. First, while the repeatability of

behavioral modifications noted in response to ocean acidification has been questioned (Clark et al., 2020), behavioral effects of oxazepam have been found repeatedly for several species in many independent studies (Brodin et al., 2013; Brodin et al., 2017; Huerta et al., 2016; Klaminder et al., 2014; Saaristo et al., 2019; Vossen et al., 2019). Second, the way in which oxazepam acts on the GABA-a receptor is well known (Pritchett et al., 1989), but the inversed ionic currents through the GABA-a receptor due to acidosis remains largely hypothetical (Heuer et al., 2016; Nilsson et al., 2012). Finally, ocean acidification and its effect on behavioral traits have mainly been studied in laboratory trials, whereas oxazepam has been shown to also affect behavior in more natural settings (Hellström et al., 2019; Klaminder et al., 2016; Klaminder et al., 2019). However, these latter studies have used oxazepam exposure concentrations of around 200 µg/l, which is about 1000 times higher than average concentrations measured in contaminated freshwater systems (Fick et al., 2017). Yet, effects on boldness traits from oxazepam have been observed in laboratory trials at concentrations of 0.5-2 µg/l (Brodin et al., 2013; Brodin et al., 2017; Vossen et al., 2020), a concentration range that overlaps with concentrations measured in effluent waters (i.e., up to 1.8 µg/l) (Loos et al., 2013) and is close to that found in small contaminated rivers (0.2-0.6 µg/l) (Brodin et al., 2013; Klaminder et al., 2015).

Aim of this thesis and hypotheses

This thesis aims to improve our understanding about the fate and behavioral effects of pharmaceuticals in natural ecosystems. By conducting experiments both in laboratory settings as well as in whole lake ecosystems, I probe the validity of laboratory predictions for more complex natural environments. I have two main hypotheses: i) laboratory incubations can be used to predict the fate of pharmaceuticals in natural systems and ii) common laboratory behavioral trials can be used to explain behavioral alterations caused by oxazepam exposure in natural settings. These hypotheses were tested in four papers. Papers **I** and **II** focus on understanding differences between laboratory settings and complex aquatic ecosystems in regard to dissipation and food web uptake of five pharmaceuticals in natural environments. Papers **III** and **IV** focus on how well laboratory behavioral assays predict behavior in situ (i.e., in a natural environment such as a pond).

Objectives

The main objectives of this thesis are as follows:

- To determine how well pharmaceutical dissipation rates from laboratory incubations describe dissipation rates in situ (Paper **I**);
- To assess whether five common pharmaceuticals (diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine) are accumulated in organisms at different trophic levels in an aquatic food web (Paper **II**);
- To investigate how well common laboratory assays on Eurasian perch (*Perca fluviatilis*) behavior (activity, boldness, exploration, sociality, predator avoidance, thigmotaxis, and scototaxis) translate to behaviors in a natural setting (Paper **III**); and
- To determine how GABAergic behavioral modifications caused by oxazepam measured in laboratory settings on isolated fish are expressed in situ in Eurasian perch (*Perca fluviatilis*) and to determine the potential effects relative to changes in water temperature (Paper **IV**).

Materials and Methods

Laboratory dissipation experiment and analysis

To test my first hypothesis, laboratory incubations were used to determine how environmental conditions (temperature, solar radiation, organic solutes, and sediment sorption) affect dissipation rates of the studied drugs, either alone or in interaction with one another. The laboratory incubations were performed in pyrex vials (20 ml) where pharmaceutical solutions (diclofenac, diphenhydramine, hydroxyzine, trimethoprim and oxazepam) were added at nominal concentrations of 400 ng/l for each drug (four replicates per treatment). This factorial design study included the following treatments: solar radiation, organic solutes, temperature, and sediment. Solar radiation was generated using a Q-Panel UVA-340 lamp (wavelength spectra from 295 to 365 nm and an effect of 36 W QLAB 2012) where untreated samples were covered with light-blocking tape. Treatment with organic solutes was induced using either humic water (pH = 6.2, TOC = 19.5 mg/l total phosphorous (TP) = 49 µg/l, total dissolved nitrogen (TDN) = 599 µg/l) collected from Sävarån (63°54.805'N 20°34.303'E), a small river receiving no treated wastewater input, or groundwater (pH = 7.4, TOC 1.5 mg/l, TP = 11 µg/l, TDN = 92 µg/l) from an untreated experimental pond. Temperature treatments were obtained by performing the assays in two separate climate rooms (22°C and 3°C). After collected from the experimental pond, sediment was sieved (1 mm mesh) and air dried before being added to the incubation vials. Sampling of water was performed weekly for the 28 days of incubation. Initial solution concentrations were measured before the experiment began and were used as a measure for starting (t = 0) concentrations for each treatment. Figure 1 illustrates how field conditions were mimicked in the laboratory assays.

Analyses for pharmaceutical concentrations considered in Papers **I**, **II**, and **IV** were performed using a triple stage quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela and a Surveyor LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland). Analytical methods for water samples have previously been described (Lindberg et al., 2014) as have the methods for biota analysis (Brodin et al., 2013; Brodin et al., 2014).

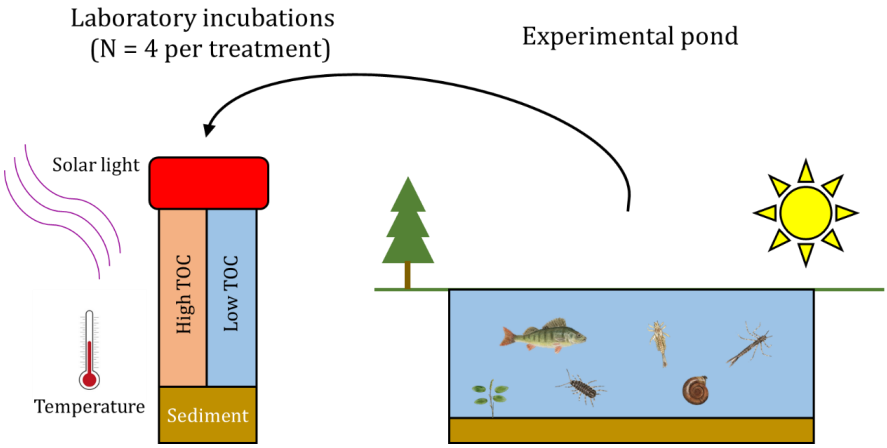

Figure 1. Conceptual view of how field conditions were mimicked in the laboratory assays (Paper **I**). Based on the experimental pond ecosystem environment (right), I created a full factorial laboratory design where incubations (N=4 per treatment) were subjected to combinations of treatment simulating solar light, two different concentrations of organic solutes, sediment and two temperature regimes (3˚C and 22˚C).

The in situ environment used for pharmaceutical dissipation experiments

The experiments described in Papers **I** and **II** were performed in a large experimental pond at Röbäck (63°48.572'N 20°14.584'E) in Umeå, Sweden. The pond dimensions are 10 m by 40 m with a mean depth of 1.3 m (Figure 2). The pond, a naturally developed ecosystem, is the home to aquatic invertebrates such as predatory damselflies (*Zygoptera*), grazing mayflies (*Ephemeoptera*), detrivorous waterlouse (*Asellus aquaticus*), and Ramshorn snails (*Planorbidae*). The only fish species in the pond is a naturally reproducing population of Eurasian perch (*Perca fluviatilis*). For the experiments described in Papers **I** and **II**, the pond was spiked with trimethoprim (antibiotic), diclofenac (anti-inflammatory), oxazepam (anxiolytic), hydroxyzine (antihistamine), and diphenhydramine (antihistamine) by dispensing a stock solution using a pipette at ~50 locations around the pond to ensure complete dispersal. The pond was then sampled on a daily to weekly basis for water chemistry and the organisms listed above. Biota were sampled for 66 days using hand netting in the littoral zone for macroinvertebrates and using umbrella traps for perch. Water was sampled for 247 days using a Ruttner sampler.

Figure 2. The Röbäck experimental pond used to determine the environmental fate of pharmaceuticals (Papers **I** and **II**). Notice that the pond function as a functional aquatic ecosystem where fish (perch) can survive all year around. Photo: Johan Fahlman

The in situ environment for behavioral experiments

For the purposes of my second hypothesis, the experiments described in Papers **III** and **IV** were largely performed in two lakes (Figure 3) outside of Åmsele, Sweden (64°29.25'N 19°25.82'E). Both lakes are kettle-holes, with an area of roughly 4000m^2 each, and a maximum depth of about 6 m (Paper 4, Figure S1). The lakes are both bordered by quagmire and surrounded by coniferous forests. Both lakes were fishless prior to the experiments described in Papers **III** and **IV**, which was confirmed by gillnetting with no catch. The main purpose of the lake experiments was to study fish behavior in a natural setting; these in situ experiments used high-end acoustic telemetry. Fish tracking in the lakes was performed using the VEMCO HR2 system with eight receivers (plus satellite receivers to cover an island in the lower lake, see Paper IV, Figure S1). The transmission interval of the system was 2 s, and accuracy of the tracking was determined using horizontal positioning error (HPE) and root-mean-square error (RMSE), with a median spatial accuracy of 0.5 m (Paper **III**, Figure S1). To address the issues described in Paper **IV**, after finishing the data collection described in Paper **III**, one of the lakes (Upper Lake, NW corner) was treated with oxazepam at a concentration of 11.4-24.1 µg/l.

Figure 3. The Åmsele lakes used in the tracking of in situ behavior of perch (Paper **III** and **IV**). Upper Lake to the left and Lower Lake to the right. Photo: Micael Jonsson

Methods used for tracking of in situ behavior

The perch described in Papers **III** and **IV** were caught in Lake Stöcksjön (63°45'45.9"N 20°11'54.1"E), and the pike were caught in Lake Tavelsjön (64°0'2.4"N 20°3'5.1"E). The pike were kept in a holding net, tagged, and transported to the study lakes within 24 hours, and the perch were transported to Umeå Marine Research Facility (UMF) for tagging and housing. All fish were tagged using VEMCO V4 180 kHz acoustic transmitters surgically inserted into their abdominal cavity. During the procedure, the fish were anesthetized using MS-222. The incision was closed with a suture. The perch were left to recover for a minimum of 14 days before any further handling and were healthy and feeding before further testing.

The perch used in Papers **III** and **IV** were, in order to test the predictive ability of laboratory behavioral analysis in situ for the purpose of Paper **III**, subjected to six behavioral trials before their release into the two lakes described in section 3. The tests (Figure 4) and their respective endpoints were open field test (activity, thigmotaxis), novel tank diving test (activity, boldness), novel object test (activity, boldness), social preference test (activity, sociality), predator avoidance test (activity, boldness), and scototaxis test (boldness). Before each test, which lasted 15 minutes, the fish were left alone for one hour as a habituation measure. All tests were performed during the same day for a given individual and in the same order, although starting tests varied due to logistical reasons. Scototaxis analysis (where only one area was available) was performed on a separate day.

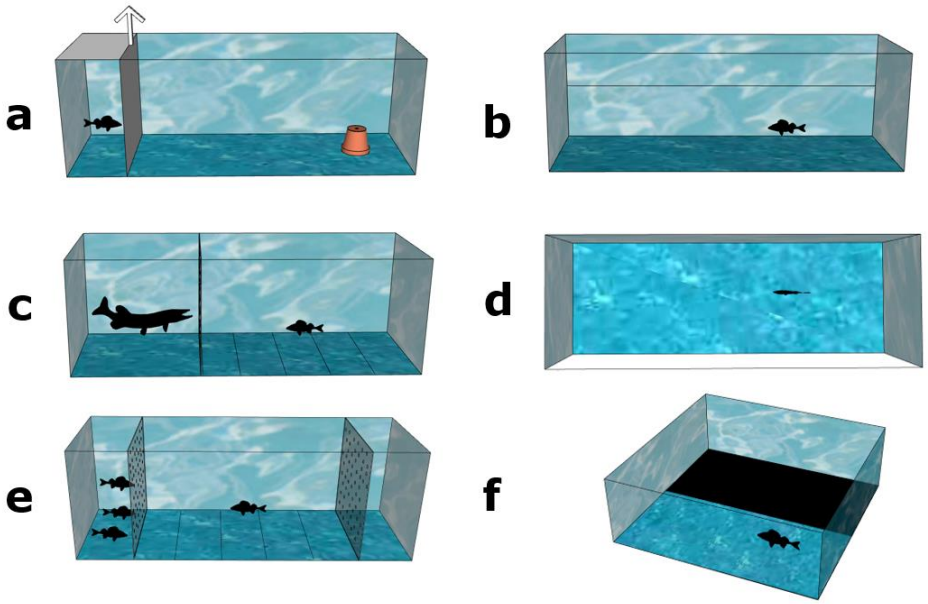

Figure 4. Overview of laboratory test arenas used in Paper **III** in order (a through f). The arenas are as follows, in consecutive order: a) Novel object emergence test, where the latency to leave the refuge to inspect the object was used as a measure of boldness; b) Novel tank diving test, where the near surface swimming depth was recorded as a proxy for bold behavior; c) Predator proximity test, where distance to a pike was used as a boldness measure; d) Open field test, where swimming distant to the walls was used as a measure of bold behavior; e) Social preference test, where distance from conspicifics was used as a proxy for asocial and bold behavior; and f) Scototaxis test, where selective swimming above a white bottom instead of a black bottom (considered refugia) was used as an anti-anxiety behavior. In all tests, activity was measured as time spend in motion. (Paper **III**)

Using the data from the VEMCO receivers mentioned above, I measured three behavioral traits in situ for the released perch in the experiments described in Papers **III** and **IV**: 1) *activity* (swimming speed in m minute^{-1}), measured during the first 12 hours (only Paper **III**); 2) *pelagic use* (distance to shoreline in m); and 3) *home range* (95% area utilized calculated as mean convex polygon in m^2). In Paper **III**, *predator avoidance* (distance to closest predator in m) was also used as a field measurement. In Paper **III**, *dispersion* from the release site during the first three hours (unit: *n* detections >15m from release site) was also used as a measurement as well as *social association* to conspecifics (unit: proportion of time spent within 1 m of any conspecific during at least 15 consecutive minutes). In Paper **IV**, the first three measurements were also used for pike. Additionally, Paper **IV** measured social network density, a population-level measurement of the present connections within the social network relative to the number of possible connections (Wasserman and Faust, 1994), as well as degrees, a measure of the number of social connections an individual has during a specific time frame. These measures were extracted as a running measurement using the *spatsoc* package for R. Point-based spatial grouping with a distance threshold of 1 m was used to define social connections (Robitaille et al., 2019).

Results and Discussion

Fate of pharmaceuticals in natural systems

In Papers **I** and **II**, I show that the fate of pharmaceuticals in natural systems is linked to the drug's intrinsic chemical properties and the ecosystem's properties. The half-lives ($t_{1/2}$) in the pond ecosystem varied between 8 and 53 days for the studied pharmaceuticals (oxazepam, trimethoprim, diclofenac, diphenhydramine, and hydroxyzine). Oxazepam was the most persistent pharmaceutical (detectable for over 247 days) and diclofenac the least (detectable for up to 29 days). When comparing the laboratory results with the pond results, I found that the small incubations based on sediment from the pond could be used to predict fairly well the dissipation in the pond during the initial ~10 days of the study. Of the drugs tested, trimethoprim's dissipation laboratory rate was closest to the apparent dissipation rate measured during the first ten days in the field. However, this drug remained in its therapeutic form in the pond for well over 100 days, which was a somewhat surprising result considering that its laboratory half-life suggested complete removal after less than 50 days (10 half-lives). Clearly, my first hypothesis seems valid in a short-term (weekly time scale) perspective, but invalid in a long-term (monthly time scale) perspective.

As with my study, previous studies have found that trimethoprim, one of the most commonly prescribed antibiotics globally (Drugs.com, 2020), is environmentally persistent (Alexy et al., 2004; Halling-Sørensen et al., 2000). As development of bacterial resistance to antibiotics is considered one of the current threats to human health (Neu, 1992), I find the strong persistence of trimethoprim (Paper **I**) of great concern as it seems that this drug may contribute to antibiotic resistance in the environment. For example, it is well known that antibiotics like trimethoprim can result in selection pressure for antibiotic resistance in *E. coli* (Toprak et al., 2012); however, none of the studied organisms in Paper **II** seem to have accumulated this drug.

The most successful models derived from laboratory estimates (explaining both short- and long-term dissipation relatively well) were hydroxyzine and diclofenac. The least persistent drugs in the pond remained at detectable levels for less than 100 days. The low environmental persistence of diclofenac is well documented (Jaeger et al., 2019; Packer et al., 2003; Zhang et al., 2017), and even though it has been shown to have adverse effects on terrestrial wildlife (Oaks et al., 2004), it does not seem to enter the aquatic food web as shown in Paper **II.** Regarding the latter finding in combination with its low persistence in the water column, diclofenac seems to pose a much lower threat to aquatic ecosystems than to terrestrial ecosystems, where devastating impacts have been observed.
Indeed, my laboratory-based predictions did not capture the long-term dissipation trends occurring after the first 10 days for oxazepam, diphenhydramine, and trimethoprim (Paper **I**). The estimated half-lives from the laboratory incubations generally underestimated the long-term half-lives. For example, the best laboratory estimate of oxazepam half-life (Low TOC treatment) was 22 days, which should have caused oxazepam to be undetectable (<LOQ = 5 ng/l) after about 220 days (10 half-lives), although it was still detectable at day 247. An estimated half-life of oxazepam from the apparent dissipation from the pond water corresponds to a $t_{1/2}$ of around 70 days. Interestingly, this estimate from the pond ecosystem seems to be conservative as the oxazepam used in the spiked lake described in Paper **IV** remained at detectable levels (0.2 µg/l) three years after addition (unpublished data) – i.e., an apparent dissipation corresponding to a half-life within that lake of around 160 days. Clearly,

oxazepam can be very persistent in certain natural environments, and my finding that oxazepam can linger in the environment for much longer than predicted from the incubations is intriguing and deserves further study.

So, what processes control the dissipation of pharmaceuticals in natural aquatic environments that explain why laboratory predictions sometimes underestimate the persistence of pharmaceuticals in the environment? Solar radiation and sediment sorption were two factors identified that drive in situ dissipation during the first weeks, which is unsurprising as both these factors have previously been shown to affect the fate of pharmaceuticals (Calisto et al., 2011; Löffler et al., 2005). My PLS analysis on the laboratory dissipation results (Paper **I**, Figure 3) clearly highlights that environmental factors (temperature, sediments, organic solutes, and solar radiation) appear as (or more) important for pharmaceutical dissipation (average loading 0.14) than the properties of the drugs themselves (average loading 0.10). Here, sediments and solar radiation were the most influential. The results of the PLS analysis tell me that the dynamic nature of the pond environment may play a larger role than the intrinsic chemical properties of the drugs. Clearly, my static small-scale incubations were not successful in replicating the dynamic environmental factors. Although a number of environmental factors were simulated in my laboratory incubations, the mismatch between laboratory dissipation rates and in situ dissipation rates clearly indicate that I was unable to fully replicate all processes active in the pond. One condition that is apparently not replicated in my incubations is related to the size of the vials compared to the size of the pond ecosystem itself. The depth of the pond (1.3 m) reduced the importance of UV light in situ in comparison to the laboratory environment, as UV light penetrates water to about a 10-cm depth (De Haan, 1993). This means UV light penetrated all the way through the small incubation vials used in my study, while only the surface of the pond was similarly affected. Additionally, sunlight hours declined rapidly during the pond experiment, dropping from nine hours per day at the start of the experiment to only four hours at the end of the 247-day sampling effort, a change that drastically reduced the influence of sunlight at the end of my field experiment. Another effect of scale is the importance of natural sediment sorption/desorption processes, which have proved to bind pharmaceuticals for decades (Klaminder et al., 2015). It seems plausible that drugs initially adsorbed to the sediment could re-enter the water column via desorption processes or due to the actions of sediment-dwelling organisms, either contributing to the degradation of organic sorption sites or by facilitating resuspension through bioturbation. Regarding the latter, it seems evident that benthic organisms may play a crucial role in the fate of at least diphenhydramine, hydroxyzine, and oxazepam, considering the measured high uptake in organisms from this habitat (Paper **II**). Indeed, it seems clear that the full complexity of the natural system could not be replicated in my incubations, so I underestimated the tail of the drugs lingering in the water. However, part of this error might be an artefact of the assumed first-order dissipation model itself. That is, by assuming a first-order reaction similar to most other studies doing risk assessment with pharmaceuticals (OECD, 2020b), I created a model that cannot reproduce the long tail effect with drugs lingering in the water column. In other words, dissipation of pharmaceuticals in natural environments is poorly described by a first-order reaction and there seems to be an inherent flaw in this model approach.

Much of the current focus on pharmaceutical persistence is on the intrinsic molecular structure of the drugs (Caliman and Gavrilescu, 2009; OECD, 2020b; Sanganyado et al., 2017), and current assessments of the environmental impact of drugs are mainly discussed using their dissipation in laboratory settings (EMA, 2006). If my results are representative for other drugs, they clearly show that the site-specific

conditions prevailing in aquatic environments receiving inputs need to be considered when conducting sound predictions of the dissipation rate of pharmaceutical substances. For example, a drug can disappear within a few days from warm and shallow waters, where photo-degradation proceeds rapidly, but the very same drug can remain for months or even years in freshwaters where low temperatures constrain microbial processes and ice blocks solar radiation. I argue for using laboratory conditions that resemble high latitude environments when conducting environmental risk assessments of pharmaceuticals in boreal and arctic ecosystems. Although the importance of environmental specific degradation rates for pharmaceuticals is an important conceptual insight, it is not controversial as this importance has previously been shown for organic substances in other fields of research (Massicotte et al., 2017).

Food web uptake of pharmaceuticals

The food web uptake of the studied drugs showed trends that differed to some extent from the observed dissipation rates in the water column. Three of the five studied pharmaceuticals could be detected in all biota: oxazepam, diphenhydramine, and hydroxyzine. These three drugs were all relatively persistent in the pond, remaining detectable for between 60 and 247 days, and moderately bound to sediment and/or biota (Log K_{ow} between 2.2 and 3.3) (Wishart et al., 2018). However, trimethoprim, which remained in the water for months, was not detected in any of the studied organisms; hence, the dissipation rate alone had limited predictive power for the uptake of the studied drugs. Diclofenac and trimethoprim were not detected in any of the studied organisms probably because of these drugs' weak affinity to biota (Log K_{ow} <2 when adjusted for pH in the pond). Diphenhydramine and hydroxyzine concentrations in biota decreased over time, following the water concentration (Paper **II**, Figure 2). In general, the benthic species (i.e., the water louse and Ramshorn snail) had the highest BAFs, while the top predator (i.e., perch) had the lowest (Paper **I**, Figure 3). This finding indicates that diet and habitat use may play important roles in the bioaccumulation of pharmaceuticals. Interestingly, it is likely that concentration of oxazepam BAF increased in the perch (Paper **II**) but decreased in the water (Paper **II**, Figure 4a). This finding strongly indicates a trophic transfer of oxazepam within the food web. Previous laboratory studies have found that oxazepam can be transferred between trophic levels, but never to perch (Heynen et al., 2016c). Therefore, oxazepam exposure assessed solely by fish uptake of the drug from water (OECD, 2020a) or by the use of Log K_{ow} (Arnot and Gobas, 2006) would not work well. Additionally, the high BAF of oxazepam in perch found in this study is supported by earlier observations (Muir et al., 2017), where a BAF of 927 was observed for ∑Diazepam (diazepam and oxazepam) in the blood plasma of wild carp (*Cyprinus carpio*). This finding makes it likely that earlier work on oxazepam uptake in fish may have underestimated the actual uptake in nature by not including uptake via dietary intake (e.g. Brodin et al., 2013; Heynen et al., 2016b; Klaminder et al., 2014). Another interesting finding is that the BAF of the studied antihistamines hydroxyzine (BAF >1000) and especially diphenhydramine (BAF ~ 100 000) in Ramshorn snail are at the lowest trophic level. As detritivores such as the Ramshorn snail are rarely tested in laboratory assays, further focus on these organisms seems warranted, especially when considering the strong bioaccumulation of antihistamines and that antihistamines affect insect behavior (Jonsson et al., 2019; Jonsson et al., 2014) as well as carbon cycling (Jonsson et al., 2015).

Effect of oxazepam on fish behavior

In situ fish behavior resembles laboratory fish behavior when multiple behavioral tests are combined into multidimensional behavioral profiles (Paper **III**). Clearly, multiple behavioral assays are needed to understand in situ behavior as behavior in nature is complex and affected by many stimuli, both internal and external. Moreover, behavior of tested perch displayed a high contextual plasticity in the laboratory trials. This contextual plasticity represents a previously recognized challenge when attempting to identify behavioral traits of general relevance (Stamps, 2016; Stamps and Biro, 2016). The observed plasticity is cause for concern, as single test behavioral assays are customary for currently outlined ecotoxicologic tests (OECD, 2020a). However, behavioral responses due to oxazepam contamination have been shown to be repeatable (Brodin et al., 2013; Brodin et al., 2017; Klaminder et al., 2014), which strongly suggests that the observed behavioral effects from oxazepam are significant in laboratory environments but that multiple tests may be needed to predict in situ behavior.

Hypothesis **II** was mainly based on earlier laboratory studies that used the same novel object, activity, and sociality tests described in Paper **III** (Brodin et al., 2013; Brodin et al., 2017; Klaminder et al., 2014). In other words, the behavioral tests serving as a base for my main hypothesis proved to have limited connection to lake performance if interpreted alone. Nevertheless, it seems that oxazepam alters perch behavior in laboratory settings given previous studies show behavioral impacts on many species and in different behavioral assays (Brodin et al., 2013; Brodin et al., 2017; Klaminder et al., 2014; Saaristo et al., 2019; Sundin et al., 2019). In other words, because Paper **III** clearly shows a link between laboratory behavior and in situ behavior for perch, it seems rationale to expect that perch behavior in the studied lake would be altered by oxazepam, especially since the effects of oxazepam on perch behavior have been reproduced in laboratory tests (Brodin et al., 2013; Klaminder et al., 2014; Saaristo et al., 2019). In addition, oxazepam exposure seems to affect the activity, home range, sociality, and habitat use for pre-treated fish released into a natural lake (Klaminder et al., 2016).
It is indisputable that oxazepam induces behavioral modifications in fish studied in the laboratory and in fish handled in laboratory environments before being released into freshwater ecosystems.

However, the whole-lake oxazepam treatment did not have any obvious expected effects on the studied behaviors previously theorized to be linked to oxazepam exposure (activity, habitat use, home range, and predator avoidance). Instead, significant effects were observed for measured perch activity and predator avoidance, where the swimming activity remained constant in the exposed lake while increasing in the control lake, and predator avoidance increased slightly following oxazepam exposure (Figure 5). In other words, a contrasting effect to what would be expected considering that previous laboratory studies have found that oxazepam increases perch activity and decreases anti-predator behavior. However, the difference in activity between the two lakes is very slight, and my interpretation is that small environmental differences between the two lakes probably caused the observed effects. Such small environmental differences could be caused by food resource distributions coupled to lake morphologies, or due to pike with predator cues of different strength, which could be the underlying reason for the changes in predator avoidance following oxazepam exposure. Here, the need to restock lost predators may have contributed to small differences in predator avoidance behavior in the two lakes. Nevertheless, I cannot exclude that the measured behavioral effects of

oxazepam exposure is indeed an effect of the drug, especially as Paper **III** indicates that in situ behavior can be very counterintuitive with respect to performance in laboratory settings.

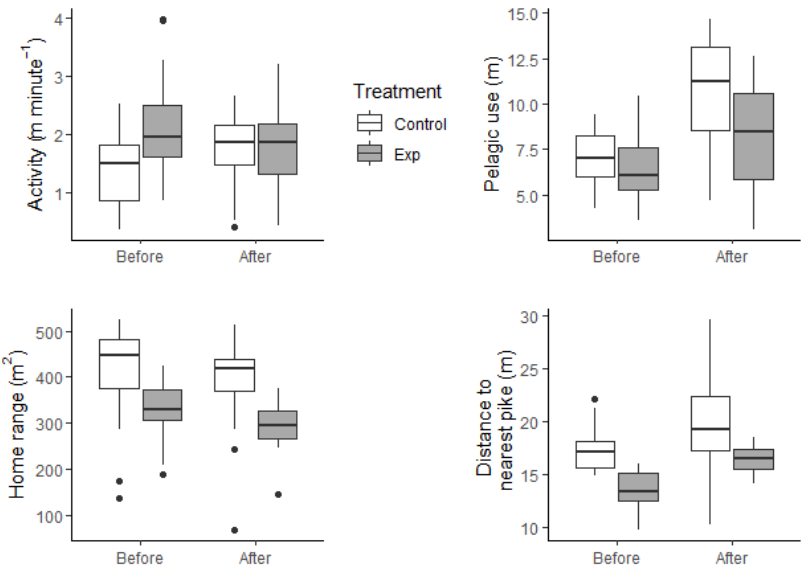

Figure 5. Daily averages of measured behavioral traits of perch from the two studied lakes in paper **IV.** Boxplots displays the effect of oxazepam on swimming activity (left, upper panel), use of pelagic habitats (right, upper panel), home-range size (left, lower panel) and distance to predators (right, lower panel). White boxes are measurements in the control lake (Lower Lake) and grey boxes in the impact lake (Upper Lake). Data are presented in Paper **IV**, Fig. 4.

Behavioral effects of oxazepam on perch in natural systems has been previously shown although only on pre-exposed recently handled perch at high concentrations (about 200 µg/l). Paper **IV** describes the first study to test the effects of oxazepam exposure on the behavior of habituated perch in an exposed and a natural environment. One major difference between Paper **IV** and previous studies, both laboratory- and field-based, is that my fish were acclimatized to the environment for an extended period (11 days) before the oxazepam exposure. Long acclimatization times in the new environment have been pointed out as a very important component in ecotoxicological behavioral assays as the perceived level of stress is reduced over time (Melvin et al., 2017). Therefore, one possible explanation for the limited effect on in situ behavior from oxazepam is that this drug does not affect behavior of habituated fish, resulting in less stressed fish, as strongly as for wild caught fish subjected to artificial stress in laboratory settings. If true, my interpretation suggests that the previously observed effect of oxazepam on wild caught fish (*Danio rerio*) but not on laboratory-reared conspecifics (Vossen et al., 2020) might be due to the fact that the laboratory strain are habituated and less stressed and somewhat paradoxically a better analogue for wild fish living in natural environments.

Another plausible explanation for the lack of major behavioral effects from oxazepam is related to the social network within the lakes. The lack of the expected exposure effect in Paper **IV** may be attributed

to collective decision making and the density of the shoal (measured as social network density), which had significant effects on all measured traits in the study. Social context has previously been proven paramount for fish decision making (Krause et al., 2002; Magnhagen, 2012; Petit and Bon, 2010), fish behavior in laboratory settings (Jolles et al., 2019), and effects of psychoactive substances on fish (Martin et al., 2019). My results clearly show the top-down influence of shoaling on individual fish behavior, with high plasticity on individual social degree throughout the study (Paper **IV**, Figure S2). I also observed circadian rhythms driving social network density, with shoaling densities intensifying around dusk and dawn and almost disappearing at night (Paper **IV**, Figure 2). My findings are in line with earlier work suggesting that decision making is driven by spontaneous shifts in the social structure of fish communities, where leader-follower roles are continuously changing (Rands et al., 2003). Other studies have found contrasting results, indicating that social structures in animal communities are more static (Jolles et al., 2019) and possibly correlated to individual boldness (Dahlbom et al., 2011). Earlier work also demonstrates the protection against erratic individual behavior by the collective inertia of social groups (Lu et al., 2008) as well as by the lack of effects of oxazepam exposure on fish groups (Hellström et al., 2019; Lagesson et al., 2018). Thus, we cannot exclude that the social context of the lake acts as a buffer against oxazepam-induced behavioral alterations. However, as the multidimensional description of perch behavior from Paper **III** is related to in situ behavior, it is evident that individual decision making plays a part in the expression of natural behavior within the studied populations. As oxazepam has been known to alter individual behavior in laboratory studies (Brodin et al. 2013), one could assume that these effects would also be visible in the field. However, it appears as if the effect of oxazepam is too weak to alter behaviors linked to shoaling, a behavior that is driven by strong selection pressure (Alexander, 1974).

Apart from social context, water temperature proved to be an important factor controlling in situ behavior of perch, affecting their activity, habitat use, and home range (Paper **IV**, Table 1). The influence of temperature (and other abiotic factors) on fish behavior has been shown in previous laboratory (Biro et al., 2010; Saaristo et al., 2019) and field studies (Nakayama et al., 2018; Nakayama et al., 2016). Additionally, in laboratory and mesocosm studies, social context has been known to alter fish behavior (Gómez-Laplaza and Morgan, 2003; Gómez-Laplaza and Morgan, 2000) as well as cortisol levels (Heynen et al., 2016a). However, to my knowledge, Paper **IV** is the first study to investigate the importance of social context in fish decision making together with abiotic factors and pharmaceutical exposure in an in situ setting.

A conceptual summary of drivers of perch behavior

Based on the observed effects of oxazepam treatment, temperature and social context in paper **IV,** in combination with findings of other studies, I suggest a conceptual framework for perch *in situ* behavior outlined in Figure 6. My synthesis suggests that factors such as social context and water temperature play an important role across all study scales, from laboratory to field settings, whereas oxazepam only causes major changes on perch behavior for isolated fish in laboratory settings and not for shoaling fish in ponds and lakes. Effects measured in these scales are only seen for artificially-stressed fish released into novel natural environments after exposure to concentrations about 100 times higher than ever measured in a contaminated river system. With my results in mind, where social context and temperature seem to be the main drivers of perch behavior, I find it unlikely that major behavioral

effects will occur in the wild in response to the dilute concentrations of oxazepam typically occurring in contaminated waters (i.e., about 10-300 times lower than in my lake experiment) (Fick et al., 2017; Loos et al., 2013). My interpretation is supported by previous studies that found no effect on spawning behavior (Hellström et al., 2019) or growth and survival (Lagesson et al., 2018) at the mesocosm scale.

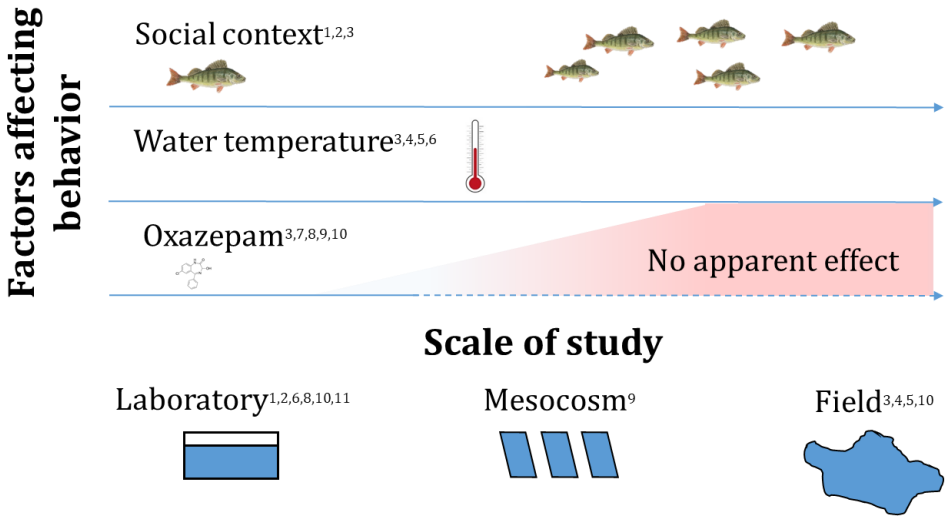

Figure 6. Conceptual synthesis of factors affecting perch behavior in this thesis based on empirical data and available literature. This concept suggests that the listed drivers of behavior have relevance at different spatial and temporal scales. Social context and abiotic factors, here represented by water temperature, appear to influence all study scales. Meanwhile, individual behavioral dimensions that have been proven important at the laboratory scale become harder to distinguish at larger spatiotemporal scales, similar to the effect of oxazepam, which has been difficult to prove significant in field contexts at environmentally relevant concentrations. 1 (Gómez-Laplaza and Morgan, 2003); 2 (Gómez-Laplaza and Morgan, 2000); 3 (Paper **IV** of this thesis); 4 (Nakayama et al., 2018); 5 (Nakayama et al., 2016); 6 (Biro et al., 2010); 7 (Brodin et al., 2013); 8 (Brodin et al., 2017); 9 (Hellström et al., 2019); and 10 (Klaminder et al., 2016).

Concluding remarks

Diclofenac, the only pharmaceutical studied in this thesis on the EU watch list (Carvalho et al., 2015), remained undetectable in the food web and displayed rapid dissipation within the water column. Thus, diclofenac, which has been shown to have dramatic effects on terrestrial ecosystems (Oaks et al., 2004), does not appear to be a large threat to aquatic ecosystems. The studied antibiotic, trimethoprim, persisted for months in the water column, although it did not enter the studied food web. However, the main environmental issue relating to this persistent antibiotic is its potential role in creating antibiotic resistance in microbial communities, (Toprak et al., 2012). Furthermore, the antihistamines (hydroxyzine and diphenhydramine) tended to be relatively persistent in the water column and showed strong accumulation in benthic organisms. Although antihistamines are not frequently mentioned as pollutants, they have been known to bioaccumulate in riparian food webs (Richmond et al., 2018), affecting stream ecosystem functions (Jonsson et al., 2015) as well as insect behavior (Jonsson et al., 2019; Jonsson et al., 2014). Indeed, these previous studies in combination

with my findings suggest that the fate of antihistamines in aquatic ecosystems warrants further study. Finally, the studied benzodiazepine, oxazepam, appeared to be very persistent in the water column, a condition that proved hard to predict using laboratory incubations. Oxazepam was also bioavailable for months and even displayed increased BAF over time, indicating a possible trophic transfer of oxazepam in the studied food web.

In pharmaceutical risk assessment, potential persistence and effects on biota are key components (Daughton and Ternes, 1999; Hernando et al., 2006). Having accurate estimations of these two factors are crucial when estimating the potential risk of a given drug (Johnson et al., 2020). Therefore, laboratory trials studying the fate of pharmaceuticals in the environment have a paramount function in risk assessment, so it is crucial that these trials are accurate representations of natural conditions (Challis et al., 2014). One conclusion of this thesis is that the fate of pharmaceuticals is highly contextual and that spatiotemporal dissipation dynamics are affected by not only substance, but also the environment, conditions that are difficult to replicate in laboratory incubations. Yet, it is possible to make fairly accurate predictions regarding in situ pharmaceutical dissipation on a weekly time-scale from findings in laboratory settings. At this time-scale, most pharmaceuticals will have been transported from WWTPs via rivers to lakes or oceans where dilution possibly mitigates their deleterious effects (Freeze and Cherry, 1979). However, if risk assessments need sound estimates of persistence in these latter environments, more long-term field experiments are needed. There is still a lack of knowledge when it comes to understanding processes that control dissipation of pharmaceuticals in aquatic ecosystems with longer residence time, such as lakes (Klaminder et al., 2015), oceans (Benotti and Brownawell, 2009), and groundwater (Sui et al., 2015). I argue that we need further research on the matter if we want a sound and comprehensive understanding of pharmaceutical fate in all natural systems.

While the current testing for the effects of pharmaceuticals in the environment via OECD-style laboratory assays (OECD, 2020a) appears to be effective when it comes to identifying toxicity levels, behavioral assays are clearly much more sensitive tools when it comes to understanding the sub-lethal effects of pharmaceutical exposure on fish (Brodin et al., 2014). Although behavioral endpoints are useful tools for detecting non-lethal effects of chemical stressors, the results of this thesis indicate translating results from laboratory trials into natural contexts is complicated. My findings suggest that the predictive power of behavioral traits can be increased if multiple trials are used to account for contextual plasticity and repeatability of behavior, artificial stress in the laboratory environment is limited by allowing for extended habituation time and possibly more natural testing arenas, social context is included in the laboratory context, and behavioral impacts from abiotic conditions, such as temperature, are considered.

Although oxazepam was shown to be persistent and bioavailable for aquatic organisms, oxazepam-induced behavioral changes appear to be less of a threat than, for example, the disruption of reproductive hormones (Kidd et al., 2007) and the increase in antibiotic resistance (Kemper, 2008; Kümmerer, 2009). I base this notion on the fact that I found no conclusive behavioral effects of oxazepam exposure at a population level despite using exposure levels 300 times higher than what has been measured in the environment.

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References

Adriaenssens B, Johnsson JI. Shy trout grow faster: exploring links between personality and fitness-related traits in the wild. Behave. Ecol. 2011; 22: 135-143.

Aitken M, Kleinrock M, Simorellis A, Nass D. The Global Use of Medicine in 2019 and Outlook to 2023: Forecasts and Areas to Watch. IQVIA Institute, NC, USA 2019.

Al-Ahmad A, Daschner F, Kümmerer K. Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. Archives of environmental contamination and toxicology 1999; 37: 158-163.

Alexander RD. The evolution of social behavior. Annual review of ecology and systematics 1974; 5: 325-383.

Alexy R, Kümpel T, Kümmerer K. Assessment of degradation of 18 antibiotics in the closed bottle test. Chemosphere 2004; 57: 505-512.

Arnot JA, Gobas FA. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environmental Reviews 2006; 14: 257-297.

aus der Beek T, Weber FA, Bergmann A, Hickmann S, Ebert I, Hein A, et al. Pharmaceuticals in the environment—Global occurrences and perspectives. Environmental toxicology and chemistry 2016; 35: 823-835.

Bates MN. Mercury amalgam dental fillings: an epidemiologic assessment. International journal of hygiene and environmental health 2006; 209: 309-316.

Bell AM, Hankison SJ, Laskowski KL. The repeatability of behaviour: a meta-analysis. Animal behaviour 2009; 77: 771-783.

Benotti MJ, Brownawell BJ. Microbial degradation of pharmaceuticals in estuarine and coastal seawater. Environmental Pollution 2009; 157: 994-1002.

Biro PA, Beckmann C, Stamps JA. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. Proceedings of the Royal Society B-Biological Sciences 2010; 277: 71-77.

Biro PA, Post JR. Rapid depletion of genotypes with fast growth and bold personality traits from harvested fish populations. Proc. Natl. Acad. Sci. USA 2008; 105: 2919-2922.

Boxall AB, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, et al. Pharmaceuticals and personal care products in the environment: what are the big questions? Environmental health perspectives 2012; 120: 1221-1229.

Brandão F, Rodrigues S, Castro B, Gonçalves F, Antunes S, Nunes B. Short-term effects of neuroactive pharmaceutical drugs on a fish species: biochemical and behavioural effects. Aquatic toxicology 2013; 144: 218-229.

Brodin T, Fick J, Johnson M, Klaminder J. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 2013; 15: 814-815.

Brodin T, Nordling J, Lagesson A, Klaminder J, Hellström G, Christensen B, et al. Environmental relevant levels of a benzodiazepine (oxazepam) alters important behavioral traits in a common planktivorous fish,(Rutilus rutilus). Journal of Toxicology and Environmental Health, Part A 2017; 80: 963-970.

Brodin T, Piovano S, Fick J, Klaminder J, Heynen M, Jonsson M. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Philosophical Transactions of the Royal Society B: Biological Sciences 2014; 369: 20130580.

Caliman FA, Gavrilescu M. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment–a review. CLEAN–Soil, Air, Water 2009; 37: 277-303.

Calisto V, Domingues MRM, Esteves VI. Photodegradation of psychiatric pharmaceuticals in aquatic environments–Kinetics and photodegradation products. Water research 2011; 45: 6097-6106.

Calisto V, Esteves VI. Psychiatric pharmaceuticals in the environment. Chemosphere 2009; 77: 1257-1274.
Carson R. Silent spring: Houghton Mifflin Harcourt, 2002.
Carvalho RN, Ceriani L, Ippolito A, Lettieri T. Development of the first watch list under the environmental quality standards directive. JRC Science Hub 2015.
Challis JK, Hanson ML, Friesen KJ, Wong CS. A critical assessment of the photodegradation of pharmaceuticals in aquatic environments: defining our current understanding and identifying knowledge gaps. Environmental Science: Processes & Impacts 2014; 16: 672-696.
Clark TD, Raby GD, Roche DG, Binning SA, Speers-Roesch B, Jutfelt F, et al. Ocean acidification does not impair the behaviour of coral reef fishes. Nature 2020: 1-6.
Conrad JL, Weinersmith KL, Brodin T, Saltz J, Sih A. Behavioural syndromes in fishes: a review with implications for ecology and fisheries management. Journal of fish biology 2011; 78: 395-435.
Dahlbom SJ, Lagman D, Lundstedt-Enkel K, Sundstrom LF, Winberg S. Boldness Predicts Social Status in Zebrafish (Danio rerio). Plos One 2011; 6: 7.
Daughton CG, Ternes TA. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environmental health perspectives 1999; 107: 907-938.
Davies NA, Hodson ME, Black S. The influence of time on lead toxicity and bioaccumulation determined by the OECD earthworm toxicity test. Environmental pollution 2003; 121: 55-61.
De Haan H. Solar UV-light penetration and photodegradation of humic substances in peaty lake water. Limnology and Oceanography 1993; 38: 1072-1076.
Dell'Omo G. Behavioural ecotoxicology: John Wiley & Sons, 2002.
Dixson DL, Munday PL, Jones GP. Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. Ecology letters 2010; 13: 68-75.
Drugs.com. Antibiotics Guide, 2020.
EMA E. Guideline on the environmental risk assessment of medicinal products for human use. EMEA/CHMP/SWP/4447/00. Committee for Medicinal Products for Human Use (CHMP), London, UK 2006.
FASS. Environmental classification of pharmaceuticals - Guidance for pharmaceutical companies, 2012.
Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. Aquatic toxicology 2006; 76: 122-159.
Fick J, Brodin T, Heynen M, Klaminder J, Jonsson M, Grabicova K, et al. Screening of benzodiazepines in thirty European rivers. Chemosphere 2017; 176: 324-332.
Freeze RA, Cherry JA. Groundwater, 1979.
Gómez-Laplaza L, Morgan E. The influence of social rank in the angelfish, Pterophyllum scalare, on locomotor and feeding activities in a novel environment. Laboratory animals 2003; 37: 108-120.
Gómez-Laplaza LM, Morgan E. Laboratory studies of the effects of short-term isolation on aggressive behaviour in fish. Marine & Freshwater Behaviour & Phy 2000; 33: 63-102.
Gunnarsson L, Jauhiainen A, Kristiansson E, Nerman O, Larsson DJ. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environmental science & technology 2008; 42: 5807-5813.
Halling-Sørensen B, Lützhøft H-CH, Andersen HR, Ingerslev F. Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. Journal of antimicrobial chemotherapy 2000; 46: 53-58.
Hamilton TJ, Holcombe A, Tresguerres M. CO2-induced ocean acidification increases anxiety in rockfish via alteration of GABAA receptor functioning. Proceedings of the Royal Society B: Biological Sciences 2014; 281: 20132509.
Hanson M, Wolff B, Green J, Kivi M, Panter G, Warne MSJ, et al. How we can make ecotoxicology more valuable to environmental protection. Science of The Total Environment 2017; 578: 228-235.
Heberer T. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology letters 2002; 131: 5-17.

Hellström G, Brodin T, Jonsson M, Olsen H, Leander J, Fahlman J, et al. Environmentally relevant concentrations of the common anxiolytic pharmaceutical oxazepam do not have acute effect on spawning behavior in mature male Atlantic salmon (Salmo salar) parr. Journal of Applied Ichthyology 2019.

Hellström G, Klaminder J, Finn F, Persson L, Alanara A, Jonsson M, et al. GABAergic anxiolytic drug in water increases migration behaviour in salmon. Nature Communications 2016; 7.

Hernando MD, Mezcua M, Fernández-Alba AR, Barceló D. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 2006; 69: 334-342.

Heuer R, Welch M, Rummer J, Munday P, Grosell M. Altered brain ion gradients following compensation for elevated CO 2 are linked to behavioural alterations in a coral reef fish. Scientific Reports 2016; 6: 33216.

Heynen M, Backström T, Fick J, Jonsson M, Klaminder J, Brodin T. Home alone—The effects of isolation on uptake of a pharmaceutical contaminant in a social fish. Aquatic toxicology 2016a; 180: 71-77.

Heynen M, Brodin T, Klaminder J, Jonsson M, Fick J. Tissue-specific uptake of the benzodiazepine oxazepam in adult Eurasian perch (Perca fluviatilis). Environmental Chemistry 2016b; 13: 849-853.

Heynen M, Fick J, Jonsson M, Klaminder J, Brodin T. Effect of bioconcentration and trophic transfer on realized exposure to oxazepam in 2 predators, the dragonfly larvae (Aeshna grandis) and the Eurasian perch (Perca fluviatilis). Environmental toxicology and chemistry 2016c; 35: 930-937.

Huerta B, Margiotta-Casaluci L, Rodríguez-Mozaz S, Scholze M, Winter MJ, Barceló D, et al. Anti-anxiety drugs and fish behavior: Establishing the link between internal concentrations of oxazepam and behavioral effects. Environmental toxicology and chemistry 2016; 35: 2782-2790.

Jaeger A, Posselt M, Betterle A, Schaper J, Mechelke J, Coll C, et al. Spatial and temporal variability in attenuation of polar organic micropollutants in an urban lowland stream. Environmental science & technology 2019; 53: 2383-2395.

Johnson AC, Jin X, Nakada N, Sumpter JP. Learning from the past and considering the future of chemicals in the environment. Science 2020; 367: 384-387.

Jolles JW, King AJ, Killen SS. The Role of Individual Heterogeneity in Collective Animal Behaviour. Trends in Ecology & Evolution 2019.

Jones KC, De Voogt P. Persistent organic pollutants (POPs): state of the science. Environmental pollution 1999; 100: 209-221.

Jonsson M, Andersson M, Fick J, Brodin T, Klaminder J, Piovano S. High-speed imaging reveals how antihistamine exposure affects escape behaviours in aquatic insect prey. Science of the total environment 2019; 648: 1257-1262.

Jonsson M, Ershammar E, Fick J, Brodin T, Klaminder J. Effects of an antihistamine on carbon and nutrient recycling in streams. Science of the Total Environment 2015; 538: 240-245.

Jonsson M, Fick J, Klaminder J, Brodin T. Antihistamines and aquatic insects: bioconcentration and impacts on behavior in damselfly larvae (Zygoptera). Science of the total environment 2014; 472: 108-111.

Kemper N. Veterinary antibiotics in the aquatic and terrestrial environment. Ecological indicators 2008; 8: 1-13.

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, et al. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences 2007; 104: 8897-8901.

Kidd KA, Paterson MJ, Rennie MD, Podemski CL, Findlay DL, Blanchfield PJ, et al. Direct and indirect responses of a freshwater food web to a potent synthetic oestrogen. Philosophical Transactions of the Royal Society B: Biological Sciences 2014; 369: 20130578.

Klaminder J, Brodin T, Sundelin A, Anderson N, Fahlman J, Jonsson M, et al. Long-term persistence of an anxiolytic drug (oxazepam) in a large freshwater lake. Environmental Science & Technology 2015; 49: 10406-10412.

Klaminder J, Hellström G, Fahlman J, Jonsson M, Fick J, Lagesson A, et al. Drug-Induced Behavioral Changes: Using Laboratory Observations to Predict Field Observations. Frontiers in Environmental Science 2016; 4.

Klaminder J, Jonsson M, Fick J, Sundelin A, Brodin T. The conceptual imperfection of aquatic risk assessment tests: highlighting the need for tests designed to detect therapeutic effects of pharmaceutical contaminants. Environmental Research Letters 2014; 9: 084003.

Klaminder J, Jonsson M, Leander J, Fahlman J, Brodin T, Fick J, et al. Less anxious salmon smolt become easy prey during downstream migration. Science of the Total Environment 2019; 687: 488-493.

Kosjek T, Perko S, Zupanc M, Hren MZ, Dragičević TL, Žigon D, et al. Environmental occurrence, fate and transformation of benzodiazepines in water treatment. Water research 2012; 46: 355-368.

Kowalczyk A, Martin TJ, Price OR, Snape JR, van Egmond RA, Finnegan CJ, et al. Refinement of biodegradation tests methodologies and the proposed utility of new microbial ecology techniques. Ecotoxicology and environmental safety 2015; 111: 9-22.

Krause J, Ruxton GD, Ruxton GD, Ruxton IG. Living in groups: Oxford University Press, 2002.

Kümmerer K. Antibiotics in the aquatic environment–a review–part II. Chemosphere 2009; 75: 435-441.

Kunkel U, Radke M. Biodegradation of acidic pharmaceuticals in bed sediments: insight from a laboratory experiment. Environmental science & technology 2008; 42: 7273-7279.

La Farre M, Pérez S, Kantiani L, Barceló D. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. TrAC Trends in Analytical Chemistry 2008; 27: 991-1007.

Lagesson A, Brodin T, Fahlman J, Fick J, Jonsson M, Persson J, et al. No evidence of increased growth or mortality in fish exposed to oxazepam in semi-natural ecosystems. Science of the Total Environment 2018; 615: 608-614.

Lam MW, Young CJ, Brain RA, Johnson DJ, Hanson MA, Wilson CJ, et al. Aquatic persistence of eight pharmaceuticals in a microcosm study. Environmental Toxicology and Chemistry: An International Journal 2004; 23: 1431-1440.

Li Z, Sobek A, Radke M. Flume experiments to investigate the environmental fate of pharmaceuticals and their transformation products in streams. Environmental science & technology 2015; 49: 6009-6017.

Lindberg RH, Östman M, Olofsson U, Grabic R, Fick J. Occurrence and behaviour of 105 active pharmaceutical ingredients in sewage waters of a municipal sewer collection system. Water research 2014; 58: 221-229.

Löffler D, Römbke J, Meller M, Ternes TA. Environmental fate of pharmaceuticals in water/sediment systems. Environmental science & technology 2005; 39: 5209-5218.

Longcore T, Rich C. Ecological light pollution. Frontiers in Ecology and the Environment 2004; 2: 191-198.

Loos R, Carvalho R, António DC, Comero S, Locoro G, Tavazzi S, et al. EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water research 2013; 47: 6475-6487.

Lu J, Liu J, Couzin ID, Levin SA. Emerging collective behaviors of animal groups. 2008 7th World Congress on Intelligent Control and Automation. IEEE, 2008, pp. 1060-1065.

Magnhagen C. Personalities in a crowd: What shapes the behaviour of Eurasian perch and other shoaling fishes? Current Zoology 2012; 58: 35-44.

Martin JM, Saaristo M, Tan H, Bertram MG, Nagarajan-Radha V, Dowling DK, et al. Field-realistic antidepressant exposure disrupts group foraging dynamics in mosquitofish. Biology letters 2019; 15: 20190615.

Massicotte P, Asmala E, Stedmon C, Markager S. Global distribution of dissolved organic matter along the aquatic continuum: Across rivers, lakes and oceans. Science of the Total Environment 2017; 609: 180-191.

Melvin SD, Petit MA, Duvignacq MC, Sumpter JP. Towards improved behavioural testing in aquatic toxicology: acclimation and observation times are important factors when designing behavioural tests with fish. Chemosphere 2017; 180: 430-436.
Meredith-Williams M, Carter LJ, Fussell R, Raffaelli D, Ashauer R, Boxall AB. Uptake and depuration of pharmaceuticals in aquatic invertebrates. Environmental pollution 2012; 165: 250-258.
Metcalfe N, Van Leeuwen T, Killen S. Does individual variation in metabolic phenotype predict fish behaviour and performance? Journal of fish biology 2016; 88: 298-321.
Mittelbach GG, Ballew NG, Kjelvik MK. Fish behavioral types and their ecological consequences. Canadian Journal of Fisheries and Aquatic Sciences 2014; 71: 927-944.
Moretz JA, Martins EP, Robison BD. Behavioral syndromes and the evolution of correlated behavior in zebrafish. Behavioral ecology 2007; 18: 556-562.
Muir D, Simmons D, Wang X, Peart T, Villella M, Miller J, et al. Bioaccumulation of pharmaceuticals and personal care product chemicals in fish exposed to wastewater effluent in an urban wetland. Scientific reports 2017; 7: 1-11.
Munday PL, Cheal AJ, Dixson DL, Rummer JL, Fabricius KE. Behavioural impairment in reef fishes caused by ocean acidification at CO2 seeps. Nature Climate Change 2014; 4: 487-492.
Munday PL, Dixson DL, McCormick MI, Meekan M, Ferrari MCO, Chivers DP. Replenishment of fish populations is threatened by ocean acidification. Proceedings of the National Academy of Sciences 2010; 107: 12930-12934.
Nakayama S, Doering-Arjes P, Linzmaier S, Briege J, Klefoth T, Pieterek T, et al. Fine-scale movement ecology of a freshwater top predator, Eurasian perch (Perca fluviatilis), in response to the abiotic environment over the course of a year. Ecology of Freshwater Fish 2018; 27: 798-812.
Nakayama S, Laskowski KL, Klefoth T, Arlinghaus R. Between-and within-individual variation in activity increases with water temperature in wild perch. Behavioral Ecology 2016: arw090.
Neu HC. The crisis in antibiotic resistance. Science 1992; 257: 1064-1073.
Niemela PT, Dingemanse NJ. On the usage of single measurements in behavioural ecology research on individual differences. Animal Behaviour 2018; 145: 99-105.
Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sørensen C, Watson S-A, et al. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. Nature Climate Change 2012; 2: 201-204.
Nriagu JO. Tales told in lead. Science 1998; 281: 1622-1623.
Nunes B, Gaio A, Carvalho F, Guilhermino L. Behaviour and biomarkers of oxidative stress in Gambusia holbrooki after acute exposure to widely used pharmaceuticals and a detergent. Ecotoxicology and environmental safety 2008; 71: 341-354.
Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, et al. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 2004; 427: 630-633.
OECD. Guidelines for the Testing of Chemicals, Section 2; Effects on biotic systems. 2020a.
OECD. Guidelines for the Testing of Chemicals, Section 3; Environmental fate and behaviour. 2020b.
Packer JL, Werner JJ, Latch DE, McNeill K, Arnold WA. Photochemical fate of pharmaceuticals in the environment: Naproxen, diclofenac, clofibric acid, and ibuprofen. Aquatic Sciences 2003; 65: 342-351.
Petit O, Bon R. Decision-making processes: the case of collective movements. Behavioural processes 2010; 84: 635-647.
Petrović M, Škrbić B, Živančev J, Ferrando-Climent L, Barcelo D. Determination of 81 pharmaceutical drugs by high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole–linear ion trap in different types of water in Serbia. Science of the total environment 2014; 468: 415-428.
Petrovska BB. Historical review of medicinal plants' usage. Pharmacognosy reviews 2012; 6: 1.
Posselt M. Transformation of Micropollutants in the Hyporheic Zone. Department of Environmental Science, Stockholm University, 2020.
Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, et al. Importance of a novel GABA A receptor subunit for benzodiazepine pharmacology. Nature 1989; 338: 582-585.

Rands SA, Cowlishaw G, Pettifor RA, Rowcliffe JM, Johnstone RA. Spontaneous emergence of leaders and followers in foraging pairs. Nature 2003; 423: 432-434.
Reale D, Reader SM, Sol D, McDougall PT, Dingemanse NJ. Integrating animal temperament within ecology and evolution. Biological Reviews 2007; 82: 291-318.
Retief FP, Cilliers L. Lead poisoning in ancient Rome. Acta Theologica 2006; 26: 147-164.
Richmond EK, Rosi EJ, Walters DM, Fick J, Hamilton SK, Brodin T, et al. A diverse suite of pharmaceuticals contaminates stream and riparian food webs. Nature Communications 2018; 9: 4491.
Robitaille AL, Webber QM, Vander Wal E. Conducting social network analysis with animal telemetry data: Applications and methods using spatsoc. Methods in Ecology and Evolution 2019; 10: 1203-1211.
Saaristo M, Lagesson A, Bertram MG, Fick J, Klaminder J, Johnstone CP, et al. Behavioural effects of psychoactive pharmaceutical exposure on European perch (Perca fluviatilis) in a multi-stressor environment. Science of the Total Environment 2019; 655: 1311-1320.
Sandheinrich MB, Atchison GJ. Sublethal copper effects on bluegill, Lepomis macrochirus, foraging behavior. Canadian Journal of Fisheries and Aquatic Sciences 1989; 46: 1977-1985.
Sanganyado E, Lu Z, Fu Q, Schlenk D, Gan J. Chiral pharmaceuticals: A review on their environmental occurrence and fate processes. Water research 2017; 124: 527-542.
Sartin JS, Perry HO. From mercury to malaria to penicillin: The history of the treatment of syphilis at the Mayo Clinic—1916–1955. Journal of the American Academy of Dermatology 1995; 32: 255-261.
Schaper JL, Posselt M, McCallum JL, Banks EW, Hoehne A, Meinikmann K, et al. Hyporheic exchange controls fate of trace organic compounds in an urban stream. Environmental science & technology 2018a; 52: 12285-12294.
Schaper JL, Seher W, Nützmann G, Putschew A, Jekel M, Lewandowski J. The fate of polar trace organic compounds in the hyporheic zone. Water research 2018b; 140: 158-166.
Sih A, Bell A, Johnson JC. Behavioral syndromes: an ecological and evolutionary overview. Trends in ecology & evolution 2004a; 19: 372-378.
Sih A, Bell AM, Johnson JC, Ziemba RE. Behavioral syndromes: an integrative overview. The quarterly review of biology 2004b; 79: 241-277.
Sih A, Cote J, Evans M, Fogarty S, Pruitt J. Ecological implications of behavioural syndromes. Ecology letters 2012; 15: 278-289.
Sprague J. Measurement of pollutant toxicity to fish—III: Sublethal effects and "safe" concentrations. Water research 1971; 5: 245-266.
Stalling DL, Mayer Jr FL. Toxicities of PCBs to fish and environmental residues. Environmental health perspectives 1972; 1: 159-164.
Stamps JA. Individual differences in behavioural plasticities. Biological Reviews 2016; 91: 534-567.
Stamps JA, Biro PA. Personality and individual differences in plasticity. Current Opinion in Behavioral Sciences 2016; 12: 18-23.
Sternbach LH. The benzodiazepine story. Journal of medicinal chemistry 1979; 22: 1-7.
Stumm-Zollinger E, Fair GM. Biodegradation of steroid hormones. Journal (Water Pollution Control Federation) 1965: 1506-1510.
Sui Q, Cao X, Lu S, Zhao W, Qiu Z, Yu G. Occurrence, sources and fate of pharmaceuticals and personal care products in the groundwater: a review. Emerging Contaminants 2015; 1: 14-24.
Sundin J, Jutfelt F, Thorlacius M, Fick J, Brodin T. Behavioural alterations induced by the anxiolytic pollutant oxazepam are reversible after depuration in a freshwater fish. Science of The Total Environment 2019; 665: 390-399.
Tanoue R, Margiotta-Casaluci L, Huerta B, Runnalls TJ, Eguchi A, Nomiyama K, et al. Protecting the environment from psychoactive drugs: Problems for regulators illustrated by the possible effects of tramadol on fish behaviour. Science of the Total Environment 2019; 664: 915-926.
Ternes TA. Occurrence of drugs in German sewage treatment plants and rivers. Water research 1998; 32: 3245-3260.

Topp E, Sumarah MW, Sabourin L. The antihistamine diphenhydramine is extremely persistent in agricultural soil. Science of the total environment 2012; 439: 136-140.
Toprak E, Veres A, Michel J-B, Chait R, Hartl DL, Kishony R. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. Nature genetics 2012; 44: 101.
Ullrich SM, Tanton TW, Abdrashitova SA. Mercury in the aquatic environment: a review of factors affecting methylation. Critical reviews in environmental science and technology 2001; 31: 241-293.
Vossen LE, Cerveny D, Österkrans M, Thörnqvist P-O, Jutfelt F, Fick J, et al. Chronic exposure to oxazepam pollution produces tolerance to anxiolytic effects in zebrafish (Danio rerio). Environmental Science & Technology 2019.
Vossen LE, Červený D, Sarma OS, Thörnqvist P-O, Jutfelt F, Fick J, et al. Low concentrations of the benzodiazepine drug oxazepam induce anxiolytic effects in wild-caught but not in laboratory zebrafish. Science of The Total Environment 2020; 703: 134701.
Walters E, McClellan K, Halden RU. Occurrence and loss over three years of 72 pharmaceuticals and personal care products from biosolids–soil mixtures in outdoor mesocosms. Water research 2010; 44: 6011-6020.
Wania F, Mackay D. Peer reviewed: tracking the distribution of persistent organic pollutants. Environmental science & technology 1996; 30: 390A-396A.
Warner RE, Peterson KK, Borgman L. Behavioural pathology in fish: a quantitative study of sublethal pesticide toxication. Journal of Applied Ecology 1966: 223-247.
Warnick SL, Bell HL. The acute toxicity of some heavy metals to different species of aquatic insects. Journal (Water Pollution Control Federation) 1969: 280-284.
Wasserman S, Faust K. Social network analysis: Methods and applications. Vol 8: Cambridge university press, 1994.
Webster E, Mackay D, Wania F. Evaluating environmental persistence. Environmental Toxicology and Chemistry: An International Journal 1998; 17: 2148-2158.
Wishart DS, Feunang YD, Guo AC, Lo EJ, Marcu A, Grant JR, et al. DrugBank 5.0: a major update to the DrugBank database for 2018. Nucleic acids research 2018; 46: D1074-D1082.
Wolf M, Weissing FJ. Animal personalities: consequences for ecology and evolution. Trends in Ecology & Evolution 2012; 27: 452-461.
Zhang N, Li J, Liu G, Chen X, Jiang K. Photodegradation of diclofenac in aqueous solution by simulated sunlight irradiation: kinetics, thermodynamics and pathways. Water Science and Technology 2017; 75: 2163-2170.

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