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REVIEW

Human and environmental risk assessment of pharmaceuticals: differences, similarities, lessons from toxicology

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Abstract The presence of human and veterinary pharmaceuticals in the environment has caused increasing concern due their effects on ecological receptors. Improving the risk assessment of these compounds necessitates a quantitative understanding of their metabolism and elimination in the target organism (toxicokinetics), particularly via the ubiquitous cytochrome P-450 (CYP) system and their mechanisms of toxicity (toxicodynamics). This review focuses on a number of pharmaceuticals and veterinary medicines of environmental concern, and the differences and similarities between ecological and human risk assessment. CYP metabolism is discussed with particular reference to its ubiquity in species of ecological relevance. The important issue of pharmaceutical mixtures is discussed to assess how emerging technologies such as ecotoxicogenomics may assist in moving towards a more mechanism-based environmental risk assessment of pharmaceuticals.

Keywords Ecological risk assessment · Human risk assessment · Cytochrome P-450 · Toxicokinetics · Toxicodynamics · Uncertainty factors · Ecotoxicogenomics

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Introduction

The growing occurrence of human and veterinary pharmaceuticals in the environment is causing increasing concern, and improving their ecological and human risk assessment constitutes a challenge for the scientific community [1]. Historically, each therapeutic class has been designed, for humans, mammals and poultry, to target specific organs, metabolic pathways and receptors resulting in the modulation of physiological functions of the organism so that a disease or infection can be treated and a healthy state restored. Of critical importance, the cytochrome P-450 (CYP) system forms the largest oxidative class of enzymes for the metabolism of xenobiotics (including pharmaceuticals and other environmental contaminants) and endogenous substrates. Over 6000 different CYP genes have been identified in animals, fungi, plants, protists, bacteria and archaea ([2], http://drnelson.utmem.edu/Cytochrome P450.html). One can speculate that these enzymes may be well conserved in evolution, showing a high degree of gene and protein homology between vertebrate families and species; however, in many cases qualitative and quantitative species-specific expression have been established [3]. The

identification of such species-specific CYP isoforms in ecological species and the consequence of metabolism seems to be a prerequisite for improving the risk assessment of pharmaceuticals, since CYP metabolism may moderate or induce toxicity. Also, environmental exposure to pharmaceuticals mostly involves complex mixtures. Quantitative metrics based on a mechanistic understanding of the potential interactions between these compounds and mixtures at the level of their metabolism and general elimination, including CYP metabolism (toxicokinetics) and how they may exert their toxicity at the target organ/cell/receptor (toxicodynamics), would be of great benefit to risk assessors [4]. Currently, the metabolism and potential toxicological effects of many pharmaceuticals, as well as their interactions, upon vertebrates and invertebrates, are unfamiliar [5, 6].

This review will, in the first instance, present a number of pharmaceuticals that have been shown to be of particular environmental concern, and then examine the differences and similarities between ecological and human risk assessment. The ubiquity of CYP metabolism will then be illustrated with examples from test species of ecological and human health relevance. Finally, the issue of mixtures will be explored to critically evaluate how future work may contribute to improving science-based risk assessment of pharmaceuticals during environmental risk assessment, with particular emphasis placed on ecotoxicogenomics.

Pharmaceuticals of environmental concern

Examples of human and veterinary pharmaceuticals of environmental concern are presented below for both aquatic and terrestrial organisms, but comprehensive accounts can be found elsewhere [5, 7–9].

Antidepressants and antiepiletics

Of all the pharmaceuticals released into the environment, the antidepressant fluoxetine has been shown to be one of the most potentially toxic human drugs to aquatic species. Phytoplankton has been shown to be the most sensitive group [9], with acute toxicity occurring at EC_{50} levels as low as 0.024 mg/l after 48 hours of exposure, and lethal concentrations (LC_{50} values) at 2 mg/l [10]. Recently, fluoxetine has been detected in tissues of fish species (*Lepomis macrochirus, Ictalurus punctatus, Cyprinus carpio* and *Pomoxis nigromaculatus*) residing in a municipal effluent-dominated stream in north Texas at levels of at least 0.1 ng/g [11]. Diazepam, a widely used antiepileptic, has also been graded as being potentially highly toxic to aquatic organisms, with acute toxicity occurring below levels of 100 mg/l [9].

Analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs)

Members of these therapeutic classes have been found to exert variable toxicity. The classic NSAID ibuprofen has exhibits toxic effects on riverine microbial communities at levels of 10 μg/l [12].

Paracetamol (acetaminophen) has been found at relatively high concentrations in surface water (up to 10,000 ng/l) [7], and it is mostly converted to inactive compounds by conjugation with sulfate and glucuronide, with a small portion being metabolized via CYP2E to a highly reactive intermediary metabolite, *N*-acetyl-*p*-benzo-quinone imine. This reactive metabolite is normally detoxified by conjugation with glutathione, however toxic concentrations can occur at high paracetamol doses through saturation of the glucuronide, sulfate and glutathione pathways. It has been shown to be highly toxic to domestic cats lacking glucuronidation, so the sulfation pathway saturates at relatively low doses [13]. However, effects of paracetamol on other Felidae have not been investigated in detail, probably because exposure to toxic concentrations would be unlikely in natural environments. This compound was found by the United States Department of Agriculture in trials to be effective at controlling local populations of the invasive brown tree snake (*Boiga irregularis*) in Guam. Brown tree snakes are a major invasive pest and have been responsible for the loss of at least 12 species of bird on Guam. Snake control uses rodent baits containing 40 mg of active paracetamol [14]. Due to the potential (but hitherto unexplored) hazard of paracetamol to non-target vertebrates, including birds, which are potential scavengers both of the snake carcasses and of the rodent baits, a comprehensive risk assessment was necessary for control of tree snakes using paracetamol. Risk to ground-dwelling species was removed by aerially suspending the baited rodents, whilst risk to Corvidae was reduced by enclosing the rodent baits within plastic pipes. Corvids were also observed to regurgitate paracetamol-contaminated food, which reduces their risk of intoxication when scavenging snake carcasses [15].

The NSAID diclofenac has acute toxicity to algae and invertebrates below concentrations of 100 mg/l [16]. Toxic effects for this drug have also been found in terrestrial vertebrates, and it has been implicated in catastrophic declines in the populations of three species of vultures, *Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris* in India and Pakistan [17, 18]. Exposure to diclofenac has been shown to be the main cause of these increasing numbers of deaths, as it causes renal failure and visceral

gout in vultures when the scavengers feed on the carcasses of domestic cattle treated with a normal veterinary dose of the drug shortly before death [19]. Lowest observed effect concentrations for diclofenac were low, 0.007 mg/kg in vultures, and resulted in toxic effects with renal failure [17]. Concern over the high toxicity of this NSAID led the Indian government to ban its use by September 2005, as diclofenac and the consequential loss of vultures has caused major ecological effects over the subcontinent and poses a potential threat to human health. The collapse of the vulture populations has benefited feral dog (*Canis familiaris*) and rat (*Rattus* species) populations due to the increases in carcasses usually preyed upon by the *Gyps* species. This raises public health and social concerns, as the risk of transmission of diseases such as rabies, increases tremendously, and in smaller Indian communities vultures are used to dispose of human corpses in burying rituals. Recenty, authors have suggested the importance of finding an alternative NSAID, and meloxicam was selected as a potential candidate. The compound was fed to the African white-backed vulture, *Gyps africanus*, documented as being susceptible to diclofenac toxicity, and Asian vultures at levels above the likely maximum level of exposure for wild populations. All birds survived with no obvious clinical effects, suggesting that meloxicam is of low toxicity to *Gyps* vulture species and would be an environmentally suitable and sustainable substitute for diclofenac [20].

The NSAID-related nephrotoxicity is well known in humans and the mechanism of toxicity is attributed to the pharmacological activity of the compounds through non-selective inhibition of cyclooxygenase (COX). This inhibition of cyclooxygenase impairs the synthesis of prostaglandins, which alters pathological processes that would normally impair a variety of renal functions. Application of more selective COX inhibitors, such as COX-2 inhibitors, were thought to provide a promising way to reduce these adverse effects; however, they equally appear to initiate nephrotoxicity [21].

Beta blockers

The beta blockers propranolol and metoprolol are strong membrane stabilisers [22] and show acute and chronic toxicity to aquatic organisms with lowest observed effects concentrations (LOECs) for growth and fecundity of 0.44 and 12 mg/l and 0.11 and 6 mg/l in *Daphnia magna*. LOECs of 0.055 mg/l and 3.1 mg/l were found for physiological biomarkers of heart rate following subchronic and acute exposures to propranolol and metoprolol, respectively, and these were associated with lower heart rate. These effects on the heart rate of *D. magna* suggest that both drugs exert sublethal toxicity at lower concentrations than observed in the classical endpoints, and reiterates the debate on endpoint choice in ecotoxicology [23].

Antimicrobial and antibiotic drugs

Antibiotics present in the environment can produce resistance in microbial assemblages, which can have potentially drastic effects upon human health. Resistance has already been identified in aquatic biota. Algal species have been identified as being particularly sensitive to fluoroquinolone and sulfonamide antibacterials, with NOECs (no observed effect concentrations) below 1 mg/l; in contrast, invertebrate species are much less sensitive [5]. Oxolinic acid was tested on *D. magna* and 48-hour EC_{50} values were 4.6 mg/l [24]. In fish species, there is little evidence for realistic concentrations of antibiotics causing adverse effects [25]. Ash et al. [26] carried out a study on water samples taken from streams in the United States of America and found evidence of bacterial resistance to imipenem, as well as to the beta-lactams ampicillin, cefotaxime and ceftazidime.

Synthetic estrogens

Many synthetic estrogens have been found in aquatic environments at biologically active concentrations. The pharmaceuticals Ethinylestradiol (EE2) and 17β-estradiol (E2) have been found at active concentrations (5 ng/l), and this is of potential concern for wildlife, particularly fish. These synthetic estrogens are widely used in the oral contraceptive pill, and enter aquatic environments via sewage treatment works [6]. When a breeding population of zebrafish were exposed to environmentally relevant levels of EE2, a 56% reduction was found in reproductive behaviour and fecundity [27]. Male rainbow trout have proved even more sensitive to these pharmaceuticals, and concentrations of only 0.1 ng/l have been shown to produce vitellogenesis [28]. The Japanese rice fish (*Oryzias latipes*) has also been shown to be highly sensitive to ethinylestradiol, with induced intersex being observed when exposed to levels of 0.03 mg/l [29].

The main active ingredients of the contraceptive pill are E2, estrone, and estriol (the natural estrogens), and it is these metabolites that have proven to be most polluting to the aquatic environment and that cause the resulting adverse affects upon the inhabiting organisms [30]. EE2 is a particularly potent endocrine modulator, and often found at biologically active concentrations [27], with even very low concentrations of around 1 ng/l or less resulting in possible adverse biological effects [6]. A significant reduction of up to 50% of fertilisation rate was observed in a study with zebra fish (*Danio rerio*) over a multigenerational study, highlighting the harmful effects that this

pharmaceutical can have when released into the environment [32].

Similarities and differences and between human and ecological risk assessment

Human and ecological risk assessment (HRA and ERA) both deal with the interaction of toxic substances with living organisms; the processes involved are similar for all biological receptors through basic building blocks (DNA, proteins, membranes, cells, etc.) and physiological processes (respiration, transport, signalling). This notion is reflected in the common terminology, theoretical concepts, toxicity measures (e.g. no observed effect levels or concentrations), disciplinary origins (biology and chemistry), and it provides the basis for the extrapolation of toxicity data, performed in both risk assessment approaches. Extrapolation also accounts for similar processes, i.e. interindividual variability, interspecies variability, differences in exposure time, differences in endpoints, potential synergistic effects, systematic errors, assumptions and random errors. Another similarity is that both HRA and ERA extrapolations have to deal with uncertainty.

Differences exist between the endpoints: protection of the individual (HRA) and the ecosystem (ERA) (with the exception of the protection of wildlife in ERA). Hence, HRA pharmacological and toxicological studies on pharmaceuticals would look at all potential adverse effects, whereas only relevant endpoints for the population level would be taken into account in ERA (e.g. growth rate, reproduction and lethality). This explains the special interest of ecotoxicology in pharmaceuticals that are potentially endocrine disrupters—they may influence parameters relevant to population survival, such as reproduction and moulting. Another consequence of these differences is that ERA deals with an extra level of biological integration, i.e. the ecosystem or community level, with the common axiom that protecting the most sensitive species protects the whole ecosystem (although it is often unfeasible to identify the latter) [33, 34].

As an alternative, two different species-to-system extrapolation techniques are often used:

- Divide the lowest available single-species toxicity value by an assessment factor, resulting in a value that is considered safe for the ecosystem. The value of the assessment factor depends on the measured toxicity endpoint and the availability of single-species toxicity data. The procedure is generally referred to as the assessment factor or AF approach [16].
- A species sensitivity distribution is fitted over the available single-species toxicity data and subsequently the p^{th} percentile (often the fifth percentile) of the distribution is determined. This is considered a safe exposure value for the ecosystem. The procedure is based on the assumptions that (1) the spectrum of species sensitivities can be accurately described by a statistical distribution (e.g. a lognormal or loglogistic distribution), and (2) the ecosystem is sufficiently protected if $1-p$ percent of the species is protected (if p represents the fifth percentile, this would imply that 95% of the species are protected). The procedure is generally referred to as the species sensitivity distribution or SSD approach [35].

Both approaches deal with interspecies variability in sensitivity. The AF approach uses a fixed set of assessment factors to cover interspecies variability (including the associated uncertainty). The SSD approach uses the standard deviation of the SSD as a data-specific estimate of interspecies variability.

Interspecies differences are assessed in HRA, as a proxy for the uncertainty in the extrapolation from test species to man, and this covers only four species (rat, mouse, dog and rabbit), a much narrower range than in ERA [33]. In HRA, chemicals are classified as genotoxic and nongenotoxic carcinogens, and their regulation by governmental bodies differs. The former are regulated using dose–response relationships from experimental animal data combined with low-dose extrapolation in order to relate a human health risk to an estimated exposure or an estimated exposure to a human health risk [36]. For nongenotoxic carcinogens, safe levels of exposure for food and water contaminants (expressed in mg/kg of diet or volume of water per day) have been traditionally derived using a threshold approach (i.e. assuming the existence of a threshold below which no toxicity occurs). Surrogates for the thresholds, such as the lowest and/or no observed adverse effect level (LOEL, NOEL) or the benchmark dose (BMD), are determined from chronic or subchronic animal studies using the most sensitive of the four species. These values are then divided by an uncertainty factor of a 100-fold to allow interspecies differences (tenfold) and human variability (tenfold) in the absence of substance-specific data [4]. The scientific validity of these factors has been assessed using a database quantifying interspecies differences and human variability in elimination (toxicokinetics, TK), such as liver weight, liver blood flow, renal blood flow, absorption, elimination, and sensitivity to a chemical in relation to toxicity (toxicodynamics, TD). The tenfold default factors could be further subdivided to allow for both TK and TD, and based on the database, values of $10^{0.6}$ (×4.0), $10^{0.4}$ (×2.5) and $10^{0.5}$ (×3.16) $10^{0.5}$ (×3.16) were derived for interspecies differences and human variability, respectively. The main advantage of this subdivision is to

allow chemical-specific TK and mechanistic data to contribute quantitatively to the derivation of uncertainty factors [37]. For example, when animal or human data on a particular chemical are available for the TK or TD aspect, the default factors can be replaced by a chemical-specific adjustment factor (CSAF), usually derived from a physiologically-based pharmacokinetic model (PB-PK) [4]. This is of particular relevance to pharmaceuticals, since extensive data are available about their metabolism and the molecular interactions involved in absorption, transformation, intoxication and elimination in both humans and test species [37, 38].

A recent approach has been developed using the pharmacokinetic and metabolism literature for pharmaceuticals to classify compounds according to their metabolic route so that pathway-related uncertainty factors can be derived for interspecies and human variability in toxicokinetics (TK) in order to replace the kinetic default uncertainty factor (3.16). These uncertainty factors were derived using probe substrates of the major human phase I metabolism (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, ADH, hydrolysis), phase II conjugation reactions (glucuronidation, glycine conjugation, *N*-acetylation (NAT), sulfate conjugation) and renal excretion. Common pathways for CYP1A2, glucuronidation and renal excretion between test species and humans exist [39–41]. High interindividual differences in TK were observed for chemicals metabolised by polymorphic routes of metabolism (CYP2D6, CYP2C19, CYP2C9), resulting in uncertainty factors larger (>20) than the current default uncertainty factor allowing for TK differences (3.16) to provide a sufficient degree of protection for individuals of the human population. Neonates have also been shown to be the most potentially susceptible subgroup and would not be covered by the current toxicokinetic uncertainty factor for any elimination pathway with available data (although reliable data are not currently available for polymorphic metabolism in this subgroup) [42–48; for reviews see 4, 38, 49]. Lower activities could translate into a greater susceptibility if the parent compound were the active toxicant, but lower susceptibility if the compound underwent metabolic activation. In the absence of data on the activity of the relevant pathway(s) of elimination in neonates and the consequences of metabolism (i.e. detoxication or activation), an extra assessment factor higher than that in adults for polymorphic metabolism (CYP2D6, CYP2C19, NAT) may be an option for risk assessors and risk managers to consider [38].

Quantification of interindividual variability is generally not performed in ERA, as the aim is to protect species. However, studies on interspecies and interindividual variability of pharmaceuticals in humans provide relevant information about potential effects of pharmaceuticals in the environment, i.e:

- The low enzyme activity in human neonates may well be a phenomenon which is conserved evolutionarily, stressing the importance of including early life stages in ecotoxicity tests.
- The high interindividual variability found for polymorphic metabolic pathways in humans also applies to other species such as rat and dog [50, 51]. The emphasis in ERA on the protection of species instead of individuals may thus result in the elimination of sensitive genotypes and ultimately in a reduction of genetic diversity within the species. If these polymorphisms are conserved evolutionarily—i.e. present in various species within the ecosystem—these may even result in the elimination of sensitive pathways from the system, eventually resulting in a loss of genetic diversity and resilience within the ecosystem.

In the past, relatively little attention has been paid to these similarities with regard to toxicity, mainly because of a lack of mechanistic understanding. In standard toxicity testing, the exposed organism was treated as a black box with the main parameters of interest being the external exposure, concentration and effect intensity, and this hampered the extrapolation between individuals and species, and resulted in much uncertainty. However, recent advances in molecular and biological sciences have improved our understanding, and substance properties, genes, proteins, molecular receptors, and transport, transformation and excretion pathways involved in toxicity are slowly being revealed. This creates the opportunity to improve extrapolations based on molecular and physiological similarities and differences between human and ecological receptors. The species barrier will become less important because of the increasing role of mechanistic information. Especially for pharmaceuticals, a wealth of toxicological information is available for humans and standard laboratory animals such as rat, dog and other vertebrates with regard to TK and metabolism such as phase I (including CYP enzymes) and phase II enzymes (conjugation reactions) [34]. A recent study has assessed the functional relationship between the environmental partitioning coefficient (Kd) and volumes of distribution determined from pharmacokinetic studies on 13 human pharmaceuticals in three model systems combining solid phases and solutions. Regression coefficients R^2 of 0.62–0.72 showed that the use of human data would be of great help when prioritising pharmaceuticals as environmental contaminants in risk assessment. The authors have proposed to explore these relationships for a wider range of compounds and environmental systems [52].

Ubiquity of CYP metabolism and potential impact for ecological risk assessment

Over the last 20 years, the availability of cell lines (expressing specific enzymes), liver microsomes and enzyme inhibitors have assisted scientists wishing to characterise CYP metabolism in vitro [53] in conjunction with clinical pharmacology trials to generate in vivo metabolism and excretion data [4]. The CYP superfamily, which has over 6000 different genes identified in animal, fungi, plants, protists, bacteria and archaea, constitutes the major oxidative system for most pharmaceuticals, environmental contaminants and endogenous compounds, and is associated with a strong hierarchy among animals, bacteria and fungi ([2], http://drnelson.utmem.edu/CytochromeP450.html). Hence, these enzymes are attractive candidates for developing molecular markers so that the fate of pharmaceuticals in the environment can be predicted (i.e. to identify the consequence of metabolism as a toxification/detoxification route) [54]. CYP enzyme contents have been reported to be quantitatively similar in chickens compared to some vertebrate species including humans, cats, pigs, snakes, frogs and trouts, but were three- to fourfold lower than those recorded in dogs, guinea pigs, hamsters, monkeys, mice, rabbits, rats, horses and ruminants [55].

The CYP3A subfamily is expressed in all vertebrates such as teleosts, diapsids, reptiles, birds, and mammals as the dominant CYP form in the digestive and respiratory tracts. In humans, this subfamily represents over 50% of the total CYP content. It has been shown to go through independent diversification with an ancestral single CYP3A vertebrate gene. For example, guinea pig CYP3A genes have diversified within the rodents whereas the rat, mouse, and hamster CYP3A genes are mixed among different rodent CYP3A subclades, indicating complex speciation and gene duplication processes [56]. The presence of CYP3A in the carp, the ball python (*Python regius*) and the harbour seal (*Phoca vitulina*) and grey seal (*Halichoerus grypus*) [56–58] are just a few other examples of the ubiquity of this family in vertebrates. In the future, ecotoxicologists and conservation biologists could develop molecular markers for CYP3A and other well conserved CYP isoforms such as CYP1A to improve the risk assessment of pharmaceuticals on wildlife [59].

Identification of CYP isoforms can also give scientists insights into the potential interaction between pharmaceutical and physiological functions. This is of particular relevance to fish and the aquatic environment since pharmaceuticals can enter rivers through sewage treatment discharges. The effect of synthetic estrogens was studied in the male sea bass (*Dicentrarchus labrax*) to identify their effects upon vitellogenin synthesis and hepatic phase 1 and 2 enzymes. Inhibition was observed for CYP1A-linked EROD and phase 2 gluthathione *S*-transferase, without affecting the CYP3A-linked enzymes. In this case, specific expression of CYPs and phase 2 enzymes are altered through exposure to endocrine disrupters, leading to potential adverse effects and reduced detoxification capability [60].

In rats, marked age and sex differences in the effect of E2 (10 μmol/kg daily for three days) on CYP3A were shown in neonates during development, with an increase in hepatic levels of the enzyme observed in both males and females, and with a greater effect seen in females (days 4–6). The same pubertal exposure also increased hepatic CYP3A activity, but only in females [61]. EE2 is also known to induce CYP3A9 in female rats [62]. The well conserved mechanism of induction of CYP3A involves the Pregnane X nuclear receptor, and this induction affects the sex hormone receptor indirectly to rapidly produce higher concentrations of active metabolites, leading to endocrine disruption [63].

In invertebrates, the expression of certain CYP enzymes and their concentrations have been been shown to be different even within species. Studies on two subalpine populations of *Daphnia pulex* revealed differences in the expressions of CYP4C32 and CYP4AP between the two populations, and the authors speculated that these may be due to polyphenol richness in vegetation surrounding the two populations. This study also highlights the fact that CYP4 can also be used as a potential molecular marker in the aquatic environment [54].

Ecological risk assessment of pharmaceutical mixtures

In terms of mixtures, inhibition or induction of a particular CYP isoform by members of the mixture may have toxicological consequences, and with the availability of in vitro techniques these fundamental mechanisms can be investigated routinely. Recently, in vivo changes in the area under the curve (AUC) of midazolam (reflecting clearance) after single intravenous or repeated oral administration of the CYP3A inhibitor itraconazole could be predicted with good accuracy by measuring in vivo AUCs, the in vitro maximum metabolic reaction velocity V_{max}, the Michaelis–Menten constant (K_m) for midazolam, the CYP3A liver content and the unbound concentration of itraconazole in the liver [64].

Sex differences in expression of CYP3A can be a challenge when investigating these types of interactions, since male rats express CYP2C11 isoforms whereas female rats express CYP3A isoforms more readily. Female rats pretreated with dexamethasone (inducing CYP3A) and then exposed to intravenous or oral midazolam and the inhibitor ketoconazole (orally) have recently been shown to provide

a good model for CYP3A drug–drug interaction in humans. In this study, midazolam metabolism after intravenous dosing remained unchanged after ketoconazole treatment, whereas oral clearance was reduced fivefold [65]. Similar effects of ketoconazole were also shown on CYP3A metabolism in the liver microsomes of seals [57]. These results show that hepatic first-pass metabolism of CYP3A substrates is compromised in the presence of inhibitors after oral administration due to the inhibition of CYP3A in the intestine. These situations are of particular relevance to pharmaceutical contamination in the wild, since exposure would occur mostly via the oral route.

Recent in vitro studies have also shown the inhibitory potency of selected pharmaceuticals (antidepressants, anti-inflammatory drugs, lipid regulators) on CYP enzymes and phase-2 glucuronidation in the carp. The experiments were conducted using liver subcellular fractions with substrate for each CYP isoform and the selected drug. Overall, antidepressants (fluoxetine, fluvoxamine, paroxetine) inhibited more than 90% of CYP1A activity (92–94% inhibition) and 70–80% of CYP3A-like activity, while anti-inflammatory drugs (ibuprofen, diclofenac, naproxen, ketoprofen) and lipid regulators (gemfibrozil) were potent CYP2M- like inhibitors (40 and 90%, respectively) and inhibitors of glucuronidation (50–90%). [58].

However, such investigations are limited since functional assays are mostly available for some vertebrates (but very few for wildlife) and basic knowledge on CYP isoforms in invertebrates is very scarce. A typical example in ecological species is the low acute ecotoxicity of the NSAIDs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid, (via nonpolar narcosis) in *Daphnia* (EC_{50} 68–166 mg/l) and algae (72–626 mg/l) for single compounds. However, prediction of mixture toxicity using the concentration addition model showed high toxicity of the mixture at concentrations of single compounds that would have no or very slight effects. Hence, a more accurate prediction of mixture toxicity is crucial to environmental risk assessment [66].

State-of-the-art technologies that have emerged from molecular biology and analytical chemistry, such as genomics, transcriptomics, proteomics and metabol(n) omics, hold great potential for the characterisation of new CYP isoforms at a fundamental level. These may also assist scientists to quantify changes in overall and specific CYP expression after exposure to mixtures and to quantify the toxicological consequences that these may have. The first wave of toxicogenomics projects focused on technical validation of methods [67]. Later larger scale initiatives generated vast databases of transcriptomic or metabol(n) omic profiles for species exposed to environmental toxicants and pharmaceuticals. The aim of these studies has been to identify single or more likely patterns of multiple molecular markers (gene expression or metabolite changes) that can be related to particular adverse histopathological effects (e.g. renal or hepatotoxicity) [68, 69].

To date these studies have focused on laboratory strains of rat, but application to wildlife remains a possibility. Indeed, some studies have already made this leap. For example, nuclear magnetic resonance spectroscopy-based metabol(n)omics has been applied to identify small metabolite markers of histopathological effect following arsenate exposure in the wild mammal species *Clethrionomys glareolus* [70]. In this study, the metabolomics approach was particularly favored since there were no analytical restrictions on measuring low molecular weight metabolites due to the fact that there was limited genomic information available for the species. For transcriptomics studies, the presence of existing sequence resources means that it is already feasible to conduct a limited set of cross-species experiments [71]. As sequencing projects (both genome and expressed sequence tag) continue to diversify to cover species across the full diversity of life [72], such studies should become more integrated, offering a greater opportunity to identify the most vulnerable species and populations.

As well as biomarker identification, another great hope for ecotoxicogenomics is that it can answer questions regarding the mode of action of compounds. In their intended species, pharmaceuticals have usually been designed with a specific biochemical target in mind, and when they reach the environment, the biochemistry of the exposed species may not recognize the molecular target. In these cases, determining the mode of action may be difficult. In some cases, a particular compound with a known specific mode of action in the target species will show only baseline toxicity linked to membrane disruption while these effects may be specific in another species through interaction with the intended molecular target. For risk assessment of mixtures in particular, knowledge concerning mode of action is vitally important. This is because the choice of which of the two main models for mixture toxicity to apply is driven by prior knowledge of mode of action. Thus, concentration addition is applied for chemicals with the same mode of action, while independent action (response addition) is applied when compounds act differently.

Initial studies that have used toxicogenomics to investigate mode of action have already taken place. As outlined above, toxicogenomic methods have been applied to identify molecular signatures of renal and hepatic tissue damage in rat [68, 69] and microarrays have also been used to discriminate between the effects of genotoxic and nongenotoxic carcinogens [73]. In environmental species, microarrays have been used to identify toxicant-specific gene expression changes that are characteristic of putative modes of action for different toxicants in rainbow trout. In earthworms, Bundy et al. [74] used a metabol(n)omic

approach to separate the metabolic effects of three fluoroanilines. This separation of metabolic profiles was likely to represent different biochemical effects of the three compounds characteristic of their particular, and different, modes of action. In this latter example, the potential value of the toxicogenomics approach is clear: Any joint assessment of mixture effects for these compounds would have employed the concentration addition model for mixture toxicity, since the compounds used were all representative of the same groups. In fact, as the compounds have separate modes of action, it is probable that the independent action model would be more appropriate.

The identification of mode of action is an example of the potential value of the toxicogenomics approach in risk assessment. As illustrated in Fig. 1, if the analysis of the transcription products for the metabolites (transcriptomics) of a species indicates overlap of response profiles (e.g. for compounds A and B), this suggests these chemicals have the same biochemical effects and so any joint assessment of mixture effects for these compounds should employ the concentration addition model for mixture toxicity. In contrast, the separation of response profiles (e.g. for compounds A and C and compounds B and C) suggests different biochemical effects and so any joint assessment of mixture effects for mixtures of these compounds should employ the independent action model. In this case, which is representative of the situation found in earthworms exposed to different fluoroanilines by Bundy et al. [74], the toxicogenomics approach (using metabonomics) increases the probability of employing the correct model for mixture assessment, thus improving both the accuracy and validity of the risk assessment.

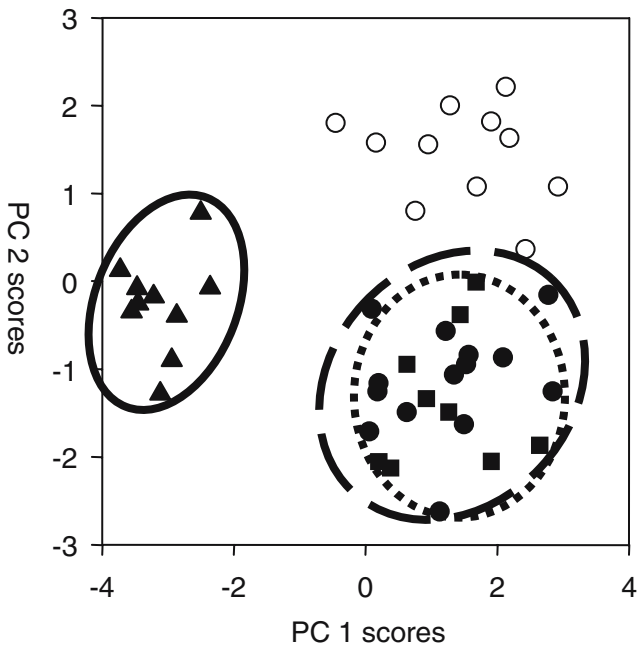

Fig. 1 Theoretical example of the outcome of a pattern-recognition-based analysis (e.g. a principal component analysis scores plot) for transcriptomic or metabolic data for a species exposed to three chemicals (compound A (●), compound B (■), compound C (▲), and control (○)). Examples show the separation of response profiles from the control in all cases, but the overlap of responses for chemicals 1 and 2 suggests the same mode of action, while the separation for chemical C suggests a different mode of action from the other two compounds

Conclusion

The presence of human and veterinary pharmaceuticals in the environment is of increasing concern due to their effects on ecological receptors, and the scientific community is developing new tools and methods to improve their risk assessment. The EU project *Environmental risk assessment of pharmaceuticals* (ERAPharm) aims to develop such methods under the priority "Global change and ecosystems" of the Sixth Framework Programme of the European Union [75]. Moving to a more science-based ecological risk assessment for these substances requires the use of substance-specific and species-specific data on metabolism and fate as well as mechanisms of toxicity, although this requires substantially more information than currently available. The CYP enzymes are of critical importance, since they metabolise most pharmaceuticals and can be used as molecular markers in wild species to identify the consequences of metabolism [detoxification or production of toxic metabolite(s)]. Interspecies and intraspecies differences in qualitative and quantitative CYP expression should be considered in order to identify sensitive species and avoid ecological disasters such as the decline of vultures in Pakistan and India due to diclofenac toxicity. CYP genetic polymorphism is a source of uncertainty in human risk assessment and polymorphism has also been shown in dogs for the metabolism of the COX-2 inhibitor celecoxib [50] and diazepam in the rat [51]. However, the consequences of such polymorphism on the sensitivity of ecological species to pharmaceuticals have not been assessed to date. Of a complex nature, but of high relevance, is the assessment of ecological risk in relation to pharmaceutical mixtures. In this case, risk assessment of environmentally relevant mixtures would benefit from the use of quantitative metrics based on a mechanistic understanding of the potential interactions between the components at the toxicokinetic and toxicodynamic levels.

New technologies have emerged to assist scientists in dissecting these mechanisms, such as ecotoxicogenomics, probabilistic methods and quantitative structure–activity relationships, and these may be of great value not only to ecological risk assessment but also to human risk assessment of pharmaceuticals in drinking water [76]. Such issues are currently being investigated in the Sixth Framework EU

project NOMIRACLE (Novel Methods for Integrated Risk Assessment of Cumulative Stressors in Europe http://viso.jrc.it/nomiracle/), which aims to develop new methods to improve the risk assessment of mixtures including pharmaceuticals. Moreover, options have been explored to harmonise the derivation of uncertainty factors used in human and ecological risk assessment [33, 34].

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