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Toxicology and Risk Assessment of Pharmaceuticals

Daniel R. Dietrich[1], Bettina C. Hitzfeld[2] and Evelyn O'Brien[1]

[1]Environmental Toxicology, University of Konstanz, Germany; [2]Swiss Agency for the Environment, Forests and Landscape, Bern, Switzerland

1 Introduction

For many years, the main focus of environmental pollution has been on chemical and pharmaceutical manufacturers. Spectacular accidents, such as that at the BASF facility in Ludwigshafen, Germany in 1948, the Union Carbide plant in Bhopal, India in 1984 and the Sandoz facility in Schweizerhalle, Switzerland, 1986, amongst others, provided the impetus for improved safety and spillage regulations as well as driving the efforts for environmental modeling and improved analysis of environmental samples. More recently, attention has turned to the far less dramatic, but nonetheless important consideration of the potential effects of medication residues on the aquatic environment. As a complete analysis of national and international regulations governing all polar organic pollutants is beyond the scope of this chapter, only regulations and concerns with specific relevance for pharmaceuticals will be discussed here. Waste Water Treatment Plants (WWTPs) [1] have been identified as the major point source of pharmaceuticals in surface waters, but diffuse intake via run-off can also be an important route for veterinary pharmaceuticals [2]. The realization of the latter situation has led to a parallel increase in the number of surveys designed to investigate the levels of contamination of surface waters and attempts to characterize the potential risks posed by these substances to the aquatic environment [3-7]. These studies have led to the recognition that while the concentrations in the WWTPs are generally related to the population equivalents of the respective region and the highest concentrations of pharmaceuticals are measured at sites where WWTP effluent is introduced into the receiving water body, the concentration of the substances found downstream of the WWTPs is a function of the tributary rivers that can cause either an increase or a decrease in the concentration of a given substance in the main river, depending on their own pre-load [1]. While many pharmaceutically-active substances can indeed be broken down in modern sewage treatment plants, by their very nature and, perhaps more importantly, due to their continual infusion into the environment, several of them can be classified as persistent polar organic pollutants. The efficiency of the removal of pharmaceuticals from wastewater in WWTPs can vary based on a number of factors including the population served, precipitation volumes, ambient temperature and nutrient loads. The particular substance classes delivered in sewage to the WWTP can also

influence the extent of substance removal. For example antimicrobials and antineoplastics in sewage are by their very nature toxic to the microbes present in the sludge. Therefore, some pharmaceuticals not only reduce the efficiency of their own removal, but can also impair the removal of all other substances. Metabolically conjugated derivatives of pharmaceuticals can be released from WWTPs and as such, are not detected/accounted for in the determination of the concentrations of the parent compounds. Consequently, unless metabolically converted parent compounds have been specifically investigated and analytically accounted for, the absolute concentration of pharmaceuticals can be higher than anticipated with present analytical schemes. Furthermore, as many of these conjugated compounds can be converted back to the parent substance, any derived environmental effect or risk assessment should consider the concentrations of both the parent compound and of reversibly-conjugated parent compounds.

In recent years, over eighty compounds have been found by researchers in rivers, lakes and sediments in several countries [8] and it is likely that the number of compounds found will rise exponentially with improvements in analytical methods. Despite the plethora of reports of pharmaceuticals being found (a summary of these can be found in an article by Daughton and Ternes published in 1999 [9]), these reports represent only a fraction of the pharmaceuticals on the market and most agents have not as yet been investigated. Data on maximal environmental concentrations measured in surface waters is not available for every country or in certain cases is not in the public domain. For example, data to the occurrence and concentration of several common surface water contaminants could be found for Germany but not for French surface waters and vice versa [10]. This dearth of information in the public domain makes risk assessment dependent on the availability of correct estimates, with respect to market proportion and total amounts produced, from the manufacturer.

Environmental risk assessment is usually performed in a tiered fashion as described in the following sections and shown in Figures 3, 4 and 5 and requires data on biological effects and on expected substance exposure levels. Exposure assessment looks at the release and fate of the substance into the environment and determines a predicted environmental concentration (PEC). The effect is extrapolated from the lowest NOEC of toxic effects in three trophic levels (algae, *i*nvertebrate, fish) with the inclusion of safety factors (usually a total of 1000 is assumed). In an initial assessment, (first tier), a PEC/PNEC ratio is calculated, where PNEC is the predicted no effect concentration, and the decision on the safety of a substance is based on this ratio: If the PEC/PNEC ratio is <1, then it is assumed that the substance poses little environmental risk. If the PEC/PNEC ratio is >1, then a risk is assumed and a refined risk assessment is usually performed. In an effort to collate data on PEC/PNEC ratios, Webb [11] collected data on consumption of pharmaceuticals in order to derive predicted environmental concentrations. No metabolism and 100% loss to drain derived from the use in humans, no removal in wastewater treatment plants and no dilution in surface waters were assumed. This very crude assessment showed that of all 60 investigated drugs, which account for about 50% of known pharmaceutical usage in tonnes, all but eight have

PEC/PNEC ratios less than one. The eight substances were paracetamol (analgesic, antipyretic; PEC/PNEC 39.92), aspirin (analgesic, antipyretic, antiinflammatory; PEC/PNEC 1.00), dextropropoxyphene (narcotic analgesic; PEC/PNEC 2.06), fluoxetine (antidepressant; PEC/PNEC 14.19), oxytetracycline (antibiotic; PEC/PNEC 26.8), propanolol (antihypertensive, antianginal, antiarrhythmic; PEC/PNEC 1.16), amitriptyline (antidepressant; PEC/PNEC 1.29) and thioridazine (antipsychotic; PEC/PNEC 2.59). Based on the current draft EU technical guidance document (see below), a refined risk assessment would therefore be advised for these compounds. Taking into account elimination in wastewater treatment and dilution in surface water (a factor 10 is normally assumed) would in most cases result in refined PEC/PNEC ratios below one. Paracetamol, for example, is eliminated by up to 98% in wastewater treatment plants, mostly by biodegradation, and this would lead to a refined PEC/PNEC ratio of 0.08. Furthermore, paracetamol is usually not detected in surface waters and the calculation of a MEC/PNEC ratio (where MEC is the maximum environmental concentration) was given as <0.02. Equally, for all of the other eight substances such a refined risk assessment would result in PEC/PNEC ratios below one.

A similar study using a slightly different approach (no metabolism in man, no elimination in sewage treatment, but 10-fold dilution factor in surface waters) came to similar conclusions [12]. In this study, assessment of paracetamol (PEC/PNEC 1.29), amoxycillin (PEC/PNEC 588.02), oxytetracycline (PEC/PNEC 3.60), diclofenac (PEC/PNEC 3.16) and mefenamic acid (anti-inflammatory; PEC/PNEC 1.03) gave rise to PEC/PNEC ratios >1 when using the most sensitive endpoint in acute toxicity testing. Of course, acute endpoints are not very sensitive especially when considering compounds with specific mechanisms as discussed below.

Unfortunately, aquatic toxicity data have not been published for the majority of those substances, which have been confirmed to be present in the aquatic environment. However, some theoretical estimation of possible toxic interactions can be gleaned from the comparison of mammalian toxicity data (kinetics and dynamics) with human therapeutic plasma levels and those expected to occur in fish plasma [13]. Huggett and co-workers used the ratio of human therapeutic plasma concentration to steady state fish plasma concentration as an indicator of potential interaction in fish, whereby the lower the ratio the higher the likelihood of a possible long-term effect in fish upon chronic exposure. Although such calculations are initially helpful, only experimental validation can strengthen the prediction capability of such estimations. In view of the paucity of data available for such model estimations, current risk assessment strategies rely on much cruder risk estimation schemes for prioritizing pharmaceuticals and are primarily dependent on a drug-by-drug detailed evaluation of environmental risk as well as on rather arbitrary non-scientifically validated cut-off or decision “triggers”. In the following sections the different risk assessment procedures under discussion or already implemented in various countries and regions will be presented. The European and Canadian systems are summarized and specific differences between these and the U.S. and Japanese regulations are outlined.

2 A Comparison of International Risk Assessment Procedures

The European Union Technical Guidance Document (TGD)

A council directive of the European Commission (2001/83/EC) stipulates that an environmental risk assessment (ERA) be carried out before marketing authorization can be given for medicinal products for human use [14]. In contrast to the Canadian system (see below), this regulation deals with the use, storage and disposal of the product and synthesis and manufacture are considered by separate legislation, as are veterinary products. Furthermore, whereas the Canadian regulations govern new substances and medications with new indications, the guidelines of the EU require the generation of an ERA for new substances, those with new indications (type two variations) and for existing active pharmaceutical ingredients (APIs) with a specific mode of action, effectively including all pharmaceutically-active substances. The two-phased stepwise procedure for the generation of this ERA is described be the "Guideline on Environmental Risk Assessment of Medicinal Products for Human Use", [15] written by the European Agency for the Evaluation of Medicinal Products, which was re-released in draft form for consultation in January 2005.

In the draft guidance document of the European Union, phase I aquatic risk assessment begins with an estimation of the concentration to which aquatic organisms may be exposed. This is generally expressed as risk quotient of either the PEC or MEC, and the PNEC. If, as briefly described above, this ratio exceeds one, then an ecological risk is assumed. Worst-case scenarios involving no human metabolism and no degradation within the WWTP of the compound under review are generated. The total population of the country in question is factored into the equation as well as the estimated daily production of wastewater per head of population and the market penetration (fpen) of the compound. The so-called predicted environmental concentration in surface water ($PEC_{surfacewater}$) is calculated based on several factors as indicated in figure 1. An even distribution of product use throughout the year and throughout the geographic area are assumed.

$$PEC_{surface\ water} = \frac{DOSEai * Fpen}{WASTEinhab * DILUTION * 100}$$

Figure 1: Formula for $PEC_{surfacewater}$ estimation. DOSEai: maximum daily dose of active ingredient per inhabitant; Fpen: percentage of market penetration (defaults to 1% based on a survey of approx 800 APIs currently marketed); WASTEinhab: amount of wastewater per inhabitant per day, (defaults to 200 l/inh./day); DILUTION: dilution factor, (defaults to 10).

If the initial calculated $PEC_{surfacewater}$ is less than 0.01 μg/L, then the substance is considered not to represent a risk to the aquatic environment and no actual toxicity testing is required. A $PEC_{surfacewater}$ greater than 0.01 μg/L makes the compound subject to phase II analysis. Based on this formula and the associated defaults, the maximum dosage not resulting in a $PEC_{surfacewater}$ of greater than 0.01 μg/L can be calculated as 2 mg/patient/day. Substances with a PEC of greater than 0.01 μg/L proceed to phase II, tier A of the assessment procedure (see Figure 3). However these action limits are not applicable when an expert evaluation of preclinical safety and ecotoxic potential suggests potential atypical ecotoxic effects. In phase II tier A, environmental fate and effects testing are required and the PEC must be refined using substantiated information on predicted use and market share. This assessment should include physico-chemical parameters such as water solubility, K_{ow}, vapor pressure etc. as well as information pertaining to the biodegradability, photolysis, hydrolysis and aerobic and anaerobic transformation potentials. These tests should be carried out in accordance with the relevant OECD guidelines as outlined in Table 1. A standard base set of acute aquatic toxicity data at three trophic levels (algae, OECD 201; daphnia, OECD 211; fish early life stage, OECD 210 and activated sludge respiration, OECD 209) is also required in order to generate the PNEC via the application of an assessment factor (AF) to the lowest determined LC_{50}, EC_{50} or NOEC value. AFs are applied in order to account for the extrapolation from acute to chronic toxicity, interspecies variations in sensitivity, intraspecies variability and the extrapolation from laboratory to field scenarios. Generally, when just the basic set of acute toxicity data is available, an AF of 1,000 should be applied, however, when more information on the effects of long-term exposure is available, the AF may be as low as 10.

$$PNEC_{surfacewater} = \frac{\text{Lowest acute } LC_{50} \text{ or } EC_{50} \text{ or NOEC}}{AF}$$

Figure 2: Formula for $PNEC_{surfacewater}$ estimation

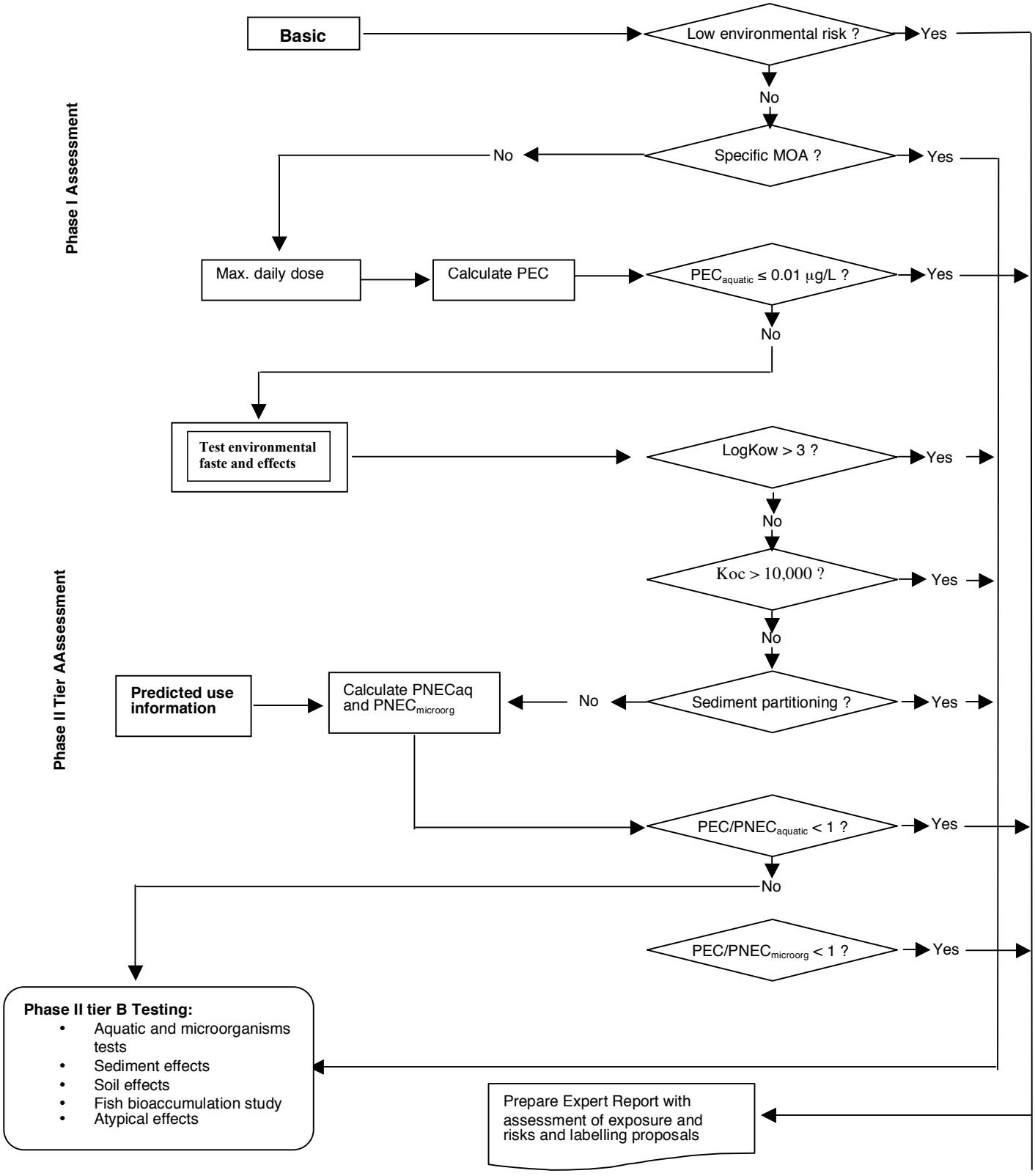
Tier A also requires the estimation of the $PNEC_{microorganisms}$, as substances which are toxic to microorganisms are likely to have detrimental effects on the potential for substance removal in the WWTP. A $PEC_{surfacewater}$: $PNEC_{microorganism}$ ratio of greater than one, results in a requirement of tier B testing. If, on the other hand neither $PEC_{surfacewater}$: $PNEC_{microorganism}$ nor $PEC_{surfacewater}$: $PNEC_{aquatic}$ are greater than one, and the $\log K_{ow}$ is less than three, it is assumed that the substance is unlikely to have a negative impact on the aquatic environment and no further testing is required. A K_{oc} of greater than 10,000, signifying a high affinity of the substance for sewage sludge indicates a need for an assessment of the substance in the terrestrial environment. Such testing schemes are also outlined in the TGD, but will not be dealt with further in this chapter. Details are available under http://www.emea.eu.int. Tier B of phase II of the risk assessment process aims to assess which compartments of the environment are particularly at risk and employs specific test systems to assess the individual compartments (biota, microorganisms, aquatic organisms, terrestrial organisms and sediment dwellers). Tier B furthermore, considers human and environmental metabolites if they constitute more than 10% of the API.

Data required/Test	OECD Guideline
Water solubility	105
Dissociation constant	112
UV-visible adsorption spectrum	101
Melting temperature	102
Vapor pressure	104
K_{ow}	107 or 117
Adsorption-desorption using batch equilibrium method	106
Ready biodegradability	301
Aerobic and anaerobic transformation in aquatic sediment systems	308
Photolysis (optional)	Seek regulatory guidance or use OECD monograph no. 61
Hydrolysis as a function oh pH (optional	111

Table 1: Set of physico-chemical data and fate studies required at the start of phase II, tier A of the EU Technical Guidance Document.

Perhaps two of the most critical aspects of the ERA procedure currently proposed by the EU are the use of a cut-off approach based on the predicted environmental concentration and the use of acute toxicity testing to generate the predicted no-effect concentration of a substance. Although these premises at an initial glance appear logical, closer examination reveals several problems. The former dictates that if the PEC exceeds 0.01μg/L, a complete assessment of the potential of a substance to be problematic must be carried out. The majority of PECs overestimate the concentrations of pharmaceutically-active substances reaching the aquatic environment by approximately one order of magnitude, although the PECs of substances may also indeed be greatly underestimated due to the availability of the active ingredient in other formulations and in over-the-counter preparations. One notable exception is the finding that estrogen has been detected at concentrations of 130μg/L in an area where a PEC, based on prescription contraceptives, of just 1ng/L had been predicted [16]. The higher concentration was probably due to a combination of the excretion of natural estrogen by humans combined with estrogens of animal origin. These examples illustrate the manifold uncertainties in this approach. In addition, the relationship between the PEC and the maximal environmental concentrations (MEC = measured ec) in either treatment plant effluent (MEC_{effl}) or in surface water (MEC_{sw}), for any given substance is dependent on the particular sewage treatment processes employed, with longer retention times and more complicated processes generally resulting in a lower delivery of pharmaceutically active compounds to the aquatic environment. While this leads to a general increase in the level of safety built into the risk assessment, it may result in unnecessary testing of compounds, which are in fact harmless.

Figure 3: Schematic of the environmental risk assessment proposed in the EU-TGD


Phase I Assessment
Basic
Low environmental risk ?
Yes
No
Specific MOA ?
No
Yes
Max. daily dose
Calculate PEC
PEC aquatic ≤ 0.01 µg/L ?
Yes
No
Test environmental faste and effects
LogKow > 3 ?
Yes
No
Koc > 10,000 ?
Yes
No
Phase II Tier AAssessment
Predicted use information
Calculate PNECaq and PNEC microorg
No
Sediment partitioning ?
Yes
PEC/PNEC aquatic < 1 ?
Yes
No
PEC/PNEC microorg < 1 ?
Yes
Phase II tier B Testing:
Aquatic and microorganisms tests
Sediment effects
Soil effects
Fish bioaccumulation study
Atypical effects
Prepare Expert Report with assessment of exposure and risks and labelling proposals

Stuer-Lauridsen and co-workers attempted to carry out a risk assessment for the 25 most used human pharmaceuticals in Denmark [16]. In this study, the authors applied the criteria proposed by the EU draft guideline document for new pharmaceuticals. Briefly, a risk quotient was generated using the PEC and PNEC and a safety factor of 1000 (AF = 1000) was employed in the generation of the PNECs. Using this approach, all of the evolving PECs exceeded 0.01µg/L, making all of these substances candidates for phase II testing under the proposed EU regulations. Interestingly, several compounds, for which estimates indicated an extremely high likelihood of relevant concentrations in WWTP effluent have to date not been detected in the environment [17-19]. Two possible explanations exist for this - either 1) the substance in question cannot as yet be detected in water bodies at environmentally relevant concentrations or 2) the substance is not to be found in the aquatic environment, regardless of the calculated predictions. The latter would call into question the validity of using estimates to determine the "safe" concentrations.

Lack of relevant data on the toxicity of most pharmaceuticals to aquatic organisms as well as of data pertaining to environmental concentrations of these substances, logically prevents the calculation of risk quotients based on actual measurements. At best, only predictions can be made. This is exacerbated by the lack of standardized analytical methods for the detection of these substances at environmentally relevant concentrations, as already mentioned.

A further problem arises in when one attempts to assess potential chronic effects of pharmaceuticals in the aquatic environment. The use of the maximum suggested AF of 1000 to account for acute to chronic extrapolation is, in many cases, not sufficient as acute to chronic ratios (ACRs) ranging between 0.79 to 5,000 have been demonstrated within a single species [20,21]. This becomes even more critical when one considers certain hormonally active substances where ACRs may be far greater. Indeed an ACR of 800,000 for ethinylestradiol in rainbow trout was described by Webb [22], clearly illustrating that little if any useful information for the extrapolation of potential chronic toxicity, can be gleaned from acute toxicity data. This further highlights the problems associated with using cut-off triggers and ratio approaches rather than improved scientific understanding of potential toxicological effects. Fortunately, the new release of the EU Draft guideline strongly embraces expert evaluation of mammalian toxicokinetic and toxicodynamic data as a means for deriving potential ecotoxic effects. Indeed, as many enzyme/receptor systems are highly conserved across the different phyla, specific target interactions of given pharmaceuticals in species other than the humans are to be expected, although the dynamically required doses and the array of effects may be different. Indeed, the reported nephrotoxic effects of diclofenac in rodents and other mammals, was also demonstrated to occur in fish [23-25], albeit at much lower i.e. close to environmentally relevant concentrations. Indeed, despite that the PEC calculations using the EU guideline provisions predicted an environmental concentration of 0.54 µg diclofenac/L, reported values of diclofenac, with a $\log K_{ow}$ of 4.51, reach concentrations

of up to 1.2 µg/L and higher [26]. The no observed effect concentration (NOEC) in the study by Hoeger *et al*. [23] was determined to be 0.5 µg/L for monocyte infiltration/accumulation in livers of brown trout exposed for 21 days, although mild effects were seen in two of six animals in the 0.5 µg/L group.

The calculation of a PNEC requires the application of an assessment factor of 10 to account for inter-/intraspecies variations and extrapolation from laboratory data to field impact. The "Draft Guideline on the Environmental Risk Assessment of Medicinal Products" [15] states, however, that the application of an AF of 1000 on acute data will not be protective for pharmaceuticals, especially as the acute to chronic calculations have been demonstrated to show extreme divergence in predictive capability within one species and even poorer prediction for multiple species. Based on this it is considered justified to base the PNEC not on acute, but rather on chronic data. As the data from Hoeger *et al*. and Schwaiger and co-workers illustrate, [23-25] neither present an acute nor a chronic exposure scenario but rather a subchronic situation, an AF of 100 would most likely be an acceptable calculation scenario. A PNEC of 0.005 µg/L (NOEC/100) would therefore be derived as a conservative scenario. In conjunction with a PEC of 0.54 µg/L, this results in a PEC/PNEC ratio of >100 calling for further investigations and a refined risk assessment for this substance. As diclofenac is a cycloxygenase inhibitor, pharmaceuticals of the same effect class should be investigated conjointly as single entities as well as mixtures rather than individually for risk assessment purposes. A similar premise should be applied to other pharmaceutical classes.

US-EPA

The environmental risk assessment process in the U.S.A., which has been in existence and under constant review since 1977 under the auspices of the National Environmental Policy act of 1969, begins with a new drug application, which is submitted to the FDA [27]. Many of the steps involved are similar to the proposed European scheme and also display similarities to the Canadian registration system, thus only major differences will be outlined in this section. Within the application, the manufacturer is required to provide an estimate of the amount of the drug entering the environment. This is termed the "expected introductory concentration" (EIC) and is based on total five-year production estimates. Virtually all of the environmental assessments of pharmaceuticals in the US have resulted in a "finding of no significant impact" (FONSI), which had been set with a cut-off threshold of 1 µg/L. This threshold was derived from the fact that no effects were observed on standard environmental test organisms at drug concentrations less than 1 µg/L in acute and chronic tests of over 60 compounds. If the EIC is lower than 1 µg/L, the drug is classified as acceptable, receives a so-called categorical exclusion and no environmental testing is required. Remarkably, once a drug has received this status, no subsequent monitoring to confirm that the expected environmental concentrations hold true is carried out. Considerations such as the specific mode of action of the agent in question are not considered.

An EIC of greater than 1μg/L, results in a requirement for a formal environmental risk assessment. Similar to the EU system, this consists of a tiered system of ecotoxicity tests. However, in contrast to the EU TGD, the base set of data normally includes an assessment of the potential effects of the pharmaceutical on microbial respiration as well as standard tests systems involving at least one algal, invertebrate and fish species. This approach places more emphasis on the ability of a substance to inhibit the activity of microbes present in sewage treatment works and hence to reduce the removal capability of the facility for this and other substances.

In contrast to the EU-TGD, chronic testing is not indicated unless the drug has the potential to bioaccumulate. This is described by the log octanol-water partition co-efficient ($\log K_{ow}$). In contrast to the European system, where a $\log K_{ow}$ of 3 indicates a requirement for chronic toxicity testing, the $\log K_{ow}$, which automatically leads to chronic toxicity testing in the USA is 3.5. Details on the course of the chronic testing scheme, are available on the EPA homepage (http://www.epa.gov/epahome/resource.htm).

One of the largest differences to the European system, is that the US-EPA suggests the use of a watershed-based ERA approach i.e. the use of a geographically defined area and local information such as the definition of activities within the watershed area and assessment of stressor transfer across watershed boundaries. This approach could have high relevance for the European mainland where countries may possess several land borders, yet be joined by a water body or river e.g. the river Rhine, which forms both borders and links between several European countries. Similar approaches within individual countries could also prove useful.

Japan

Based on recommendations of the OECD and in consultation with the national Industrial Structure, Health Sciences and Central Environment councils, the Japanese government concluded that the stipulations of the Japanese Chemical Substances Control Law of 1973 should be extended to include an evaluation and regulation of the adverse effects of chemical substances on living organisms in the environment. Further, the efficiency and effectiveness of risk management strategies should be improved [28]. The new regulations were promulgated in May 2003. As for the US-EPA, only the major differences with the European and Japanese processes will be outlined in the following section.

New chemical substances with a total yearly domestic manufacture or import volume of less than one tonne are exempt from prior evaluation processes, as are substances with a total yearly domestic manufacture or import volume of up to ten tonnes, if these substances are judged to be persistent but without the potential to bioaccumulate.

In recognizing the importance of endocrine active compounds, the Ministry of Economy, Trade and Industry (METI) has started a program of three dimensional-QSAR analyses, in order to generate a screening system for compounds with the potential for endocrine disruption (EDC). *In vitro* screening techniques are currently being evaluated in parallel [29] and are subject to a harmonized pre-validation and validation exercise under the auspices of the OECD [30]. These pre-screen test scenarios focus on the mode of action of the putative EDC for example sex hormone receptor recognition or binding, arylhydrocarbon receptor (AhR) recognition or binding or effects on aromatase activity. Although primarily directed at human risk assessment these *in vitro* methodologies as well as QSARs are considered transferable for use in environmental risk assessment. Despite these recent and promising developments it has been difficult to assess whether or not METI has regulated any pharmaceutical with regard to its inherent and specific pharmacological and thus consequently putative toxicological properties in the environment e.g. endocrine modulating capabilities.

Canada

Several considerable differences exist between the Canadian regulations and those described for the European Union, the U.S.A. and Japan. These differences and their benefits/disadvantages will be discussed in the following section. In Canada, the regulation of pharmaceuticals falls under the control of the Canadian Environmental Protection Act, (CEPA), in combination with the Food and Drug Act (F&DA). These laws aim to ensure that all new substances are assessed with respect to their potential to harm the environment before introduction onto the Canadian market and systematic assessment of substances began in September 2001 [31]. The CEPA classifies a substance as being toxic if "it enters or may enter the environment in a quantity or concentration or under conditions that 1) have or may have an immediate or long term harmful effect on the environment or its biological diversity; 2) constitute or may constitute a danger to the environment on which life depends; or 3) constitute a danger in Canada to human life or health". In contrast to other sovereign regions, where environmental and human toxicity concerns are dealt with by two separate government agencies, these assessments are carried out by Health Canada in co-operation with Environment Canada. This is likely to improve both the availability and transfer of information as well as to expedite the evaluation process.

The principle component of the CEPA is formed by the New Substance Notification Regulations (NSNRs). These regulations aim to prevent the entry into the Canadian marketplace before an assessment of their toxic potential has been carried out. In contrast to the European Union guidelines, where industrial chemicals and even veterinary and human pharmaceuticals are regulated by different documents, the CEPA encompasses all new chemical substances including those destined for research and development, export only, Canadian market as well as those which will only be site-only intermediates during the manufacture of other compounds. A new substance is defined as any chemical, polymer or living organism, which is not on the Domestic Substance

List (DSL). The DSL is a list of approximately 24,000 substances known to be or have been on the Canadian market. Substances contained in this list are not subject to notification. A list of substances in products regulated under the Food and Drugs Act (F&DA) that were in commerce between January 1, 1987 and September 13, 2001 is available at www.hc-sc.gc.ca/ear-ree/1987-2001_webpost_e.html. These substances are eligible for addition to the DSL and are not subject to notification under NSNR.

Substances not on the DSL but listed on the Non-domestic Substance List (NDSL, substances not on the DSL but believed to be in international commerce) are subject to notification, however, the comprehensiveness of the information required is reduced due to the consideration of information and experience in the USA. The Unites States Toxic Substances Control Act acts as a basis for this list. In co-operation with the US-EPA, the NDSL has undergone annual revision to add or remove substances, however, substances restricted, either in their manufacture or import, may not be added to the NDSL.

Notification is required for any substance not on the DSL and also for substances where a Significant New Activity (SNAc) is proposed. As for the system proposed by the European Union, the assessment consists of a stepwise process, which can be represented by a flow chart (see Figure 4). However in contrast to the EU procedures, the dossier of information to be submitted for assessment does not require an estimation of the PEC although certain information on the potential for environmental release or delivery to municipal WWTPs and identification of the facilities and water bodies is requested. Decisions are instead based on the yearly or accumulated amount of production or import. New substances are allocated into one of three classes (polymers and biopolymers, living organisms or chemicals and biochemicals) each with specific requirements for their registration. Pharmaceuticals fall under the category of chemicals and biochemicals, which is then further divided based on the volume of manufacture or import and proposed use (see Figure 5). The regulations governing transitional substances, substances produced for research and development, export only chemicals, site-limited intermediates are dealt with under separate sections (Schemes A, B, C and D, respectively) of the guideline document. Pharmaceuticals default into the class of “all others”, and as the analysis of the steps involved in the other categories mentioned is not the intention of this chapter, only the testing requirements for Scheme E will be outlined here. The reader is directed to the guideline document [31] and the CEPA text (available at http://laws.justice.gc.ca/en/C-15.31/SOR-94-260/69450.html#rid-69496), for more detailed information.

Figure 4: Notification requirements for new chemicals. Adapted from the Guidelines for the Notification of New Substances: Chemicals and polymers [31]

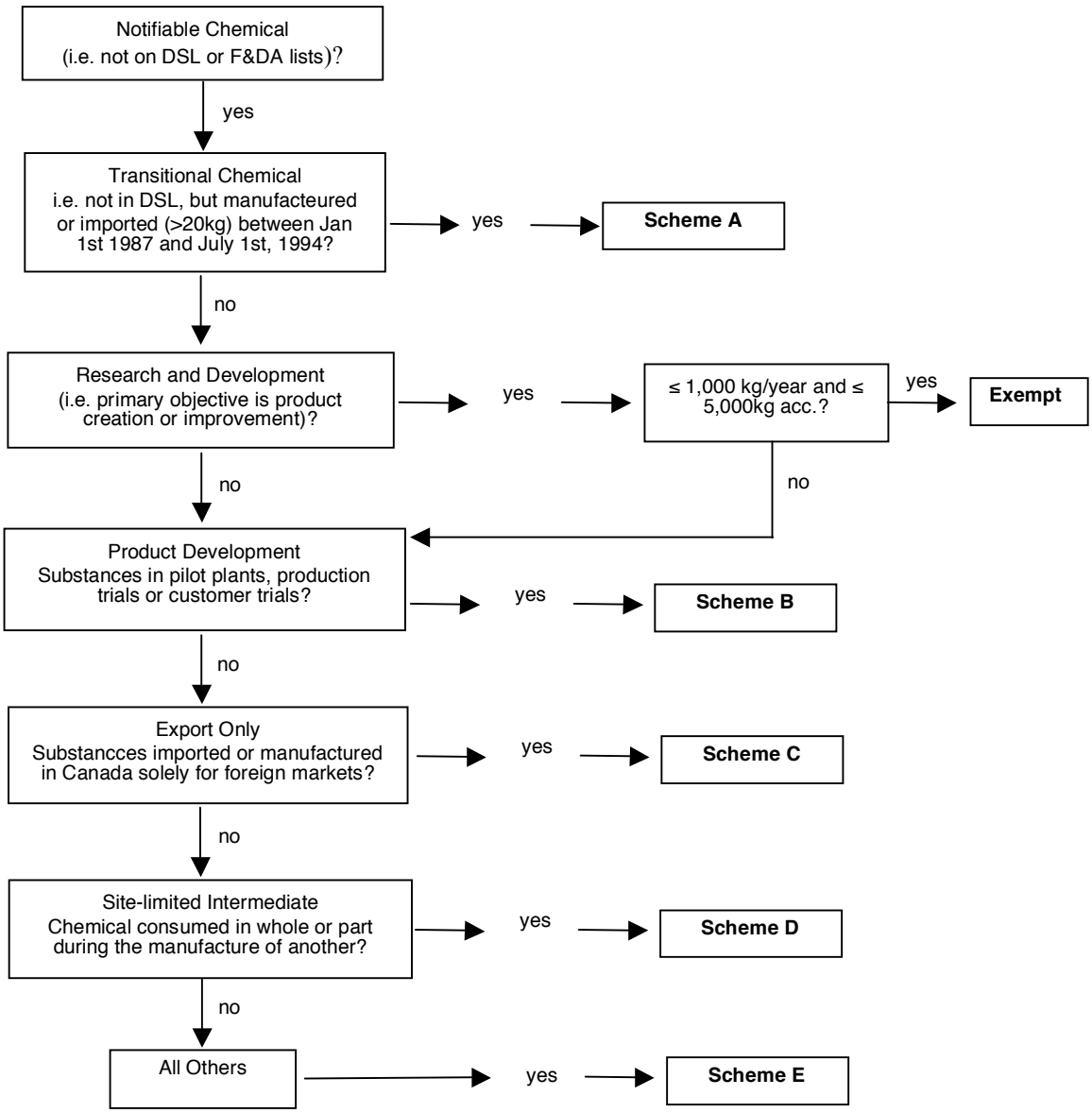
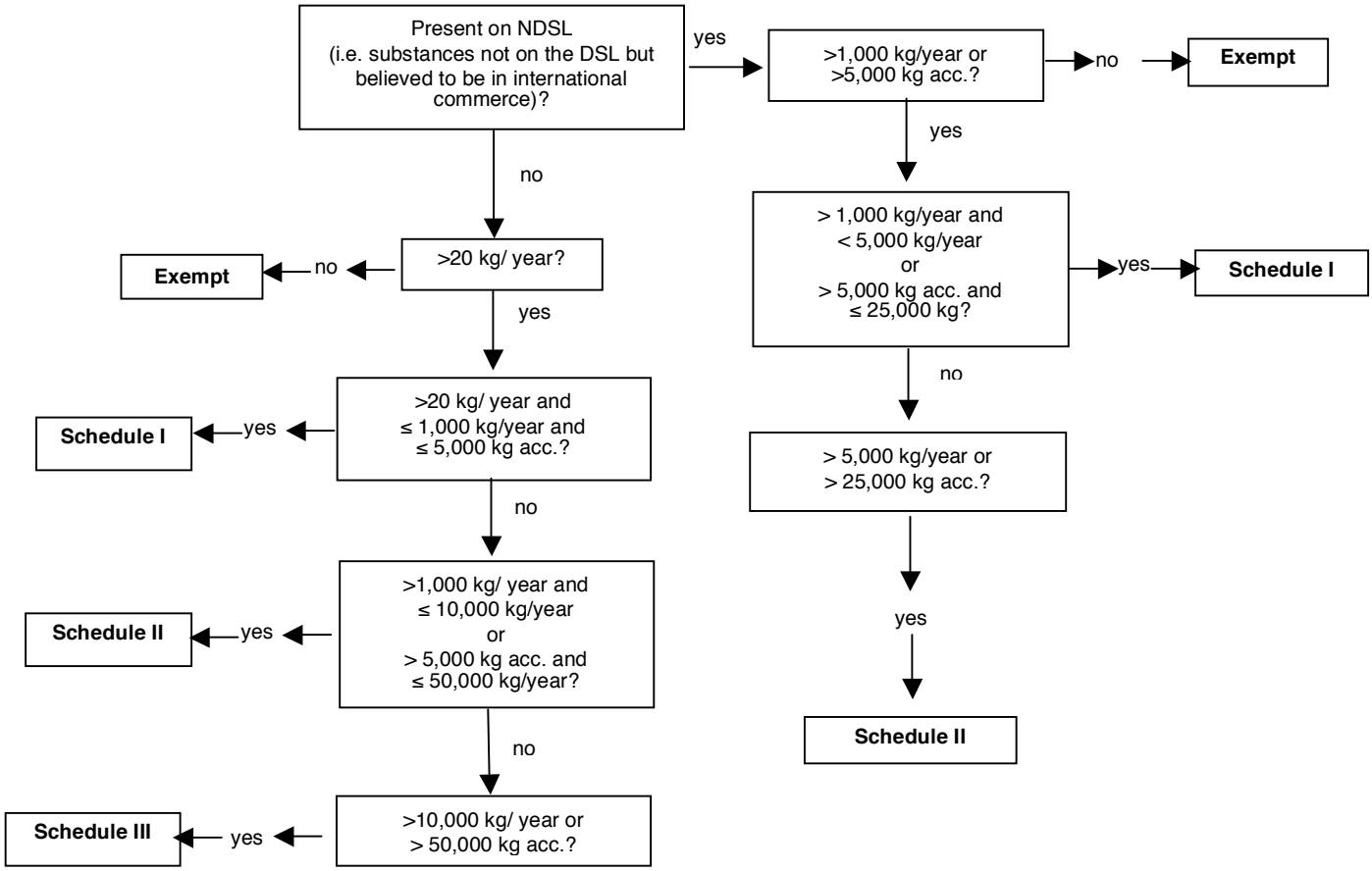


Figure 5: Testing schedule for new substances as stipulated by Canadian law



Part A of the notification submission consists of administrative and substance identity information. Part B contains technical information, which must include, but is not limited to physical-chemical properties, mammalian toxicity, ecotoxicity, exposure

information and a list of other agencies notified. Within Scheme E, the actions taken and level of investigation required for the NSR depends solely on the amount of the substance to be produced either within one calendar year or the total accumulated production (see Figure 5). Substances are allocated to one of a total of nine schedules (I – IX), depending on the type of chemical in question and the estimated production or import. Of these, schedules I – III are those with relevance for pharmaceuticals and the stringency of the criteria and information to be provided increases with increasing schedule number, based on total substance amounts. Schedules I – II require standard information such as physico-chemical data, octanol-water partition coefficient, vapor pressure, water solubility etc. as well as acute and repeated-dose mammalian toxicity, skin irritation and sensitivity data and mutagenicity testing (details can be taken from the original source [31], p58 ff.). Schedule III (production/import > 10,000 kg/year or > 50,000 kg accumulated), is the only schedule requiring aquatic toxicity testing, although the number of tests required is minimal, comprising just fish and *Daphnia* acute toxicity tests with LC_{50} determination and biodegradation tests. All testing should be carried out in accordance with the OECD guidelines valid at the time of testing (the specific OECD standard protocols for each test are listed in the guideline document) and the conditions of the study must be applicable to Canada (e.g. species tested, soil or sediment used). All relevant information to the environmental toxicity of the substance should be detailed including literature reviews and database searches, and data generated for notifications in other countries is acceptable. Structure-activity relationship analyses and environmental fate modeling should also be provided. The NSN must also contain detailed description of an analytical protocol for the detection of the substance at concentrations at or below the lowest reported LC_{50} value in fish or *Daphnia*. The latter illustrates a more focused approach on the part of Environment Canada to the development of appropriate analytic techniques than do any of the other regulatory documents.

In contrast to other regulations, where the trigger for testing is the PEC, under Canadian law it is the quantity of substance imported or manufactured, which if exceeded, requires the notifier to provide an NSN. Based on the information provided by the notifier, the government authorities make their assessment of the substance. If the substance is assessed as being innocuous, then production and/or import can proceed and if all criteria are met, the substance may subsequently be added to the DSL as described above and thus become exempt from future notification requirements. If on the other hand the substance is suspected to have toxic potential, the government may request additional information from the notifier, impose specific conditions on the manufacture and import of the substance of indeed prohibit the use of the substance.

3 The toxicological data set for environmental risk assessment

Although many countries now have or are in the process of preparing regulations pertaining to aquatic toxicity testing for pharmaceuticals, several problems regarding the practicality and applicability of these regulations still exist in this area. Some of these and some approaches for improvement will be outlined in the following section.

Extrapolation from acute to chronic toxicity

Extrapolation from acute to chronic for risk estimation is a particularly critical aspect for current guidelines on aquatic toxicity testing. This is strikingly illustrated by the findings of Ferrari and co-workers, who assessed the chronic and acute toxicity of three pharmaceuticals (carbamazepine, clofibric acid and diclofenac) in several different standard test systems [21]. Enormous differences in the EC_{50}, NOEC and LOEC values for carbamazepine were apparent even in the acute assays (microtox, 30 min, *D. Magna*, 48 h, *C. dubia*, 48 h) depending on the type of test employed. Furthermore, test species were found to display stark variation in their relative sensitivity to the three substances tested, further illustrating that the choice of test system is critical for the generation of data which can be used to perform a reliable risk assessment for the aquatic environment. These differences were even more pronounced in the chronic test systems with NOECs for carbamazepine ranging from >100,000μg/L in the *Pseudokirchneriella. subcapitata* 96 h test to 25 μg/L for *Ceriodaphnia dubia* seven-day test. In contrast, Pfluger and Dietrich have reported moderate toxicity for carbamazepine (74 – 138mg/L) in *D. magna* (48 h), *Danio. rerio* (96 h exposure) and *Xenopus. laevis* (96 h) [32], while Ferrari and co-workers [21] reported a NOEC of 25 mg/L in a *D. rerio* early life stage test (ten days). Indeed, a recent review of available acute and chronic aquatic ecotoxicity data by Webb [11] has shown that >90% of the observations of acute ecotoxicity for more than 100 pharmaceuticals were at concentrations above 1 mg/L and that all environmental values were at concentrations <1 μg/L. Ten compounds showed acute toxicity with specific test systems at concentrations lower than 1 mg/L (Table 2).

Interestingly, four of the ten substances belong to the antidepressant or antipsychotic classes. The range of reported ecotoxicity effect concentrations from >15 000 mg/L for atropine sulfate (anticholinergic/mydriatic; LC_{50} *Artemia salina*) to 0.003 mg/L for fluvoxamine (antidepressant; LOEC parturition *Sphaerium striatinum*) is extremely wide and illustrates that pharmaceutical compounds cannot be treated as a general class but must be looked at from the perspective of their mode of action and chemical properties. Indeed, the mode of action as suggested by the Specific Serotonin Reuptake Inhibitors (SSRI), fluoxetine and fluvoxamine, is maintenance of an unusually high level of serotonin over prolonged periods thus largely suppressing daily and/or seasonal fluctuations [33].

Table 2: Ten compounds showing acute toxicity at concentrations lower than 1 mg/L

Pharmacuetical	Class/Indication	Test System and Result
Alendronate	metabolic bone disease	MIC green algae: >0.5 mg/L
Amitriptyline	antidepressant	LC_{50} 24 h B. calyciflorus: >0.5 mg/L
Carvediol	antihypertensive /antianginal	LC_{50} Fish: 1 mg/L
Ethinylestradiol	estrogen	EC_{50} Algae: 0.84 mg/L
Fluticasone	antiasthmatic	EC_{50} Daphnia spp.: 0.55 mg/L
Fluoxetine	antidepressant	EC_{50} Algae: 0.031 mg/L EC_{50} Daphnia spp.: 0.94 mg/L
Fluvoxamine	antidepressant	LOEC 4h S. striatinum: 0.003 mg/L
Midazolam	anesthetic	EC_{50} D. magna: 0.2 mg/L
Paclitaxel	antineoplastic	LC_{50} D. spp.: >0.74 mg/L
Thioridazine	antipsychotic	EC_{50} 24 h D. magna: 0.69 mg/L

As previously suggested by many authors [32,34-36] it is of essence not only to understand the mode of action (MOA) but also the ramifications of the presence of similar or identical enzymes and receptors across different phyla. The usefulness of acute ecotoxicity data in the risk assessment of pharmaceutical compounds is therefore questionable and effects that might occur due to specific mechanisms will remain undetected. Chronic bioassays performed over the full life cycle of the organisms or covering several trophic levels are considered to be more appropriate if probably prohibitive in terms of financial and temporal costs. A survey of the currently available data showed that chronic ecotoxicity data is available for approximately twenty substances, of which ethinylestradiol is the most intensely studied. Ethinylestradiol is a special case amongst the pharmaceuticals, as it is intended to be active in the low ng/L concentration range in mammals and thus it is not surprising to find similar endocrine mediated effects in non-mammalian species such as fish and other aquatic species [37]. This is a typical compound for which the trigger values of the EU TGD would not function. Nevertheless, the EU TGD does specify MOA as a separate criterium. However, the risk assessment process does not envision the fact that other naturally occurring estrogens in the environment may have an additive or more than additive effect [36]. It should however, also be remembered that many substances have been demonstrated to have a variety of completely unforeseen effects in non-target organisms for example the inhibition of estrogen-induced vitellogenin production in isolated trout hepatocytes, by micromolar concentrations of paracetamol [38].

Despite this, MOA considerations rather than tiered testing would arguably provide more credible and useful results. For example use of antibiotics in a *Vibrio fischerii* (Microtox) luminescence assay not surprisingly will provide for toxic effects. Similarly, blue green algae (cyanobacteria) employed for first tier testing of antibiotics e.g.

ciprofloxacin will yield effects at lower concentrations than the corresponding test systems with green algae. As the effects at low concentrations are expected in these specific test systems, and essentially provide only confirmatory data that the test systems are working, little information can be gained for environmental risk assessment purposes. These findings support the consideration of the MOA of the pharmaceutical in question in the definition of the tests to be carried out. Indeed, Henschel and colleagues reported non-standard tests, which take the MOA into account, to be more sensitive than the standard test constellation of algae, *Daphnia* and fish, for three out of four tested substances [39].

Ferrari *et al*. [10] have considered chronic and acute endpoints and performed an environmental risk assessment for carbamazepine, clofibric acid, diclofenac, ofloxacin, propanolol and sulfamethoxazole taking into consideration conditions both in Germany (D) and France (F). While ofloxacin (PEC/PNEC 8.75), propanolol (PEC/PNEC 4.25) and sulfamethoxazole (PEC/PNEC 11.4) may pose an acute risk under conditions in France, only sulfamethoxazole (PEC/PNEC 59.3) would show a PEC/PNEC ratio >1 in Germany. On the other hand, carbamazepine (PEC/PNEC 2.4 (F), 3.82 (D)), propanolol (PEC/PNEC 104 (F), 11.9 (D)) and sulfamethoxazole (PEC/PNEC 2.72 (D)) would also display increased risk when considering chronic endpoints.

A further study looked in greater depth at the specific situation in the German federal state of Brandenburg, taking yearly consumption, number of inhabitants, amount of wastewater per inhabitant as well as human metabolism, elimination in the sewage treatment plant into account on the exposure side. The effect side considered the lowest known effect concentration and/or log$K_{ow,}$ half-life in surface waters, biological degradation, elimination in the WWTP and possible carcinogenic/mutagenic/reprotoxic and endocrine effects in mammals [40]. For some pharmaceuticals QSAR calculations were used. Using this type of assessment, eight substances were expected, based on their respective PECs, to be found at concentrations greater than 1 μg/L in surface waters: Metformin-HCl (antidiabetic), phenoxypropanol-isomers, cocospropylendiaminguaniacetat, glucoprotamine, polyvidon-iodine (all antiseptics), iodixanol (contrast media), metoprololtartrate (β-blocker, anti-hypertensive) and furosemid (diuretic). PNEC values, on the other hand, were shown to be low (< 1 μg/L) for ethinylestradiol, ciprofloxacin-HCl (antibiotic), carbamazepin (anti-epileptic), clofibric acid (lipid lowering agent metabolite), benzalkoniumchlorid and glucoprotamine (all antiseptics). A comparison of PEC and PNEC values to assess the risk to organisms in surface waters shows that 11 substances show PEC/PNEC ratios >1. These substances are listed in Table 3.

Unfortunately all of the PEC/PNEC calculations depend on the determination of the PNEC as described earlier. Consequently all of the PNEC calculations (including the application of the assessment factors) completely rely on the initial quality of the original data and the relevance of the endpoint determined.

Table 3: Selected substances with PEC : PNEC ratios greater than one

Substance Class	Specific Substance
Antibiotic	ciprofloxacin-HCl, clarithromycin
Antiepileptic	carbamazepine
Antiseptic	benzalkoniumchlorid
	cocospropylen-diaminguaniacetat glucoprotamine
	laurylpropylendiamin
	polyvidon-iodide
Hormone	ethynilestradiol
Antidiabetic	Metaformin-HCl
Lipid lowering metabolite	Clofibric acid

At this point one may ask the question what value endpoint determinants e.g. NOEC and LOEC really have for environmental risk assessment. Indeed both NOEC and LOEC are entirely determined by the experimental design of the study at hand and thus largely arbitrary. A much more conclusive and reliable approach is to use the EC_5 or EC_{10} determinations while applying the strictest statistical quality criteria to the curve determinants [41]. Use of EC_5 or EC_{10} values for the LOEC calculation would certainly merit more trust as the subsequent assessment factors would ensure limitation of uncertainties to the uncertainty factors for which they were envisioned e.g. intra- and interspecies differences etc. and not to experimental design flaws of the original study.

QSARs

As mentioned above ecotoxicicity data are often non-existent, or at least not in the public domain, and as it not feasible to perform the necessary tests in an adequate time frame, other approaches have been used or should be developed to assess the environmental risk of pharmaceuticals. One of these approaches is using (Q)SARs ((quantitative) structure activity relationships) at least for prescreening or prioritising of substances. Further approaches, such as (Q)PPRs ((quantitative) property property relationships), (Q)AARs ((quantitative) activity activity relationships), (Q)SPRs ((quantitative) structure property relationships) or even (Q)SBRs ((quantitative) structure bioaccumulation relationships) consider other properties of the chemical and are also being employed. QSARs are currently being used amongst others by industry in internal screening programs and by the EU in risk assessments of biocides, new and existing chemicals (http://ecb.jrc.it/Documents/TECHNICAL_GUIDANCE_DOCUMENT/EDITION_2/tgdpart3_2ed.pdf) and by US EPA in risk screenings for example for premanufacture notices. In ecotoxicology, one of the most widely used QSARs is ECOSAR which was developed by US EPA and has been validated by US EPA, OECD and the EU. ECOSAR uses both baseline toxicity as well as expert-system-based ecotoxicity assessment for certain chemical groups. Furthermore, by combining ECOSAR with other models in EPISuite such as BIOWIN (biological degradation), BCF ($logK_{ow}$-based extrapolation of the bioconcentration factor) and the WWTP fugacity models (modeling of fate in a

three-step sewage treatment plant) one may characterize the ecotoxic properties of substances and their environmental fate (EPISuite can be downloaded free of charge at http://www.epa.gov/oppt/exposure/docs/episuitedl.htm). The EU proposes to use QSARs for modelling of biocides and new and existing chemicals based on nonpolar narcosis in acute and (sub)chronic algae, *Daphnia* and fish assays, on polar narcosis in acute *Daphnia* and fish assays and BCF for substances with $\log K_{ow}$ <6. Pharmaceuticals are complex molecules and relatively little experience has been gained to date concerning the predictability of ecotoxicological risks of pharmaceuticals or pharmaceutical classes [42]. ECOSAR classifies pharmaceuticals as neutral organics and uses mainly lipophilicity to develop models for toxicity prediction. Comparisons of effect concentrations of pharmaceuticals with modeled values are therefore necessary. Performing such a comparison with 20 pharmaceuticals present in surface waters and for which experimental data for at least algae, *Daphnia* or fish are available, Sanderson *et al*. [43] could show that in 80% of the cases the modeled ECOSAR EC_{50} values (the lowest predicted ecotoxicology value was chosen) were lower than the measured effect concentration and therefore over-protective. This, however, also implies that the predictive value of the QSAR is not high. By applying the same methodology on a larger set of pharmaceuticals with the expectation that ECOSAR is applicable to pharmaceuticals for ranking purposes, almost 3000 substances in 51 classes were screened [44]. Cardiovascular, gastrointestinal, antiviral, anxiolytic sedative hypnotics and antipsychotics, corticosteroids, and thyroid pharmaceuticals were predicted to be the most hazardous classes. This does of course not imply that the other pharmaceutical classes will be acquitted based solely on the QSAR predictions [44]. Pharmaceuticals generally have very specific modes of action (MOA) and a QSAR taking into account the MOA might give a better predictivity in terms of hazard identification. This implies however that the QSAR is well characterized, as it was shown that more complex QSARs that take MOA into account are outperformed by more simple QSARs in terms of prediction accuracy [45]. In conclusion, QSARs may be a useful tool for preliminary screening and prioritization for environmental risk assessment, but should always be coupled with experimental data and expert knowledge. This very conclusion is also recognized in the recent revision of the EU TGD where expert evaluation of available data has gained a central role in the risk assessment process. Furthermore, the term "expert" is substantially defined, in that the background and experience of experts employed in the risk assessment process are required entities for the provision of high stringency and consistency in expert knowledge.

"Omics

"Omic" technologies have not halted before environmental risk assessment and their use and applicability is currently being discussed in regulatory bodies and intergovernmental organizations such as the OECD. "Omics" technologies, including genomics, proteomics, transcriptomics and metabolomics/metabonomics, are tools being evaluated for chemical hazard and risk assessment in order to better understand species and subgroup sensitivities, assess mixtures and combinations and offer the long-term

possibility to reduce animal-intensive methods for screening and testing. It should, however, be understood, that current approaches in the research community are very varied and at different stages of methodological development. A further critical issue is the development of bioinformatics tools that can handle and analyze and distil the huge amount of data that can be generated using omics techniques. A valuable effort has been made by the MGED (Microarray Gene Expression and Data) Society (http://www.mged.org) in initiating a standardized format for reporting ecotoxicogenomics data, MIAME (Minimum Information About a Microarray Experiment), that is already a prerequisite for publication of (eco)toxicogenomics data in many scientific journals. Although (toxico)genomics techniques are currently being used in the development of pharmaceutical compounds, there are currently no publications relating to ecotoxic effects of pharmaceuticals using genomic techniques. In all of this one must never forget that all of the "omics" techniques have one essential limitation in that they all describe very short time windows in the exposure experiment of a given species. In order to provide more insight into the processes involved, numerous time-points would be necessary, however this is currently prohibitive due to the sheer amount of data and huge financial costs involved.

Toxicity of Mixtures

The problems associated with assessing the aquatic toxicity of a single compound burgeon when one considers the possibility of interactive/synergistic effects of the myriad of different pharmaceuticals currently on the market. In reality, aquatic organisms are exposed to a veritable cocktail of contaminants and synergistic effects have indeed been reported for compounds with the same mode of action, for example the non-steroidal anti-inflammatory COX inhibitors. A study carried out by Cleuvers and colleagues [46] using standard *Daphnia magna* toxicity tests, showed that the toxicity displayed by a mixture of diclofenac, ibuprofen, naproxen and acetylsalicylic acid was far higher than that predicted by simple addition of the effects of the individual substances. In contrast, concentration addition was shown to accurately predict the toxicity of NSAID mixtures in acute algal (*Desmodesmus subspicatus*) toxicity testing. The authors suggested that nonpolar narcosis rather than a specific mode of action was responsible for the observations and supported this with quantitative structure activity relationship (QSAR) analysis and further suggested that significant combination effects could also be expected for similar compounds at concentrations below their NOECs. Indeed a study by Renner demonstrated a mixture of ibuprofen, prozac and ciprofloxacin to be toxic to plankton, aquatic animals and fish at concentrations up to 200 times lower than the standard human dose [47]. Such combination effects are probably even more pronounced in a chronic exposure situation. Thus, the use of individual NOECs as a basis for risk assessment is questionable.

A further class of compounds could enhance the toxic potential of substances not normally toxic in single exposure regimens. Verapamil, reserpine and cyclosporine exert some of their therapeutic effects by functioning as so-called efflux pump inhibitors.

Similarly, non-pharmaceutical compounds e.g. nitromusk and polyclic musk compounds and personal care products can inhibit multidrug/multixenobiotic resistance (MDR/MXR) efflux transporters [48,49]. This could potentially also have serious consequences with respect to mixture toxicity. Indeed the teratogenic effects of a range of compounds (vinblastine, mitomycin C, cadmium chloride, methylmethanesulfonate, chloroquine and colchicines) in mussel larvae, have been shown to be exacerbated with verapamil (20μM) co-exposure [50]. The question, however, remains as to the doses that are required for transport inhibition and whether these doses realistically are found in the environment at the same time with other pharmaceuticals or polar organic pollutants.

4 Conclusions

In conclusion, estimation of the potential of an individual pharmaceutical to cause detrimental effects in the aquatic ecosystem does not include an assessment of the potential of the compound to interact and possibly cause additive, more than additive or even synergistic effects with other formulations of the same substance or with other substances with similar modes of action. In the same token, counteractive i.e. inhibitive effects by competition for the same enzyme/receptor must also be taken into consideration. The analysis of structure activity relationships (SARs) could help to abrogate part of this problem, as discussed above. However, only high quality data sets using realistic compound concentrations, coupled with simultaneous analysis of the bioavailable compound concentrations and accounting for the possible MOA as well as cross-reactions and interacting enzyme/receptor systems can shed light into this challenging field. As of yet, despite presently numerous and exponentially increasing number of publications, there is a great paucity of qualitative acceptable data sets that can be readily employed for environmental risk assessment purposes.

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