Chapter 6

Ecotoxicology, Environmental Risk Assessment & Potential Impact on Human Health

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Abstract

This chapter examines potential risks posed by active pharmaceutical ingredients (APIs) present in the aquatic environment to humans and aquatic life. We first describe the mechanisms by which pharmaceuticals enter the vertebrate body, produce effects and leave the body. Then we describe theoretical and practical issues limiting the certainty which can be expected from risk estimates. This is followed by a description of considerations applicable to evaluation of human risks, along with a summary of some important studies examining those risks. A similar discussion of theory and data relevant for estimating risks to aquatic life is then presented. We finish by discussing potential contributions of antibiotics in the environment to the spread of antibiotic resistance. We conclude that there are too few data to definitively address every concern, particularly risks to aquatic life and contributions to development of antibiotic resistance. However, available data suggest risks to humans are very low for all APIs and risks to aquatic life are very low for most APIs. Although aquatic risks cannot be as confidently ruled out for a few APIs, potential risks are probably limited to particularly contaminated regions in close vicinity to concentrated pollution sources, such as wastewater treatment plant outfalls.

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6.1 Introduction

Active pharmaceutical ingredients (APIs) are designed to affect the physiology of human as well as veterinary patients, and many do so at very low doses. Therefore, it is not surprising that the frequent detection of pharmaceutical residues in wastewater, surface water, groundwater and drinking water has stimulated extensive discussion[1-4] about potential impacts on the health of humans and the aquatic environment.

In this chapter we discuss the estimation of potential risks posed to humans and aquatic life by APIs present in the aquatic environment. We will not address risks from excipients or contrast media, because many of the special considerations applicable to APIs are not relevant to such compounds. We begin by describing some principles of pharmacology that are useful for understanding effects of APIs and for understanding some approaches to estimating risks. Then we review limitations in the environmental occurrence and dose-response data available for risk estimation. We examine some approaches to substitute more readily accessible, but probably less reliable, estimates of exposure rate and dose-response information. We subsequently describe specific considerations associated with estimating human risk as well as results from some studies examining these risks. This is followed by a similar discussion for risks associated with aquatic life. Finally, we consider residues entering the environment as

a result of antibiotic use and their potential contribution to the development or spread of antibiotic resistance.

6.2 Some Relevant Pharmacology

The study of the desired biological effects of chemical compounds is termed pharmacology. Principles of pharmacology are closely related to principles of toxicology (the study of undesired biological effects of compounds) and are useful for understanding potential risks posed by pharmaceuticals in the environment. The processes underlying pharmacology are divided into pharmacokinetics and pharmacodynamics.

6.2.1 Pharmacokinetics

Pharmacokinetics describe the absorption, distribution throughout the body, metabolic transformation and excretion of an API. Most APIs produce their intended physiological effects by interacting with specific molecular receptors in a target tissue. Here, we use 'molecular receptor' generically to refer to any endogenous molecule (which might be an ion channel, g-protein coupled receptor, protein kinase, *etc.*) whose function is altered by interaction with an API. In order to reach the target tissue the drug must first be absorbed into the body and travel from the site of absorption to the target tissue while avoiding degradation or excretion. The first step in this process for most drugs (parenteral administration can be an exception, but is not a part of

typical environmental exposure scenarios) is the drug crossing a boundary tissue, such as surface of the gut, nasal sinuses, lung or skin. This involves crossing the physical barrier provided by the boundary tissue, as well as avoiding detoxification mechanisms often abundantly expressed in boundary tissues.[5] For orally administered drugs, this also usually involves passage through the liver, in which metabolic transformation and detoxification processes are particularly active. The efficiency with which APIs enter the general circulation after administration is called 'bioavailability', which usually varies depending on the route of administration. Bioavailability of a given dose of API administered by a particular route is expressed as a percentage of the amount of drug seen in blood plasma after intravenous dosing. Intravenous dosing is a particularly direct route into the body that bypasses many of the barriers to API entry and, therefore, typically represents the maximum possible bioavailability.

The process by which an API entering the systemic circulation is removed from the body, either by physiologically mediated chemical transformation or by excretion, is called 'clearance'. Bioavailability and clearance both have major influence on the time course of plasma concentrations after API administration and, therefore, how much of the API is seen by target tissues. Many xenobiotic clearance mechanisms exist in vertebrates. They are found in various tissues, but tend to be particularly active in boundary tissues and liver. Clearance mechanisms include two groups, called phase I and phase II reactions. Phase I reactions involve enzymatic oxidations, reductions or hydrolysis of xenobiotics (such as APIs). These modifications change the chemical structure of xenobiotics, tending to increase their water solubility and providing sites

for conjugation during phase II reactions, both of which encourage rapid clearance from the body. Although phase I reactions often reduce the biological activity of toxicants, they can also activate some APIs which are administered as inactive 'pro-drugs'. In other cases, both the parent as well as one or more major metabolites have substantial physiological activity and jointly contribute to therapeutic effects. Phase II reactions usually involve covalent addition of large hydrophilic moieties (glucuronic acid, sulfate, acetyl, glutathione, glutamine and glycine additions are common), which facilitate excretion through the kidney by increasing water solubility. Conjugates with a molar mass above 500 Daltons are usually excreted via the biliary tract into the gut. In addition to these covalent modifications of xenobiotics, transport proteins with wide substrate specificity transfer a variety of endogenous or exogenous molecules out of cells and out of the body.

For many APIs, once in the central circulation, a large fraction (for some APIs, more than 99 %) binds to plasma proteins or blood cells, leaving only a small portion of plasma API freely dissolved in the plasma water. The proportion freely dissolved often varies from species to species, reflecting species-specific differences in the composition of plasma. This may be an important process to account for because, for many drugs, only the fraction freely dissolved in plasma water can be efficiently taken up by tissues and interact with the target molecule. After freely dissolved API passes from the central circulation into a tissue, most APIs elicit biological effects by binding to and altering the activity of a target molecule. This triggers a cascading series of

events at progressively higher levels of biological organisation, which culminates in the desired therapeutic or adverse effect.

6.2.2 Pharmacodynamics

Many APIs can affect more than one molecular receptor, but usually do so with differing potencies. Potency differences between different receptors can provide selectivity for the therapeutic effects over side effects. This specific binding to a molecular receptor is often described as a 'lock-and-key' interaction, where several specifically positioned residues in the drug molecule simultaneously pair up with complementary residues in the drug receptor, forming hydrogen bonds, polar interactions, or hydrophobic interactions between the drug molecule and the molecular receptor. For most APIs, the cooperative activity of these relatively weak interactions results in a stable, but non-covalent, interaction between the API and receptor. In other cases, covalent bonds form between the API and the molecular receptor. In either case, binding of drug to receptor then alters the function of the receptor, for instance by changing the receptor's preference for different structural conformations in ways that alter functional properties, or by sterically blocking binding of normal physiological ligands. These changes in the receptor's function alter its interactions with other cellular constituents, which leads to alterations in overall cellular function. For most drugs, the alterations in cellular function give rise to changes in tissue and organ function, which then culminate in the desired therapeutic effects. Drugs such as antimicrobials and cytotoxic drugs are an exception, where the

intended therapeutic effect of killing pathogen or cancer cells might only require cellular effects, rather than *e.g.* tissue or organ-level effects.

Drugs often have unintended physiological effects (termed 'side effects'). Side effects usually result from the systemic distribution of an API (due to its favorable pharmacokinetic properties) and the presence of API-mediated receptors in many tissues other than the target tissue. The same molecular to physiological cascade response initiated by the API in the target tissue may manifest in non-target tissue as a different physiological effect (the side-effect). In addition, certain features which help an API bind to the targeted protein receptor may facilitate binding and interaction with non-targeted proteins, sometimes with affinity similar to that of binding to the intended target. Non-target binding is more likely when the target receptor molecule (typically a protein or polynucleotide) belongs to a larger family of structurally related large molecules with similar potential API binding sites. Even if the API very specifically interacts with its targeted receptor, it can still produce side effects because the targeted receptor has multiple functions, either at different points in development, in different tissues, or even within the same tissue. Sometimes the cascade of molecular effects triggered by alteration in the function of the drug target include effects in addition to the therapeutic effect, which can give rise to clinically noticeable side effects. APIs can also suppress or induce enzymes involved in the clearance of a variety of xenobiotics from the body, or compete for plasma protein binding sites with other xenobiotics. These effects give rise to interactions that can alter the dose response to either the API or the other xenobiotic.

Mechanistic considerations suggest that, for receptor mediated API effects, lower concentration thresholds may exist below which there is no effect on organismal fitness. API binding to a receptor usually initiates a cascade of events at increasing levels of biological organisation, spreading from molecule to cell, to tissue, to organ, and finally affecting the whole organism. These interactions between physiological components are usually regulated by various compensatory systems (typically involving feedback loops) that provide stability and robustness to higher level physiological processes. These homeostatic mechanisms allow organisms to adapt to natural variations in environmental conditions, such as changes in diet, ambient temperature or water availability. This physiological adaptability suggests that there may exist API exposure levels which have no effect on higher levels of biological function. Therefore, even though principles of statistical mechanics suggest that the effect on the molecular receptor population may be non-zero at any non-zero API concentration, the resulting small receptor effects may not be large enough to challenge homeostatic processes or thereby affect overall organismal fitness. These considerations apply to not only the discussion of the possibility of toxicity thresholds, but also to the translation of studies reporting biomolecular changes resulting from toxicant exposures into estimates of organismal or population risks.

A few APIs, particularly some used in cancer chemotherapy, are potent DNA-damaging agents (mutagens). Rather than interacting with a specific receptor, these APIs chemically modify DNA, potentially causing heritable mutations in germline tissues, as well as raising the risk of cancer in the exposed individual. Risks associated with

mutagen exposure are typically modelled[6] similarly to exposure to ionising radiation, assuming a 'one hit' model[7], where a single DNA lesion in a single cell can be sufficient to give rise to an adverse event (usually cancer in the exposed individual is discussed). This suggests that even a single molecule of genotoxic API might be able to give rise to an excess adverse event and, therefore, there is no safe threshold below which no excess risk exists.

Besides producing generally reproducible effects in the general population, rare but serious idiopathic reactions can occur in a small proportion of individuals exposed to some APIs. These reactions usually involve an inflammatory response, occur at least a week after drug is first administered, and have often been suggested to be immune mediated. Immune involvement suggests these responses are probably ultimately receptor-mediated, though not by the same receptor involved in therapeutic effects. A variety of tissues can be adversely affected, including the liver, bone marrow, kidney, skin, muscle, *etc*. Details of the dose response for this class of reactions is not as well documented as it is for other API activities, but almost all cases reported involve therapeutic dose rates.

6.3 General Approaches and Data Availability

Typically, chemical risks are estimated by comparing environmental exposure rates to exposure rates capable of eliciting adverse biological effects. Comparison is often expressed as a ratio of environmental exposure rate to biologically effective exposure

rate, here referred to as the risk quotient (RQ). Sometimes the reciprocal of the RQ is reported, which is sometimes called the margin of exposure (MOE) or margin of safety (MOS). For estimation of risks to aquatic life, water concentrations are often used in place of exposure rates and risk is then estimated using a ratio of an exposure concentration to a minimally toxic water concentration. Since exposure concentrations are expected to vary from place to place and time to time, it is important to consider the likely distribution of aquatic concentrations. Similarly, different species are expected to potentially have differing sensitivities to any particular toxicant, so it would be useful to know the distribution of species sensitivities.

6.3.1 Dealing with Data Distributions

When enough measurement data are available, aquatic concentration distributions (which are the typical basis for exposure estimates) are sometimes estimated by fitting available measurement data to a parametric function (often, a statistical distribution with well-known properties), or by using the empirical distribution directly (if there are enough data to construct one). In the absence of enough data, the concentration distribution is often estimated using a predictive mechanistic model. Similarly, when data are abundant, toxicity distributions can be estimated by fitting parametric functions to toxicity data gathered in different species. Ideally (but rarely) toxicity data will be representative of the variety of species and developmental stages potentially exposed to the API. If toxicity data are in short supply, distributions can be estimated from species-specific predictions made by QSAR-based predictive models.

Although estimates based on empirical occurrence or toxicity data are more reliable, for the vast majority of APIs data are not available and are expensive to gather. By contrast, the less reliable estimates from predictive modelling can be carried out at little expense for virtually all APIs in current use.

The simplest approach to using a range of available occurrence and toxicity data is to compare the highest reported occurrence concentration to the lowest reported no-effect concentration or the lowest reported-effect concentration. By contrast, in one form of probabilistic analysis,[8] an upper percentile (but not the most extreme value) of estimated exposure concentrations (the 90th percentile, for instance) is compared with a lower percentile (but not the most extreme value) of toxic threshold concentrations (*e.g.* the 5th percentile). The exact cutoffs are typically subjectively chosen and often vary between studies. Nevertheless, the approach can be used to conduct a transparent and reproducible analysis. One potential benefit provided by this type of probabilistic risk estimate, when there are many data, is greater robustness to outliers (potentially present due to experimental artifacts) in the available occurrence or toxicity data. On the other hand, if the more extreme measured occurrence and toxicity values are real, the particular probabilistic approach described above potentially fails to protect 5 % of species in about 10 % of exposures situations, which may be insufficiently protective for some practical applications (such as protection of an endangered species). The details of the upper percentiles of the exposure distribution and lower percentiles of the concentration-response distribution usually matter a lot where human exposures are concerned, because the

fate of each individual is valued. By contrast, in many cases, protection of aquatic life may be consistent with accepting the loss of some individuals represented by the tails of the exposure or susceptibility distributions, as long as enough unaffected individuals remain for the population to thrive.

For the vast majority of APIs, there are not enough data to empirically estimate extreme percentiles of either the occurrence or toxicity distributions. For example, if one has toxicity values for an API in three biological species, one cannot directly estimate a 5th percentile of species toxicity values, as empirical estimation would require at least 20 data points (in which case the lowest of the 20 data points would be used to represent the 5th percentile). One might be able to estimate the 5th percentile toxicity with fewer data by extrapolation from the available data, but this involves assumptions about the shape of the distribution beyond the available data. In this case, a parametric function (log normal, Weibull and logit distributions are popular) thought to represent the true underlying form of the distribution is parameterised using the available data.[8-12] The more extreme percentiles desired for calculation of the risk estimate are then extrapolated from the available data using the fitted idealised distribution to estimate the shape of the tail of the true underlying distribution. When data are sparse, this type of probabilistic approach can be more protective than using the most extreme measured value because, if fewer than 20 data points are available and a log-normal distribution is assumed, the 5th percentile concentration estimate from the fitted distribution is always lower than the lowest measured concentration. On the other hand, it is difficult to determine how well the

assumed distribution corresponds to the real world beyond the range of the available data. With limited data it is typically the case that several distributions can be found that fit the data indistinguishably well, even though the distributions make very different predictions in the tails – precisely the region one might be most interested in for risk estimation.

6.3.2 What is a 'Safe' Concentration?

For mutagenic toxicants, the one-hit mechanistic model described in section 2.2 suggests a linear relationship between dose and frequency of adverse events. Applying the one-hit model to mutagenic toxicants involves fitting a linear relationship between dose and probability of an adverse event beyond the background probability of that event. The background probability is important to account for, because typically one assesses cancer incidence in a strain of test animal which is particularly prone to that type of cancer, even in the absence of carcinogen exposure. The linear fit is assumed to have a zero intercept; that is, it is assumed to pass through the point defined by zero exposure and zero probability of effect. Fitting a straight line through this point implies a 'no threshold' model, where there will be some excess risk for any exposure level other than zero, rather than a safe threshold below which no risk exists. However, the assumptions of linearity at low exposure rates may be overly conservative, if pharmacokinetics are non-linear, if multiple hits per cell are needed in order to elicit the adverse effect,[13] or if up-regulation of compensatory DNA repair systems in response to mutagen exposure actually results in a paradoxical net decrease in adverse effect probability[6] (due to improved resistance to both mutagen-

induced and background mutations). Generally, there are not enough data available to decisively resolve these low-dose issues for a particular toxicant, so the one-hit model is often adopted as a conservative default. Since a non-zero probability of adverse effects is implied by any non-zero exposure concentration, 'safe' exposure rates are defined in terms of an acceptable (or 'negligible') excess risk. Typically, an increase in cancer incidence of one per 100,000 or one per million exposed individuals is chosen.

The mechanistic considerations described in section 2.2 suggest that higher-level physiological effects of most non-mutagenic APIs drop off rapidly (perhaps to zero) at low concentrations due to homeostatic regulation. Therefore, one might like to define a 'safe' concentration below which we can know there is absolutely no risk. However, basic statistical considerations tell us that we can only estimate the upper limit of risk at a non-zero concentration of toxicant and that limit will always be some value greater than zero. The familiar 'no observed effect concentration' (NOEC) is sometimes interpreted as implying zero risk, but the observability of the effect to which the acronym refers is contingent on the size of the effect being considered and the power of the test used to estimate the NOEC. The power of a test is, in large part, a function of the number of individuals tested and the inter-individual variability of the response measurement. If the test is repeated with a much larger number of individuals or a more precise measurement system, smaller effect sizes will be discernable, potentially at a previously declared NOEC. The NOEC is contingent on the size of smallest effect that can be observed given the power of the test system and,

since the NOEC must coincide with a measured concentration, is also contingent on the spacing of the test concentrations. This means that one cannot statistically prove absolutely zero risk at any non-zero concentration, even if there is no risk. Furthermore, this is true for mutagenic as well as non-mutagenic toxicants, regardless of how much testing is performed. Additional testing can only decrease the size of the effect that can be discounted.

6.3.3 Data Feast and Famine

In the case of pharmaceuticals, there are many data on toxicity in mammalian test species (generated during pre-clinical evaluation) as well as in human patients (from clinical experience), which provide evidence for estimating potentially toxic concentrations. Animal safety data for APIs almost always include evaluation of chronic effects, including mutagenicity and full lifecycle reproductive testing. Comparable data are very rare for other commercial chemicals. The data in humans are collected using a much greater number of individuals than is usually employed in animal toxicity testing of industrial chemicals. Human data also often include an evaluation of chronic effects, particularly for drugs with very high usage rates, which tend to be prescribed for chronic conditions. Furthermore, use of human safety data for estimating human risks does not require interspecies extrapolation for estimating human risks. Compared to the more generic endpoints represented in typical safety data collected during preclinical animal testing, effects data collected in humans often includes endpoints that are more subtle, occur at lower exposure rates and are more reflective of the specific mechanism of action of the pharmaceutical.

There is also an unusual abundance of data on the clinical usage rates of pharmaceuticals and there are only a small number of fairly simple scenarios describing how most pharmaceuticals enter the environment. These two factors facilitate mechanistic modelling of the distribution of pharmaceutical concentrations in the environment resulting from community or hospital use.[14] The complexity of these models can vary greatly, depending on the availability of data and the willingness of the researcher to make assumptions where data are lacking. Typically, *per capita* consumption rates and wastewater production rates are used to arrive at the simplest predicted environmental concentrations (PECs). More elaborate models may try to account for metabolic inactivation in patients, removal during wastewater treatment plant (WWTP) treatment, dilution into receiving waters or bioconcentration into fish. Comparisons between measured concentrations and predictions from mechanistic modelling suggest that, for most APIs, mechanistic modelling is adequate for estimating typical (*i.e.* near the average or median) exposure concentrations to within an order of magnitude or so.[4, 15-20]

Residual discrepancies in predictions from mechanistic model predictions can usually be explained by overestimation of metabolic inactivation, overestimation of WWTP removal rates and unaccounted sources of geographic or temporal variability. Metabolic inactivation can be overestimated due to uncertainty in reported values or because some metabolic modifications, such as glucuronidation, can be reversed during WWTP treatment. WWTP removal rates are often indirectly estimated using hydrophobicity, which can be an unreliable predictor. Removal rates can also vary

greatly between WWTPs. Even within a single WWTP, removal efficiency can fluctuate widely across time of day and across seasons.[21] By making assumptions of no metabolism and no WWTP removal, as well as applying an uncertainty factor (UF) of about ten-fold to account for geographic and temporal variations in API or water usage, underestimates of potential exposure rates *via* ambient or drinking water can largely be avoided.[22] This is encouraging, because it suggests that mechanistic modelling can be used to conservatively estimate the upper bounds of expected aquatic exposure rates to all drugs in use, not just the few whose concentrations have been measured in the environment.

6.4 Potential Risks to Humans

The most likely routes of human exposure to pharmaceutical residues in the environment are thought to be ingesting drinking water and fish. Exposure through recreational activities (*e.g.* swimming) are also possible, but are expected to typically be of much lower magnitude. The average relative contribution of dietary fish intake is expected to increase with an API's bioconcentration factor (BCF), which is defined as the ratio of steady state API concentration in fish tissues divided by API concentration in water. Assuming typical consumption rates in the US (for adults, about 2 l/day drinking water and about 17.5 g/day fish; for children, about 1 l/day drinking water and about 6.5 g/day fish), approximately equal contributions of drinking water and

fish consumption have been predicted to occur at a BCF of 115 for adults and 150 for children.[19]

In order to estimate risks to humans, exposure rates are often compared to acceptable daily intake (ADI) rates for the drug, which are usually developed from pre-clinical animal safety data or from minimum human therapeutic dose rates. Comparisons between ADIs derived using non-human safety data and ADIs derived from human therapeutic dose rates suggest the latter are often lower.[11, 23] This may be because most APIs exert therapeutic effects at doses lower than those eliciting clinically significant toxicity. ADIs derived from therapeutic dose rates have the added advantage of avoiding uncertainties involved in extrapolating from test animal species to humans.

The ADI is often calculated by applying a series of UFs to either a minimum toxic dose rate or a minimum therapeutic dose rate. This is intended to account for uncertainties accompanying extrapolations, such as from a lowest observable effect level (LOEL) to no observable effect level (NOEL), extrapolation from one species (*e.g.* lab mouse) to another (*e.g.* human), and extrapolation from acute tests to potential chronic exposures. Since the number of extrapolations performed for a particular API depend on the data available for that API, UFs provide a means to account for variations in data availability underlying toxicity estimates for different APIs. On the other hand, the magnitude of UFs is somewhat arbitrary and it is important to remember that the risk quotients resulting from their application often reflect the lack of specifically applicable data for an API, rather than the existence of evidence suggestive of risk.

This may be a very desirable property for prioritisation exercises, but may be misleading when communicating risks to wider audiences. In order to facilitate comparison of ADIs with water concentrations, human API exposure rates are estimated using the product of the concentration of API in drinking water multiplied by daily water consumption (typically assumed to be between one and two litres per day).

A number of research groups have compared human exposure estimates with estimates of acceptable intake rates. Some of the larger studies are summarised in the following two subsections. The first subsection describes risk estimates obtained using predicted environmental concentrations (PECs) generated using mechanistic models, while the second describes results obtained using measured environmental concentrations (MECs) instead of PECs.

6.4.1 PECs vs ADIs

An early attempt to evaluate potential risks to humans[19] estimated exposure *via* both drinking water and dietary fish intake for 26 selected APIs. Drinking-water concentrations were predicted assuming no physiological degradation and no removal during wastewater or drinking-water treatment. A hydrological model was used to estimate dilution of WWTP effluent into surface waters. The hydrological model was parameterised with data from eleven watersheds across the US, reflecting surface-water flows during the lowest average seven-day flow expected to occur in a ten-year period (7q10 flow). Bioconcentration in fish was estimated using a widely cited model

that makes predictions based on a substance's hydrophobicity.[24] ADIs were generated using API-specific UFs (adjusted depending on the type of data available for that API) ranging from one to one thousand. The highest RQs (ratio of estimated daily exposure divided by ADI) found for any stream locations were for ciprofloxacin (RQ = 0.3, UF = 1, reflecting potential effects on human gut bacteria), ranitidine (RQ = 0.1, UF = 100), metformin (RQ = 0.1, UF = 90) and warfarin (RQ = 0.1, UF = 90). However, comparison of available MECs to ADIs resulted in RQs less than 0.04 for all the APIs. This difference may be accounted for by API removal in-stream and during drinking-water treatment, which was not accounted for by the PECs, or by the use of low-flow dilution rates for generating PECs, rather than more typical flow rates.

A later analysis[23] of atomoxetine, duloxetine and olanzepine used the same hydrological model to estimate dilutions of effluents into streams, but added API-specific estimates of degradation in patients and QSAR-based predictions of WWTP removal rates. Comparing exposure estimates based on the 99th percentile in-stream PECs to ADIs (estimated as the ratio of the minimum daily therapeutic dose rate divided by a UF of 1000) suggested exposure rates were no more than 1/147 of the corresponding ADI.

Simple PECs,[25] ignoring potential reductions of API concentrations in WWTPs, surface water or DWTPs, were generated for 371 high-use APIs in the US. Although these PECs incorporate conservative assumptions about API removal rates, the PECs were based on national annual average drug usage and wastewater production rates and, therefore, may underestimate peak concentrations resulting from temporal or spatial

variability. The highest estimates of maximum potential drinking-water exposure in this study were less than 1/250 of minimum daily therapeutic dose.

Hydrological models for subsets of watersheds in the US and EU were employed[26] to estimate 90th percentile low-flow in-stream PECs and fish intake for 44 APIs. For most APIs, potential exposures were compared directly (without applying an uncertainty factor) to the minimum therapeutic dose rate. For antibiotics, PECs were instead compared with microbial effect concentrations. For anticancer drugs, a one hit model was used with the ADI (0.15 µg/day) calculated based on an 'acceptable risk' of one per million excess incidence of cancer. The only APIs with RQs greater than 0.01 were amoxicillin (RQ = 0.07, AF = 50), mercaptopurine (RQ = 0.04 based on a one per million excess cancer risk), hydrochlorthiazide (RQ = 0.03, AF = 30) and metformin (RQ = 0.02, AF = 90).

Probabilistic assessment[11] of human risks from carbamazepine, meprobamate and phenytoin exposure through drinking water and fish ingestion was conducted using available measured concentrations in surface water and drinking water to parameterise a log-normal distribution. The 99th percentile concentration from this distribution was used as an estimate of drinking-water concentrations, and fish intake was estimated from this concentration using a hydrophobicity-based bioconcentration model.[24] Employing UFs ranging from 30 to 90, 99th percentile risk quotients were found to be below 0.0001.

Site-specific PECs were generated for 589 APIs for six Australian hospitals,[27] including specific PECs for the hospital effluent and for influent to the receiving WWTP. The WWTP influent concentrations lower due to dilution of the hospital effluent by other inputs to the WWTP. Using PECs for hospital effluent and ADIs derived by dividing therapeutic dose rates by 1000 (or 10,000 for cytotoxic drugs), maximum RQs greater than or equal to one were reported for fifteen anesthetics, antibiotics and chemotherapy agents. By contrast, comparison with WWTP influent PECs resulted in only one RQ greater than one (for the cytotoxic agent vincristine, RQ = 2.5) and two other RQs greater than 0.1 (for the antibiotics piperacillin and tazobactam).

6.4.2 MECs vs ADIs

In 2003, the highest measured API concentrations reported in German drinking water were compared[28] with corresponding therapeutic dosage rates for more than 50 APIs. The highest relative exposure rates found (corresponding to a total of 0.02 daily doses consumed over 70 years of drinking water at the highest reported concentration) were for phenazone.

A later summary[29] of measured groundwater and drinking-water concentrations of 26 APIs across the globe suggested that it would take between 3.4 and 34,000 years (depending on the API) of drinking-water exposure to accumulate a single minimum daily dose of API. The researchers voiced some residual concern about ethinyl estradiol and norethindrone, because these drugs are counter-indicated during

pregnancy, but exposure might add up to 12 % of one dose or 1.5 % of 1 dose over 9 months of pregnancy (still very low relative to therapeutic exposures).

Concentration of 15 selected APIs in 222 finished drinking-water samples collected at various times from 19 DWTPs in the US have also been compared with ADIs.[30] For most APIs, ADIs were generating by applying uncertainty factors between 1000 and 10,000 to minimum therapeutic dose rates or animal safety data. For genotoxic APIs a one-hit model was used to estimate ADIs corresponding to a one per million excess cancer risk. Dividing the MECs by the corresponding ADIs resulted in RQs below 0.01 for all the APIs.

Measured concentrations of 52 APIs and hormones in 71 surface water and 70 groundwater samples collected in France suggested[31] maximum potential exposures for levonorgestrel (corresponding to 38 doses over 70 years of exposure), ethinyl estradiol (15 doses per 70 years), progesterone (one dose per 70 years), lorazepam (0.2 doses per 70 years), oxazepam (0.15 doses per 70 years) and diclofenac (0.1 doses per 70 years).

Measured concentrations of 56 prioritised APIs in 24-hour composite samples of effluent collected from 50 very large WWTPs in the US were recently reported.[32] Assuming drinking-water concentrations equal to maximum effluent MECs, the authors concluded drinking-water exposures would be less than one dose equivalent accumulated per decade for all the analytes except lisinopril (slightly less than one dose per year) and hydrochlorothiazide (about one dose per six years).

6.4.3 Genotoxicity

Several research groups have looked specifically at human risks from environmental exposure to genotoxic APIs. Various acceptable intake rates have been proposed. One commonly used cutoff (0.15 µg/day) was proposed[33] for genotoxic contaminants in food, and is supposed to correspond to an excess cancer risk of one per million. Another commonly used cutoff (1 µg/day) was adapted from regulatory limits[34] of cyclophosphamide oral exposure estimated to result in an excess cancer risk of no more than one per 100,000 exposed individuals.

Cyclophosphamide concentrations in hospital effluent up to 4.5 µg/l have been found[35]. However, only up to 143 ng/l was measured in the influent of the receiving WWTP (presumably reduced in large part due to dilution by wastewater from other inputs), and maximal concentrations in the WWTP effluent were only 17 ng/l. No mutagenic activity could be detected using a bacterial assay with prior metabolic activation, even in hospital effluent. Metabolic activation, by pre-incubating assay material with liver enzymes, is used because some genotoxic APIs (such as cyclophosphamide) are pro-drugs that require metabolic transformation in order to display genotoxic activity.

Similarly, comparison[36] of cyclophosphamide MECs reported from Europe and North America with a 1 µg/day threshold of concern suggested probable exposure rates were well below the threshold, with the highest reported WWTP effluent MEC being 146 ng/l and the highest reported surface-water MEC being 10 ng/l.

Mechanistic predictions[37] of wastewater concentrations of cyclophosphamide suggest combined drinking-water and dietary fish exposures to cyclophosphamide may be up to 18 ng/day. Based on available literature, total excess cancer deaths from cyclophosphamide therapy worldwide have been estimated to be about 800 per year. Using a linear extrapolation from this figure, along with estimates of doses used during treatment and numbers of individuals treated, the researchers concluded that environmental exposures might result in a one per million excess cancer risk.

An in-stream PEC distribution for 5-fluorouracil was generated[38] using a hydrological model parameterised using flow patterns of a large watershed in England. The model included estimates of degradation in patients, but not wastewater removal rates. Low-flow conditions were used to model dilution throughout the watershed. Resulting surface-water PECs for 5-fluorouracil were between 5 and 50 ng/l. Consumption of 2 l/day at the higher concentration would result in intake rates slightly below a 0.15 µg/day threshold of concern.

A very similar dilution model[39] was used to calculate in-stream PEC distributions for five APIs that are genotoxic alkylating agents. These PECs accounted for patient excretion rates, wastewater removal rates and in-stream dilution, assuming low-flow conditions. The estimated 90th percentile combined intake of all five APIs *via* drinking water was about 40 ng/day, below a 0.15 ug/day threshold of concern.

6.4.4 Hormonal Disruption

Hormonally active pharmaceuticals mimic the actions of, and are often chemically identical to, endogenous hormones. Humans are normally exposed to a background level of endogenously produced hormones at all points in development, and many common foodstuffs naturally contain substantial quantities of hormonally active material. These low-level background hormonal exposures, which are fairly well characterised, are typically considered safe and normal. This suggests the possibility of using the background exposure levels as benchmarks for comparison with potential environmental exposures to hormonally active pharmaceuticals. The most widely used classes of hormonally active APIs are estrogens, progestins, androgens, thyroid hormones and corticosteroids. Estrogens are used in contraceptive formulations as well as for hormone replacement therapy. Progestins are used in contraceptives and also are used during fertility treatments. Androgens and thyroid hormones are used for hormone replacement therapy. Corticosteroids are primarily used to treat inflammation and also are used for hormone replacement.

Among the hormonally active APIs potentially found in the aquatic environment, estrogens are by far the most frequently studied. Estrogenic APIs include the synthetic hormone ethinyl estradiol (primarily administered for birth-control purposes) and a variety of naturally occurring animal estrogens (usually administered for hormone replacement therapy). Estrogenically activity hormones are endogenously produced and excreted by people as well as by other vertebrates. A substantial proportion of estrogenic activity in wastewater can be accounted for by endogenously produced

estrogens naturally excreted by people. Estrogens are naturally present at appreciable concentrations in many foodstuffs, particularly dairy products, eggs and meat.

Potential exposure rates to ethinyl estradiol have been estimated[37] on the basis of simplified water and fish PECs, which assumed no API removal during wastewater treatment. Ethinyl estradiol intake through drinking water and fish was predicted to potentially reach about 85 ng/day, which is considerably lower than endogenous estrogen production, even in demographic groups which produce very little estrogen. For instance, estradiol production in prepubescent boys is about 6 µg/day, which is considerably lower. Comparison of endogenous estradiol production directly to potential oral ethinyl estradiol intake was justified by pointing out that the latter's greater potency is largely due to improved bioavailability, which does not affect plasma levels of endogenously produced hormones.

Comparison[28] of MECs reported in drinking water with therapeutic dose rates for ethinyl estradiol suggests that maximum potential drinking-water exposures are at least 7,000-fold below therapeutic dosing rates. Potential drinking-water exposures are also several orders of magnitude below endogenous production in prepubescent boys, below dietary intake of naturally occurring estrogens and below ADIs developed for acceptable dietary exposures to estrogenic food additives.

PECs for ethinyl estradiol, estradiol, estrone and estriol have also been calculated[40] using a hydrological dilution model parameterised for low-flow conditions in eleven different watersheds in the US. The PECs accounted for degradation in patients and

removal during WWTP treatment. Potential combined drinking-water exposure (expressed as estradiol equivalents) to these four compounds was reported to be about two orders of magnitude below estimated dietary intake of naturally occurring estrogens, and several thousand-fold below ADIs developed for dietary exposures to estrogenic food additives. Potential combined exposure was also more than 100-fold below allowable levels for occupational exposure to ethinyl estradiol.

In vitro (using an estrogen-sensitive cell-based assay) measurements of aggregate estrogenic activity in water (raw and finished) from 17 DWTPs have been compared[41] with estrogenic activity in various foodstuffs. Estrogenic activity of drinking water was reported to be similar to the level of activity in apple juice, baby formula and milk. Estrogenic activity in surface water was found to be higher, being similar to activity observed in coffee and tea. Estrogenic activity in human breast milk, soy milk and soy-based infant formula were many orders of magnitude higher than any water sample tested.

Published MECs of ethinyl estradiol, estradiol, estriol and estrone measured in the US (including samples from wastewater, environmental waters and drinking water)[42] also suggest potential human exposures are low relative to therapeutic or dietary exposures. Consumption of two litres per day of water at the highest reported concentration of ethinyl estradiol in WWTP effluents, ambient water or drinking water (omitting a single extremely high outlier from the several hundred available data points) would be equivalent to administration of about 1/1000 of a minimal therapeutic dose. Total normalised estrogenic activity possible in drinking water can

be estimated based on relative potency in a variety of assays and the highest MECs for these four analytes in any environmental waters (minus the aforementioned outlier). Resulting worst-case total daily estrogenic exposure from all four estrogens in water are comparable to what is expected from drinking cow's milk.

Fewer data are available on the occurrence of APIs that act as progestins, androgens, thyroid hormones or corticosteroids. Although the latter are widely prescribed, their concentrations have been reported much less frequently than the concentrations of estrogenic APIs. Progesterone has been reported[31] in water at concentrations up to 11 ng/l, much of which may reflect natural human excretion of endogenous hormone. This is well below levels reported in milk[41] (about 3,100 ng/l). Synthetic progestins have also been reported in the environment, including norethindrone[31] (up to 8 ng/l), levonorgestrel[31] (up to 11 ng/l in drinking water) and medroxyprogesterone[43] (up to 15 ng/l in WWTP effluent). Similarly, testosterone has been detected[43] in surface waters at up to 6.1 ng/l, while concentrations in milk are about[41] 30 ng/l.

6.4.5 Susceptible Sub-populations

Some human sub-populations are more susceptible to effects from some APIs because of an enhanced dose response, which is usually due to enhanced bioavailability or reduced clearance of API. Affected sub-populations can include pregnant women, infants, small children, the elderly, liver patients, kidney patients and individuals carrying certain rare genetic variations. Some other human sub-populations are more susceptible to undesirable effects of some APIs because the molecular receptor of the

API has a different physiological role in that sub-population. This often applies to humans during early stages of development, including children, infants and fetuses *in utero*. For these sub-populations, detailed dose-response data are usually not available and cannot be directly measured due to ethical concerns. However, most often these effects seem to be mediated by the same molecular API receptors that are responsible for therapeutic effects; therefore, one might expect a similar probability of response to a given plasma concentration of API in both targeted patients and in sensitive sub-populations. That is, the sensitivity of the sub-population does not typically appear to result from a different concentration response at the molecular receptor. Therefore, it is possible that the plasma concentration model, which is described in section 5 on risks to aquatic life, might be usefully adapted to screening-level risk assessment of APIs in these types of susceptible sub-populations.

The probability of triggering allergies or other idiopathic reactions at different concentrations of API is not completely known. Nevertheless, some clinical experience and research on allergen-related food safety may provide some guidance on safe exposures. It has been reported that the potential for idiopathic drug reactions is much rarer with drugs that are administered at no more than 10 mg/day. Nevertheless, the smallest dose potentially eliciting a penicillin allergy has been estimated[4] to be as low as 0.24 µg although 6 µg, or about 1/10,000 of a minimal human dose, is more often cited.[37] For food allergies, estimates of safe levels include[44] an oral threshold dose (corresponding to an estimate of no more than one reaction per million exposed individuals) as low as 100 ng for peanut-allergen protein (one of

the most potent known food allergens). This figure was extrapolated from a variety of published data using a fitted parametric function, and substantially higher 'safe levels' have been proposed (for example, 3 µg peanut-allergen protein[45]). Limitations in available data, coupled with uncertainties about the proper distribution to use when extrapolating beyond the available data, once again complicate efforts to arrive at a definitive risk estimate.[46]

PECs generated[37] assuming no removal during wastewater treatment suggest the possibility of penicillin V exposures up to 86 µg/day from drinking water. However, most reported MECs suggest efficient removal of penicillins. For instance, one study,[4] using an immunoassay with a 10 ng/l detection limit failed to find penicillin or recognisable (using an antibody-based assay) metabolites in drinking water. Similarly, published MECs for penicillins V and G in German drinking water were found[28] to all be below 50 ng/l detection limits. A review[22] of published MECs measured in the US reported that penicillins V and G have also never been detected in US effluents, surface water or groundwater, despite several attempts to look for them.

Penicillins are discussed above because of their well-known potential to elicit allergic reactions in susceptible individuals. However, idiopathic reactions have been reported for other drugs. Potential drinking-water concentrations for various APIs have been reported between the 'safe' estimates of 50 ng/l (corresponding to a 100 ng/day intake safe level suggested for peanut-allergen protein) and 3,000 ng/l (corresponding to the considerably higher 6 µg/day penicillin intake suggested as safe even in allergic individuals). Much more rarely, concentrations of some APIs have been reported

above both of these levels. For instance, up to 3,100 ng/l of ibuprofen has been measured[47] in groundwater samples collected in the US. Other APIs detected in potential drinking water above 50 ng/L include carbamazepine concentrations as high 900 ng/l in French groundwater samples[31] and phenazone in German drinking-water samples[48] as high as 400 ng/l. There are no data to suggest these levels really have any potential to elicit idiopathic reactions, but it is difficult to completely rule out any possibility, given our uncertainty about the shape of the response distribution at very low doses.

6.4.6 Conclusions on Human Risks

Studies using predicted or measured wastewater concentrations as a surrogate for drinking-water concentrations generally conclude that maximum human exposure rates to APIs in drinking water and fish are on the order of 100-fold below therapeutic dose rates. By contrast, studies based on groundwater or drinking-water concentrations generally suggest maximum possible exposures are actually on the order of 1000-fold below therapeutic dose rates. Both types of studies suggest typical exposures are probably at least ten-fold lower than this and are well below one dose per lifetime for most APIs. Risks from individual genotoxic APIs are usually estimated to be approximately one excess cancer case per million exposed individuals or lower. Likely exposure rates to estrogenic APIs *via* drinking water are no higher than exposures that would be expected from apple juice and considerably lower than potential exposures from several other common foodstuffs. These data suggest risks to humans from exposure to APIs in fish or drinking water are very low. The main

residual uncertainties involve the possibility of very rare allergic reactions or greater than additive interactions between different co-occurring APIs.

These conclusions are in agreement with the assessment presented in a recent WHO report[49] about pharmaceuticals in drinking water. This report concluded that targeted investigations conducted in the above-mentioned countries found that traces of pharmaceuticals in drinking water are largely present at several orders of magnitude (more than 1000-fold) below the lowest therapeutic dose and largely below the calculated ADIs. The substantial margins of safety for individual compounds suggest that appreciable adverse impacts on human health are very unlikely at current levels of exposure in drinking water.

6.5 Potential Risks to Aquatic Life

Numerous publications have emerged in the last two decades describing levels of pharmaceuticals in the environment as well as eco-toxicological effects of pharmaceuticals on environmental species of various taxa. We shall not reiterate all this knowledge, but will focus on the efforts to combine these different pieces of information to risk-estimation approaches. We shall also describe some of the difficulties in achieving valid and scientifically sound estimates of environmental risk.

6.5.1 Limitations of Available Ecotoxicity Data

The investigations of environmental effects of pharmaceutical compounds cover a large range of different taxa, study protocols and endpoints. When evaluating eco-toxicological studies available for estimating risks, one must consider whether the study protocols and endpoints selected are relevant for risk assessment. Some studies provide insight into mechanistic responses in organisms or show adaptive effects which have no population relevance. Others follow novel approaches such as behavioural testing, but the historical database for those endpoints in a given species is small or non-existent. As a result, the reproducibility and variability of these measures is poorly characterised and protocols for ensuring reliability have not yet been developed. More importantly, the relevance of these non-traditional outcome measures to estimating population impacts is largely unknown.

When data are available, one often sees uncorroborated outliers that are several orders of magnitude different from the rest of the data points.[50] This may be more likely when considering non-traditional endpoints (like behaviour), perhaps in part due to lower reliability of the measures employed, as well as the incomparability of these endpoints with traditional endpoints of growth, survival and reproduction. Given how sparse ecotoxicity data for most APIs are, and the low levels of replication used in many ecotoxicity studies, it is usually difficult to discern whether such extreme results are likely to be reproducible. Nevertheless, outlier data points can drive many estimates if non-robust methods are used for risk estimation. This suggests the importance of 'duplicative' research to corroborate data points that have particular

influence on risk estimates, and the importance of conducting studies to determine population impacts associated with non-traditional endpoints.

When estimating potential toxicity to aquatic life, challenges posed by the large number of pharmaceuticals to be evaluated are compounded by the huge number of species, developmental stages and endpoints that might be affected. Furthermore, toxicological measurements often involve more time and expense than occurrence measurements, meaning there are typically even fewer available measurements of toxicity in aquatic species than there are measurements of aquatic occurrence. Estimates suggest there is some ecotoxicity data for only 1 %[51] to 10 %[52] of APIs in any organism. A more recent survey[53] reported that although EU environmental risk assessments (ERAs) are available for about 650 human pharmaceuticals, only about 120 of these include chronic ecotoxicity data on at least three species, as well as inhibition tests on sewage sludge microorganisms, environmental fate data and a crude estimate of exposure. This suggests that both effects and fate data in regulatory ERAs are only available for about 6 % of the approximately 2000 APIs[54] registered for use in Europe. Another 530 compounds were reported to not require generation of experimental data either because of low environmental exposure (the guideline established a PEC surface-water trigger value of 10 ng/l), lack of environmental relevance (vitamins, electrolytes, amino acids, peptides, proteins, carbohydrates, lipids, vaccines and herbal medicines do not require a formal regulatory environmental risk assessment), or were related to existing products and not supposed to substantially increase current environmental concentrations.

Chronic toxicity data, particularly on legacy compounds, are considerably rarer than the already rare acute toxicity data described above. Furthermore, the majority of toxicity data are reported as LC_{50}s and EC_{50}s rather than NOECs or LOECs. Most of the available values are greater than 1 mg/l.[52, 55] Even most reported chronic NOECs are above 100 ug/l,[52] which is also well above typical API concentrations, even in WWTP effluents.

While probabilistic sampling and subsequent statistical modelling of chemical concentrations in surface waters or POTW effluents is relatively straightforward (though imperfect), there is no scientific consensus on how to effectively sample or model toxicological data across biological species. Ideally, test species would be selected to be as representative as possible of all the species that might be exposed to the toxicant. Unfortunately, toxicant sensitivity can vary greatly between what are apparently closely related species. This lack of reliable association between species spacing in a taxonomic tree and differences in sensitivity, combined with practical limitations on the volume of toxicity testing that can be conducted, makes it difficult to ensure that the more sensitive species are represented in any test set. As a result, it is virtually impossible to get completely convincing representation of the entire tree of life for any toxicant. Instead, we are usually confronted with very sparse data of uncertain relevance for characterising the most sensitive species and endpoints.

A number of relatively large studies have evaluated risks to aquatic life by comparing predicted environmental concentrations for a broad swath of APIs with predicted no-effect concentrations (PNECs) developed from available ecotoxicity estimates. A few

typical ones are described in the next subsection. We then summarise results from some large studies using measured environmental concentrations to estimate ecological risks. Most of the researchers whose work is summarised have voiced concerns about the reliability of PNEC estimates due to the shortage of dose-response data of any type in enough ecologically relevant species. More particularly, a shortage of chronic data reflective of the API's mode of action is frequently noted. This broad overview provides some perspective on the relative concerns voiced for different classes of APIs. It also helps provide some motivation for our subsequent, more detailed examination of estrogens, antidepressants and antibiotics.

6.5.2 PECs vs PNECs

Surface-water PECs were estimated[56] for 111 frequently dispensed APIs in Germany, incorporating estimates of human metabolism, assuming no WWTP removal but ten-fold dilution into receiving waters. Comparison to PNECs calculated by applying a 1000-fold uncertainty factor to EC_{50}s resulted in RQs greater than one for ciprofloxacin (RQ = 12, based on bacterial inhibition), ethinyl estradiol (RQ = 5.5) and clarithromycin (RQ = 1.5, based on bacterial inhibition). This suggests worst-case potential exposures to a few APIs may involve concentrations well below EC_{50}s, but still within a 1000-fold uncertainty factor designed to account for extrapolations across species, within species, and from EC_{50}s to NOECs.

PECs were calculated[57] for 27 of the 100 most-dispensed drugs in Sweden, incorporating hydrophobicity-based estimates of WWTP removal rates and an

assumption of ten-fold dilution of WWTP effluent into receiving waters. Comparison with PNECs derived by applying API-specific factors to available ecotoxicity data resulted in risk quotients above one for estradiol (RQ = 180, UF = 50, applied to a NOEC for vitellogenin gene induction, rather than a traditional apical endpoint), estriol (RQ = 1.6, UF = 100, applied to a fish reproduction NOEC), ethinyl estradiol (RQ = 10, UF = 50, on a chronic fish NOEC for reproduction) and acetaminophen (also known as paracetamol, RQ = 1.41, UF = 1000, applied to an invertebrate 24-hour LC_{50}).

PECs have also been generated[15] for 112 APIs widely dispensed in France, incorporating estimates of physiological degradation in patients and, when available for a particular API, experimentally determined WWTP removal rates. These PECs also assumed ten-fold dilution of WWTP effluent into surface waters. PNECs were calculated by applying an API-specific UF to available effect concentration estimates. The highest estimated risk quotient was for amoxicillin (RQ = 62, UF = 10, based on inhibition of cyanobacterial growth). Risk quotients were below one for the other APIs, but above 0.1 (with UFs between 10 and 100) for aspirin, ofloxacin, propranolol, carbamazepine, furosemide, clarithromycin, diclofenac and sertraline.

PNECs derived from QSARs have been used[58] to estimate the toxicity of 100 drugs dispensed within a general hospital and a psychiatric clinic in Switzerland. Site-specific PECs were calculated from local API and water-usage rates, assuming no metabolic degradation or WWTP removal. PNECs were calculated by dividing log D-based QSAR estimates of EC_{50}s by 1000. Highest risk quotients for hospital effluent were found for amiodarone (RQ = 85) and risk quotients for nine other APIs exceeded one. By

contrast, using PECs for the effluent of WWTPs receiving the hospital effluents, the highest risk quotient was about one (amiodarone), followed by 0.17 (clotrimazole).

Surface-water PECs (parameterised using French dispensing rates) have also been reported[59] for 60 anticancer drugs. The PECs incorporated estimates of human metabolism, experimentally determined WWTP removal rates (when available for an API) and an assumption of ten-fold dilution of WWTP effluent into surface waters. Comparing the PECs, along with available MECs, with reported ecotoxicity values suggests there is little risk from any of the APIs considered in isolation, but the authors expressed concerns about the limited amount of data available for assessing mixture effects.

6.5.3 MECs vs PNECs

A probabilistic approach has been used[12] to estimate risks from 67 APIs in the US and EU. A 95th percentile concentration for each API was estimated either empirically (if enough data were available) or by fitting a log-normal distribution to available MECs. A distribution was also fitted to QSAR predictions of toxicity in different species in order to estimate a 10th percentile EC_{50}. The 95th percentile surface-water concentrations were found to be at least an order of magnitude below 10th percentile effect concentrations, and no MECs exceeded the lowest reported EC_{50}s.

An early comparison[51] of published ecotoxicity values for a very broad range of APIs with maximal concentrations reported in wastewater effluent suggests that, for most APIs, maximal effluent concentrations are at least one to two orders of magnitude

lower than available LOECs for aquatic life. However, the lowest available diclofenac LOEC (reflecting fish toxicity) overlapped with frequently seen (but higher than median) wastewater concentrations. For propranolol and fluoxetine the lowest LOECs (describing effects in zooplankton) were similar to the highest reported effluent concentrations.

A more recent large-scale comparison[60] of published occurrence concentrations with aquatic-effect (including biochemical effects with unknown consequences for individual and population fitness) concentrations, suggested that the highest reported aquatic concentrations for most APIs are at least an order of magnitude below effect concentrations. However, highest-reported WWTP effluent concentrations exceeded lowest-reported effect concentrations for ethinyl estradiol, diclofenac, ibuprofen and fluoxetine.

Log-normal distributions fitted to available aquatic concentration data have been used to estimate[8] the 95th percentile (relatively high) of environmental concentrations of 22 APIs selected on the basis of high use and relatively high ecotoxicity. A log-normal distribution fitted to available ecotoxicity data was then used to estimate the 5th percentile (relatively low) toxic concentration estimate. The only APIs with RQs greater than 0.01 were ciprofloxacin (RQ = 0.1), ofloxacin (0.1), furosemide (0.024), ibuprofen (0.014) and propranolol (0.011).

RQs generated[61] using MECs of 32 APIs or metabolites in samples from Spanish surface waters and PNECs derived by applying varying UFs to available ecotoxicity

data only exceed one for the antibiotics clarithromycin (RQ = 38, UF = 1000), azithromycin (RQ = 30, UF = 1000), trimethoprim (RQ = 8, UF = 1000) and sulfamethoxazole (RQ = 7, UF = 1000).

Measured concentrations of 73 APIs in three hospital effluents, as well as influent and effluent of the receiving WWTP, have been reported.[62] Dividing WWTP effluent MECs by PNECs (EC50s divided by a UF of 1000) resulted in risk quotients greater than one for the antibiotics ciprofloxacin (RQ = 279), sulfamethoxazole (RQ = 90), ofloxacin (RQ = 20), azithromycin (RQ = 15) and clarithromycin (RQ = 6).

MECs of 26 APIs in effluent samples collected from seven WWTPs[63] include ofloxacin MECs greater than 1/100 of the lowest published ofloxacin EC_{50}. In addition, MECs for gemfibrozil, ibuprofen, ciprofloxacin, lomefloxacin, norfloxacin and sulfamethoxazole occasionally exceeded 1/1000 of the lowest corresponding EC_{50} value.

6.5.4 Estrogenic APIs

The ambiguities that can result from model fitting, even with relatively large data sets, are exemplified by the estrogens ethinyl estradiol and estradiol. One analysis,[9] using a Weibull distribution fitted to reproductive and developmental toxicity data from 26 species reported in 52 different studies, estimated a PNEC for ethinyl estradiol of 0.35 ng/l. This PNEC theoretically corresponds to the lower bound of a 50 % bootstrap-based confidence interval for the concentration affecting 5 % of exposed species. A similar study,[64] including more-recent data for ethinyl estradiol as well as data for

estradiol, estrone and estriol, reported PNEC values of 0.1, 2, 6 and 60 ng/l, respectively.

Based on practically the same data set and derivation method, but using a slightly different curve-fitting as well as an additional uncertainty factor of two, the European Commission derived[65] an environmental quality standard (which is comparable to a PNEC) of 0.035 ng/l and 0.4 ng/l for ethinyl estradiol and estradiol, respectively. This demonstrates that, even for relatively large overlapping data sets, the derivation of PNECs can differ several-fold, depending on the models used, which makes risk assessment for those compounds more complex.

The relevance of laboratory studies of ethinyl estradiol for predicting outcomes in the field was corroborated[66] by dosing an experimental lake in Canada. Ethinyl estradiol was introduced into the lake system for two consecutive years during the vegetational season and effects were investigated on a number of species representing the food web of those lakes. At an average ethinyl estradiol concentration of 4 - 6 ng/l, several direct as well as indirect population effects were seen on fish and invertebrates. This work showed that observations previously made in the laboratory agreed well with this ecosystem approach when the same fish species (fathead minnow) was used in both sets of experiments.

Although many analytical data have been published, the estimation of risks associated with ethinyl estradiol in the aquatic environment is further complicated because estimated PNEC values are at or below the typical detection limits. An analysis[67] of the

available literature data on ethinyl estradiol water concentrations in Europe and the US found that more than 85 % of samples were below the limit of detection (usually between 0.1 and 1 ng/l). Models incorporating hydrological parameters for watersheds in either the US or Europe predict water concentrations of 0.2 - 0.3 ng/l, which are slightly above the estimated chronic PNECs. However, given the very conservative approaches built into these exposure models (*e.g.* parameterisation for 90th-percentile low-flow conditions), it is difficult to evaluate whether typical water concentrations of ethinyl estradiol have an impact on aquatic life, apart from some potential exposure hot spots. This residual uncertainty persists despite the fact that occurrence and toxicity data are much more abundant for ethinyl estradiol than they are for the vast majority of pharmaceutical compounds.

6.5.5 Antidepressants

Amongst the other groups of pharmaceuticals studied recently for their potential environmental impacts are therapeutic classes such as antidepressants, antibiotics, cytostatics, antiviral and anti-inflammatory compounds. Particular attention has recently been focused on antidepressants like selective serotonin-reuptake inhibitors (SSRIs) or serotonin-noradrenaline reuptake inhibitor (SSNRIs), due to their potential effects on behaviour of environmental organisms.

The effects of the SSRI fluoxetine on the behaviour of fish was evaluated[68] by exposing mating pairs of fathead minnow for four weeks to fluoxetine concentrations of 0.1, 1, 10 and 100 µg/l. Endpoints such as mating behaviour, sex steroids, feeding behaviour

and reproductive success were measured. Changes in male mating behaviour were reported at concentrations from 1 µg/l; however, for some of the parameters the effect did not show conventional concentration dependence. Looking to population relevance of the observed effects, survival of female fish and, in consequence, reproductive success was only disturbed at the highest concentration of 100 µg/l. The authors claim that environmentally relevant concentrations of fluoxetine (0.012 - 1.4 µg/l are cited) cause adverse effects in fish. However, the population-relevant endpoints of survival and reproduction clearly indicate that there is at least a factor of ten difference between the traditional NOEC suggested by this study and the upper range of environmental concentrations. Certainly, these measurements of non-traditional endpoints suggest further research on environmental risks of fluoxetine, but they do not establish an environmental risk for this compound.

The effects of the antidepressant venlafaxine on stress responses resulting from handling were investigated[69] in rainbow trout juveniles exposed to concentrations of 0.2 and 1 µg/l venlafaxine. Transient changes of some biochemical parameters (sodium-potassium ATPase activity, plasma glucose levels) were observed after 7 days exposure at 1 µg/l. Although this is within the range of reported environmental concentrations (the authors cited 0.2 - 2.2 µg/l close to WWTP outfalls), the relevance of these biochemical endpoints to fitness of individual fish and population stability is not known.

A more population-relevant observation for venlafaxine fish toxicity was made[70] by exposing male fathead minnow to two concentrations (0.3 and 1.1 µg/l) over a period

of 21 days. Survival of fish was reduced in both treatments; however, the response did not show a monotonic association between concentration and response intensity. These data are difficult to apply to risk assessment because of the limited number of treatment concentrations employed and the absence of a LOEC concentration. Nevertheless, valuable information has been gained for mode-of-action analysis on the basis of the various reported parameters.

6.5.6 Plasma Concentration Model

The 'read-across' approach[71-74] has been suggested as a means to compensate for shortcomings in the ecotoxicity data available for most APIs. This approach suggests that one might be able to use human pharmacological parameters in order to inform assessments of potential risks to aquatic life, because of the substantial degree of shared physiology across the tree of life. One method proposed for doing this is to compare expected plasma concentrations in fish exposed to a given water concentration of API with therapeutically effective plasma concentrations in people. This approach assumes that potentially increased sensitivity in non-human species results primarily from differences in pharmacokinetics rather than differences in pharmacodynamics. This suggested approach was subsequently elaborated[75] by addition of a bioconcentration model[76] which is used to predict fish plasma concentrations based on water concentrations and the API's hydrophobicity.

The bioconcentration model employed was originally developed[24] using a training set of highly hydrophobic pollutants that are not ionisable within the typical pH range of

surface waters. This may be an issue for some APIs (which tend to be more hydrophilic), particularly those that might be ionisable. Ionisation can drastically affect partitioning and introduces a strong pH-dependence on partitioning rates. APIs that are weak bases or weak acids may gain or lose protons near neutral pH, resulting in a substantial proportion of the molecules being ionised (carrying a charge) in equilibrium with the remaining un-ionised molecules. The ratio of ionised to un-ionised material at a particular pH is expressed as the K_d. The effect of pH on partitioning should be better accounted for[72, 77] by accounting for the K_d in addition to the K_{ow} of the neutral species.

The scant evidence addressing pH effects seems to provide some empirical support for this rationale: a study[77] examining the effects of pH on the plasma concentration of fish exposed to water containing sertraline (which is ionisable near neutral pH), found that plasma concentrations were predicted substantially better when pH-dependent ionisation of sertraline was accounted for. The authors also reported that plasma concentrations correlated with binding of sertraline to the fish version of the sertraline molecular receptor in the exposed fish's brains. Plasma concentrations were also associated with measureable behavioural changes and also help explain the ten-fold change in lethal concentration observed between pH 6.5 and 8.5. The behavioural effects were, however, only seen at water concentrations well above those measured in the aquatic environment. Another application of the plasma model to 42 different human pharmaceuticals[78] also discussed improvements in predictions of steady-state fish plasma concentrations when accounting for pH-dependent ionisation.

In addition to pH, other environmental factors (such as presence of colloidal substances, which can reduce the bioavailability of toxicants that are bound to them, or low dissolved oxygen, which can greatly increase the brachial ventilation rate in gills, thereby potentially increasing extraction rate of toxicants from the water) apparently can result in large differences in bioconcentration rates of the same API under differing ambient conditions.[79] Furthermore, hydrophobicity-based bioconcentration models seem to dramatically under-predict bioconcentration in plasma of some APIs.

For instance, some reports suggest that propranolol,[80] levonorgestrel[81] and cilazapril[81] plasma concentrations may be 15- to 260-times greater than would be predicted using the usual hydrophobicity-based model. For levonorgestrel, this behaviour may reflect specific binding of levonorgestrel to sex-hormone globulin,[81] which may provide higher capacity binding and higher affinity binding than a simple hydrophobicity-based model might predict.

In contrast to the propranolol results described above, 40-day exposures of trout to propranolol[82] suggest that propranolol plasma concentrations are in good agreement with predictions from the hydrophobicity-based bioconcentration model. Given the widespread use of this model, the lack of consensus and the limited quantity of available data, this appears to be another area where more high-quality data exploring the effects of various parameters, such as exposure duration, pH, temperature, other water constituents, *etc*., would be welcome. Beyond the plasma-concentration model discussed here, changes in toxicant bioavailability under varying

environmental conditions would be expected to affect other important parameters, such as toxicity at a given toxicant concentration. This issue is widely recognised in ecotoxicology, but is quite complicated and currently not characterised well enough for most toxicants to provide reliable predictions under real-world conditions. Despite all the potential issues with the plasma model described to this point, the simple approach suggested by Huggett *et al*. usually seems to give reasonable order-of-magnitude predictions of plasma concentrations of most APIs under most conditions that have been examined.[74, 77, 79-81, 83]

Comparison[81] of concentrations of 14 APIs in wastewater effluent with concentrations in the plasma of trout exposed *in situ* to the effluent stream for two weeks suggested that, for 12 of the analytes, plasma concentrations were within an order of magnitude of what would be expected from the hydrophobicity-based bioconcentration model. By contrast, levonorgestrel and cilazapril plasma concentrations were much higher than expected. Levonorgestrel fish-plasma concentrations exceeded the human therapeutic plasma concentration, while haloperidol, risperidone and cilazapril concentrations were within 1/10 of the corresponding therapeutic plasma concentration, raising some concern about potential low-level effects.

The same research group applied a hydrophobicity-based bioconcentration model to published MEC data in order to estimate plasma concentrations that could be reached in exposed fish.[84] This analysis suggested that only for estrone were reported MECs high enough to potentially result in fish-plasma concentrations exceeding the human therapeutic plasma concentration. However, exposing[83] trout to diclofenac

concentrations similar to the highest-reported environmental concentrations has been found to result in plasma concentrations in exposed fish that are similar to the human therapeutic plasma concentration.

6.5.7 Conclusions on Risks to Aquatic Life

There are only a handful of APIs for which there exist enough ecotoxicity data to generate robust species-sensitivity distributions. Of greater concern, no measured ecotoxicity data whatsoever is available for most APIs. When data are available, they usually take the form of acute EC_{50}s, rather than the chronic NOECs that are more directly applicable (does not require use of uncertainty factors to account for exposure duration and response intensity) to estimating risks. Comparisons between PECs or MECs and the limited available ecotoxicity data suggest that typical (*i.e.* near the average or median) pharmaceutical concentrations are well below effect concentrations for all pharmaceuticals. By contrast, for a few APIs peak environmental concentrations occasionally exceed lowest-reported effect concentrations. This situation has most often been reported for certain antibiotics, estrogens, analgesics, antidepressants and blood-pressure medicines. It is not clear whether these peak exposures, which are apparently geographically and temporally limited, might have any effects on populations of aquatic life. However, little direct evidence suggests important effects on ecological health.

6.6 Antibiotics, Clinical Resistance and Potential Risks to Beneficial Microbes

Many antimicrobial substances are naturally produced by animals, plants and, most famously, by microbes. Antimicrobials are produced by multicellular organisms primarily as a defense against pathogens. The role of antibiotics produced by microbes is more controversial, but is often suggested to include inhibiting the growth of competitors. Interestingly, in laboratory cultures, concentrations of antibiotics capable of inhibiting growth of other species are typically only produced when there is a high concentration of the producer bacteria, but not during the exponential growth phase, when competition might be suspected to be more intense. Evidence suggests that antibiotics produced by microbes often serve purposes other than inhibiting competitors, such signalling for intracellular regulation, as well as intercellular signalling (such as quorum sensing) between related bacteria.[85] Many antibiotics used in clinical practice are chemical derivatives of naturally occurring antibiotics. Others, such as fluoroquinolones, are structurally unrelated to naturally occurring antibiotics. Antibiotic resistance to both classes of antibiotics is common in patient populations across the globe.

Microbial resistance to antibiotics is also widespread in nature and clearly predates human use of antibiotics.[85] Virtually all isolates of some bacterial species (*e.g. Pseudomonas aeruginosa*, a known pathogen) carry chromosomally encoded resistance genes. This sort of resistance, which is a typical characteristic of a species rather than a differential characteristic of particular strains, is termed 'intrinsic resistance'. Structural features typical of a bacterial species sometimes explain intrinsic resistance. For example, the cell wall of gram-negative bacteria confers

resistance to wide range of antibiotics by limiting permeability of the bacterial cell to the antibiotic. Intrinsic resistance is also often due to characteristics of the molecular target of the antibiotic in that species, such as variations in ribosomal RNA sequence, or differences in the API binding site of a critical enzyme that interferes with API binding. Other cases of intrinsic resistance may be explained by the bacterial species possessing enzymes that efficiently clear API from the cell. The main function of such enzymes in intrinsically resistant species may not be conferring antibiotic resistance. Some of these enzymes have instead been implicated in regulation of cell-wall synthesis, signal-molecule export, or general detoxification of the bacterial cell.[86]

In contrast to intrinsic resistance, which is a typical characteristic of a species, acquired resistance refers to resistance that is initially absent or rare in a species but becomes substantially more common after selection by antibiotic exposure.[87] Spread of acquired resistance can occur either by clonal expansion of resistant organisms under selective pressure (such as widespread antibiotic use), or by horizontal genetic transfer (HGT) of resistance genes between cells of different bacterial strains or species.[87] The relative importance of these two mechanisms can vary greatly, depending on the bacterial species involved, and both appear to contribute to clinical resistance in very important human pathogens. For instance, widespread dispersal of multidrug-resistant strains of tuberculosis is one of the greatest global health challenges, but there is no evidence of involvement of HGT in the development of tuberculosis drug resistance. Instead, it is thought to involve step-wise selection of antibiotic-resistant mutants in treated patients, followed by direct patient-to-patient

transmission of resistant bacterial strains.[85] By contrast, phylogenetic analysis has suggested that vancomycin (an antibiotic of last resort) resistance in pathogenic *Staphylococci* may have arisen by HGT from soil bacteria that naturally harbor a diverse set of resistance genes.[86] Phylogenetic analysis has also been used to suggest[88] that ubiquitous bacteria, such as *Bacillus circulans*, can shuttle resistance genes between soil, residences, clinics and the gut.

HGT can take place[87] by conjugation (direct transfer of DNA between two bacterial cells in a process analogous to mating), transfection (uptake of DNA directly from the environment, perhaps released from other cells after death), or transduction (transfer of genetic material by bacteriophages, which are viruses that infect bacteria). Bare DNA is naturally taken up by some bacterial species and can sometimes be incorporated directly into the receiving bacterium's chromosome, resulting in stable retention of a functional resistance gene. However, this process is usually a very inefficient. Resistance genes are transferred much more efficiently between bacteria after the genes are incorporated into a mobile genetic element, such as a plasmid, transposon or integron. Mobile elements contain other sequences that facilitate efficient propagation within bacteria and transfer between bacteria.[89] In addition to one or more resistance genes, these mobile elements often carry genes that provide other adaptive functions, such as enzymes that allow bacteria to assimilate nutrients that would otherwise be unavailable. The range of species between which HGT occurs can be broad (it has occasionally been documented to occur between gram-positive and gram-negative bacteria, which is the primary taxonomic division of eubacteria),

but is usually limited by the range of species in which the sequences facilitating mobile element propagation and transfer properly function. Therefore, HGT is most often observed within a genus, reminiscent of limitations in cross-species genetic exchange in sexually reproducing species. The acquired resistance genes can persist long after selection by antibiotics is over[89, 90] and often there appears to be very little selective pressure for their loss.

Antibiotics, antibiotic-resistant pathogens and phages carrying resistance genes are shed by patients into sewage collection systems. All three are subsequently found in biosolids[91] and municipal wastewater effluents,[92] even after tertiary treatment.[93] Similar releases have been reported from land-applied manure from cattle treated with antibiotics.[94] HGT of resistance genes into resident WWTP bacterial populations has also been reported.[95] Mapping the environmental distribution of resistance genes (for instance, downstream of WWTPs) has suggested an association with anthropogenic point sources.[92, 93, 96] Bacteria with acquired resistance genes have also been reported in surface water and finished drinking water.[97, 98] The mechanism by which the resistant bacterial populations might come to reside in the finished drinking water is not clear.

The greatest concern about antibiotic residues in the environment is potential acceleration in the rate of emergence of resistance and its spread among pathogens. It seems mechanistically plausible that frequent widespread environmental introduction of resistant bacterial strains and resistance genes *via* municipal wastewater effluents as well as land-applied sludge, might contribute to reservoirs of

resistance in the environment, either due to colonisation by resistant pathogens or, perhaps more likely, by transfer of resistance genes into non-pathogenic endemic bacterial species where they might be maintained for long periods. It also seems plausible that these endemic reservoirs of resistance might subsequently transfer resistance genes to pathogens to which humans may then be exposed. Nevertheless, this model is largely speculative. The significance of the contribution of these wastewater-related mechanisms to the emergence and spread of resistance is not proven, but also cannot be readily dismissed. It is clear that resistant strains of some pathogens originating from wastewater may be able to persist and be transported *via* the aquatic environment to a point where human exposures are possible. The roles of these pathways relative to better-documented pathways, such as contact with patients infected with resistant pathogens, or with surfaces in public places (including health-care facilities) where resistant pathogens from patients can in many cases persist for some time, are not known. Perhaps wastewater contributions to the spread of clinically important resistance are more likely for pathogens typically transmitted by water, than *e.g.* for pathogens typically spread by aerosols or body fluids.

The potential for antibiotic residues in water to select for resistance also cannot be ruled out, but we know of no data suggesting that this route has ever given rise to clinically important resistance. Clinical resistance of a microbial strain is defined by the minimum inhibitory concentration (MIC) of the antibiotic for that strain and the breakpoint concentration of the antibiotic. The MIC is the lowest concentration of antibiotic that prevents microbe growth in a short-term assay. The breakpoint is the

highest concentration of antibiotic that can be maintained in patient tissues without incurring unacceptable risk of side effects. If the MIC is well below the breakpoint, the pathogen is considered sensitive, otherwise it is deemed resistant. Comparing aquatic concentrations to breakpoints suggests that concentrations in wastewater, surface water, groundwater and drinking water are orders of magnitude below corresponding breakpoint concentrations. By contrast, it is common to find concentrations in wastewater and surface water that are close to or even somewhat above MICs (see the summaries of results for antibiotics in sections 4 and 5). This suggests the possibility of some selection of low-level resistance, but not the direct selection of highly resistant bacteria.

Nevertheless, a variety of data suggest that even concentrations of antibiotics below the MIC can have effects that may contribute to the spread of resistance. On the other hand, it is not clear how far below the MIC such effects can occur and it is not clear how much these phenomena actually contribute (if at all) to clinically encountered resistance. For example, one study[99] reported that spread of resistance genes among *Staphylococcus aureus* by phage can be accelerated over 100-fold by sub-MIC concentrations of antibiotic. Others[100] have reported concentrations of antibiotic about 1/10 of the MIC can affect reproduction and frequency of conjugation. Clonal selection for resistance has been described[101-103] at about 1/20 to 1/10 of the MIC. Similarly, a 10-fold increased conjugation frequency in bacteria living in the guts of mice fed sub-inhibitory levels of tetracycline has been reported.[104]

A less commonly voiced, but perhaps important, concern is that antibiotic residues in the aquatic environment (or in WWTPs) may have a negative effect on beneficial microbes. Such microbes play many important environmental functions, including primary production and decomposition. Many also participate in important symbiotic relationships with fungi, plants or animals. Soil bacteria can be affected by antibiotic addition, but typically this is reported[105] at very high antibiotic concentrations around 1 mg/kg soil. Activated sludge bacteria are usually only affected at concentrations greater than 100 mg/kg, although denitrifying aquatic bacteria have been reported[106] to be sensitive to concentrations as low as 100 µg/l. These sorts of results are well above typical reported aquatic concentrations and are probably only relevant in scenarios involving large livestock/poultry production facilities, some aquaculture scenarios and manure application. However, the lowest reported MICs (as low as 500 ng/l) for some antibiotics have been determined in non-pathogenic autotrophic bacteria (cyanobacteria) and these MICs are occasionally exceeded by some reported aquatic concentrations. Nevertheless, more typical water concentrations seem to be well below even cyanobacterial MICs.

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