# Towards Sustainable Pharmaceuticals in a Healthy Society

## MistraPharma Research

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First edition

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# Preface

Last year the MistraPharma programme published a book "A Healthy Future – Pharmaceuticals in a Sustainable Society" that summarized our current knowledge of the problems that surround pharamaceuticals and sustainable development. The book you now hold in your hands gives an overview of the achievements obtained during the first two years of Mistra Pharma.

The programme is well underway, and many important results have already been reported. The work to identify pharmaceuticals of environmental concern (the "priority list") has progressed well, and a short-list of 120 substances will be published later this year.

I will highlight two results from the MistraPharma research in light of a current problematic issue within chemicals control, namely combination effects of chemicals, in particular combination effects of endocrine disruptors. We are very concerned with estrogenic effects of chemicals. The report in *chapter 2* further increases this concern since frogs seem to be more sensitive to estrogenic environmental pollutants than previously known. It is important to continue studies in this field as amphibians are declining dramatically world-wide.

Synthetic estrogen in contraceptives is known to contribute to the feminization of male fish. A new MistraPharma study indicates that levonorgestrel, a gestagen present in certain contraceptives, may impair the reproduction of fish at concentrations present in effluent water from sewage treatment plants (*chapter 1*).

Contraceptives are not the only source for environmental contamination of endocrine disrupting substances. Several other chemicals that we use in

society today may act in combination with e.g. hormones from contraceptives.

In December 2009 the Council of the European Union invited the Commission to make recommendations as to how exposure to multiple endocrine disruptors should be further addressed within relevant existing Community legislation, inter alia in the context of its forthcoming report on the implementation of the Community strategy on endocrine disruptors to be completed by 2010.The Council further stressed that further action in the field of chemicals policy, research and assessment methods to address combination effects of chemicals is required. The MistraPharma research activities are important contributions to this effort.

The book has a rather broad scope on sustainable use of pharmaceuticals and I hope that you will find it interesting. Actually, you can contribute to the work by proposing data to be added to the WikiPharma database (*chapter 6*).

Ethel Forsberg

Director-General of the Swedish Chemicals Agency
Chairperson of MistraPharma´s Programme Board

# Global outlook - the Swedish Medical Products Agency's perspective

Global challenges for a sustainable development are our responsibility; a global development including respect of human rights and democracy, and where growth leads to reduced poverty. A sustainable global development set out by the EU Treaty as the overarching long-term goal of the EU means that resources will be used in a way that is long-term effective ensuring the needs of current generations without compromising the ability of future generations to meet their needs. This includes management of common resources where economic growth, social cohesion and environmental protection go hand in hand and are mutually supportive.

Medicinal products are one of the individual product groups that have had the greatest positive impact on public health and human welfare. In the responsibility of the National Competent Authorities as laid down by the EU, the aim of a sustainable development is not clear. Today, authorities may not include environmental risk assessment in the risk/benefit analysis when assessing whether a human drug will be approved for marketing authorisation. This means that today's legislation does not allow a denial of authorisation of medicinal products for humans due to any risk of negative environmental effects. To develop the legislation in a science based way input from research activities such as MistraPharma is important.

In Europe, drug-related environmental problems are mainly linked to the use of medicinal products. However, in low cost countries a significant part of the current manufacture takes place and many large companies are planning to place even more of their production there. The results from MistraPharma researchers indicate that emissions from the manufacture of medicinal products in India reach a level which might seriously affect human and animal health, as well as their environment. With this background,

the discharge of substances from the pharmaceutical production in the third world is of sincere concern. An important aspect in this context, is the MistraPharma work on improved waste water treatment as one possible risk management strategy.

Sustainable development is a relatively new concept in the pharmaceutical sector, perhaps due to the great importance of medicinal products for public health. We are now increasing our understanding of the link between health and the environment; a good environment is a prerequisite for good public and animal health. An example of the connection between health, environment and the economy is the spread of antibiotic-resistant bacteria, where high discharge of substances from the pharmaceutical production increases the risk of spreading antibiotic resistant bacteria. Both the EU and WHO ranks the rapid development of antibiotic resistance as one of the three greatest threats to human health, making one of humanity's most important medicines ineffective. Increased mortality, extended care and increased health care costs entail a large load on the already strained health economy.

An increased understanding of the work towards a sustainable development became clear during the Swedish Precidency and the EU Conference on sustainable development. During the Conference representatives of the Member States of health, medicines and the environment, EU institutions, academia and industry met. It was gratifying to see the constructive spirit among the participants and the willingness to discuss a way forward, and the great interest in the MistraPharma research.

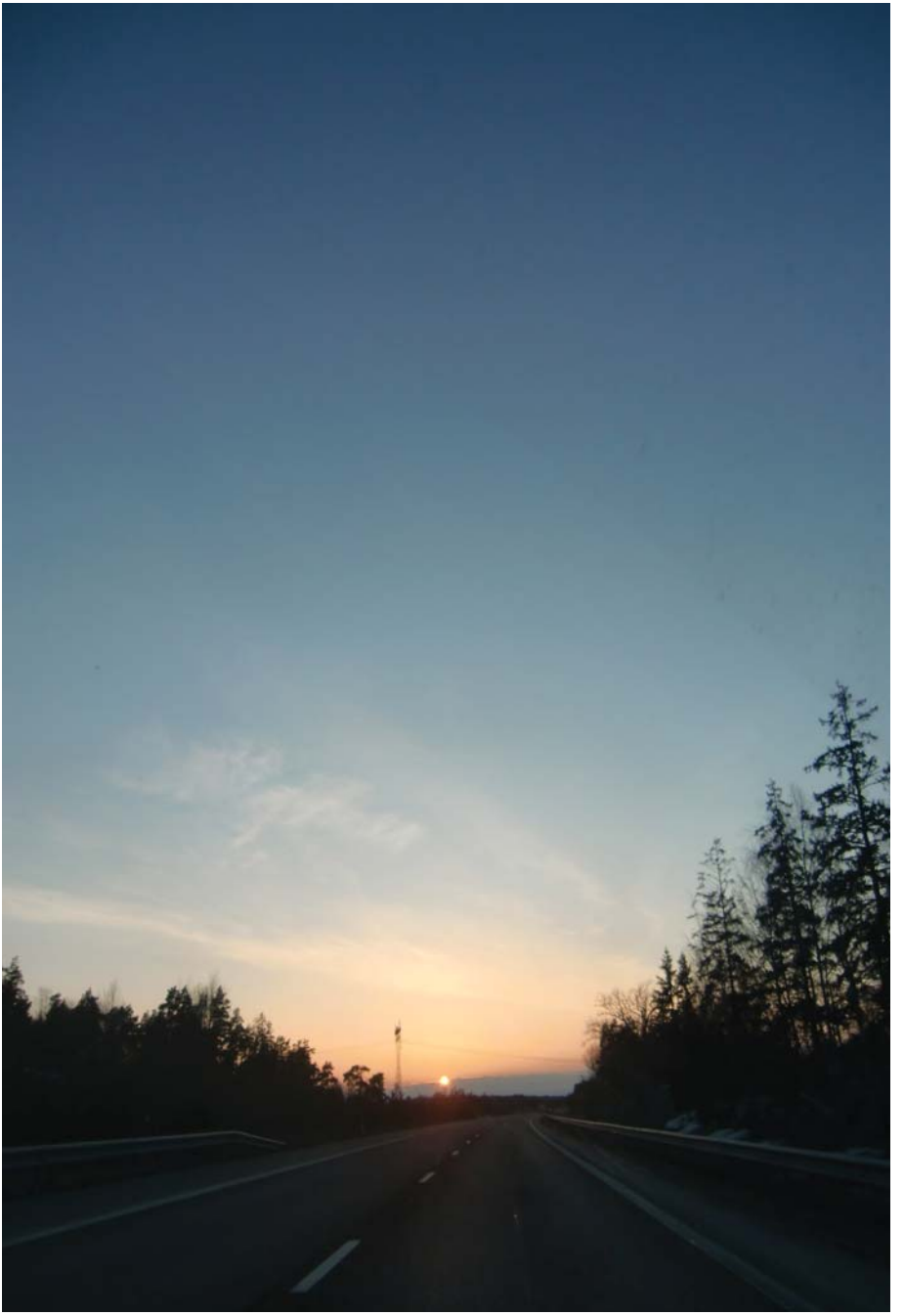
In December the Medical Products Agency (MPA) report was presented to the Government. The report highlighted the need to generate more knowledge about the environmental risks that pharmaceuticals may pose. It also identifies opportunities for strengthening the environmental requirements pertaining to the manufacture of medicinal products and active pharmaceutical ingredients, in a national and international context. The main proposal included a requirement for an environmental certification of the production facilities to be introduced to the legislation on Good Manufacturing Practice.

I believe in the future. During the last year I have seen positive changes. The willingness of Member States and the European Medicines Agency (EMA) to move towards a sustainable development is increasing, e.g. EMA now includes environmental issues in their proposal for a roadmap to 2015. Responsibility for pharmaceuticals within the EU Commission has now moved to DG SANCO which will further increase the opportunity for success.

For 2010 I hope we will be able to expand the global aspect of sustainable development, introducing countries outside the European Union. The initiated collaboration between the MPA and our sister agency in New Delhi, India, during the Indo-Swedish health week arranged by the Governments of India and Sweden, gives me great hope since sustainable development is one of the agreed areas for further collaboration. Further on the spring agenda is a meeting in Washington on sustainable development of pharmaceuticals including a presentation of the MistraPharma research and a possibility of collaboration with the USA.

Together we should take the responsibility of sustainable development for pharmaceuticals into the future thus ensuring that the international policies set up by the UN and the EU will result in concrete measures.

Christina R Åkerman
Director General of the Swedish Medical Products Agency
Member of the MistraPharma´s Programme Board



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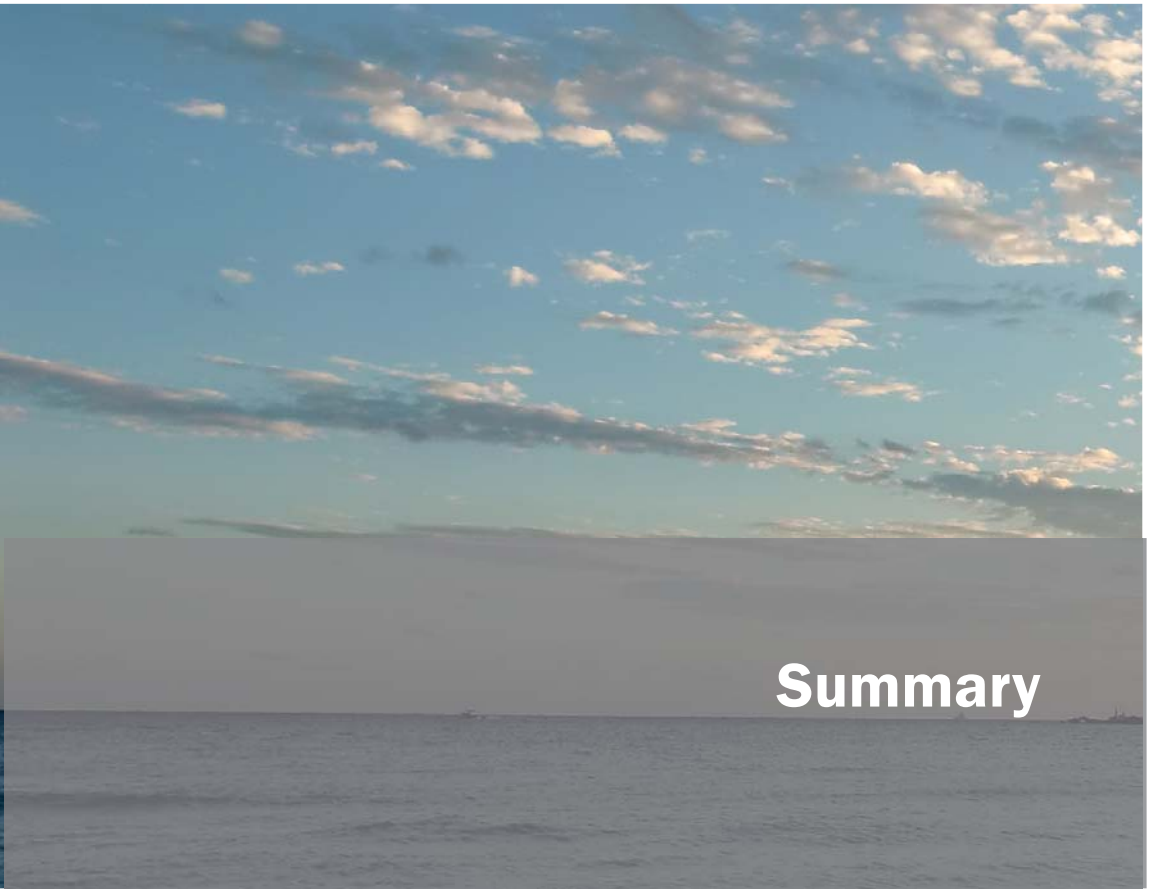


# Summary

The research programme "MistraPharma – Identification and Reduction of Environmental Risks Caused by the Use of Human Pharmaceuticals" is financed by the Swedish Foundation for Strategic Research (Mistra). The programme consists of seven partners: Göteborg University, the Royal Institute of Technology, Lund University, Umeå University, Uppsala University, Stockholm University, and Goodpoint AB.

The overall objectives of MistraPharma are:

1. To identify human pharmaceuticals that pose a significant risk to organisms in the aquatic environment
2. To propose recommendations to improve wastewater treatment technologies
3. To suggest strategies for early identification of human pharmaceuticals that may pose a threat to aquatic species, and

4. To strengthen the network between stakeholders in this field

MistraPharma started in January 2008 and its first phase will be finalized at the end of 2011. In this volume we summarize the programme's main achievements so far.

In ***chapter 1*** (*Bioconcentration of pharmaceuticals*, by Jerker Fick, Roman Grabic, Richard Lindberg, Joakim Larsson and Mats Tysklind) an essential part of the MistraPharma research approach is described, namely the procedure of prioritizing pharmaceuticals based on empirical bioconcentration studies and the "plasma model"[1]. The plasma model is based on the assumption that two species sharing the same drug target, i.e. a receptor or an enzyme, will require about the same plasma concentrations of a pharmaceutical to activate a pharmacological response. Comparing the human therapeutic plasma

concentration for a particular pharmaceutical to the measured steady state levels in fish blood plasma thus makes it possible to estimate the likelihood that the fish is pharmacologically affected by that drug. Within MistraPharma all the 1200 active pharmaceutical ingredients available on the Swedish market were initially considered. Using a theoretical model, risk estimates for these substances were calculated and based on this 120 active pharmaceutical ingredients were prioritized for further scrutiny.

Empirical determination of bioconcentration for 25 of these pharmaceuticals was investigated in rainbow trout exposed to effluent water from three Swedish sewage treatment plants. Out of the 25 selected pharmaceuticals, 17 were detected in fish plasma. One of the pharmaceuticals, levonorgestrel, was detected in fish plasma at levels that exceeded the human therapeutic plasma concentration[2]. Levonorgestrel is a synthetic progestin used in different types of contraceptives. Other researchers recently showed that exposure to as little as 0.8 nanogram ($10^{-9}$ gram) levonorgestrel per litre water significantly reduced the fertility of fish[3]. In accordance, the study by Fick *et al.* showed that an effluent concentration of 1 nanogram per litre water resulted in a plasma concentration in exposed fish that was higher than the human therapeutic plasma concentration. This significant finding will certainly be further investigated within MistraPharma.

One type of investigation that will be applied to the substances identified as potential environmental risks is biological testing. In ***chapter 2*** (*The frog test system*, by Cecilia Berg) the frog test model is introduced.

In the frog test system larvae are exposed to the test substances from hatching until completed metamorphosis. Effects are evaluated at certain larval stages, at metamorphosis and when the frogs have reached sexual maturity. Major endpoints are expression of genes important for sex differentiation and development, aromatase activity (aromatase catalyses the conversion of androgens into estrogens), and the activity of specific neurotransmitters in the brain. The testicles, ovaries, oviducts, thyroid gland and other suspected target organs are evaluated for structural changes using microscopy. Fertility studies include endpoints such as sexual behaviour, egg production, hatchability, embryo mortality, and sperm quality.

Results obtained within MistraPharma show that frogs are more sensitive to estrogenic environmental pollutants than what was previously known. Larval exposure to the estrogenic pharmaceutical ethynylestradiol ($EE_2$) at concentrations found in the environment caused permanent male-to-female sex-reversal and reduced fertility. This demonstrates that also for

amphibians the differentiation of the gonads is sensitive to disturbance by estrogenic environmental pollutants. Overall it is concluded that the frog is a promising model for research on developmental reproductive toxicity.

In ***chapter 3*** (*Genomics as a guide for environmental risk assessments of pharmaceuticals,* by Filip Cuklev, Lina Gunnarsson, and Joakim Larsson) genomics is introduced as a method for risk identification and risk assessment.

Genomics includes studies of the genome on a large scale. The genome is the sum of an organism's genetic information, encoded in the DNA molecule. The DNA contains the genes, and the genes will generate proteins via mRNA. Proteins determine the structure and function of the cells, and as a result, everything that occurs in an organism is related to an effect of a protein. Thereby the study of mRNA can reveal effects at the protein level and thus effects on physiological processes.

Many pharmaceuticals are designed to induce a specific pharmacological response through interactions with target proteins, e.g. receptors, while affecting other physiological processes as little as possible. Many of the proteins in wild organisms are similar to the proteins in humans. This means that a human pharmaceutical may interact with a similar protein in other species and thereby affect the non-target organism. Consequently, the presence of human drug target proteins in a wildlife species increases the likelihood that this species will be affected by human pharmaceuticals present in the environment. Analyses of gene sequences indicate that fishes and frogs have a corresponding target protein for 80% of the investigated human drug targets, while for certain other organisms many human drug targets are lacking. For risk identification purposes, testing should focus on organisms that have the appropriate drug target, and data generated from species that lack the drug target should not be generally extrapolated to other species.

Microarray technology allows the study of thousands of potential gene responses simultaneously. A microarray analysis may serve several purposes. First, it conveys information about the *mode of action* of the pharmaceutical in the exposed species, and second it can aid in the identification of substances within a mixture that are present at sufficiently high levels to affect organisms. This approach was recently illustrated within MistraPharma for genes regulated by $EE_2$ and other estrogens. A third potential use of microarray analyses is to reveal information about effects at low concentrations. Such information can be used to prioritize further testing and risk assessment. Two techniques closely related to genomics are *proteomics* (the study of pro-

teins) and *metabolomics* (large-scale study of metabolites i.e. small molecules such as amino acids, sugars, lipids, and energy-carriers, resulting from the body's metabolism). These techniques are also described and discussed in this chapter.

It is concluded that the increasing knowledge about the genomes of different species will contribute to more precisely defined gene functions across organisms. This may facilitate extrapolation of data between species and thereby provide a more solid framework for comparisons between the modes of action in humans versus those in environmental species.

Within MistraPharma effects of human pharmaceuticals are studied both in the laboratory and in the field. In ***chapter 4*** (*Laboratory vs. field studies to assess environmental hazards posed by pharmaceuticals for human use*, by Ingvar Brandt and Björn Brunström) the strengths and weaknesses of laboratory studies compared to field studies are discussed in relation to environmental risk assessment.

The purpose of performing ecotoxicological laboratory studies for research purposes is generally to characterize the effects in a test organism caused by exposure to different concentrations of a single substance, i.e. a dose-response relationship. Laboratory experiments can also be used to investigate effects caused by exposures to mixtures of substances in a systematic way with the purpose of identifying interactions between the different chemicals. Mixtures of pharmaceuticals, biocides and other chemicals are released in effluent water from sewage treatment plants, usually in low concentrations. Given that the exposure in the environment is complex, and that it changes in time and space, field studies generally give only limited information about causal relationships between exposure and effects. Furthermore, several types of effects are in practice impossible to study under field conditions, including many behavioral changes.

Biomarkers are important tools to predict effects in the environment. Currently only a few sufficiently robust and specific biomarkers for environmental effects are available. One example of such a biomarker is induction of the egg yolk protein vitellogenin which is a mechanism-based biomarker suitable both for laboratory and field studies. To develop novel biomarkers, highly inducible fish CYP1 genes have been cloned within MistraPharma and large scale gene expression profiling is used for monitoring.

In this chapter it is concluded that the development of biomarkers based on the pharmaceuticals mode-of-action is an important task that can help

bridging the gap between laboratory data and effects in the environment and in that way the scientific basis of environmental risk assessment can be improved.

In ***chapter 5*** (*Standard and non-standard tests for risk assessment purposes,* by Christina Rudén, Marlene Ågerstrand, Malena Göransson, and Magnus Breitholtz) the process of regulatory environmental risk assessment is introduced, from test requirements, via interpretation and evaluation of data, to risk assessment and finally decision-making.

From a regulatory perspective, pharmaceuticals have a number of inherent properties that make them unique among other groups of chemicals. They are designed to interact with biological processes in a specific way already at low doses, and they should be sufficiently persistent to remain un-metabolised long enough to reach the target organ in the human body. In addition, they are relatively well investigated with respect to their pharmacological effect, mode-of-action and potential side effects. These unique features of pharmaceuticals give rise to both possibilities and challenges; It is of course a main advantage that there is a lot of knowledge about their biological effects but at the same time, currently available standard ecotoxicological test methods are in many cases not sufficiently sensitive to the types of very specific effects that we expect from pharmaceuticals in non-target species. This is a crucial deficiency of the process for regulatory environmental risk

assessment for pharmaceuticals since current legislation only requires data from standard tests.

In this chapter three potential ways to improve the situation are proposed:
1. To develop new standard tests
2. To adjust existing standard tests i.e. supplementing them with additional endpoints
3. To increase the use of non-standard tests

A development along the lines of the first two alternatives might be possible, but it would take a significant amount of time and resources. Therefore it is concluded that the option to increase the use of non-standard tests for regulatory risk assessment purposes should be further explored. This would include further development of criteria for evaluating the reliability and relevance of non-standard tests, and to ensure that non-standard tests are reported in a transparent and comprehensive way, much like required when using the standard test methods. A systematic use of non-standard tests in environmental risk assessment could contribute to making the process more scientifically robust.

In ***chapter 6*** (*WikiPharma – A database with environmental effect data for pharmaceuticals,* by Linda Molander, Marlene Ågerstrand, and Christina Rudén) the MistraPharma database "WikiPharma", is described. In WikiPharma data on ecotoxicological effects of pharmaceuticals have been gathered. The aim of WikiPharma is to provide a comprehensive and easily accessible source for ecotoxicological data for pharmaceuticals and it is publicly available at no cost at *www.wikipharma.org*.

The database is constructed as a ''wiki" so that the users can propose data to be added. Data that have either been published in the open scientific literature or that have been sufficiently peer-reviewed and made publicly available by other means can be included. The database currently contains data for 130 active pharmaceutical ingredients extracted from 172 different sources. For each test there is information about tested species, test method, tested concentrations, exposure duration, reported effects, and effect concentrations ($LC_{50}$, $EC_{50}$, or NOEC/LOEC).

In this chapter an overview of the database structure and its contents is provided, and instructions on how to search the database and how to propose additional data are also given.

***Chapter 7*** (*The Swedish Environmental Classification and Information System for pharmaceuticals. An evaluation of the system's achievements so far,* by Marlene Ågerstrand and Christina Rudén) presents the results from a detailed evaluation of the Swedish Environmental Classification and Information System for pharmaceuticals (SECIS).

In the chapter the system is introduced and an overview of the classifications made so far is reported. The classifications are also analyzed using the following questions:

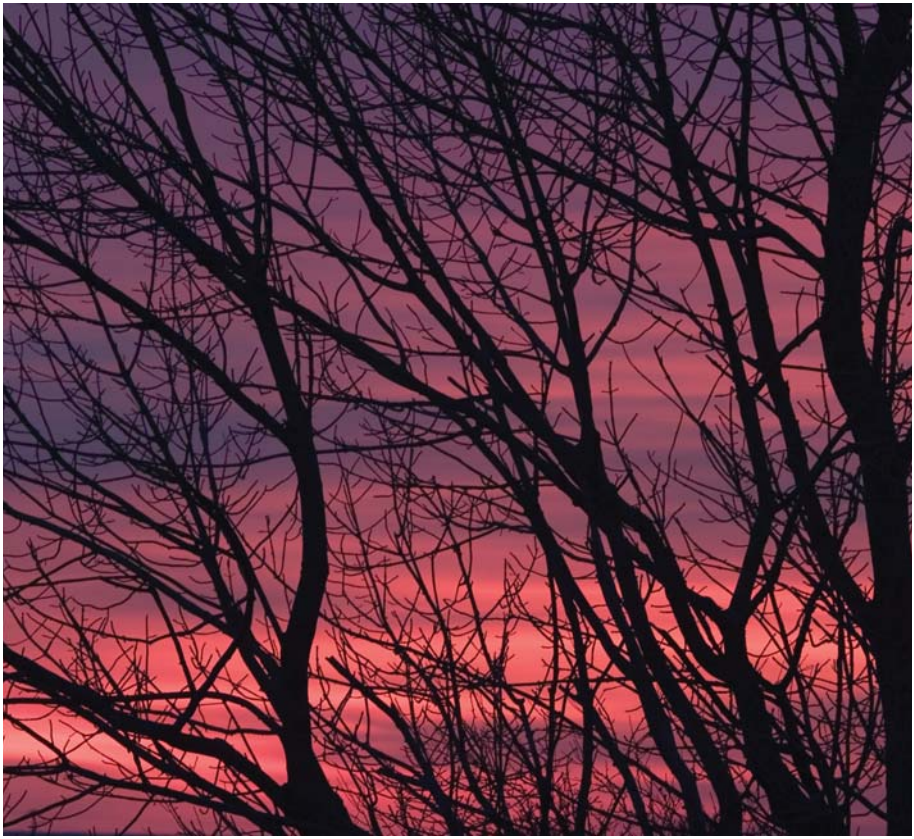
1. How do the companies select data for classification purposes? Do they follow the system's guidance document?
2. Are the data-sets sufficiently complete? Would additional data, from the scientific literature affect the companies' classifications?

For these analyses all substances classified according to SECIS and for which there are data available in WikiPharma were identified. In total 36 substances and 48 classifications (each substance can be classified by more than one company).

Data from standard tests are most commonly used. Data from non-standard tests were used in only 6 of the 48 cases under scrutiny (12%). In addition, short-term data are more abundant than data from long-term studies (79% short-term studies and 19% long-term studies). In 42% of the analyzed assessments (20/48) data from the open scientific literature was referred to, and in 27 of the 48 cases company owned data were the only source of information.

For each classification it was also investigated whether the data-set could be supplemented with information from the open scientific literature and whether a supplemented data-set would give rise to alternative classifications. This analysis showed that a supplemented data-set could change the risk assessment (PEC/PNEC) for 18 of the 48 assessments (37%), and the alternative assessment would have resulted in an altered risk classification in 13 of these 18 cases (72%). For 10 of these the risk category would have been higher compared to the classification based on the data-set that the company chose.

*Chapter 7* concludes by presenting three recommendations:
1. SECIS allows for both standard and non-standard test data when calculating PNEC
2. Long-term ecotoxicity data are preferred when calculating the PNEC



3. Companies are recommended to take external ecotoxicity data into account when classifying their products

***Chapter 8*** (*Swedish wastewater treatment plants and their ability to remove pharmaceuticals*, by Per Falås and Jes la Cour Jansen) focus on existing Swedish wastewater treatment technologies and their ability to remove pharmaceuticals.

In the early 1950s there were only a few wastewater treatment plants in Sweden but today there are more than 2 000 municipal plants designed to remove organic material and nutrients. Chemical phosphorus removal was introduced in the 1970s and extended nitrogen removal was introduced in many plants in the 1990s. Extended nitrogen removal is restricted to southern Sweden, where it is mainly found at larger plants and at plants in coastal areas.

In Sweden, most of the domestic wastewater generated in urban areas is transported to wastewater treatment plants. Therefore, wastewater treatment plants have a unique potential to serve as a barrier for discharge of environmentally hazardous micropollutants. There are three main ways for removal of micropollutants at wastewater treatment plants: sorption to particulate matter, biological degradation/transformation and evaporation. The effectiveness of the treatment depends on the inherent properties of the pollutant, as well as by the treatment processes and the operation of the plant.

In Sweden there are more than 450 wastewater treatment plants dimensioned for more than 2 000 person equivalents, and these plants treat approximately 90% of the wastewater volume from urban areas. In this chapter the main techniques and their efficiency to remove organic matter, nutrients, and other pollutants including pharmaceuticals are discussed.

It is concluded that Swedish wastewater treatment comprises a broad spectrum of treatment technologies that are known to remove pharmaceuticals to a greater or lesser extent. There is also variability in the removal efficiency for different types of pharmaceuticals. Given this variability it is crucial that any decision on supplementing current wastewater treatment technologies with the aim to reduce pharmaceuticals should rely on an assessment of the environmental risks associated with these substances. Furthermore, the present wastewater treatment and the possibility to integrate new technologies must also be considered in such decisions.

In the final chapter, ***chapter 9*** (*The vision – sustainable pharmaceutical management in a sustainable society,* by Åke Wennmalm, Bengt-Erik Bengtsson and Bo Gunnarsson) the process towards a sustainable development and use of pharmaceuticals is discussed. It is argued that sustainability in this area should be characterised by managing economic, environmental and social issues, and include the entire life cycle of the pharmaceutical, from development and production to use and disposal.

Driving forces, fore and against a sustainable development are discussed, such as market forces, demands from consumers and prescribers, political decisions, and novel decision mechanisms including globally acting international and non-political organisations.

Finally a number of positive examples are presented, such as research efforts contributing to new knowledge about risks, the recently introduced European legislation requiring environmental risk assessments for new

human pharmaceuticals, and a requirement for all member states to establish take-back systems for unused or expired medicines. It is furthermore noted that the European Commission has stated that "Pollution of waters and soils with pharmaceutical residues is an emerging environmental problem and also an emerging public health concern". (COM [2008]666, paragraph 1.3.3). Some significant Swedish initiatives are also mentioned: screening of pharmaceuticals in Swedish surface waters, launched by the Swedish Environmental Protection Agency, and two proposals from the Swedish Medical Products Agency: first to include environmental aspects in the criteria for Good Manufacturing Practice (GMP) and second to change current legislation so that environmental risks should be allowed to serve as a basis for refusal of a marketing application.

*Enjoy your reading!*

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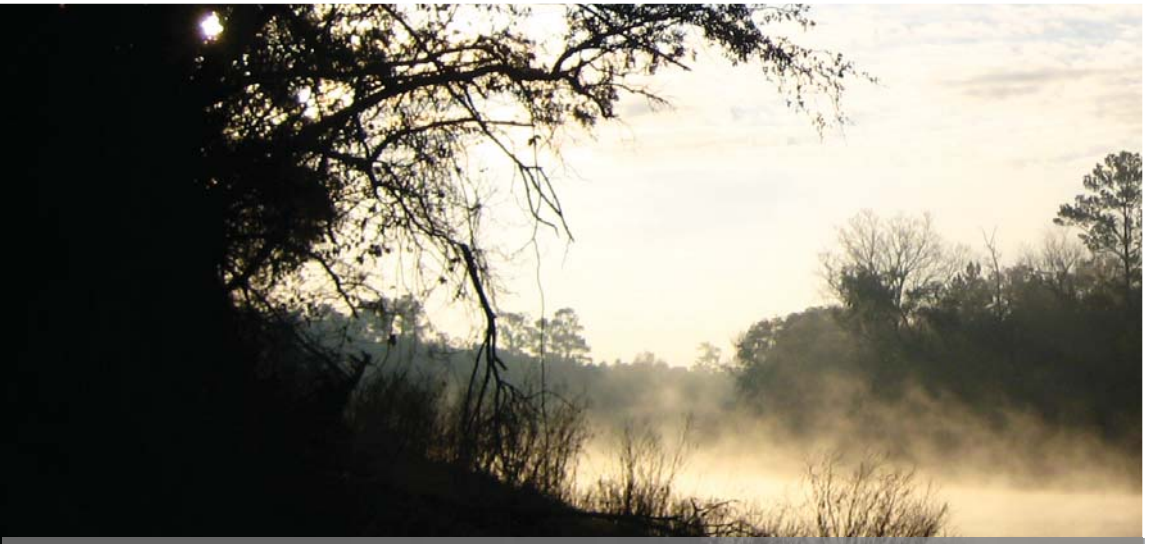

# Sammanfattning

Forskningsprogrammet "MistraPharma – Identification and Reduction of Environmental Risks Caused by the Use of Human Pharmaceuticals" finansieras av Stiftelsen för miljöstrategisk forskning (Mistra). Programmet består av sju partners: Göteborgs universitet, Kungliga Tekniska Högskolan, Lunds universitet, Umeå universitet, Uppsala universitet, Stockholms universitet och Goodpoint AB.

MistraPharmas fyra övergripande mål är att:
1. identifiera humana läkemedelssubstanser som kan utgöra en väsentlig risk för vattenlevande organismer
2. utarbeta rekommendationer för förbättrad avloppsvattenrening
3. föreslå förbättrade strategier för att tidigt kunna identifiera läkemedelssubstanser som kan orsaka oönskade miljöeffekter

(4) stärka nätverket mellan forskare och andra aktörer inom detta område

MistraPharma startade i januari 2008, och pågår i en första fas till och med december 2011. I denna skrift sammanfattar vi de huvudsakliga resultat som hittills genererats inom programmet.

I ***kapitel 1*** (*Bioconcentration of pharmaceuticals*, av Jerker Fick, Roman Grabic, Richard Lindberg, Joakim Larsson och Mats Tysklind) beskrivs en av programmets centrala processer: prioritering av läkemedelssubstanser baserat på empiriskt uppmätt biokoncentration och den så kallade ”plasma-modellen”[1]. Enligt plasmamodellen jämförs koncentrationen av ett läkemedel i blodplasma hos en exponerad fisk med motsvarande koncentration hos en behandlad människa.  Kvoten som bildas kan ge en indikation om risk.

Ju närmare koncentrationen i fiskplasman är den plasmakoncentration som finns hos en behandlad människa, eller om fiskens plasmakoncentration överstiger den terapeutiska, desto större är sannolikheten att fisken påverkas på något sätt.

Inom MistraPharma beaktades inledningsvis samtliga cirka 1 200 läkemedels-substanser på den svenska marknaden. Genom teoretiska beräkningar gjordes en första uppskattning av vilka av dessa substanser som skulle kunna utgöra en risk i den akvatiska miljön. Baserat på denna process prioriterades 120 substanser för vidare studier inom programmet.

Empiriska biokoncentrationsstudier har sedan genomförts med fisk som exponerats för renat avloppsvatten. I den första av dessa studier analyserades 25 läkemedelssubstanser. Av dessa återfanns 18 i vattnet och 17 i fiskplasman. Det mest uppseendeväckande resultatet gäller levonorgestrel som är ett vanligt gestagen i p-piller och andra typer av antikonceptionsmedel. Levonorgestrel återfanns i fiskplasman i koncentrationer som klart överstiger plasmakoncentrationen hos en behandlad människa. Det har av andra forskare nyligen visats att exponering för 0.8 nanogram (miljarddels gram) levonorgestrel per liter vatten kraftigt reducerar fertiliteten i fisk[2]. I vår studie uppmättes koncentrationen av levonorgestrel i renat avloppsvatten till 1 nanogram per liter vatten[3].

Dessa resultat tyder alltså sammantaget på att levonorgestrel finns i utgående vatten från reningsverk i en koncentration som har potential att i betydande grad reducera fertiliteten hos fisk. Dessa indikationer kommer naturligtvis att följas upp i detalj inom programmet.

En typ av uppföljning kommer att ske genom biologiska tester. En av de biologiska testmodeller som vi använder inom programmet är långtidsexponering av groda vilket beskrivs i ***kapitel 2*** (*The frog test system*, av Cecilia Berg). I grodmodellen exponeras grodyngel från kläckning till dess att metamorfosen är klar och djuren är färdigutvecklade. Effekter analyseras vid specifika yngelstadier, vid metamorfos och när grodorna är könsmogna. Viktiga testvariabler är uttryck av hormonreceptorer, hormonellt betydelsefulla enzymer (aromatas), och halter av olika neurotransmittorer i hjärnan. Testiklar, ovarier, äggledare, sköldkörtel, och andra centrala målorgan undersöks i mikroskop med avseende på strukturella förändringar, och fertiliteten undersöks med avseende på parningsbeteende, ägg- och spermieproduktion, spermiekvalitet, äggens kläckningsfrekvens, och embryodödlighet.

Resultat som genererats hittills visar att grodor är känsligare för östrogent verkande ämnen (etinylöstradiol, $EE_2$) än vad som tidigare var känt. Exponering av *Xenopus tropicalis* (Västafrikansk klogroda) för etinylöstradiol resulterade i att hangrodor utvecklades till honor och en betydande andel av de vuxna grodorna blev infertila. Grodan är i denna modell ungefär lika känslig som fisk för denna typ av substanser och de effekter som identifierats uppkom vid miljörelevanta koncentrationer. En övergripande slutsats är att grodan kan bidra med viktiga kunskaper om effekter av hormonellt verkande kemikalier i den akvatiska miljön.

I ***kapitel 3*** (*Genomics as a guide for environmental risk assessments of pharmaceuticals* av Filip Cuklev, Lina Gunnarsson, och Joakim Larsson) introduceras genomik som metod för riskidentifiering och riskbedömning.

Genomik innebär att man studerar genomet i större skala. Generna finns på DNA-molekylen. DNA transkriberas till en mRNA molekyl som översätts till aminosyror och proteiner. Proteiner i sin tur, styr hur cellerna i en organism byggs upp och fungerar.

Läkemedel är utvecklade för att interagera specifikt med levande organismer, till exempel med olika proteiner. Många proteiner återfinns både i människor och i djur. Studier gjorda inom MistraPharmas projekt i Göteborg indikerar till exempel att cirka 80% av de undersökta humana målproteinerna också finns hos fisk och groda, medan en del andra organismer har en lägre andel av dessa proteiner. Förekomsten av ett farmakologiskt målprotein i en art ökar sannolikheten för att läkemedlet ska ha en effekt i den organismen om den exponeras. Saknas å andra sidan målproteinet kan man anta att arten är mer okänslig för effekten av det specifika läkemedlet. För riskidentifiering bör testning med arter som har bevarade målproteiner alltså vara en viktig prioritering och tester på arter utan bevarade målproteiner kan inte automatiskt extrapoleras till andra arter.

Microarray-tekniker möjliggör studier av tusentals gener. Analyserna kan fokuseras på förväntade responser, men eftersom generna är så många så

kan tekniken också innefatta en mer explorativ ansats. Syftet med att använda microarrayteknik kan vara flera. Dels att generera information om en substans verkningsmekanism, dels att identifiera potenta substanser i olika blandningar som komplexa avloppsvatten, givet att de gener som påverkas är specifika och kända. Detta har studerats inom projektet för gener reglerade av etinylöstradiol och andra östrogent verkande substanser. Ett tredje syfte med att använda microarrayteknik är att identifiera effekter i låga koncentrationer. Sådan information kan användas som en guide till ytterligare testning och riskbedömning. Näraliggande metoder är *proteomics* (studier av proteiner) och *metabolomics* (analys av metaboliter som bildas i en organism, såsom aminosyror och olika sockerarter). Dessa metoder introduceras också i *kapitel 2*.

I kapitlet dras slutsatsen att allteftersom mer kunskap om genomet och dess funktioner genereras för allt fler arter så kommer också genomiken att bli mer och mer betydelsefull som ett verktyg för riskidentifiering och riskbedömning. Särskilt när det gäller att extrapolera effekter mellan arter och att förstå sambanden mellan verkningsmekanismer och identifierade effekter.

Inom MistraPharma studeras effekter av humana läkemedel i den akvatiska miljön, både i laboratorieexperiment och i fält. I ***kapitel 4*** (*Laboratory vs field studies to assess environmental hazards posed by pharmaceuticals for human use* av Ingvar Brandt och Björn Brunström) diskuteras förhållandet mellan data som genereras i fält och laboratoriedata för användning i riskbedömning.

Syftet med laborationsförsök är vanligtvis att identifiera effekter av en enskild substans i en specifik art och att uppskatta sambandet mellan exponeringen (dosen) och den studerade responsen. I laboratoriet kan också blandningar av substanser studeras på ett systematiskt sätt för att upptäcka eventuella interaktioner.

Ute i miljön exponeras organismerna alltid för blandningar av substanser. Blandningens sammansättning reflekterar användningen av olika typer av kemikalier i samhället och deras egenskaper, spridning, och nedbrytning. Typiskt är att varje enskild substans i de flesta fall förekommer i låga koncentrationer. Just det faktum att exponeringen är komplex, och förändras över tid, gör att det ofta är svårt att använda fältstudier för att identifiera kausala samband mellan exponering för enskilda ämnen och specifika effekter i en organism. Dessutom är vissa typer av effekter i praktiken omöjliga att studera i fält. Det gäller till exempel olika beteendeförändringar.

Utvecklandet av biomarkörer är viktigt för att kunna förutse effekter i fält. Dock finns i dagsläget endast ett fåtal biomarkörer som är tillräckligt robusta och specifika för att kunna identifiera kausala samband i fält. Ett exempel på en ändamålsenlig biomarkör är vitellogenininduktion (vitellogenin är ett ägguleprotein som induceras av östrogen och östrogenlika substanser) och inom MistraPharmas projekt i Uppsala utvecklas analys av gener för metaboliserande enzymer (CYP-gener) som en möjlig biomarkör för läkemedelsexponering. Microarray-tekniker som används inom programmet kan också komma att generera användbara biomarkörer (se *kapitel 3*).

I kapitlet dras slutsatsen att utvecklande av biomarkörer som baseras på läkemedlens verkningsmekanismer är en fortsatt viktig uppgift för att bättre kunna överbrygga gapet mellan laboratoriedata och effekter i miljön och på så sätt förbättra miljöriskbedömningar.

I ***kapitel 5*** (*Standard and non-standard tests for risk assessment purposes* av Christina Rudén, Marlene Ågerstrand, Malena Göransson, och Magnus Breitholtz) introduceras processen för regulatorisk miljöriskbedömning, från testning och testkrav, via tolkning och kvalitetsbedömning av data, till beslutsfattande.

Läkemedel skiljer sig från andra typer av kemikalier på flera sätt; de är designade för att specifikt påverka biologiska processer redan i låga koncentrationer, de är tillräckligt stabila för att passera mag-tarmkanalen, och de är dessutom välundersökta med avseende på sin farmakologiska effekt och eventuella biverkningar. Dessa egenskaper innebär både möjligheter och svårigheter i processen att identifiera risker för vattenlevande organismer. Det är en betydande fördel att substansernas egenskaper är välundersökta, men samtidigt anses nuvarande regulatorisk standardtester otillräckliga när det gäller att identifiera specifika (farmakologiska) effekter i icke målorganismer. Detta är en betydande svaghet i nuvarande lagstiftning. Den europeiska lagstiftningen kräver endast tester genomförda enligt befintliga teststandarder. För att förbättra lagstiftningen krävs att testkraven anpassas till den specifika situation som gäller för läkemedel. I kapitlet diskuteras tre huvudsakliga möjligheter:

1. Att utveckla nya standardtester anpassade för läkemedel
2. Att komplettera befintliga standardtester med nya endpoints
3. Att möjliggöra en ökad användning av icke-standardtester för regulatorisk riskbedömning

Slutsatserna är att möjligheterna 1 och 2 är resurskrävande och endast möjliga på lång sikt. Därför bör den tredje möjligheten; att öka användningen av icke-standarddata undersökas vidare. Det innefattar en utveckling av tydligare kriterier för utvärdering av enskilda studiers reproducerbarhet och relevans för riskbedömning. Det innebär sannolikt också att kraven på rapportering av icke-standardstudier behöver skärpas och bli mer lika rapporteringen av standardstudier. Med tydligare kvalitetskriterier och bättre rapportering skulle användandet av icke-standarddata för myndigheter och andra riskbedömare underlättas, vilket i vår mening skulle kunna ha potentialen att göra miljöriskbedömningarna av läkemedel mer vetenskapligt robusta.

I ***kapitel 6*** (*WikiPharma – A database with environmental effect data for pharmaceuticals* av Linda Molander, Marlene Ågerstrand, och Christina Rudén) beskrivs MistraPharmas databas WikiPharma. I WikiPharma har data om effekter av läkemedel i den akvatiska miljön sammanställts. Målsättningen med databasen är att den ska utgöra en komplett och lättillgänglig källa till information om läkemedels miljöeffekter. Den är tillgänglig via internet utan kostnad och konstruerad som en ”wiki” vilket betyder att vem som helst kan föreslå ändringar och tillägg av nya data. Data som har publicerats i den öppna vetenskapliga litteraturen, eller som har blivit expertgranskad på annat sätt, kan adderas till WikiPharma. Databasen finns på *www.wikipharma.org* och den innehåller för närvarande effektdata för 130

läkemedelssubstanser hämtade från 172 olika källor. För varje test finns information om: testad art, testmetod, testade koncentrationer, exponeringstid, rapporterade effekter, samt effektkoncentrationer ($LC_{50}$, $EC_{50}$, eller NOEC/LOEC).

I kapitlet beskrivs innehållet i databasen översiktligt och dessutom ges sökinstruktioner och information om hur man kan lägga till nya data till databasen.

***Kapitel 7*** (*The swedish environmental classification and information system for pharmaceuticals. An evaluation of the system's achievements so far* av Marlene

Ågerstrand och Christina Rudén) redovisar resultatet från en utvärdering av det svenska frivilliga miljöklassificeringssystemet för läkemedel på fass.se. Här introduceras systemet och en översikt av de klassificeringar som gjorts hittills presenteras.

Klassificeringarna analyseras också utifrån följande frågeställningar:
1. Hur väljer företagen data för klassificeringarna – följs systemets egna guidelines?
2. Hur kompletta är de data som används? Skulle ytterligare data, från den öppna litteraturen ge upphov till andra klassificeringar?

För analysen användes alla substanser som klassificerats inom fass.se-systemet och för vilka det finns data i WikiPharma-databasen, totalt 36 substanser med 48 klassificeringar (varje substans kan klassificeras av fler än ett företag).

I frågan om dataurval konstateras att data från standardtester är den vanligaste typen vid klassificering. Data från icke-standardtester användes i 6 av de 48 fallen som analyserades (12%). Det är också vanligare att data från korttidsstudier används istället för långtidsstudier (79% korttidsstudier och 19% långtidsstudier). I 42% av de analyserade fallen (20/48) användes data som hämtats från den öppna vetenskapliga litteraturen och i 27 av 48 fall användes endast information som genererats av företaget självt.

För varje klassificering undersöktes dessutom om det var möjligt att komplettera dataunderlag med ytterligare data från den öppna litteraturen och om denna komplettering skulle kunna påverka klassificeringen. De kompletterade dataunderlagen gav upphov till förändrad riskbedömning (PEC/PNEC) för 18 av de 48 bedömningarna (37%), och den alternativa bedömningen skulle ha resulterat i en ny riskkategori i 13 (72%) av dessa 18 fall. I 10 av de 13 fallen skulle det kompletterade dataunderlaget ge upphov till en högre klassificering.

*Kapitel 7* avslutas med tre rekommendationer:
1. Inför krav på att söka efter data från den öppna vetenskapliga litteraturen för varje klassificering
2. Tydliggör hur icke-standarddata kan utvärderas med avseende på relevans och reproducerbarhet
3. Underlätta användningen av icke-standarddata inom fass.se, med målet att basera varje klassificering på en relevant och tillräckligt känslig testmetod

***Kapitel 8*** ( *Swedish wastewater treatment plants and their ability to remove pharmaceuticals* av Per Falås och Jes la Cour Jansen) behandlar tekniker för avloppsrening och deras möjligheter att rena bort läkemedel.

I början av 1950-talet fanns endast ett fåtal reningsverk i Sverige men i dagsläget finns mer än 2000 kommunala anläggningar. Deras huvudsakliga uppgift är fortfarande att ta bort organiskt material och näringsämnen. Fosforrening introducerades under 70-talet och utökad kväverening infördes på många anläggningar under 90-talet. Kväverening finns framförallt i större, kustnära anläggningar i södra delen av landet.

I Sverige är en stor andel av avloppen anslutna till reningsverk vilket ger en unik potential för reningsverken att fungera som filter mot spridning av föroreningar. Tre huvudsakliga mekanismer för avlägsnade av miljöfarliga ämnen är sorption till partiklar, biologisk nedbrytning/transformation, och avdunstning. Möjligheten till rening påverkas av substansernas fysiska, kemiska och biologiska egenskaper samt av de biokemiska processerna i reningsverken, reningsverkens utformning och drift.

I Sverige finns mer än 450 reningsverk dimensionerade för mer än 2000 personekvivalenter. Dessa verk hanterar cirka 90% av den totala volymen avloppsvatten. I kapitlet beskrivs de huvudsakliga teknikerna för rening som används i dessa anläggningar och deras förmåga att ta bort organiskt material, näringsämnen och andra föroreningar inklusive läkemedel.

Sammanfattningsvis konstateras att de svenska reningsverken är många och att de är uppbyggda på olika sätt med ett brett spektrum av renings-processer. Vissa processer är bättre, andra sämre på att avlägsna läkeme-delsrester. Skillnader finns också i reningseffektivitet för olika typer av läkemedelssubstanser. Givet denna variation är det viktigt att beslut om kompletterade reningstekniker vilar på en samlad bedömning av miljö-riskerna med läkemedlen på den svenska marknaden. Med utgångspunkt från denna bedömning bör nya reningstekniker riktas mot de centrala läkemedelssubstanserna och integreras med befintliga reningsprocesser.

I det avslutande kapitlet, ***kapitel 9*** (*The vision – sustainable pharmaceutical management in a sustainable society* av Åke Wennmalm, Bengt-Erik Bengtsson och Bo Gunnarsson) diskuteras processen för läkemedelsutveckling, dess förutsättningar samt hur vägen till en hållbar läkemedelsanvändning kan se ut. Det argumenteras för att hållbarhet på detta område, på samma sätt som inom andra områden, måste inkludera ekonomiska, miljömässiga och

sociala aspekter, samt omfatta hela processen från utveckling och produktion, till användning och hantering av oanvända läkemedel.

Olika drivkrafter för, och emot, en hållbar utveckling diskuteras, såsom marknadskrafter, efterfrågan från konsumenter och förskrivare, politiska beslut, och nya beslutsmekanismer inklusive globalt verkande, politiskt obundna organisationer. Slutligen lyfts ett antal positiva exempel fram, såsom forskningsaktiviteter som bidrar till ökad kunskap för bättre riskbedömningar, nyligen genomförda Europeiska lagkrav på miljöriskbedömning för nya läkemedel, och krav på system för insamlande av oanvända eller kasserade läkemedel. Europeiska Kommissionen nämner också läkemedel i miljön som ett ”emerging environmental problem” (COM [2008]666, par 1.3.3). Bland viktiga svenska initiativ nämns till exempel screening av läkemedel i svenska vatten initierat av Naturvårdsverket, samt förslag från Läkemedelsverket att inkludera miljöhänsyn i reglerna för ”Good Manufacturing Practice” (GMP), och att tillåta att miljöhänsyn vägs in i risk-nyttavärderingen av nya humana läkemedel.

*Trevlig läsning!*

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# 1 Bioconcentration of Pharmaceuticals

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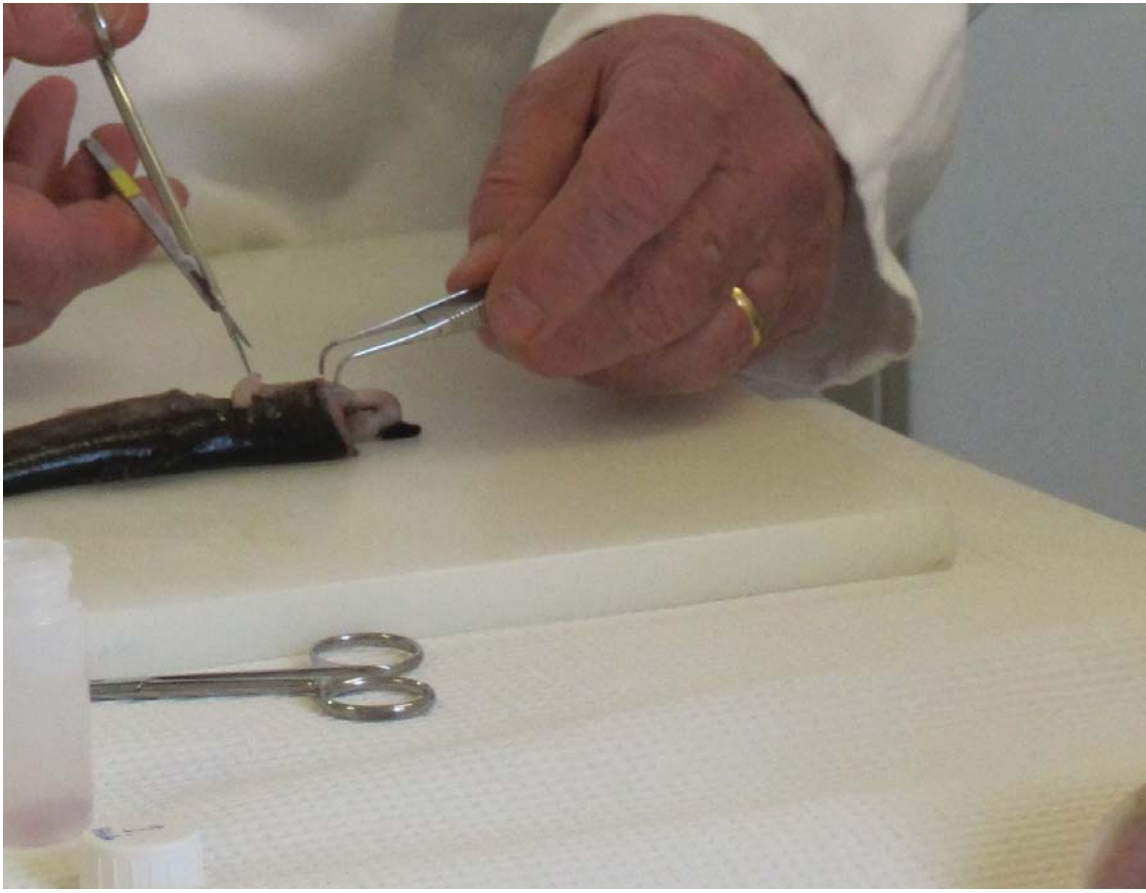
**Residues of human pharmaceuticals have been widely detected in various parts of the environment and trace concentrations are often found in sewage effluent and surface waters, typically ranging from low ng $L^{-1}$ to low µg $L^{-1}$ levels (Lindberg *et al.*, 2005; Nikolaou *et al.*, 2007; Loos *et al.*, 2009). These concentrations, however, are orders of magnitude below the therapeutic concentrations reached in human blood plasma. Thus, the potential for a physiological impact of pharmaceuticals on water-living organisms (such as fish) have been questioned. On the other hand, the levels measured in surface waters do not simply mirror the levels encountered by the receptors or enzymes present inside the fish living in these waters. Indeed, levels of pharmaceutical in for example fish blood plasma is sometimes much higher than the levels in the surrounding water. This can be explained by the concepts of bioconcentration and bioaccumulation.**



## What is bioconcentration?

Bioconcentration is a process where the level of a chemical in an aquatic organism increases by uptake from the water, eventually reaching a stable concentration higher than that of the surrounding water. Bioconcentration is often presented as a bioconcentration factor (BCF), which is the concentration of the studied chemical in the entire body or in a tissue per concentration of the chemical in water (reported as L/kg). This physical property characterizes the accumulation of pollutants through chemical partitioning from the aqueous phase into an organic phase, such as the gill of a fish. Bioconcentration values are typically derived from controlled laboratory conditions, where the chemical is absorbed from the water via the gills and/or the skin.

Bioaccumulation is a similar term which is defined as the process where the

chemical concentrations build up inside an organism regardless of exposure route, i.e. dietary absorption, transport across the respiratory surface, dermal absorption etc. Thus, bioconcentration differs from bioaccumulation because the former refers to the uptake of substances into the organism from water alone; bioaccumulation is therefore the more general term because it includes all means of uptake into the organism.

## What are the processes behind bioconcentration?

Bioconcentration is often described as a physico-chemical process that is more or less correlated to the octanol-water partition coefficient ($K_{OW}$) of the substance. Several equations have been published that describe this relation, most often with the general formula, $\log BCF = A \times \log K_{OW} - B$ (Mackay 1982; Fitzsimmons *et al*., 2001). This formula describes how chemical compounds, especially those with a hydrophobic component, partition into the lipids and lipid membranes of organisms. The concept assumes steady-state conditions, i.e. a situation when the organism is exposed for a sufficient length of time to allow uptake and excretion/metabolism to approach equilibrium. Thus, at this steady-state condition, the levels in the organisms do not change substantially. The models also more or less assumes that the chemicals are neutral, as charged molecules would have a much more restricted access to the lipid membranes of organisms. However, for some chemicals, uptake rates have been shown to remain high even after substantial ionization. Studies of water pH impact on chemical uptake for weak acids showed that the uptake rates varied little from pH 6.3 to 8.4, despite the fact that the ionization of the acids ranged from less than 1 to greater than 99.9% (Erickson *et al*., 2006). This could be explained mainly by two mechanisms, viz. reduced pH at the gill surface and that the ionized molecules contribute to the uptake by maintaining high gradients of neutral molecules across membranes (Erickson *et al*., 2006).

## Which pharmaceuticals are found in fish exposed to sewage effluents?

So far, there are very few peer-reviewed reports on the levels of pharmaceuticals in fish exposed to effluent-dominated surface water. From the limited number of available studies it has been shown that more than twenty pharmaceuticals from a wide range of therapeutic classes are present in fish, including non steroidal anti-inflammatory drugs, drugs targeting the central nervous system including selective serotonin reuptake inhibitors (SSRIs), as well as steroids. *Table 1* summarizes some reported levels in fish plasma and fish tissue.

**Table 1**. Pharmaceuticals detected in fish exposed to sewage effluent or effluent dominated surface water.

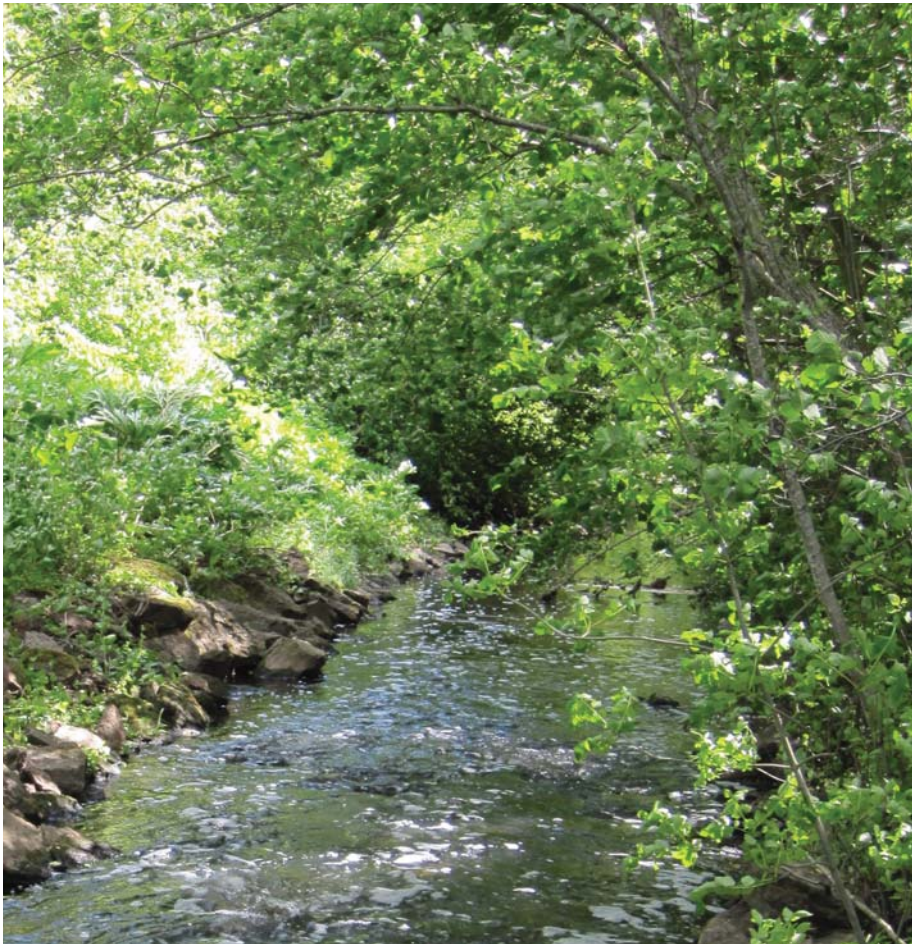
| Pharmaceutical | Detected levels ng/g | Detected levels ng/ml | Sample type | Env | References |
|---|---|---|---|---|---|
| carbamazepine | | 0.3-1.0 | blood plasma | effluent | (Fick *et al*., 2010) |
| carbamazepine | 2.3-8.0 | | muscle, liver | river | (Ramirez *et al*., 2009) |
| carbamazepine | 0.83-1.4 | | muscle | river | (Ramirez *et al*., 2009) |
| carbamazepine | 0.69-16 | | muscle, brain | river | (Brooks *et al*., 2005) |
| cilazapril | | 0.1-0.7 | blood plasma | effluent | (Fick *et al*., 2010) |
| diclofenac | | 2.2-20 | blood plasma | effluent | (Fick *et al*., 2010) |
| diclofenac | | 12 | blood plasma | effluent | (Brown *et al*., 2007) |
| diltiazem | | 0.9 | blood plasma | effluent | (Fick *et al*. 2010) |
| diltiazem | 0.13-0.9 | | muscle, liver | river | (Ramirez *et al*., 2009) |
| diltiazem | 0.11-0.27 | | muscle | river | (Ramirez *et al*., 2009) |
| diphenhydramine | 1.2-11 | | muscle, liver | river | (Ramirez *et al*., 2009) |
| diphenhydramine | 0.66-1.3 | | muscle | river | (Ramirez *et al*., 2009) |
| ethinylestradiol | | 1200 | bile | river | (Larsson *et al*., 1999) |
| fluoxetine | 19-80 | | muscle, liver | river | (Ramirez *et al*., 2009) |
| fluoxetine | 0.11-1.6 | | muscle, brain | river | (Brooks *et al*., 2005) |
| gemfibrozil | 27-90 | | muscle, liver | river | (Ramirez *et al*., 2009) |
| gemfibrozil | | 210 | blood plasma | effluent | (Brown *et al*., 2007) |
| haloperidol | | 1.2 | blood plasma | effluent | (Fick *et al*., 2010) |
| ibuprofen | | 5.5-102 | blood plasma | effluent | (Fick *et al*., 2010) |
| ibuprofen | | 84 | blood plasma | effluent | (Brown *et al*., 2007) |
| ketoprofen | | 15-107 | blood plasma | effluent | (Fick *et al*., 2010) |
| levonorgestrel | | 8.5-12 | blood plasma | effluent | (Fick *et al*., 2010) |
| meclozine | | 0.1-0.7 | blood plasma | effluent | (Fick *et al*., 2010) |
| memantine | | 2.3 | blood plasma | effluent | (Fick *et al*., 2010) |
| naproxen | | 33-46 | blood plasma | effluent | (Fick *et al*., 2010) |
| naproxen | | 14 | blood plasma | effluent | (Brown *et al*., 2007) |
| norfluoxetine | 3.2-130 | | muscle, liver | river | (Ramirez *et al*., 2009) |
| norfluoxetine | 3.5-5.1 | | muscle | river | (Ramirez *et al*., 2007) |
| norfluoxetine | 1.1-10.3 | | muscle, brain | river | (Brooks *et al*., 2005) |
| orphenadrine | | 0.9 | blood plasma | effluent | (Fick *et al*., 2010) |
| oxazepam | | 0.2-0.7 | blood plasma | effluent | (Fick *et al*., 2010) |
| risperidone | | 0.2-2.4 | blood plasma | effluent | (Fick *et al*., 2010) |
| sertraline | | 1.1-1.2 | blood plasma | effluent | (Fick *et al*., 2010) |
| sertraline | 5.0-545 | | muscle, liver | river | (Ramirez *et al*., 2009) |
| sertraline | 0.34 - 4.3 | | muscle, brain | river | (Brooks *et al*., 2005) |
| tramadol | | 1.1-1.9 | blood plasma | effluent | (Fick *et al*., 2010) |
| verapamil | | 0.7 | blood plasma | effluent | (Fick *et al*., 2010) |

Several studies have been conducted in the US where pharmaceutical residues have been measured in tissues of fish from effluent-dominated rivers (Brooks *et al*., 2005; Ramirez *et al*., 2007, Ramirez *et al*., 2009). So far these investigations have indicated that the fish is safe for human consumption but the ecological implications for the fish remains to be studied further.

In a study performed within the MistraPharma research programme, the bioconcentration of 25 pharmaceuticals were investigated in rainbow trout exposed to treated effluent from three Swedish sewage treatment plants (Fick *et al*., 2010). Out of the 25 selected pharmaceuticals, 17 were detected in fish plasma. One of the pharmaceuticals, the synthetic progestin levonorgestrel, was detected in fish plasma at levels that even exceeded the human therapeutic plasma concentration. Zeilinger *et al.* (2009) recently showed that exposure to as little as 0.8 ng / L levonorgestrel, the lowest concentration tested, resulted in strongly impaired reproduction of fish. In accordance, the study by Fick *et al*. showed that an effluent concentration of 1 ng/L resulted in a highly potent plasma concentrations in exposed rainbow trout. The MistraPharma study is the first study showing that fish exposed to sewage effluents can bioconcentrate pharmaceuticals to plasma levels equal to, or even exceeding, the human therapeutic plasma concentration. This suggests that certain pharmaceuticals could cause pharmacological effects on fish living in effluent-dominated surface waters. This study also shows that several pharmaceuticals can bioconcentrate quite significantly, as the levels found in fish plasma were up to 12000 times that of the water concentration (Fick *et al*., 2010).

## Can BCF be used in prioritization?

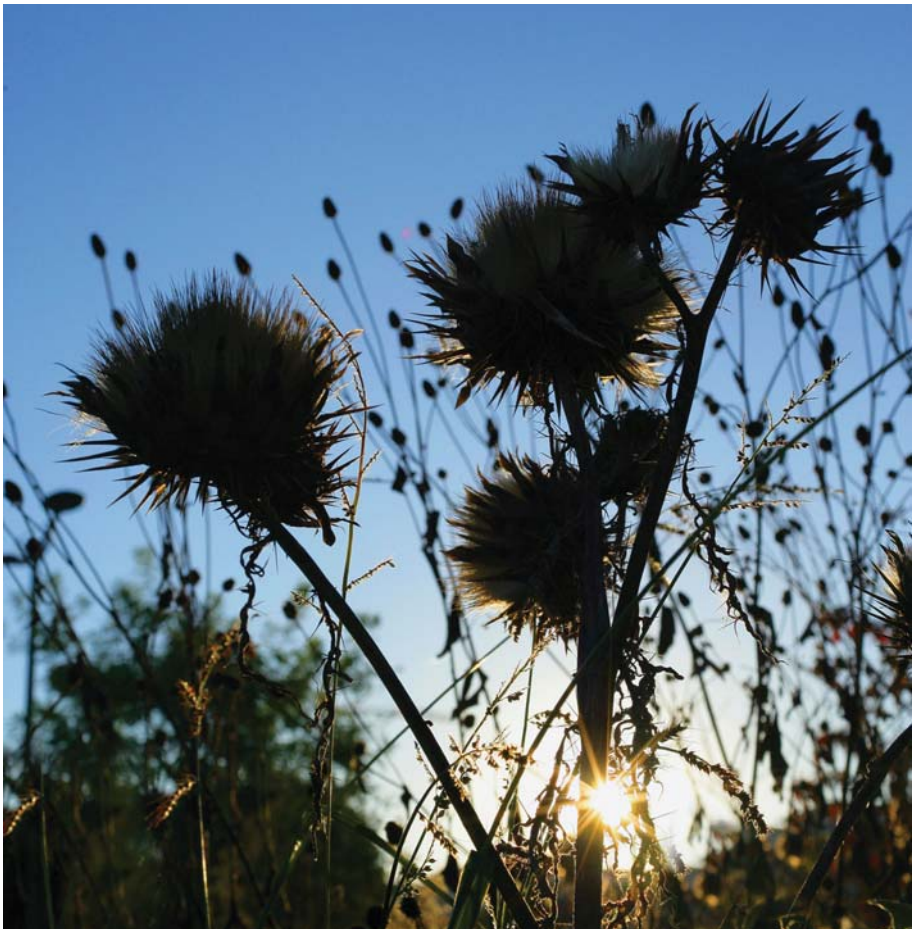
As there is a very large number of pharmaceuticals, a major challenge is how to prioritize research efforts to assess the potential risks associated with their usage. There is a need to develop novel test strategies, which has been recognized both by industry, authorities and academia (Huggett *et al*., 2003; Besse and Garric 2008; Gunnarsson *et al*., 2008; Brooks *et al*., 2009). To what extent chemicals bioconcentrate and bioaccumulate can be used directly as a tool to prioritize chemicals and this is e.g. one of the criteria used for the environmental risk assessment within REACH, the European chemical legislation (ECHA 2008). Bioconcentration studies or estimates present information on the dose that aquatic species are exposed to, which is very useful since we already have a considerable knowledge about the potency of pharma-ceuticals, at least in mammals, through their efficacy and safety testing. One option would therefore be to use existing mammalian data to assess the likelihood for a pharmacological effect in other species. It may sound



strange to compare fish and humans but due to the conservative nature of physiological processes, many aquatic species and particularly fish and amphibians, possess similar target molecules to those the drugs were intended to interact with in humans (Gunnarsson *et al*. 2008). This similarity implies that if the plasma level in fish is high enough, a similar pharmacological response could occur as in the intended target species, i.e. humans. The best available example of this are the effects of ethinylestradiol, a synthetic estrogen present in many birth control pills, on sexual differentiation and fertility of fish living downstream from sewage treatment plants (Larsson *et al*., 1999; Lange *et al*., 2009).

## What is the fish-plasma-model?

Huggett *et al.* (2003) presented a simplistic approach to predict the likelihood for pharmacological interactions in aquatic species, based on a screening-level model to predict bioconcentration followed by a comparison with human therapeutic plasma concentrations. This approach is referred to as the "the fish plasma model" (Huggett *et al.,* 2003). It assumes that two species sharing the same drug targets, i.e. receptors and enzymes etc, will require about the same plasma concentrations of a pharmaceutical to activate a pharmacological response. This approach makes it possible to generate an index of the likelihood that a fish is pharmacologically affected by a drug in the water. Huggett *et al.,* (2003) referred to this index as an "effect ratio",



whereas we have proposed the term "concentration ratio" as the index really is a ratio of two concentrations. The concentration ratio compares the blood plasma levels in humans taking a specific pharmaceutical (i.e. the human therapeutic plasma concentrations ($H_TPC$)), with measured or predicted steady state levels in fish blood plasma ($F_{SS}PC$; see *Equation 1*). Given that the target molecule(s) in the fish has roughly similar affinities to the drug as the human target(s) have, this concentration ratio will reflect the risk for a pharmacological response to develop in fish. If the concentration ratio is ≤ 1 then the concentration in the exposed fish is higher or equal to the known concentration that gives a pharmacological response in humans, i.e. the lower the ratio, the higher the risk for the fish.

$$CR = \frac{H_TPC}{F_{ss}PC}$$

**Equation 1**. CR = concentration ratio, $H_TPC$ = Human therapeutic plasma concentration, $F_{SS}PC$ = Fish steady state plasma concentration.

One of the advantages with this approach is that the $H_TPC$ is readily available in the literature for most pharmaceuticals. Since studies measuring plasma levels of pharmaceuticals in fish subsequent to exposure via water are scarce it is necessary, in most cases, to predict the plasma levels in fish using one of the several equations that are available to calculate the bioconcentration. Even though these equations are made for neutral compounds and describe the partition into the lipid membranes of organisms, it seems to be able to predict fish plasma levels of pharmaceuticals with relatively good accuracy (Brown *et al*., 2007; Fick *et al*., 2010). It should be stressed that even if a fish has the same plasma levels as a human using a pharmaceutical, this will only indicate the probability of a pharmacological response to develop, not whether this response is adverse or not.

We propose that one possible way forward for identifying drugs of environmental concern is to rank them based on their estimated concentration ratios. Concentration ratios could be derived in different ways. The most accurate, but also the slowest approach, is to derive concentration ratios from actual measurements of blood plasma levels in fish as in the studies by Brown *et al.* (2007) and Fick *et al.* (2010). Another alternative is to derive concentration ratios from *estimates* of blood plasma levels, which in turn are based on *measured* surface water levels of drugs. This approach will allow a more rapid screening of many drugs, but it also involves assumptions about how

well different drugs bioconcentrate (Fick *et al*., 2010). The most rapid approach, but also the approached with the most uncertainties, would be to base the calculation on *estimated* surface water levels, where for example usage, excretion and estimated removal efficiencies in sewage treatment plants could be taken into account. Although all of these strategies admittedly involve assumptions about conserved modes of action of drugs between fish and humans, it provides a possibility to apply a scientific basis to rank a large number of drugs prior to performing extensive biological tests with fish. Our strategy in MistraPharma to expose fish to effluents, screen for drugs in their blood plasma and compare measured levels with human therapeutic levels led to the identification of levonorgestrel as a drug of high environmental concern. The recent findings that similar water levels of levonorgestrel impairs reproduction in fish (Zeilinger *et al*., 2009) suggest that our strategy could be a fruitful and. new multi-residue analytical techniques (LC/LC-MS/MS) including more than 120 different pharmaceuticals have been developed and validated within the MistraPharma programme which will allow an expansion to a wider set of pharmaceuticals.

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# 2 The Frog Test System

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**The overall aim of the project is to evaluate effects of pharmaceuticals on development and reproduction in frogs. The project will increase our knowledge on long-term consequences of early life exposure to pharmaceuticals found in the environment, as well as the mechanisms involved. Ecologically relevant exposure conditions (route and duration) and test endpoints such as reproductive output are used. The project will thereby provide valuable information for assessing the risk of reproduction disorders in wild frog populations.**

## The sensitive early life-stages

The development of the vertebrate reproductive system starts during the early life-stages and the hormonal milieu during sex differentiation is crucial for proper differentiation and development of the reproductive organs and brain (Bernal *et al.*, 2003; Cooke *et al.*, 2004; Bakker and Baum, 2008).

Hormonally controlled processes may be disrupted by environmental chemicals that interfere with the hormone system. It is therefore important to evaluate chemicals in biological test systems to find out whether they can affect the hormone system and impair the reproductive system. Because of the sensitive embryo-foetal period it is especially important to evaluate long-term consequences of exposure during early life-stages.

## Frogs as test organisms

Amphibians represent an excellent model to investigate toxicity of hormonally active chemicals for several reasons. First, amphibians are declining dramatically world-wide (Stuart *et al*., 2004), making it important to investigate potential contributing factors to the declines. Second, there are several indications that this animal group may be specifically sensitive to compounds interfering with the hormone system. Laboratory studies have

shown that environmentally relevant concentrations of an estrogenic pharmaceutical (Pettersson & Berg 2007; Gyllenhammar *et al*., 2009a), a pesticide (Hayes *et al*., 2002), and an anti-thyroid compound, ammonium perchlorate (Goleman *et al*., 2002), can feminize gonadal differentiation in male frogs resulting in female-biased sex ratios. These findings imply that wild frog populations may be at risk from hormonally active environmental pollutants.

Third, it is a suitable model to investigate chemicals that interfere with the thyroid hormone system as the metamorphosis (transition from larva to frog) is mediated by thyroid hormone. Amphibian test systems for detection of thyroid disrupting chemicals using *X. tropicalis* and *X. laevis* are currently being developed within the Organisation for Economic Cooperation and Development (OECD) and the US Environmental Protection Agency. Fourth, the organization and components of the hormonal system including the hypothalamus-pituitary-gonadal and hypothalamus-pituitary-thyroid axes are very similar to that in higher vertebrates (reviewed in Berg *et al*., 2009).

## The clawed frog *(Xenopus)*

The African clawed frog, *Xenopus laevis*, is a commonly used model organism in developmental biology and developmental toxicology. It is the model organism in the embryo toxicity test Frog Embryo Teratogenesis Assay-Xenopus (FETAX) (Dumont *et al*., 1983). *X. laevis* is also proposed as model species in the bioassays for detection of thyroid disrupters that currently is being developed within the OECD and the US Environmental Protection Agency. A disadvantage of *X. laevis* is the comparatively long generation time (1-2 years) which makes life cycle studies and research on developmental reproductive toxicity very time consuming and expensive.

**Figure 1**. *Xenopus tropicalis.*

The emerging model species West African clawed frog ,*Xenopus tropicalis*, has several advantages over *X. laevis* as it is smaller, has a shorter generation time (about six months), and a diploid genome as opposed to the allotetraploid genome of *X. laevis* (Hirsch et al., 2002). *X. tropicalis* has been proposed as an alternative test organism for the FETAX assay (Song et al., 2003), thyroid disruption (Mitsui *et al*, 2006), as well as for endocrine disruption and developmental reproductive toxicity (Fort *et al.*, 2004; Pettersson *et al.*, 2006; Pettersson and Berg, 2007; Gyllenhammar *et al.*, 2009a;b).

## The *Xenopus tropicalis* Test System

In our test system larvae are exposed to the test substances from hatching until completed metamorphosis (*fig. 2*). Exposure via the water during the larval period is ecologically relevant as most frog species, including those that become terrestrial after metamorphosis, have an aquatic larval period. Effects are evaluated at defined larval stages (during sex differentiation), at metamorphosis and when the frogs have reached sexual maturity.

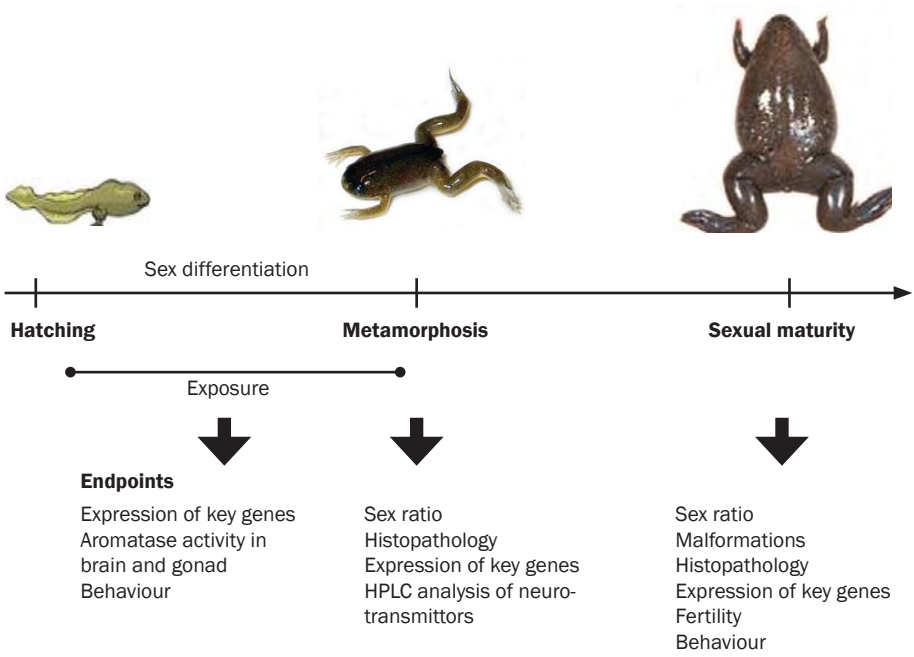


**Figure 2**. The *Xenopus tropicalis* test system for developmental toxicity.

## Endpoints

Expression of genes important for sex differentiation and development (hormone receptors and the enzyme aromatase) as well as aromatase activity will be investigated in brain and gonads at several life-stages. Aromatase catalyses the conversion of androgens into estrogens and is thereby a key enzyme in the vertebrate hormone system. Aromatase is thought to be involved in gonadal differentiation in lower vertebrates as inhibition of aromatase in embryos of birds, reptiles and fish induces testicular differentiation (Elbrecht and Smith 1992; Piferrer *et al.*, 1994; Pieau *et al.*, 1999). The prevailing hypothesis is that aromatase is involved also in amphibian gonadal differentiation but its specific role remains to be elucidated (Kelley, 1996; Urbatzka *et al.*, 2007). Given the central role in the sex hormone system aromatase activity is an interesting endpoint for endocrine disruption.

Levels of signalling molecules in the brain, neurotransmitters, are analysed in specific brain regions. The testicles, ovaries, oviducts, thyroid gland and other suspected target organs are evaluated for structural changes using a

microscopy. Fertility studies include endpoints such as sexual behaviour (*fig. 3*), egg production, hatchability, embryo mortality. Sperm quality will be assessed by evaluating quantity, morphology and motility.

Gene expression and biochemical responses (aromatase activity, neurotransmitter levels) during sex differentiation and metamorphosis will be linked to subsequent morphological and functional effects. We will thereby clarify whether early molecular or biochemical responses can be used in order to forecast later reproductive or behavioural responses. By investigating gene expression and biochemical responses during sex differentiation information will be generated regarding the mechanisms for reproductive effects.

## Results from the frog test system

Our results show that frogs are more sensitive to estrogenic environmental pollutants than previously known. Larval exposure to the estrogenic pharmaceutical ethynylestradiol $EE_2$ at concentrations found in the environment induced male-to-female sex-reversal in *Xenopus tropicalis* and *Rana temporaria* (Pettersson and Berg, 2007). *Rana temporaria* is a temperate, terrestrial frog species commonly found in Europe. Altered gonadal differentiation resulting in partially or completely sex-reversed testicles has earlier proved to be a sensitive test endpoint for estrogenic pollutants in fish, reptiles, and birds (Fry *et al.*, 1981; Bergeron, *et al.*, 1994; Gimeno *et al.*, 1996; Berg *et al.*, 1998; 1999). We have demonstrated that also in amphibians the differentiation of the gonads is a process extremely sensitive to disturbance by estrogenic environmental pollutants.

**Figure 3.** Mating pair of *Xenopus tropicalis.*

## Risk of reproductive disturbance in wild frogs

Using our model frog *Xenopus tropicalis* we showed that the $EE_2$-induced sex-reversal persisted into adulthood. Interestingly, a significant proportion of the adult frogs exposed to estrogen with ovaries lacked oviducts, making them sterile (*fig. 4*). Male-to-female sex-reversal was implied at concentrations as low as 0.006 nM $EE_2$. $EE_2$-exposed males that were not sex-reversed showed decreased fertility and reduced amount of spermatozoa in the testicles (Gyllenhammar *et al.*, 2009a). $EE_2$-levels of up to 0.9 nM have been detected in the aquatic environment in Europe and USA (Kolpin *et al.*, 2002a,b). Our findings therefore indicate that estrogenic environmental pollutants might pose a threat to wild frog populations.

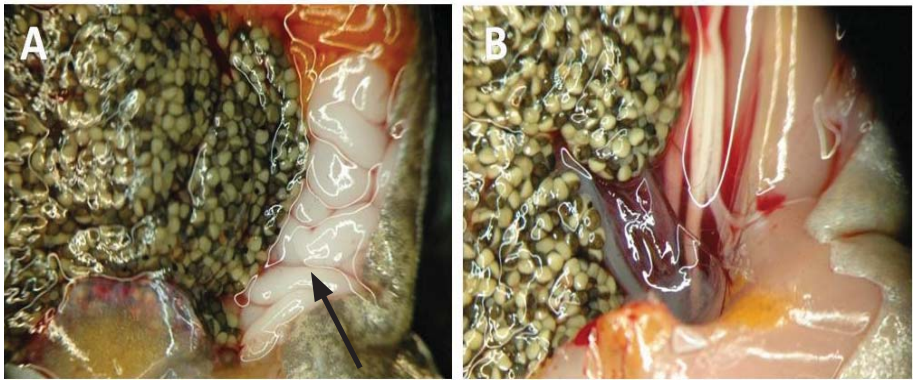


**Figure 4**. A) Adult control female *Xenopus tropicalis* with oviducts (arrow).
B) Adult "female" that lacks oviducts after exposure to EE2 during the larval period.

In wild fish, reproductive disturbances such as have been demonstrated. In the UK, high incidences of male fish were found with egg cells in their testicles as well as reduced milt production, and fertilization success compared to fish from less polluted rivers (Jobling *et al.*, 2002). In laboratory studies the lowest EE2 concentration reported to have induced female-biased sex ratios in fish is 0.001 – 0.003 nM (1 ng/L) (Örn *et al.*, 2003; Parrott and Blunt, 2005). Our studies show that the susceptibility to estrogen-induced sex reversal in *Xenopus tropicalis* is comparable to that in fish. The sensitivity to estrogen in *Xenopus tropicalis* was also shown to be comparable to that of the temperate frog *Rana temporaria*, which supports the use of *Xenopus tropicalis* as a model organism for endocrine disruption.

## Long-term consequences of early-life exposure

Early life exposure to estrogen results in serious effects on the reproductive system including impaired testicular and oviducal development. This has been demonstrated earlier in mammals, birds, and fish (Wilson *et al.*, 1986; Gimeno *et al*., 1996; Toppari *et al*., 1996; Berg *et al.*, 2001; 2004; van Aerle *et al*., 2002; Goyal *et al*., 2003; Hill and Janz 2003; Newbold 2004; Blomqvist *et al*., 2006; Holm *et al*., 2006). We are, to our knowledge, the first to demonstrate impaired fertility in adult amphibians after larval exposure to an estrogenic compound. Similarities between the testicular effects observed in the present project and those reported in fish, birds and mammals after developmental exposure to estrogens makes *Xenopus tropicalis* a promising model for research on developmental reproductive toxicity.

## Conclusion

The susceptibility to estrogen-induced sex-reversal in the model species *Xenopus tropicalis* is similar to that of a temperate European frog, *Rana temporaria*, and it is comparable to that of fish supporting the use of *Xenopus tropicalis* as a model species for research on endocrine disruption. Environmental concentrations of an estrogenic pollutant caused permanent sex-reversal and reduced fertility, indicating that reproduction in wild frogs may be at risk from exposure to estrogenic pollutants. Similarities between the effects in *X. tropicalis* and those reported in fish, birds and mammals after developmental exposure to estrogens make *X. tropicalis* a promising model for research on developmental reproductive toxicity.

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# 3 Genomics as a Guide for Environmental Risk Assessments of Pharmaceuticals

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**Pharmaceuticals are vital for the prevention, treatment and mitigation of diseases. On the downside, however, the use of pharmaceuticals has led to the unintended exposure of wildlife to residual drugs. For most pharmaceuticals, the main route to the environment is through effluents from sewage treatment works, leading primarily to the exposure of aquatic organisms. A key question is whether the pharmaceuticals reach sufficient concentrations in the environment to affect living organisms. We know that the synthetic estrogen ethinylestradiol ($EE_2$), used in many contraceptives, contributes to the feminization of male fish found downstream from sewage treatment plants (Larsson *et al.*, 1999). However the observed estrogenic effects on the gonadal development and fertility of these fish is probably due to the cumulative exposure to $EE_2$ and to other estrogenic chemicals, including natural estrogens from women's urine, estrogens used for hormone replacement therapy and various industrial**

**chemicals that are capable of weakly interacting with estrogen receptors (Tyler *et al.*, 2009). A study within the MistraPharma programme recently identified levonorgestrel, a gestagen present in certain contraceptives, at a concentration of 1 ng/L in treated sewage effluent (Fick *et al.*, 2010). Fish exposed to such effluents bioconcentrated levonorgestrel to levels similar to or exceeding human therapeutic plasma levels (Fick *et al.*, 2010; see also *chapter 1* on bioconcentration in this book). In addition, a recent laboratory study showed that levonorgestrel impairs the reproduction of fish at concentrations lower than 1 ng/L (Zeilinger *et al.*, 2009). Gestagens thus represent another example where there is strong concern for the aquatic environment.**

## Risk assessment of pharmaceuticals

Both the EU and the USA demand an environmental risk assessment be performed before the approval of new pharmaceutical products. However, the tests required were proved insufficient to discover the adverse effects of $EE_2$. Despite some refined testing approaches in recent years, particularly in Europe, the proposed test schemes are not optimized to identify sub-lethal effects, which may very well become adverse at certain steps of the life cycle and/or outside of the laboratory where there is competition for resources. Indeed, pharmaceuticals are specifically designed to have a low toxicity (at therapeutic concentrations) in the classic sense, and a focus on sub-lethal responses in the environmental risk assessment is therefore motivated. Chronic tests over several generations are indeed powerful; however, these tests can often become long, demanding and costly, particularly with fish. Methods to discover or indicate potential adverse effects after shorter exposure times would thus be valuable.

## Biomarkers

Physiological processes that are changed after shorter exposure times can indicate an increased risk for adverse effects after a more chronic exposure. Such short-term effects are often referred to as biomarkers. These biomarkers are thus used as "surrogates" for a more definite endpoint, such as disease or death. Indeed, biomarkers are used not only in environmental research but also to diagnose diseases in humans or, during clinical trials, to assess at an early stage the chances that a given drug will have beneficial effects after many years of drug therapy.

Biomarkers may be divided into four different classes, biomarkers of susceptibility, exposure, effect or disease. A susceptibility marker could be, for example, the presence of a specific version of a gene in an individual, rendering this person susceptible to developing cancer later in life. Susceptibility biomarkers can also be genetic differences that can explain or predict individual or, in an ecological context, species variability in the response to a given toxicant. Elevated body temperature (fever), a response to various infections, is commonly used as a biomarker of disease. High blood pressure can indicate cardiovascular disease development and reduced blood pressure is often used as a biomarker of effectiveness in response to treatment with cardiovascular drugs. An effect or a disease biomarker, such as fever, often tells you little about the underlying causes. In contrast, an exposure biomarker is mainly used to determine whether a person/organism has been exposed to a given chemical or group of chemicals but provides limited

possibilities to evaluate the risks for the development of adverse effects. However, there is often not a distinct line between different types of biomarkers. Biomarkers can also be used in combination, particularly on a molecular level, to become more informative. Molecular responses of an organism are often fast, and short exposure times may be sufficient to provoke a detectable response. Thus, exploratory molecular analyses may very well serve both to increase our understanding of the mode of action (including toxicity) of pharmaceuticals in aquatic organisms and to provide biomarkers after proper evaluation.

## An introduction to genomics

There is no universally accepted definition of genomics. The term often applies to studies of the genome or gene-products on a large scale. To understand the principles of genomics, one must first know the basics of how genes orchestrate the synthesis of proteins, in other words, the central dogma of molecular biology. The genome is the sum of an organism's inherited information, encoded in DNA. The DNA can be thought of as long strings of four "chemical letters", and the order and identity of these letters contain information that can be interpreted by the cells of an organism. DNA includes both genes, a region of the DNA sequence that encodes for one or more proteins, and non-coding sequences.

The central dogma of molecular biology was first described by Francis Crick in 1958 and is a framework for understanding the transfer of information between sequential information-carrying biomolecules, i.e. DNA, RNA (both nucleic acids) and proteins. The transfer describes the normal flow of biological information: DNA can be copied to DNA (DNA replication), DNA information can be copied into mRNA (transcription) and mRNA can then serve as a template for the synthesis of amino acids that are assembled into proteins (translation) (*fig. 1*). One could imagine the DNA as the blueprint of a car. The mRNAs would then correspond to the final template used to create the different parts of the car. The proteins have, like the different parts of a car, various properties. Proteins can be receptors, enzymes, etc., in other words, the essential parts of a functional organism. As a result, everything that occurs in an organism is related to an effect of a protein and therefore to DNA transcription to mRNA and mRNA translation to proteins. Thus, the study of mRNA can reveal possible effects at the protein level and thus physiological processes.

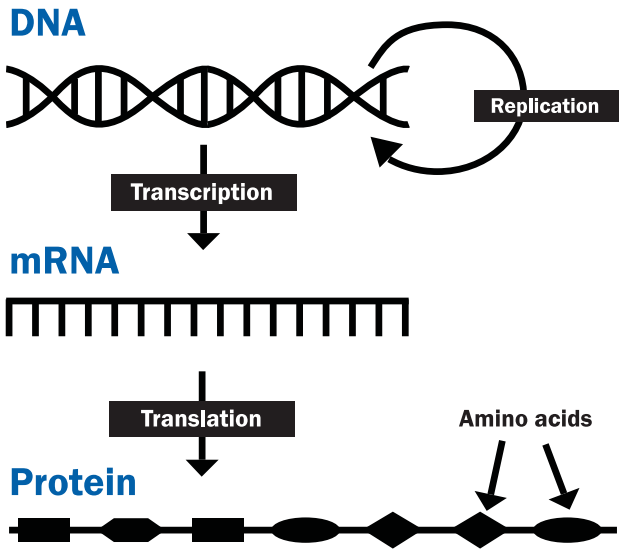


**Figure 1**. The central dogma of molecular biology.

## DNA – genomic information to predict susceptibility

Pharmaceuticals are designed or selected to induce their intended clinical effects through specific, high-affinity interactions with target proteins, e.g. receptors, while affecting other physiological processes as little as possible. Because pharmaceuticals are generally present at very low concentrations in aquatic environments, only high-affinity interactions with proteins are likely to play a role in wildlife (such as the interaction between drugs and drug targets), whereas general types of toxicity that require higher concentrations of a chemical, such as narcosis, are unlikely to occur. Many of the proteins in wild organisms are similar to the proteins in humans. This means that a pharmaceutical may interact with a similar protein in exposed wildlife species and thereby affect the organism. Consequently, the presence of similar drug target proteins in a wildlife species is associated with an increased risk for the organism to be affected by residual drugs that are present in the environment at low concentrations, i.e. a biomarker of susceptibility. Accordingly, the presence of estrogen receptors in fish indicates their susceptibility to estrogen exposure, whereas a lack of the receptors, as in algae and water fleas (*Daphnia*), indicates a relative insensitivity. This is consistent with, for example, the documented strong effects of $EE_2$ and other estrogens on fish but not on algae or water fleas.

As part of the research efforts within MistraPharma, our group investigated which organisms have protein drug targets that are similar to the human drug targets. This was achieved by comparing the genomes (total DNA sequence) of 16 species to the human genome, which encodes 1,318 known drug targets. The human genome was initially described through "The Human Genome Project" (HGP), an international research effort initiated in 1990. Today, the genome of hundreds of different organisms are known (http://www.ensembl.org/index.html), and recently the genomes of several ecotoxicologically relevant species have been sequenced as well.

Our group showed that fish and frogs have a corresponding target protein for 80% of the investigated human drug targets. However, the water flea lacked many of the drug targets, and the alga lacked almost all drug targets (*fig.* 2). Today, the most comprehensive tests within the formalized

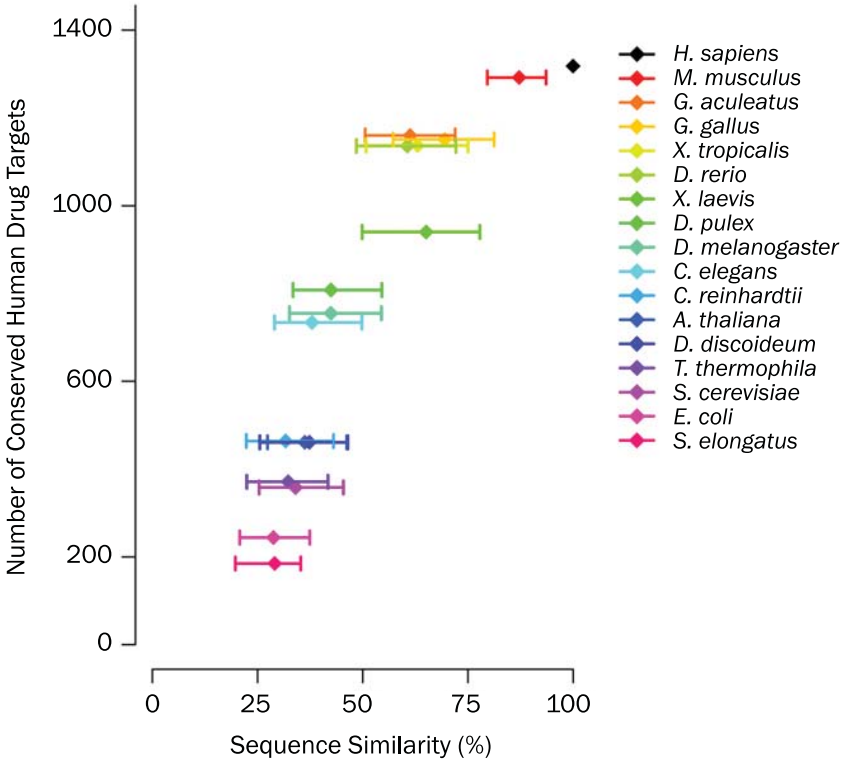


**Figure 2.** Number of predicted conserved drug targets in 16 non-target species and their sequence similarities to human drug targets. The figure is reproduced with permission from (Gunnarsson *et al.*, 2008) copyright 2009, American Chemicals Society.

environmental risk assessment procedures for pharmaceuticals are performed on water fleas and algae and not on fish. In the EU, chronic lifecycle toxicity tests are performed with water fleas and algae, whereas only semi-chronic, early life-stage tests with fish are required (EMEA, 2006). However, in the USA, the environmental risk assessment may be based on acute responses (lethality) only, and tests on fish are not mandatory. Our study shows that the environmental risk assessment of pharmaceuticals based on results only from experiments with water fleas and algae may not be protective for fish or other aquatic vertebrates. We therefore propose that environmental risk assessments for drugs with human targets should include comprehensive studies on aquatic vertebrates. All the results from our comparisons are available as supplementary material to Gunnarsson *et al*., (2008). The data may be used both for guiding the choice of test species and for avoiding particularly inappropriate species extrapolations. It should of course be stressed that our knowledge on the detailed molecular interactions of pharmaceuticals with human proteins is far from complete, and it is even less complete regarding the interactions with proteins in other species. It can thus be expected that some pharmaceuticals will interact with other proteins in wildlife other than their primary human target proteins.

## mRNA – applying microarrays to ecotoxicology

The presence of many targets for human drugs in fish is one of several reasons to place a comparably large focus on this group of organisms when assessing the environmental risks associated to these substances. Because the modes of action for many pharmaceutical substances are known in mammalian species, it is possible to create hypotheses of potential molecular responses (i.e. which gene-products to study) in fish as well. However, even if a drug target is evolutionarily well conserved, the stimulation of the target might lead to different physiological events in different organisms. Thus, there is a great value in studying a broader set of responses in the tested species than one or a few responses hypothesized from known responses in mammals. To identify which gene products are involved in the response to a given pharmaceutical, exploratory analyses need to be employed.

Microarray technology allows the study of thousands of potential gene responses simultaneously by parallel analyses of the abundance of thousands of expressed, specific mRNA sequences (transcripts). In the past decade, microarray technology has been successfully applied to various areas within biology, from cancer diagnostics to ecotoxicological research. It was first introduced in the mid 1990s, though, like most new technologies, it was initially very expensive to apply and the quality was relatively poor.

However, there has been a rapid technological development, and today the performance of many microarray platforms is very high, and the running costs are continuously decreasing. Several different types of microarrays exist, though they are all based on the basic principle of measuring mRNA abundance corresponding to individual genes by the binding, or hybridization, of mRNA molecules to specific probes on a chip. Each chip is normally equipped with thousands of spots, each containing probes made of nucleic acids. Each probe is designed to match the nucleic acid sequence of a specific mRNA molecule. The total mRNA of the organism/tissue/cell studied is first extracted and, after some additional steps, hybridized to the chip. When a specific transcript binds to its matching probe on the chip, a signal (fluorescence) can be induced and visualized by a camera (*fig. 3*). The fluorescent signal increases as the amount of specific mRNA matching a certain probe increases. Subsequently, by comparing the microarrays from exposed organisms to the microarrays from non-exposed ones, we can identify genes that are differentially expressed as a consequence of the exposure.

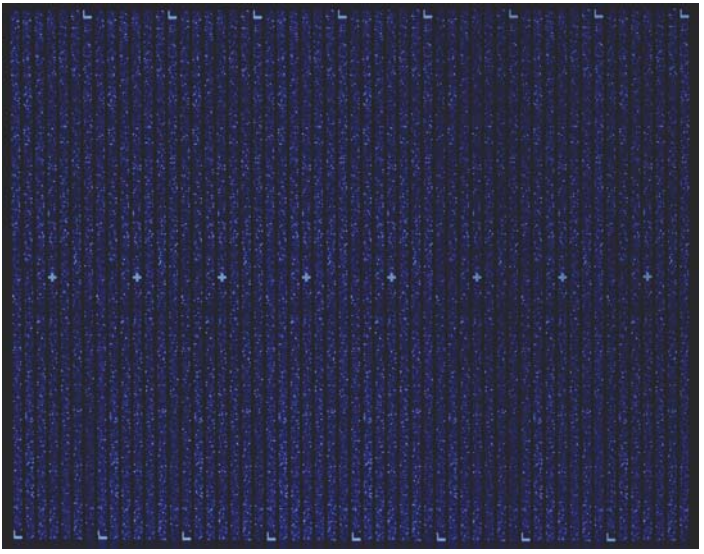

**Figure 3.** A microarray chip showing the gene expression in the liver from eight individual rainbow trout. The light intensity of each spot reflects the expression of one specific mRNA.

Because microarrays have proved useful for a variety of applications, there are several commercial microarray platforms available with probes selected to cover genes of interest or sometimes the entire genome of a species. The benefits from using these commercial arrays include their readymade protocols and support and their (often) overall high quality. However, few arrays are available for environmentally relevant species. For use in ecotoxicological research, non-commercial microarrays have been developed mainly by academia, such as those for the water flea (Soetaert *et al*., 2006) and the eelpout (Kristiansson *et al*., 2009). In addition to the eelpout array, our group has also developed microarrays for rainbow trout (Gunnarsson *et al*., 2007; 2009a) and for the barnacle (*Balanus improvisus*), a marine crustacean that lives on rocks at the shoreline also constitutes a major fouling organism on ships (Falkbring *et al*., in prep).

A microarray analysis may serve several purposes. First, it conveys information about the (toxicological) *mode of action* of the pharmaceutical in the exposed species. Such knowledge could be used to categorize individual pharmaceuticals. Pharmaceuticals acting via similar modes of action would be expected to affect organisms through what is referred to as a *concentration-addition model*. In practice, this means that even if the concentration of no single pharmaceutical in a mixture is sufficient to cause an adverse effect on the organisms alone, all pharmaceuticals (and other chemicals) acting via the same mode of action present in the same mixture (such as an effluent) will add up, leading to a combined effect. Thus, the environmental risks should be assessed based on all similarly acting chemicals together. Understanding the mode of action of pharmaceuticals in non-target species is therefore important for proper risk assessments and for taking mixture effects into account.

A second purpose of mRNA analyses is to aid in the identification of substances within a mixture that are present at sufficiently high levels to affect organisms. When it comes to pharmaceuticals, this is particularly important when evaluating the effects of complex effluents from sewage treatment plants. An increased expression of specific genes in fish exposed to sewage effluent can point to the presence of potent levels of certain pharmaceuticals or groups of pharmaceuticals (*exposure biomarkers*). However, this of course requires that we know which genes are consistently regulated by these pharmaceuticals. Thus, a comparison of the genes regulated after exposure to individual pharmaceuticals to the genes regulated after a complex effluent exposure is required. Two papers recently published by our group illustrate this approach. In the first study, we identified several genes regulated by $EE_2$ and other estrogens in the fish liver (Gunnarsson *et al*., 2007). In the second

paper, again part of the efforts within MistraPharma, we focused on analyzing the estrogen-sensitive mRNAs in fish exposed to sewage effluent treated by different methods (Gunnarsson *et al*., 2009b). This allowed an evaluation of the treatment methods that efficiently removed ecotoxicologically important estrogens based on an integrated measure of their combined effects.

A third potential use of microarray analyses is to reveal information about the potency of the pharmaceutical in the investigated organisms. A clear effect on the expression of specific mRNAs, particularly if these are related to the expected mode of action of the drug, can reveal that there is sufficient internal exposure to the drug to cause an activation of the drug target. Given the very low concentrations of most pharmaceuticals in surface water, we would expect no detectable responses at environmentally relevant concentrations for most drugs. Thus, for pharmaceuticals where we can clearly identify mRNA responses at concentrations present in effluents or surface waters, this could be regarded as a signpost, motivating further, more elaborate testing, including for example life-cycle tests. Within the MistraPharma programme we have adopted this approach (Cuklev *et al*., in preparation).

## Proteomics

Proteomics, often included within the term genomics in its wider sense, is the large-scale study of proteins, particularly their abundance, structures and functions. The proteome is the entire set of proteins produced by an organism. Because the proteins, rather than the mRNAs, are the functional units of organisms, analyzing protein levels could be argued as more relevant and more closely related to potential adverse effects than analyzing mRNA. If we had the same analytical possibilities to measure proteins at the same depth (number of different proteins), cost and speed as we have for mRNA, this could very well be true. Unfortunately, most methods to measure protein expression are not nearly as comprehensive, rapid or reproducible as microarrays. In the late 1990s, proteomics was given a lot of attention, and numerous articles promoted the potential of this "omic" technology.

Although considerable efforts were spent in development and research, the awaited excellent scientific results were largely absent. Today, much of the original hype surrounding proteomics is over. The challenges that face proteomic analyses come mostly from the highly variable properties of different proteins. In this respect, different mRNAs are so much more alike, and it is therefore much easier to develop methods that can analyze all or almost all mRNAs in parallel. Nevertheless, there are cases where analyses targeting the proteome or parts thereof are motivated. The most common approach to analyze the abundance of many proteins simultaneously in a sample is to apply two-dimensional gel electrophoresis, where proteins in a sample first are separated according to charge followed by a separation based on gross molecular mass. The gels are then stained and the intensities of matching spots between different gels/samples are compared. Rarely, more than a 1000 spots in total can be detected on such a gel, but usually substantially fewer. Regulated spots can then be cut out from the gels and

analyzed using different forms of mass-spectrometry (MS). A common problem is that each spot contains several proteins, and it is difficult to know which ones were regulated. If there is no comprehensive information on the exact amino acid sequences of proteins from the investigated species (i.e. the genome is not known), more advanced forms of MS technologies may also be required to identify the proteins. One such method is Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR/MS). Our group has used two-dimensional gel electrophoresis in combination with FT-ICR/MS to identify regulated proteins in the liver of rainbow trout caged downstream from sewage treatment plants (Albertsson *et al.*, 2007), but we do not yet know if the regulation of these proteins is caused by exposure to pharmaceutical residues or other chemicals present in the effluent (Albertsson *et al.*, 2010).

## Metabolomics

Metabolomics is the large-scale study of metabolites, which are the intermediates and products of metabolism. The metabolites referred to herein should not be confused with the metabolites (degradation products) of pharmaceuticals but rather refer to endogenous (originating from the body) small molecules such as amino acids, sugars, lipids, energy-carriers, etc. Thus, metabolomics can complement microarrays and proteomic analyses in terms of understanding the mode of action of pharmaceuticals/mixtures and to identify biomarkers. Changes detected can be claimed to reflect an actual change in the physiology of the cell/tissue/individual, more so than changes in mRNA abundance. Mass spectrometry is often used to identify and quantify metabolites after separation by techniques such as gas chromatography. However, the most common metabolomics technology is based on analyses with nuclear magnetic resonance (NMR) spectrometry. This approach does not rely on the separation of the molecules, the metabolites, and the samples can thus be recovered for further analyses. NMR is also a very robust and reproducible analysis method. The major drawback is the difficulty associated with detecting rare metabolites using this method. Our group has used NMR metabolomics to identify responses to $EE_2$ in the blood plasma of fish (Samuelsson *et al.*, 2006). The affected metabolites agreed well with what was expected, and thus the study may serve as a proof of concept for exploratory analyses using NMR metabolomics to identify metabolic changes in fish (Samuelsson and Larsson, 2008). Recently, as a collaborative effort between MistraPharma and the Stockholm Water project on pharmaceuticals in the environment, we have also applied NMR metabolomic to assess the impact on fish of sewage effluents treated with different technologies (Samuelsson *et al.*, submitted).

## The future of genomics from an ecotoxicological perspective

The number of fully sequenced organisms is increasing as we speak; thus, designing microarrays for use in ecotoxicological research is rapidly becoming less complicated. A more comprehensive knowledge about different genomes will hopefully lead to more precisely defined gene functions across organisms. This may increase the possibility for extrapolation between species and thereby provide a more solid framework for a better understanding of the modes of action of chemicals. For pharmaceuticals, this pertains particularly to comparisons between the modes of action in humans versus those in environmental species.

In pre-clinical research, the use of genetically modified mice has proven very useful. Employing cells or entire organisms with a modified genetic makeup or ability to express specific genes has currently only been used to a limited extent in ecotoxicological research and even less so to specifically understand the action of pharmaceuticals in the environment. This is clearly a field for further exploration.

The next generation of massively parallel sequencing technology (pyrosequencing) also opens up novel approaches. With a sufficient depth of the sequencing, gene expression can today be analyzed by sequencing millions of pieces of mRNA rather than by methods based on specific hybridization to probes (such as microarrays). When it comes to studying the effects of, for example, antibiotics on complex microbial ecosystems, where we have comparably little information on the species present, deep sequencing of DNA (or mRNA) provides unique opportunities to move science forward (Kristiansson et al, in preparation).

Considering the rapid development of genomic technologies and applications over the past ten years, more discoveries should be achieved in the near future that will help us to understand the risks of pharmaceuticals in the aquatic environment and to direct and evaluate mitigations.

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# 4 Laboratory vs Field Studies to Assess Environmental Hazards and Risks Posed by Pharmaceuticals for Human Use

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**Safety testing of pharmaceutical drugs, pesticides and other chemicals is classically based on studies examining single compounds. The reason for this approach is obvious; regulatory authorities approve compounds one by one. In the aquatic environment, organisms are exposed to mixtures of contaminants and adverse effects are difficult to refer to single compounds. While laboratory studies contribute information on mode/mechanism of action and concentration-response relationships, field studies generally provide phenomenological data with limited information on causality. To help understanding adverse effects in the field, a combination of laboratory and field data is therefore required. There is urgent need to develop improved methods that are useful both in the laboratory and the field. The introduction of novel mechanism-based biomarkers will hopefully help bridging the gap.**

Numerous pharmaceuticals originate from natural products formed by organisms such as fungi and plants. In some cases, the structure of the original molecule has been modified to optimize the pharmacokinetic and pharmacodynamic properties in humans. As is evident from the taxonomic origin of such compounds, the ability of organisms to synthesize them did not evolve to alter a physiological function in humans but rather to target other organisms. In contrast, most "man-made" pharmaceuticals have been designed to optimize interaction with a human molecular target, e.g. a hormone receptor, an enzyme or an ion channel.

When considering that many pharmacologically exploited human target proteins are present in similar forms in other vertebrates and even in invertebrates, also designed drugs are expected to affect aquatic wildlife. This is indeed the case; human pharmaceuticals do frequently affect vertebrates

such as amphibia, fish and birds, and occasionally also crustaceans and even algae. Notably, however, the function of a specific target molecule may differ among species.

An efficient and safe pharmaceutical should bind to the intended target molecule with high selectivity in humans. Such selectivity will determine pharmacological efficacy and safety. With exception of pharmaceuticals intended for severe diseases, high target selectivity and low toxicity originating from non-intended targets are required for a modern pharmaceutical. An obvious implication will be that effects in aquatic organisms involve molecular targets similar to the pharmacological targets exploited in human pharmacotherapy. In the search for ecotoxicological effects of human pharmaceuticals, testing according to the known pharmacological mode of action is therefore an important approach.

## Laboratory studies

The MistraPharma programme develops and applies methods to determine ecotoxic effects of human pharmaceuticals, both in the laboratory and in the field. Depending on endpoints examined (e.g. behaviour) and test species used (e.g. zebrafish), some of these methods are difficult to use in field studies. In such cases a semi-field approach may be possible; recipient water may be collected and used to expose organisms in the laboratory. In our experience, the biological response obtained when exposing fish to such water will be less pronounced compared to the response obtained in fish caged in the recipient for a longer time period.

Laboratory studies performed for research purposes generally aim at characterizing the effects of single pharmaceuticals in the test organism at a range of preselected exposure concentrations in the water. The selection of effect marker (endpoint) to study is mainly based on the pharmacological mode of action of the pharmaceutical. Important endpoints studied in fish and frogs include open-field behaviour and neurotransmittor levels in the central nervous system following exposure to neuroactive drugs, developmental disorders reflective of early life-stage exposure to sex hormone receptor agonists and antagonists, and modulation of target enzyme expression/enzyme activity. Altered expression of key genes mediating pharmacologic activity or toxic side effects is examined by means of quantitative PCR. In addition, more general methods to demonstrate biological effects are used including histopathology and large scale gene expression profiling.

In the first phase of the MistraPharma programme, our major goal has been to identify and rank pharmaceuticals with a suspected ability to affect aquatic organisms at concentrations below or close to those recorded in sewage treatment plant (STP) effluent water. The first selection of pharmaceuticals to examine is based on their simulated or measured concentrations in effluent STP water and in plasma of exposed fish (the plasma model). The ecotoxicological profiles of candidate compounds are examined at various exposure concentrations. No co-exposure to other pharmaceuticals and general pollutants present in the environment will take place at this stage. The purpose of such laboratory experiments is to define the "intrinsic" pharmacological / toxicological profile and the concentration-response curve of the pharmaceutical under study.

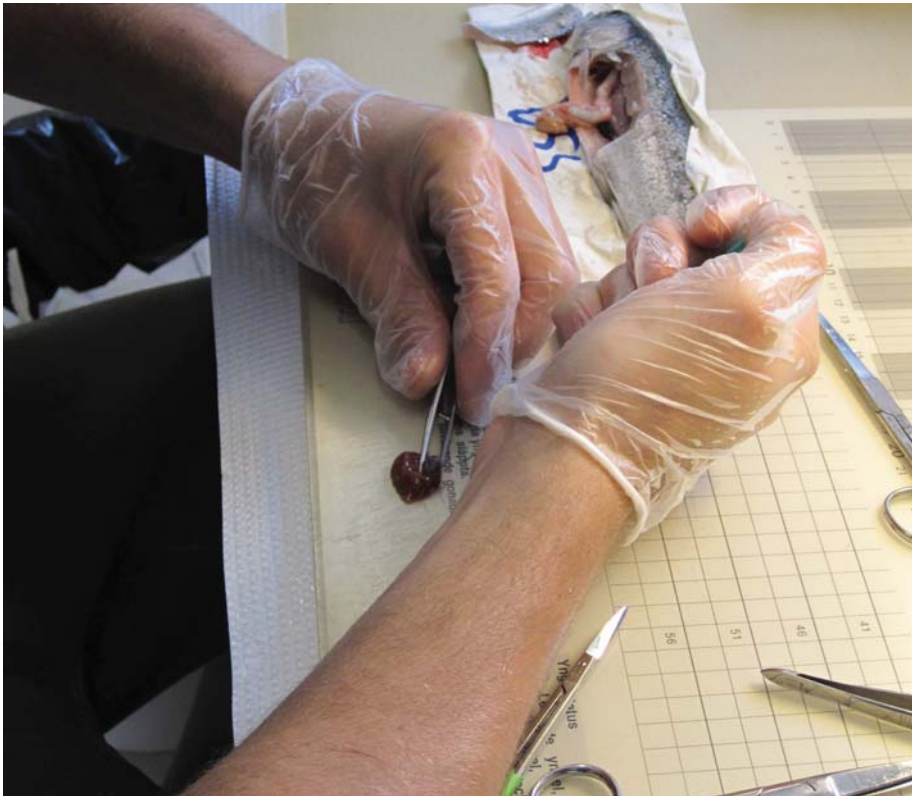
A most important finding was that early life-stage exposure to the oral contraceptive ethynylestradiol ($EE_2$) results in male-to-female sex reversal and lack of oviducts in adult frogs. It is notable that these effects occur at concentrations considerably below the highest $EE_2$ concentrations reported in urban STP effluent water (Gyllenhammar 2008, Gyllenhammar *et al*., 2009a). In addition to such dramatic anatomical alterations of the sex organs, $EE_2$ alters the levels of certain neurotransmitters in the frog brain (Berg *et al*., 2009). Another important finding was that $EE_2$ alters the methylation pattern in the promoter region of the vitellogenin I gene in zebrafish (Hjalmarsson *et al*., 2010). This observation supports the contention that pharmaceuticals in the environment may not only affect developmental and physiological processes but also impair the epigenetic control of genes regulating the reproductive physiology in fish. The possibility that pharmaceuticals and other contaminants can cause epigenetic transgenerational effects is a challenge for future risk assessment.

It has also been shown that several pharmaceuticals with similar mechanisms/modes of action in humans can induce the same biological effect in fish and frogs, although at water concentrations that are higher than those found in STP effluent water (Beijer *et a*l., 2009, Gyllenhammar *et al.,* 2009b). When considering that several pharmaceuticals and other types of chemicals sharing a common mechanism of action (e.g. inhibition of CYP19, CYP1 or CYP11B1, i.e. enzymes involved in sex hormone formation and drug metabolism) may be present in STP effluent water, it seems likely that additive effects will occur due to the co-exposure. It is therefore important to determine how mixtures of pharmaceuticals can cause characteristic effect signatures in the exposed organism. Knowledge of additive and synergistic interactions will help understanding whether sub-toxic concentrations of single compounds contribute to combined effects of pharmaceuticals in the environment. Also antagonistic interactions should be expected following exposure to mixtures of pharmaceuticals.

## Field studies

Mixtures of pharmaceuticals, biocides and other chemicals are released in STP effluent water. Single pharmaceuticals do only occasionally reach concentrations that are sufficiently high to induce severe toxicity in a sensitive species, $EE_2$ being the best characterized example so far. Although $EE_2$ may reach toxic concentration in the aquatic environment, it seems likely that the effect is enhanced by compounds such as estradiol-17ß and other estrogenic chemicals. In addition, "crosstalk" between environmental estrogens and a variety of compounds binding to the Ah-receptor will likely occur. Many pollutants target the Ah-receptor that regulates the expression of CYP enzymes. Such interactions are easier to demonstrate in controlled laboratory experiments than in field studies.

Laboratory studies can be considered as surrogates to ecotoxicological field studies. However, while laboratory studies may provide mechanistic information and dose-response relationships, field studies generally give only limited information on causality. Exceptions are studies on compounds with highly characteristic response patterns such as induction of vitellogenin and feminization of male fish, and induction of Ah-receptor regulated genes/proteins. While laboratory studies reveal causality between exposure and effect, results from field studies are generally phenomenological with restricted value for risk assessment of specific pharmaceuticals. Moreover, it is obvious that certain types of severe effects readily demonstrated in the laboratory will be difficult to demonstrate in wild populations of aquatic organisms. Psychoactive drugs altering behavioural patterns in experimen-



tally exposed fish will likely induce similar effects in wild fish. Even if such effects were detrimental to the long term survival of fish, methodological difficulties would reduce the possibility to demonstrate and interpret such effects in the field.

## Bridging the gap between laboratory and field studies

It is a major goal for ecotoxicology to help understanding how individual pollutants and mixtures adversely affect wild aquatic organisms. Given the inherent limitations of field and laboratory studies, this is a difficult task. It is a challenge to bridge the gaps between the laboratory and the field.

Major efforts have been made to develop the biomarker concept. Still, however, only a few specific, sensitive and robust biomarkers are available for monitoring of environmental contaminants in aquatic species. The egg yolk protein vitellogenin (VTG) is a mechanism-based biomarker suitable

both for laboratory and field studies. VTG is synthesized in the liver of egg laying vertebrates. Following exposure to estrogenic pharmaceuticals and chemicals, increased VTG concentrations can be recorded in the plasma of fish. VTG induction is generally measured in male fish because of their low constitutive expression of VTG. However, while VTG induction reflects the total exposure to estrogen and estrogen-like compounds, the response will not reveal the individual compounds responsible. To obtain such information, analytical determination of individual drugs and chemicals need to be carried out.

Large scale gene expression profiling is applied as a means for an improved monitoring of pharmaceuticals in the MistraPharma programme (*chapter 3*). Quantitative RT-PCR to quantify expression of mechanism-based marker genes provides another approach to refine the monitoring strategy. Genes reflective of more general biological effects are also used, e.g. genes responding to oxidative stress and other cellular stress reactions (e.g. heat shock proteins).

A set of biomarker genes has been found useful in combined laboratory and field studies. Examples are CYP genes, which may be modulated by various compounds. In order to improve monitoring of pharmaceuticals and other environmental contaminants interacting with the CYP system in fish, several new highly inducible CYP1 genes have been cloned in rainbow trout and threespined stickleback (Jönsson *et al*., 2010, Gao *et al*., in preparation). These fish species are suitable both for laboratory and field studies. Interestingly, the new CYP biomarker genes responded differently following caging of rainbow trout at different locations in the Uppsala region waters. The highest induction of gene expression was observed downstream the local STP in Uppsala (Jönsson *et al*., 2010). Experiments to characterize the response patterns following controlled laboratory exposure to selected pharmaceuticals/pollutants and mixtures are planned to reveal compound-characteristic effect signatures. These experiments will hopefully help identifying new biomarkers suitable for monitoring of a wider range of drugs and chemicals in fish. Sequencing of the genomes of various aquatic species gives opportunities to identify new drug-responsive genes to be used in the future. If successful, this can help bridging the gap between laboratory and field studies.

## Concluding remarks

Laboratory studies contribute information about mode/mechanism of action and concentration−response relationships. Field studies provide merely phenomenological data with lack of information on causality. When laboratory and field data are available, chemical analysis of pharmaceuticals in recipient water or organisms is required for risk assessment. With such information at hand, consideration of interactions and crosstalk between pharmaceuticals and other pollutants also becomes an issue.

The introduction of new mechanism-based biomarkers will hopefully help bridging the gap between laboratory and field studies. Improved testing strategies using batteries of biomarkers will support assessment of hazards and risks posed by environmentally stable pharmaceuticals. New methods based on genomic, proteomic and metabonomic approaches give hope for a rapid development.

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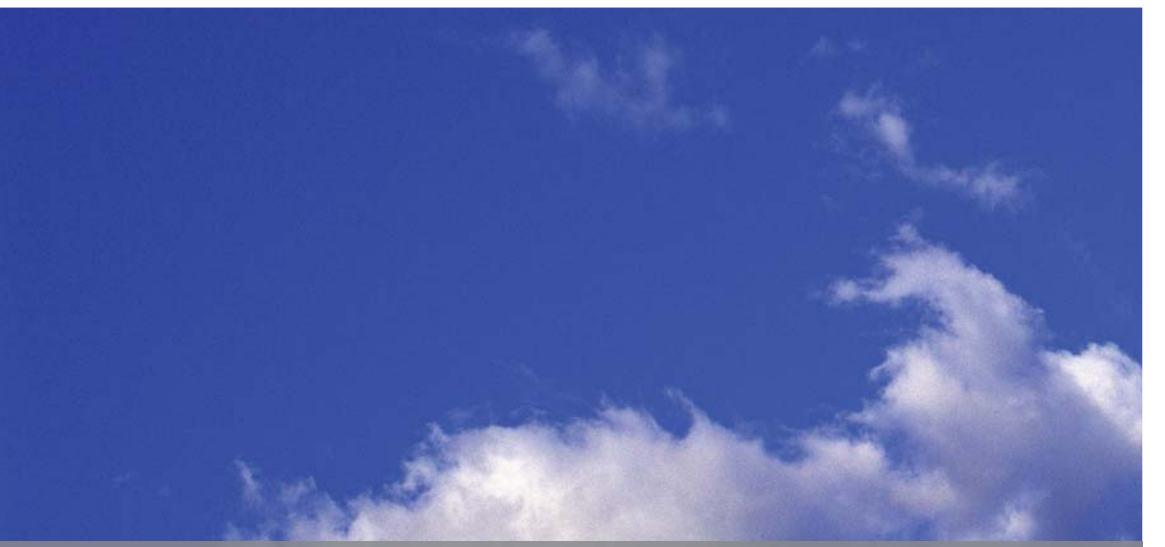

# 5 Standard and Non-standard Tests for Risk Assessment Purposes

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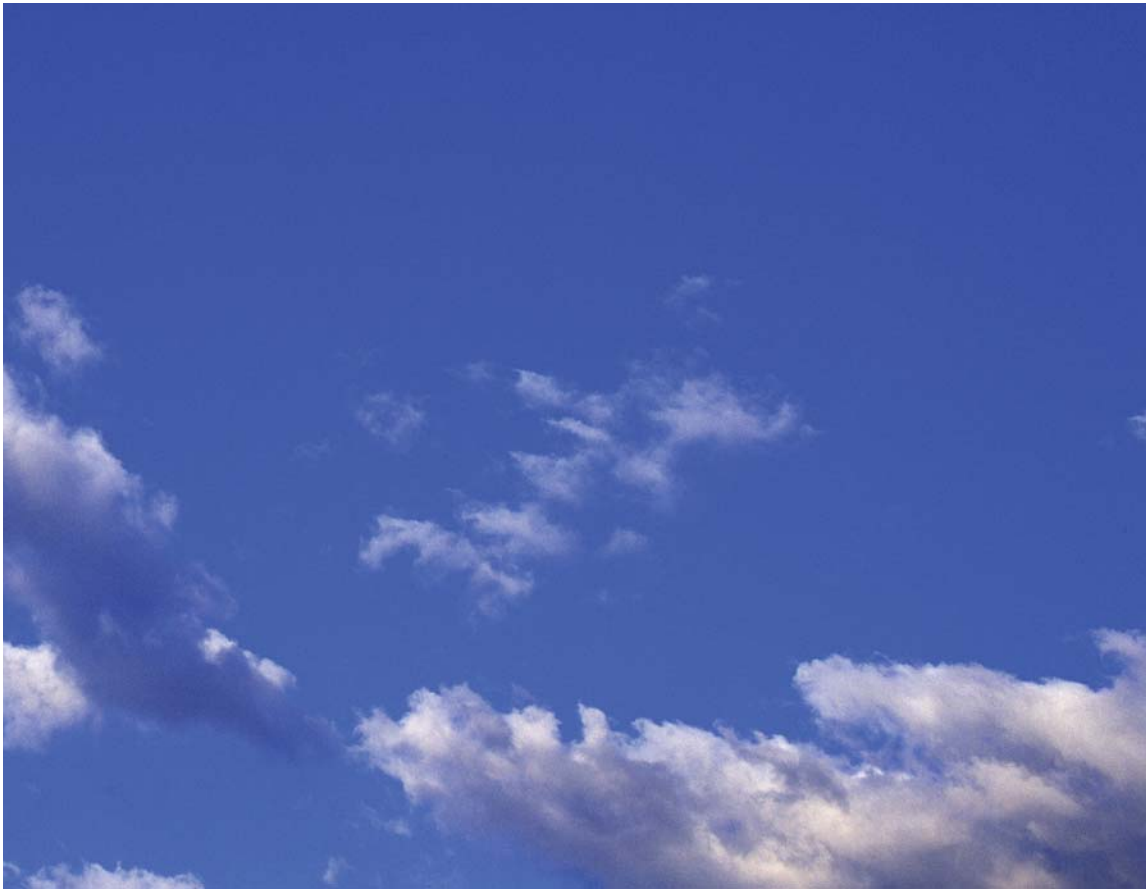
**In this chapter we will outline the process of regulatory environmental risk assessment, and discuss how this process can be applied to human pharmaceuticals and the need to adjust it to make it more efficient in identifying active pharmaceutical ingredients that can cause adverse effects in the environment. The focus is on human pharmaceuticals and on risk assessment for non-target species in the aquatic environment.**

## Introduction

Almost 200 active pharmaceutical ingredients have been identified in surface waters. For two of these, significant adverse effects have been identified in the environment. Estrogen hormones from oral contraceptives affect the reproduction of fish, and diclofenac, a non-steroidal anti-inflammatory medicine (NSAID), causes kidney toxicity in exposed vultures. However, there is concern that also other pharmaceuticals have the potential to cause



adverse effects in non-target species. Knowledge about such interactions is however still scarce, and we are facing several challenges before we can have a reasonable overview of the potential environmental risks that pharmaceuticals may cause.

From a regulatory perspective, pharmaceuticals have a number of inherent properties that make them unique among other groups of chemicals. First, pharmaceuticals are carefully designed to interact with biological processes. This means that the main purpose of an active pharmaceutical ingredient is to affect a living organism, in this case a human being. Second, this interaction should be as specific as possible, ideally influencing only one well-defined target molecule or cellular process, and have as few other side effects as possible. Third, this interaction should be achieved already at low doses (concentrations), meaning that the substance has to be relatively

potent. Fourth, to achieve this it is necessary that the active pharmaceutical ingredient is sufficiently persistent to remain un-metabolised long enough to reach the target organ in the human body.

In addition to these inherent properties, regulatory requirements to show the pharmaceuticals' efficacy and safety are relatively far-reaching. This means that their pharmacological effect, mode-of-action and potential side effects in many cases are reasonably well described in experimental models (e.g. in mice and rats). In the final stages of drug development the effects of the pharmaceutical are also evaluated in human subjects in controlled clinical trials.

These unique features of pharmaceuticals give rise to important possibilities and challenges; It is of course a main advantage that there are a lot of available knowledge about the inherent properties and the biological effects of the pharmaceutical substances, knowledge that could be taken into account when performing risk assessment for non-target species. At the same time, currently available standard test methods for deriving regulatory toxicity data for the aquatic environment are in many cases not sufficiently sensitive to the types of very specific effects that we expect from pharmaceuticals in non-target species.

Consider the following example. The EMA guideline (2006) recommends that three standard test are performed when conducting environmental risk assessments for pharmaceutical substances; a growth inhibition test on algae (OECD 201), a reproduction test on *Daphnia* (OECD 211), and an early-life stage test on fish (OECD 210). *Table 1* presents the lowest openly available standard and non-standard effect values for Ethinylestradiol, a sex hormone commonly used in contraceptives. The comparison shows that non-standard effect values are 100 to 200 000 times lower than standard effect values. A lower effect value indicates a higher risk.

**Table 1.** Comparison of the lowest standard and non-standard effect values for the sex hormone Ethinylestradiol.

| | **Test species Endpoint/standard test NOEC-value** | **Test species Endpoint/standard test LOEC-value** | **Test species Endpoint/standard test $EC_{50}$-value** |
|---|---|---|---|
| **Lowest standard test value** | *Desmodesmus spp* (algae) Growth inhibition/ OECD 201 0.1 mg/L | *Danio rerio* (fish) Reproduction/ OECD 210 0.000003 mg/L | *Daphnia* (crustacean) Reproduction/ OECD 211 0.105 mg/L |
| **Lowest *non*-standard test value** | *Danio rerio* (fish) Changes in sex ratio 0.0000005 mg/L | *Oryzias latipes* (fish) Induced intersex 0.00000003 mg/L | *Danio rerio* (fish) Fertilization success 0.0000011 mg/L |
| **Ratio between standard and non-standard values** | 200 000 | 100 | 95 455 |

NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, $EC_{50}$ = lowest identified effect concentration where fifty percent of the tested population have been found to be affected.

A crucial deficiency of the regulatory environmental risk assessment for pharmaceuticals is thus the lack of relevant and sufficiently sensitive test methods. To have appropriate test methods is of course fundamental for risk identification and thus a crucial part of the risk assessment process.

## Regulatory environmental risk assessment

Environmental risk can be defined as the likelihood of an adverse effect occurring in the environment given a certain exposure to a chemical. In order to assess the size and nature of a risk to the environment, scientific data on exposures and toxicities are analysed systematically. The basic idea of the risk assessment process is to predict the concentrations of a substance that will end up in different parts of the environment and compare these concentrations to the concentration(s), which in laboratory ecotoxicity tests, has been shown to be harmless to test organisms. Risk assessments can be performed for different environmental compartments, but in this chapter we will focus on risks for the aquatic environment.

The environmental risk assessment serves as a basis for risk management, i.e. decision-making on the need to reduce exposures. The different steps of the regulatory risk assessment, and its connection to risk management decision-making, are visualised in *Figure 1*.

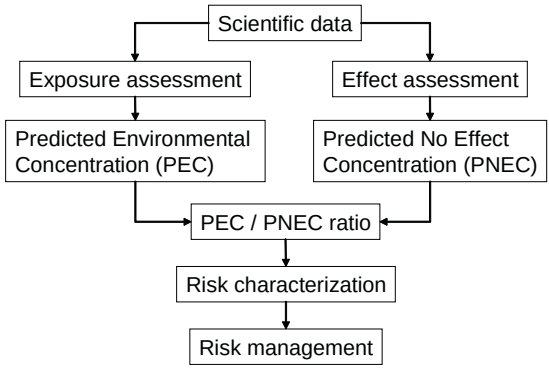



**Figure 1**. Outline of the regulatory risk assessment process and its connection to risk management (Modified from van Leeuwen and Vermeire 2007).

The aim of the first step is to identify and describe effects of concern. In the second step, the effect assessment, experimental data are reviewed to establish the lowest identified no observed effect concentration (NOEC) or the lowest identified effect concentration where fifty percent of the tested population have been found to be affected in laboratory experiments (L(E) $C_{50}$). Thereafter, this concentration is divided by an assessment factor to derive a predicted no effect concentration (PNEC). The purpose of applying an assessment factor is to compensate for variations in sensitivity within and between species, transfer from laboratory data to field conditions, and for extrapolating short-term data to chronic data. The purpose of the PNEC is thus to be used as a reference concentration below which an unacceptable effect in the environment will most likely not occur (European Commission, 2003).

In parallel, the exposure assessment is performed. The aim of the exposure assessment is to estimate concentrations in the environment resulting from different emissions, i.e. the predicted environmental concentration (PEC). A PEC is estimated by using data derived from models (prospective risk assessment concerning chemicals currently not in use) or from actual measurements (retrospective risk assessment concerning chemicals in use that have potentially already been emitted to the environment). Models are also used retrospectively when measurements are lacking.

In the risk characterisation, a risk quotient is calculated by comparing PEC with PNEC. Dependent on the PEC/PNEC ratio the decision whether a substance presents a risk to organisms in the environment is taken. A risk

ratio above 1 normally leads to further evaluations. In addition to the risk characterisation an assessment of the substance's potential to persist in the environment, and bioaccumulate in biological organisms will be performed.

The overall objective of an environmental risk assessment is to identify and characterise environmental risks and eventually to make decisions on the basis of this assessment with the purpose to prevent unacceptable harm to the environment and the large diversity of organisms represented in the ecosystems (European Commission, 2003).

## Environmental risk assessment of pharmaceuticals

In 2006 the European Medicines Agency decided that all new marketing authorisation applications for pharmaceuticals should be accompanied by an environmental risk assessment (EMA, 2006). The requirements follow the same general principles as defined in the European Commission Technical Guidance Document (TGD) (European Commission, 2003) but the process is different.

First of all the risk assessment is divided into two phases. In Phase I the PEC for surface water should be calculated and the partitioning of the substance between octanol and water – indicating its water solubility is measured (log

$K_{OW}$). If the PEC value is equal to or above 0.01 µg/L a Phase II analysis should be performed, and substances with a log Kow exceeding 4.5 should be screened for persistence, bioaccumulation and toxicity according to the TGD. Active pharmaceutical ingredients that are already known to affect the reproduction of vertebrates or invertebrates at concentrations below 0.01 µg/L should also enter Phase II.

Phase II of this risk assessment procedure is divided into two tiers, Tier A and Tier B. In Tier A data on the substance's physico-chemical properties, persistence and bioaccumulation, and ecotoxicity are reviewed and PNECs are estimated for water, groundwater and microorganisms. Standard long-term toxicity tests on algae, *Daphnia*, and fish together with recommended assessment factor(s) should be used to determine the PNEC. As mentioned above EMA recommends that tests according to OECD-guideline 201 "Alga growth inhibition test" (duration: 72 hrs), 210 "Fish early life stage toxicity test" (duration depends on test species) and 211 "*Daphnia magna* reproduction test" (duration: 21 days) are used. See *Table 2*.

**Table 2.** The process for environmental risk assessment of pharmaceuticals according to the EMA guidance document (table modified from EMA, 2006, p.4).

| Stage in regulatory evaluation | Objective | Method | Data requirement |
|---|---|---|---|
| Phase I | Estimation of exposure | Action limits:<br>(1) PEC ≥ 0.01 ng/L water<br>(2) log$K_{OW}$>4.5<br>(3) other cause for concern such as endocrine disruptors | Consumption data, log-$K_{OW}$, and other information on effects or mode-of-action |
| Phase II Tier A | Initial prediction of risk | Risk assessment: Evaluation of PEC/PNEC<br><br>Substances with a log$K_{OW}$ >4.5 should be assessed for persistence, bioaccumulation and toxicity according to the EU TGD | Standard long-term toxicity tests on algae, *Daphnia*, and fish<br><br>Data on degradability, and persistence |
| Phase II Tier B | Substance and compartment-specific refinement and risk assessment | Refined risk assessment | Extended data set on toxicity, exposure, and fate |

The PEC is derived from data on consumption of the pharmaceutical, water consumption and a dilution factor according to *Calculation 1*. Or measured environmental concentrations (MEC) can be used.

$$\mathbf{PEC} = \frac{\text{Daily dose consumed per inhabitant} * \text{Percentage of market penetration}}{\text{Wastewater per inhabitant per day} * \text{Dilution factor}}$$

**Calculation 1**. Formula for deriving a PEC according to EMA 2006.

For the risk characterisation PNEC should be based on the lowest available experimental NOEC. If the ratio PEC/PNEC is above 1, extended environmental fate and effect analysis is needed according to the rules of Tier B of phase 2 (EMA, 2006).

As mentioned above, environmental risk assessments can for most groups of chemicals, including veterinary drugs, be used as a reason to restrict or even ban the use of a substance. For human pharmaceuticals, however, this is not the case. The European legislation does currently not allow for the environmental risk assessment to affect the decision on whether to allow marketing of a human pharmaceutical. The environmental risk assessment is only intended to provide environmental information to the regulatory agency. In a report to the Swedish Government, the Swedish Medical Products Agency, recently proposed that the current EU legislation for the authorisation of medicinal products for humans should be changed so that an environmental risk assessment is also included in the approval (Swedish Medical Products Agency, 2009).

## Regulatory ecotoxicity testing

To derive the PNEC, ecotoxicological testing is performed. In the following section some central principles of such testing procedures are discussed.

In regulatory ecotoxicity testing, a small number of species and experimental models are used for hazard identification and dose/response assessment. These models are in most cases single species and the endpoints often relate to lethality or effects on reproduction or growth. Using single species models is an obvious simplification compared to the complex environment where a large number of factors (both biotic and abiotic) interact. However, it is generally accepted that also a limited selection of organisms can represent the inhabitants of an ecosystem and thus contribute meaningful information to the risk assessment. In order to increase the diversity in biology, genetic

composition, metabolism, chemical uptake routes and behaviour in the test system, toxicity tests are usually required on species from three trophic levels (normally a primary producer as well as a primary and secondary consumer).

As mentioned above, this chapter focuses on environmental effects caused by human pharmaceuticals, and for these substances, and the way that they are emitted to the environment, tests for aquatic toxicity are most relevant. In aquatic toxicity testing, the primary producers are often represented by green algae species like *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus* or the cyanobacteria *Synechococcus leopoliensis*. The primary consumers are in many cases represented by the water flea *Daphnia magna*. The secondary consumers are represented by fish and commonly used species are Zebra fish (*Brachydanio rerio*), Fathead minnow (*Pimephales promelas*) and Rainbow trout (*Oncorhynchus mykiss*). Important factors when deciding on choice of test organisms are; well known biology, cost effectiveness, availability, easy to cultivate, sensitiveness, and ecological relevance.

The existing standard test guidelines can be divided into acute and chronic tests. In acute tests, organisms are exposed to high chemical concentrations during a short period in relation to the lifespan of the selected organism and the endpoints measured are life ending, such as mortality and inhibition of growth. The exposure time for acute tests for microorganisms is usually ≤ 30 min, for algae ≤ 72 h, for invertebrates ≤ 48 h, and for vertebrates ≤ 96 h.

In chronic tests, the chemical concentrations are lower and the tests normally cover a full life cycle, the exception to this is tests performed on fish since a full life cycle test in many cases would be too costly. Endpoints often relate to reproduction failure and developmental effects. The exposure time for chronic tests for invertebrates is usually ≥ 21 days, and for vertebrates ≥ 2 weeks. Microorganisms and algae are normally not tested for chronic toxicity, but in a regulatory context a no effect value from an acute test on algae can be used as a chronic value if chronic values from other trophic levels are available.

### Standard and non-standard tests

Ecotoxicological testing can be done using a variety of methods and models. Two very general alternatives are to use a standard test or a non-standard test. Standard tests refer to tests performed and reported according to a method described and provided by an official standardisation organisation (see below). The test standard establishes a uniform specification of the

experimental set-up and execution, methods for data analyses, and the reporting format for the test data.

By non-standard tests we refer to tests performed according to any other test method that meets a set of general scientific quality criteria such as having: a defined purpose of the study, a clear description of the endpoints, inclusion of appropriate controls, appropriate identification of test substance and test organism, appropriate concentrations tested, stated exposure duration time and administration route, suitable exposure environment, and transparent reporting of effect concentrations.

The major advantages of using a standard test are that the results are directly comparable across substances and that the data they generate will be readily accepted across jurisdictions. The major disadvantage is that the standard methods do not always represent the most relevant testing approach given the type of endpoint under investigation. Therefore, results from non-standardised tests may contribute additional and significant information to a risk assessment. According to the European TGD for risk assessment, non-standardised methods should be taken into account case-by-case based on expert judgment (European Commission, 2003, part 1, p. 87). In contrast to this, the EMA guideline on environmental risk assessment proposes that standard data should be used for risk assessment of pharmaceuticals, non-standard data is not mentioned (EMA, 2006).

The main advantage with data generated and reported in accordance with detailed test standards is that it contributes to promote the reliability of the data by making it easier to repeat the experiment if needed because of the detailed test procedures and extensive reporting of data that is required. Given the characteristics and purposes of standard tests it is not surprising that standard testing is mostly performed by commercial laboratories while non-standardised methods are typically used by research scientists.

In this context, it is important to note that non-standard experiments can be just as reliable (and reproducible) as tests performed under strict implementation of test standards, and that following test guidelines will not automatically ensure that the test has sufficient relevance for risk assessment purposes.

## Standardisation procedures

As outlined above, there are important advantages of using standard tests in the regulatory system, but for some types of expected effects such standard methods are currently lacking. An important aspect is thus how new, or updated standard tests can be developed and become accepted in the regulatory system. In this section the standardisation process is introduced.

Standardisation is the process of developing and agreeing upon a standard. This process is similar within the different standardisation organisations. Here, we will briefly present the basic outline of the process to develop new standard tests as proposed by OECD (Organisation for Economic Co-operation and Development) (OECD TG 34 2005). The process starts with an initiative from a member country, the scientific community, or the organisation's own secretariat. If the initiative for a new test comes from a

member state, a national coordinator completes a so called Standard Project Submission Form (SPSF), which includes a detailed project description and other information needed by the OECD to reach agreement on the proposal. The SPSF should e.g. identify the anticipated outcomes of the project.

A clear distinction in both time and effort is made whether the assessment of the validation status concerns a new test method (prospective assessment) or an already available but not yet standardised test method (retrospective assessment). In the latter case new experimental work may not be needed and the main focus is on more refined data analysis and evaluation. In the former case, on the other hand, there is a need to present a rationale for the method (i.e. scientific basis, regulatory purpose and need for the test), the relationship between the test method's endpoint(s) and the biological phenomena (e.g. certain mechanisms of interest, such as endocrine disruption) and the intra- and inter-laboratory reproducibility (over time) using relevant reference chemical(s). A draft test guideline should be provided before the initiation of the validation steps and may be revised in an iterative process over time based on the outcomes from the various validation rounds. The numbers of participating laboratories, reference chemicals and validation rounds may vary from case to case depending on e.g. the complexity of the test and prior validation results. Normally, this part of the standardisation process is the most work intensive phase and may proceed over several years. Expert revision rounds, either in the form of workshops or via circulation, are normally undertaken each time a validation report has been prepared or when changes has been made to the draft test guideline.

Upon finalization of the validation process, the validation data and the test guideline proposal may be subject to a peer review process, which is normally made by a group of qualified and independent scientists within the field. The same information may also be sent out to a wider group of experts representing academia, governmental agencies, and industry, for comments concerning technical and scientific content as well as suggestions for improvements. Based on this activity, an overall evaluation and conclusion is presented and provides recommendations for or against proposed use. Given that the proposed test guideline is approved, a final version is provided, adopted by OECD and published to enable use of it. The process of developing a test guideline is often a extremely time consuming process and may take as long as 10 to 15 years from start to end (Breitholtz *et al*., in press). Standards are normally available free of charge but a small number of standards has to be purchased. A flow diagram describing the process for developing new test guidelines within the OECD is presented in *Figure 2*.

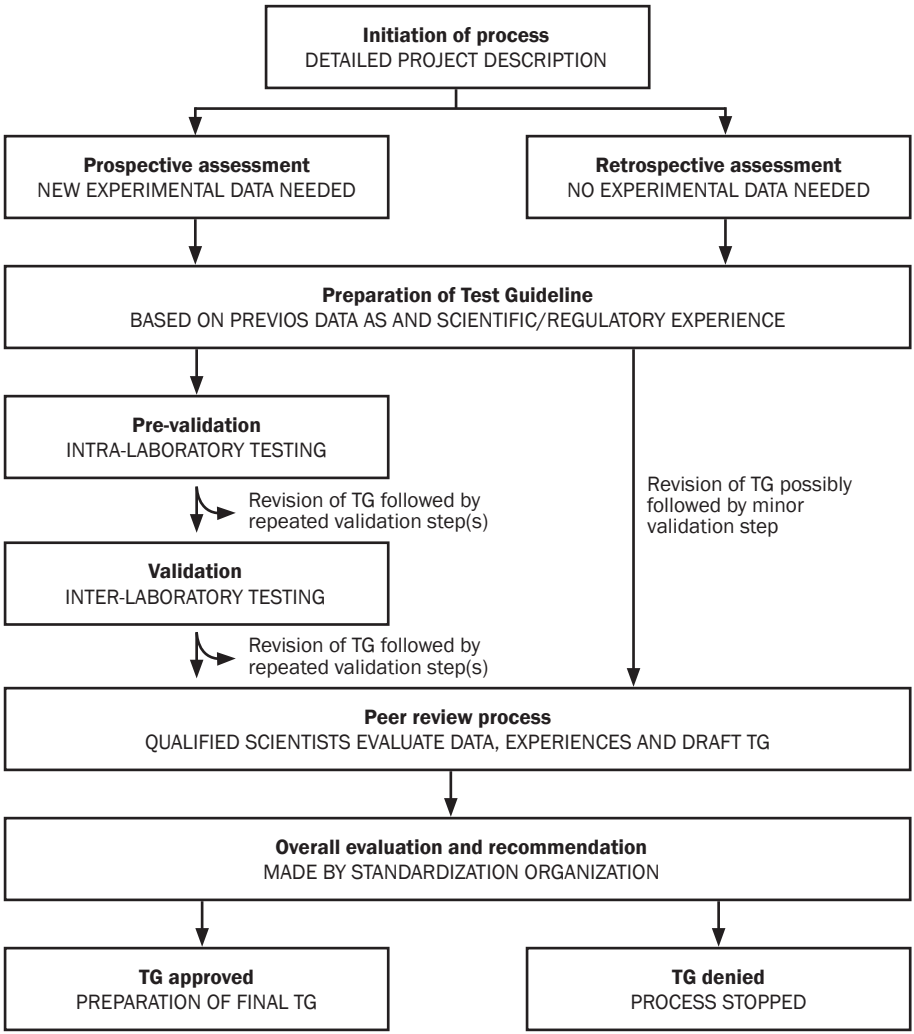



**Figure 2.** Flow diagram describing the process for developing new test guidelines within the OECD.

Besides the OECD there are also other official standardisation organisations that produce standard test guidelines often used in ecotoxicology, such as the US EPA (United States Environmental Protection Agency), ASTM (American Society for Testing and Materials), AFNOR (Association française de Normalisation), and ISO (International Organization for Standardization).

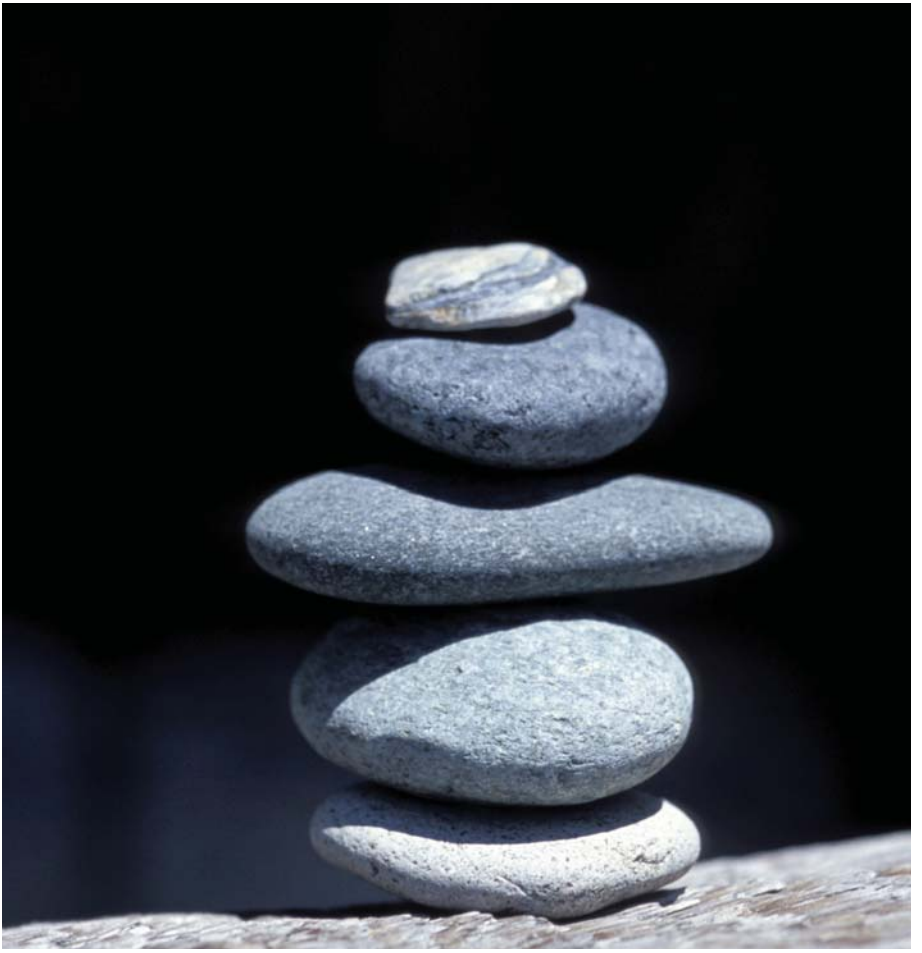
## Evaluation of data for risk assessment

A major issue in both steps of the effects assessment is to evaluate the data with regard to their completeness, reliability, and relevance.

The completeness of data is assessed case-by-case. There are primarily two sources to new environmental data on pharmaceuticals; the pharmaceutical industry and the independent research community. Pharmaceuticals were relatively recently recognised as environmental pollutants (Kümmerer, 2008). This is reflected in the fact that regulatory test requirements were just recently introduced. The number of scientific publications on environmental effects of pharmaceuticals is rising at a steady pace, but still the knowledge is strikingly limited. In general lack of data will result in higher assessment factors being used in the risk assessment.

According to the TGD, an evaluation of the data reliability should ensure "the inherent quality of a test relating to test methodology and the way that the performance and results of the test are described" (European Commission, 2003). Basically this evaluation should answer the question: Has the experiment generated and reported a true and correct result?

The assessment of the relevance of the data should describe "the extent to which a test is appropriate for a particular hazard or risk assessment" (European Commission, 2003), e.g. answer questions like these: Is the measured endpoint a valid indicator of environmental risk? Is the experimental model sufficiently sensitive in relation to detecting the expected effects? Has the



experimental model a sufficient statistical power? How representative is the experimental model to the environment that it is aimed to protect?

Evaluation of data can be done within different frameworks. In some cases it is necessary to rely solely on a case-by-case approach based on expert judgement, while in other cases a more criteria based approach can be used (European Commission, 2003). Examples of proposed systems for applying pre-set evaluation criteria can e.g. be found in Klimisch et al. (1997), Hobbs et al. (2005), and Durda et al. (2000).

A major advantage of a more criteria based approach is that a collection of pre-set evaluation criteria may contribute to an increased predictability of the data evaluation process since it becomes less dependent on the case-by-case judgments of individual experts. Pre-defined criteria will at least contribute to ensure that a minimum and similar set of aspects are considered in each evaluation. They may also contribute to increased transparency of the evaluation process to the extent that these criteria are communicated to the relevant actors.

Disadvantages of using pre-defined evaluation criteria are that they are obviously less flexible and that they in many cases will have to focus on the more general aspects of a test. Therefore they still might have to be adapted case-by-case.

Hence there is a need to strike a balance between the use of expert judgement and the reliance on pre-defined criteria. Or, put in another way, between flexibility and predictability in the data quality evaluation process. Data evaluation will always include an element of expert judgment, but it is also, in our view, important to continuously seek to increase predictability and transparency in this process.

## The way forward for environmental risk assessment of pharmaceuticals

As we see it, there are (at least) three potential ways forward to ensure that relevant and sufficiently sensitive tests are used in the regulatory environmental risk assessment of pharmaceuticals in the future:

1. To develop new standard tests, or
2. to adjust existing standard tests i.e. supplementing them with additional endpoints, or
3. to increase the use of non-standard tests

As described above standardisation is a costly and time-consuming process and since pharmaceuticals are a diverse group when it comes to how it affects biological life it is unlikely that new standards, covering all relevant endpoints, will be developed in the near future. However, if adjustments to current standard tests (e.g. additional endpoints and/or refined test criteria) could increase the biological relevance for testing pharmaceuticals, a potential way forward could be that the standardisation organisations initiate additional validation and expert comments rounds, to standardise the proposed adjustments. Still, such minor adjustments would likely not improve current standard tests to the extent that the specific biological effects of most pharmaceuticals are covered by the tests. Hence, in our view, an important way forward is to make increased use of non-standard test data to ensure a scientifically well founded environmental risk assessment of pharmaceuticals.

To enable use of non-standard tests in risk assessments two things are needed: A legislation that can cope with non-standard tests in a systematic and predictable way, and that non-standard tests are reported in a transparent and comprehensive way, much like required when using the standard test methods.

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# 6 WikiPharma – A Database with Environmental Effect Data for Pharmaceuticals

Linda Molander, Marlene Ågerstrand and Christina Rudén. KTH

**Available data about the ecotoxicity of pharmaceuticals have been collected in a database namned *WikiPharma*. The aim of WikiPharma is to provide a comprehensive and easily accessible source for ecotoxicological data for pharmaceuticals and it is publicly available at no cost at *www.wikipharma.org*. The concentration of such data in one single place is convenient when assessing environmental risks of pharmaceutical ingredients, and for identifying data gaps and research needs in this area.**

**The database is constructed as a "wiki" to enable users to propose data to be added. Data that have either been published in the open scientific literature or that have been sufficiently peer-reviewed and made publicly available by other means can be included.**

**The WikiPharma was developed within the MistraPharma research programme and in this chapter the structure, functions and contents of WikiPharma are introduced. See also Molander *et al.*, (2009) for more detailed information.**

## Scope of the database

WikiPharma was compiled with the intention to include all available ecotoxicity data for active pharmaceutical ingredients (denoted APIs). The ecotoxicity data were systematically searched for in relevant scientific databases and using the Google Scholar search tool. Furthermore, the reference lists of the retrieved publications, including review articles, were searched for information about additional references, and data from relevant books were extracted. We believe that this search strategy resulted in a reasonably good coverage of the publicly accessible ecotoxicity data.

Currently the contents of the database is limited to the APIs for which an environmental risk assessment is required according to European law, hence information on vitamins, minerals, vaccines, etc. is not included. The reason for these exceptions is that these APIs are generally considered to be of low environmental risk (EMA, 2006). Since the MistraPharma research programme does not cover the terrestrial compartment, the main focus is on effect data relevant to the aquatic environment.

For each ecotoxicity test included in the database, information about the test species (identity, sex, age or life stage, and number), type of method, test concentrations, exposure time, and reported effects and effect concentrations were extracted from the literature. The concentration that gives rise to adverse effects in ecotoxicity tests, such as mortality, immobility, growth or reproductive inhibition, can be reported in different ways. Typically it is expressed as the median lethal concentration ($LC_{50}$), or the median effect concentration ($EC_{50}$), the No Observed Effect Concentration (NOEC), or the Lowest Observed Effect Concentration (LOEC). Only tests where an effect concentration (or a no-effect concentration) was presented, or for which such an effect measure could be derived or calculated, were included in the database.

## An overview of the WikiPharma contents

In total WikiPharma currently contains effect data for 130 pharmaceutical substances, representing 36 different pharmaceutical groups. These ecotoxicity test data have been extracted from 172 different sources.

### Standardized and acute tests are in majority

As mentioned above, WikiPharma includes information about e.g. which types of test has been used, test species and endpoints investigated. Analyzing the contents of WikiPharma shows that approximately 52% of the effect data in the database originates from tests conducted according to standard methods. The most frequently occurring test standards are those issued by the Organization for Economic Co-operation and Development (OECD), the U.S. Environmental Protection Agency, and the International Standards Organization (ISO). (Modified versions of the standard test guidelines were included in this counting). Tests performed using commercial standard kits like the Microtox test, Thamnotoxkit, Rotoxkit, Spirotox, Streptoxkit and Artoxkit are also commonly occurring in the database.

An estimation of the number of short-term and long-term ecotoxicity tests gathered in the database was obtained by grouping different species

together according to their generation time. Tests with exposure times up to 48 hours for crustaceans and up to 96 hours for fish and amphibians were identified as short-term tests in accordance with Cooney (2003). For algal tests, exposure to the test substance for 3 days was counted as a short-term test when an $EC_{50}$ or $LC_{50}$ value was reported, but as a long-term test when a NOEC or LOEC value was reported. This was made in accordance with the European Commission's Technical Guidance Document on Risk Assessment (European Commission, 2003). Tests with microorganisms were counted as short-term if exposure did not exceed 20 hours. Based on the division of species into the following groups: microorganisms (mainly bacteria), algae and vascular plants, invertebrates and vertebrates (fish and amphibians) it was concluded that data generated from short-term tests constitute 52% of the WikiPharma data set. An explanation for the large amount of short-term toxicity tests may be that long-term environmental testing was not required by law before 2006 (EMA, 2006).

### Most common species

There are 90 different species represented in the database. As the majority of the tests compiled in the database were performed according to standardized test methods, most of the test organisms used are consequently also standard test species. The top 10 most frequently used test species are listed in *Table 1*.

**Table 1.** Top 10 used test species in ecotoxicity testing of pharmaceutical substances.

| Species | Number of tests* |
|---|---|
| *Daphnia magna* (crustacean) | 239 |
| *Vibrio Fischeri* (bacterium) | 104 |
| *Onchorhynchus mykiss* (fish) | 97 |
| *Pseudokirchneriella subcapitata* (algae) | 85 |
| *Poeciliopsis lucida* (fish) | 75 |
| *Pimephales promelas* (fish) | 63 |
| *Glomus intraradices* (mycorrhizal fungus) | 60 |
| *Ceriodaphnia dubia* (crustacean) | 59 |
| *Brachionus calyciflorus* (rotifer) | 55 |
| *Thamnocephalus platyurus* (crustacean) | 47 |

* Total number of tests in the database is 1716.

Crustacean species are the most commonly occurring species in the database, and among the crustaceans *Daphnia magna* and *Ceriodaphnia dubia* are the most common ones. This is not surprising since daphnids are extensively used in freshwater toxicity testing because they are abundant and widely distributed, easy to maintain in the laboratory and sensitive towards a broad range of environmental contaminants (Cooney, 2003).

The marine bacterium *Vibrio fisheri* is also recommended as test species and is found to be commonly used for ecotoxicity testing of antibiotic agents (Backhaus and Grimme, 1999).

Three different fish species are among the top ten, however the majority of the tests with *Poeciliopsis lucida* and *Oncorhynchus mykiss* were conducted in vitro, or in other words using cells from these fish. Ecotoxicity testing with fish are often used to determine adverse effects of the estrogens 17ß-estradiol and 17α-ethinylestradiol, where induction of the egg yolk protein vitellogenin is considered a valuable biomarker for assessing exposure to environmental estrogens in fish (e.g. Tyler *et al.,* 2002).

### Identified adverse effects and effect concentrations

Measured concentrations of pharmaceuticals in surface waters, groundwater and sediments normally range from nanograms up to a few micrograms per liter (Kümmerer, 2004) and for the majority of the pharmaceutical ingredients adverse effects have only been identified at concentrations far above these levels. However, for some of the substances effects are identified also at environmentally relevant concentrations.

Among the data compiled in the database the natural estrogen 17ß-estradiol and the synthetic estrogen 17α-ethinylestradiol are shown to exert adverse effects at the lowest concentrations. In WikiPharma both the lowest short-term $EC_{50}$s and long-term NOEC and LOEC values are reported for these estrogens and they range from 0.03 ng/L up to a few micrograms per liter; Approximately 90 days of exposure to 17α-ethinylestradiol at 0.03 ng/L significantly induced intersex in the Japanese ricefish *Oryzias latipes* (Metcalfe *et al.,* 2001). 17α-ethinylestradiol concentrations up to 7 ng/L have been detected in European sewage treatment plant effluents, and concentrations of these estrogens generally range between less than 0.1 ng/L to 0.5 in surface waters (Desbrow *et al.,* 1998; Länge *et al.,* 2001).

In a few aquatic ecotoxicity tests the pain killers ibuprofen and diclofenac (non-steroidal anti-inflammatory drugs) and the antidepressants fluoxetine and sertraline (selective serotonin reuptake inhibitors) showed adverse effects in the same concentration range as for the estrogens. The lowest NOEC for fluoxetine is 0.47 µg/L reported from a 56-days reproduction study with a fresh-water mudsnail (Nentwig, 2007).

Another pharmaceutical group identified in the database as producing low effect concentrations is the antibiotics, mainly represented by trimethoprime, oxolinic acid and amoxicillin. The lowest effect concentrations, which concerns changes in enzyme activity in freshwater zebra mussel and growth inhibition of blue-green algae, are reported from 0.3 up to a few µg/L (e.g. Bellini *et al.*, 2009; Andreozzi *et al.*, 2004).

## Searching the database

The database search engine is constructed so that a specific pharmaceutical ingredient or a whole drug class, e.g. non-steroidal anti-inflammatory drugs, can be selected from a drop-down-list to obtain all effect data of interest. Information from the WikiPharma database can also be retrieved by downloading the whole database (requires Microsoft Access software). The format of the data extracted from a Microsoft Access database is fully compatible with other Office software such as Excel and Word.

## Adding data to the database

The database is available to the public via the web-site: *www.wikipharma.org* and after a simple login registration, anyone may propose additions to the database contents. All data proposed to be added to the database will have to be approved by the database administrator. The administrator will check whether the proposed additions seem reasonable and compatible with the aims of the database. Data that have either been published in the open scientific literature or that have been sufficiently peer reviewed and made publicly available by other means, will be included.

In a wiki all users and contributors are responsible for quality assurance. For further scrutiny and evaluation of the data, the user is referred to the original source of the data. The goal is for WikiPharma to be an easy accessible, comprehensive, and up-to-date source for information about the environmental toxicity of APIs. If this goal will be reached is up to the users of the database.

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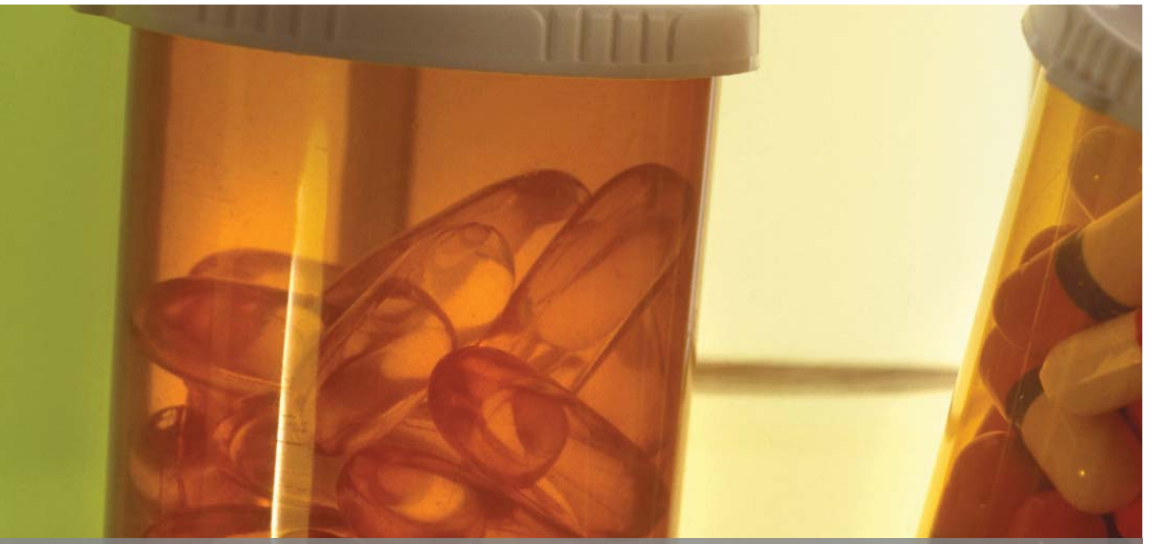

# 7 The Swedish Environmental Classification and Information System for Pharmaceuticals. An Evaluation of the System's Achievements so far

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**According to the pharmaceuticals legislation in Europe, ecotoxicity testing and environmental risk assessment is required for new marketing notifications, but the marketing of a pharmaceutical cannot be denied based on its ecotoxicological properties. Therefore, given the current situation, other risk management options might be considered. One way to reduce emissions of a particular pharmaceutical substance, if considered motivated, is to reduce prescriptions of products that contain that substance. One way to potentially affect prescriptions is to provide information about the pharmaceuticals' ecotoxicity and promote environmentally benign alternatives when available.**

**A major initiative with the purpose to provide such information is the Swedish Environmental Classification and Information System for pharmaceuticals (SECIS). In this chapter we report the results from a**

**detailed evaluation of the environmental risk assessments conducted within SECIS so far. The results reported concern the use of environmental toxicity data for the purpose of risk assessment and classification. For results concerning additional aspects and the full analyses see Ågerstrand and Rudén, 2010.**

## Background

The Swedish Environmental Classification and Information System for pharmaceuticals (henceforth called SECIS) is a voluntary system for classification of pharmaceuticals based on their effect in non-target environmental species. The system was initiated in 2005 by the Swedish Association for the Pharmaceutical Industry (LIF). In addition to LIF, several other stakeholders within the healthcare sector participated in the development of the system. The stakeholders are the Stockholm County Council, the Swedish Medical

Products Agency, Apoteket AB (the Swedish pharmacy chain) and the Swedish Association of Local Authorities and Regions (Mattson, 2007). According to LIF's webpage (*www.fass.se*), the purpose of the classification system is to provide the public and health care sectors with environmental information about all active pharmaceutical ingredients on the Swedish market by 2010.

## Environmental risk assessment according to SECIS

According to the SECIS guidance document the risk assessment within this system should follow the basic principle of environmental risk assessment, namely to combine information about the hazardous properties of an active pharmaceutical ingredient with its estimated (or measured) environmental concentrations to determine the PEC/PNEC ratio (Predicted Environmental Concentration / Predicted No Effect Concentration). The processes for environmental risk assessment for general chemicals and for pharmaceutical substances respectively are introduced in *chapter 5*.

Within SECIS the PEC/PNEC ratio is used to classify each pharmaceutical product according to pre-defined criteria into a risk category an a risk phrase. The system has four categories for environmental risk and two categories for indicating lack of data. For an overview and summary of these criteria see *Table 1*. (The Swedish Association of the Pharmaceutical Industry, 2007).

**Table 1.** SECIS classification criteria and the corresponding risk categories/risk phrases.

| SECIS Criteria | Risk category |
|---|---|
| PEC/PNEC ≤0.1 | Use of the medicine has been considered to result in insignificant environmental risk |
| 0.1< PEC/PNEC ≤1 | Use of the medicine has been considered to result in low environmental risk |
| 1< PEC/PNEC ≤10 | Use of the medicine has been considered to result in moderate environmental risk |
| PEC/PNEC >10 | Use of the medicine has been considered to result in high environmental risk |
| Data is lacking | Risk of environmental impact cannot be excluded, since no ecotoxicity data are available |
| Data availability is insufficient | Risk of environmental impact cannot be excluded, however some ecotoxicity data are available |

The PEC value shall be calculated by using the formula and the basic principles specified by European Medicines Agency (EMA) (EMA, 2002). It is also

possible to use measured environmental concentrations (MEC) if sufficient data is available (The Swedish Association of the Pharmaceutical Industry, 2007).

For the PNEC, the SECIS guidance document allows both for effect data originating from the companies' own studies and for data generated by other pharmaceutical companies or by independent research groups. All data should, ideally, be generated by methods supported by appropriate standards such as those issued by OECD (Organisation for Economic Co-operation and Development) or the FDA (United States Food and Drug Administration). The use of non-standard test data is however also permitted and companies are recommended to take data from the open scientific literature into account (The Swedish Association of the Pharmaceutical Industry, 2007). Experimental data should preferably be derived from long-term tests but the use of short-term data is allowed. Regardless of the test length, the data should originate from three trophic levels: algae, crustaceans and fish. If data is available from the species believed to be most sensitive, based for example on understanding of receptor-mediated effects, a PNEC can be calculated with data from only one or two trophic levels. When calculating PNEC, assessment factors should be used according to the Technical Guidance Document (European Commission, 2003). For a discussion on the use of standard vs. non-standard tests for environmental risk assessment, see *chapter 5*.

The proposed classification, and the environmental data on which the classification is based, are submitted by the pharmaceutical companies to LIF. LIF then forwards the information to the Swedish Environmental Research Institute (IVL), a consultant firm who is responsible for reviewing the classifications and make suggestions for revisions if considered motivated, i.e. if the classification is not made in accordance with the SECIS guidance document. IVL reports the result of the review back to LIF who then communicates this to the company. The environmental information and the resulting classification are then made public on LIF´s webpage (*www.fass.se*) by the company.

On LIF's webpage the classification and the underlying data are presented in three versions, each adjusted to the needs of different end-users; patients and other non-expert users, prescribers, and scientists and other experts.

A classification according to the SECIS guidance document furthermore includes an assessment of the pharmaceuticals' potential to persist in the environment and to bioaccumulate, but in this chapter we focus on the classification of ecotoxicity.

## Overview of the SECIS classifications made so far

In February 2010, 691 of the 1090 active pharmaceutical ingredients available on the Swedish market had been assessed within SECIS, and the results of these assessments had been published on LIF's webpage (*www.fass.se*). The 691 substances have generated over 860 assessments, i.e. some substances are produced by several companies and since the classifications are performed for products and not for substances this results in several risk assessments for active pharmaceutical ingredients that are used in several different products. A summary of the environmental risk assessments made so far can be found in *Table 2*.

**Table 2.** Overview of the environmental risk (PEC/PNEC ratio) assessments made so far. Data from LIF's webpage *www.fass.se*.

| | No. | Comment |
|---|---|---|
| Assessed substances | 691 | |
| EMA exceptions such as vitamins and vaccines | 217 | 31 % of the assessed substances |
| Assessed substances that lead to a classification | 193 | 28 % of the assessed substances, i.e. data missing for 281 substances |
| Classified as "Use of the medicine has been considered to result in insignificant environmental risk"[1] | 168 | 87 % of the classified substances |
| Classified as "Use of the medicine has been considered to result in low environmental risk"[1] | 16 | 8 % of the classified substances |
| Classified as "Use of the medicine has been considered to result in moderate environmental risk"[1] | 7 | 4 % of the classified substances |
| Classified as "Use of the medicine has been considered to result in high environmental risk"[1] | 2 | 1 % of the classified substances: estradiol and ethinylestradiol |

1) For the substances that have been classified differently by several companies the highest classification has been chosen.

In SECIS nine substances have been classified as either a moderate or a high environmental risk ie they have a PEC/PNEC ratio greater than one. These are: allopurinol, amoxicillin, estradiol, ethinylestradiol, mykofenolatmofetil, natriummykofenolat, raloxifene and sertraline. Both the EMA guideline (EMA, 2006), and the TGD (European Commission, 2003) requires an extended environmental fate and effect analysis for substance with a PEC/PNEC ratio greater than one, while this is not required in the SECIS guidance document.

The pharmaceutical industry has reported that environmental data is lacking for a substantial number of their products, i.e. 59 % of the assessed products could not be classified since they lack sufficient ecotoxicity data. (In addition, 47 % lack data to enable a classification of the substances' persistency, and 40 % lack bioaccumulation data).

## Evaluation of the system's achievements so far

There are two main aspects of SECIS considered in this chapter, namely data selection and completeness of the data-set. Data selection concerns how data were selected for the risk assessments made so far, and in particular, what types of data are included (e.g. standard or non-standard, short-term or long-term). The completeness of the data-set concerns whether all available data have been included and if not, whether a supplemented data-set would alter the PEC/PNEC ratio and the classification.

For these analyses all the pharmaceutical substances classified by at least one company (available at *www.fass.se* April 2009) *and* for which ecotoxicity data could be found in the open scientific literature were selected. This resulted in a set of 36 substances and 48 assessments that were scrutinized.

### Data selection

Each of the 48 assessments was compared in detail to the requirements of the SECIS guidance document. The comparison focused on the selection of ecotoxicity data. The three SECIS guidance document recommendations considered in this evaluation are:

1. SECIS allows for both standard and non-standard test data when calculating PNEC.
2. Long-term ecotoxicity data are preferred when calculating the PNEC.
3. Companies are recommended to take external ecotoxicity data into account when classifying their products.

(For the full analyses, including additional SECIS guidance document recommendations, see Ågerstrand and Rudén, 2010).

The 36 substances selected for evaluation according to the selection criteria defined above, have been risk assessed by 60 companies. 48 of these 60 assessments resulted in a classification. The remaining 12 assessments did not result in a classification due to lack of data. Below are the results from the comparison of the 48 risk assessments to the three guidance recommendations:

**1: SECIS allows for both standardized (such as OECD, FDA, ASTM, and ISO) and non-standardized data when calculating PNEC.**
In 33 (69%) of the 48 assessments standard ecotoxicity data was used when calculating PNEC. Non-standard ecotoxicity data was used in 6 assessments (12%), and in 9 cases it was unclear whether the data originated from standard or non-standard tests.

**2: Long-term ecotoxicity data are preferred when calculating PNEC.**
The classifications were based on long-term data in 9 (19%) of the 48 risk assessments. In one case the type of data was unclear and in 38 assessments (79%) PNEC was calculated using short-term data.

**3: Companies are recommended to take publicly available ecotoxicity data into account when classifying their products.**
Ecotoxicity data from the open scientific literature was used in 20 (42%) of the 48 risk assessments. In 16 of the 20 cases these data were used when calculating PNEC. In 27 of the 48 cases the risk assessment was based on company-owned data only, and in one case the source of the data was unclear.

From this exercise it can be seen that standard test data and short-term data are more commonly referred to compared to non-standard, and long-term data. Furthermore, ecotoxicity data from the open scientific literature was used in about 40% of the assessments. When data from the open scientific literature were considered, they were often used to calculate the PNEC. Since the guidance document requires that the lowest available PNEC is used, this indicates that data from the open scientific literature offered lower effect values (indicating a higher risk) than company-owned data in these cases.

### Completeness of data-set

The SECIS guidance document is relatively flexible in its recommendations concerning data selection and data inclusion. There are for instance no requirements for the companies to search the open scientific literature for data, this is just a recommendation. For this study we identified data from

the open literature for the 36 active pharmaceutical ingredients selected for evaluation. We gathered these data from a database developed for research purposes within MistraPharma. The database is called WikiPharma. For an overview of the database contents, the method used for compiling it, and how to access the database see chapter 6 (Molander et al., 2009 and *www.wikipharma.org*). Additional data that could be used for risk assessment and classification of the 36 substances were selected according to the rules and recommendations of the SECIS guidance document and these data were used to supplement the risk assessments under scrutiny. Based on the resulting supplemented data set, alternative PEC/PNEC ratios and classification categories were determined and compared to the results of the risk assessments and classifications conducted by the pharmaceutical companies.

This exercise resulted in altered PEC/PNEC ratios for 18 (37%) of the 48 risk assessments, and the altered PEC/PNEC ratio would give rise to a new classification category for 13 (72%) of these 18 assessments. For 10 of the 13 assessments a supplemented data-set would result in a higher classification category. (For one substance, carbamazepine, the PEC/PNEC ratio increased 3 orders of magnitude which would give a two step increase in risk classification). For one substance, propanolol, the additional data allowed for a smaller assessment factor which would lower the risk classification one step compared to the SECIS-classification.

The additional data that we used were reported in 14 studies. In 9 of the 14 studies the data originated from standard tests. Half of the studies were regarded as long-term studies and the other half as short-term. In 5 cases the additional data that were used originated from non-standard tests. These data altered the classification category in three cases.

Supplementing the data-sets thus altered the classifications in a significant percentage (72%) of the cases eligible for this analysis. This suggests that exploring the open scientific literature for additional test data might be an important complement in environmental risk assessment of these substances. The number of cases (18) is however low, so to what extent this is representative for the risk assessments within SECIS need to be further analysed.

## Discussion and conclusions

SECIS is unique in its kind. It is a national, voluntary system implemented within a global industry. Its aim is to make information available to prescribers and other end-users and it does that in a comprehensive and transpar-

ent manner. Both the risk assessments, as well as all effect data are made publicly available at LIF's website (*www.fass.se*). This is exceptional. In many cases data from such investigations are considered to be company secrets. It should be noted though that the LIF website is in Swedish only and so are one third of the risk assessments. This reduces the availability of the information to non-Swedish speaking users. However, general information about the classification system is available in English at *www.fass.se/environment*.

Overall it is concluded that LIF has reached its goal to provide environmental information regarding pharmaceutical products and in general the achievements of SECIS are remarkable. The introduction of similar systems is discussed in other countries but also on the level of the European Union (Keil, 2008).

The result of SECIS so far confirms that environmental data is lacking for a substantial number of pharmaceuticals: 59 % of the products assessed within the system did not have enough ecotoxicity data to enable a classification according to the SECIS criteria.

Non-standard data are in many cases believed to be more relevant and sensitive for ecotoxicity testing of pharmaceuticals (see *chapter 5*). Therefore the use of non-standard tests is an important aspect when evaluating this system. The majority of the classifications in SECIS are based on data from standard tests. This is not surprising since it is the type of data that regulatory authorities require and thereby the data that companies are more likely to have access to. The SECIS guidance document does not contain any information regarding what kind of non-standard data that can be used or how to evaluate these data. The criteria for inclusion and evaluation of non-standard data could thus be further clarified in order to help risk assessors produce assessments based on relevant and sufficiently sensitive test data.

The use of short-term data is so far most common but hopefully this will gradually change with the new EMA guideline which emphasizes long-term data.

Data from the open scientific literature is not used to the extent it could be, and when used it seems to offer lower effect values, i.e. indicating a higher risk than company-owned data in a significant number of cases. Supplementing the SECIS guidance document with the aim to promote companies to search for data in scientific databases, including WikiPharma, could be a way to increase this use.

## Recommendations

The classification system was only recently launched and it was developed during a relatively short period of time. It is therefore not expected that the system should be working perfectly in all aspects. Based on the results reported above we hereby propose three suggestions that we think should be further discussed in the work towards the aim to continuously develop SECIS as a classification and information system. These suggestions are:

1. Require that the open scientific literature as a rule is searched for relevant data and, when available these data should be taken into consideration in the risk assessment
2. Clarify how non-standard test data can be included and how data should be evaluated in terms of their relevance and sensitivity
3. State explicity in the SECIS guide that the aim is to always use a relevant and sufficiently sensitive test-method

Further recommendations can be found in Ågerstrand and Rudén (2010).

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# 8 Swedish Wastewater Treatment Plants and Their Ability to Remove Pharmaceuticals

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**Wastewater treatment in Sweden is generally at a high technological level, and there are many plant specific treatment solutions to meet the great variety of discharge requirements. Integration of new treatment technologies and optimisation of the present treatment must therefore be considered from a plant perspective when new and more stringent discharge demands are to be met. Growing awareness of the potential threat that pharmaceuticals pose to the aquatic environment promotes the development of new and optimised techniques suitable for removal of these substances at wastewater treatment plants.**

Today's Swedish wastewater treatment plants are designed to remove organic material and nutrients. In contrast, the first Swedish wastewater treatment plants were only built to reduce the amount of organic material, which could otherwise cause oxygen depletion in the receiving water. In the

early 1950s there were only a few wastewater treatment plants in Sweden but today there are more than 2 000 municipal plants. A rapid development of new wastewater treatment plants with biological treatment started in the mid-1950s, and this was followed by the implementation of chemical phosphorus removal in the 1970s. Extended nitrogen removal was introduced in many plants in the 1990s and these plants show a clear distribution pattern in terms of geography and size. According to Swedish regulations, wastewater discharged from plants larger than 10 000 person equivalents (pe) to coastal areas south of a line from the Norwegian border on the west coast to the municipality of Norrtälje on the east coast should undergo extended nitrogen removal. There is no such requirement for plants north of this line (Swedish EPA, 1994). *Figure 1* shows the geographical distribution of nitrogen removal requirements in Sweden.

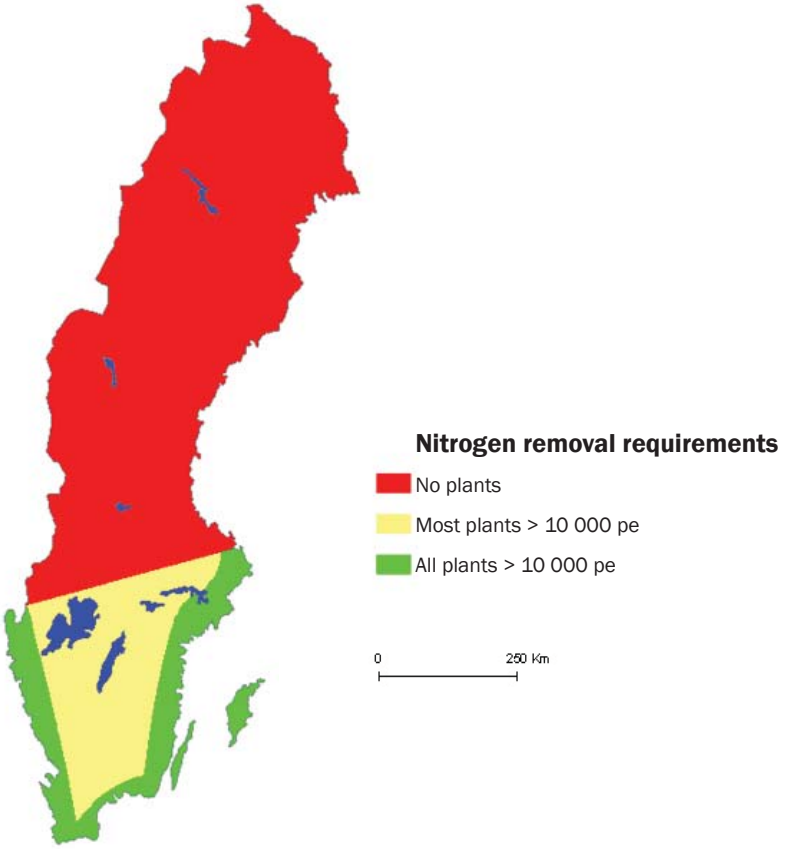



**Figure 1**. Nitrogen removal requirements in Sweden.

Historically, new treatment demands have been solved at existing plants rather than through the construction of new refined ones. This is mainly due to the economic value of all the assets associated with the wastewater treatment system and the cost of rebuilding them. Tailored solutions have therefore been required for the implementation of new treatment technologies at existing plants. Accordingly, Swedish wastewater treatment comprises a large spectrum of wastewater treatment technologies and process configurations.

## Micropollutants in the wastewater system

Wastewater of both domestic and industrial origin is treated at Swedish wastewater treatment plants. Discharge of industrial wastewater to the sewer requires approval by the body responsible for the wastewater treatment, which therefore can require that industrial chemicals are managed at the source instead of at the plant. For micropollutants associated with domestic wastewater, the sources are more diffuse and the reduction is dependent on the plant. In Sweden, most of the domestic wastewater generated in urban areas is transported to wastewater treatment plants. Wastewater treatment plants therefore have unique potential to serve as a barrier for environmentally hazardous micropollutants of diffuse origin.

*Figure 2* shows the three main ways for removal of micropollutants at wastewater treatment plants: sorption to particulate matter, biological degradation/transformation and evaporation. The ability of wastewater treatment plants to remove micropollutants is therefore affected by the physical, chemical and biological properties of the substance as well as by the biochemical processes at the plant, the plant configuration and the operation of the different treatment processes. *Figure 3* shows the main ways by which micropollutants are removed at a wastewater treatment plant with conventional activated sludge treatment.

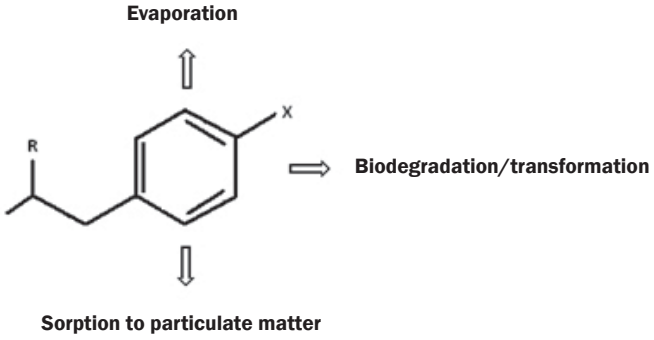



**Figure 2.** The three main ways for removal of micropollutants at wastewater treatment plants.

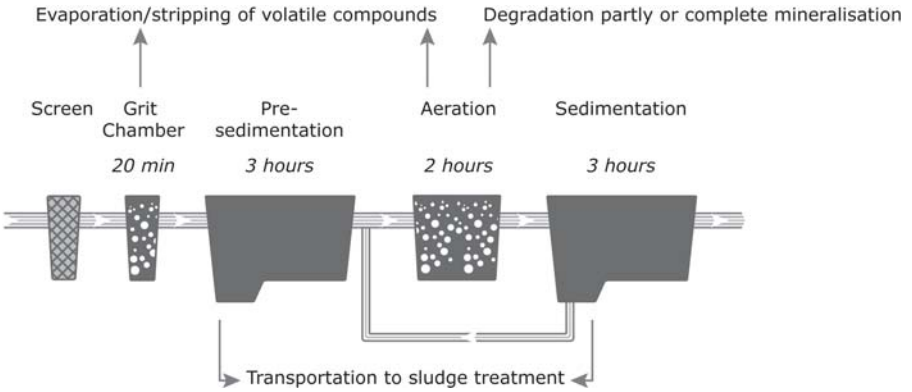



**Figure 3**. Fate and degradation/reduction mechanisms of micropollutants in a wastewater treatment plant with conventional activated sludge treatment.

## Swedish wastewater treatment

Sweden has more than 450 wastewater treatment plants dimensioned for more than 2 000 pe, and these plants treat approximately 90% of the wastewater volume from urban areas. The main treatment techniques used at these plants and how these techniques affect the removal of organic substances, nutrients and micropollutants in general will be discussed in this section.

The wastewater treatment process is initiated by screening of large debris with grids or sieves, and the subsequent treatment step is most often a grit chamber for removal of sand and other particles with high settling velocity. Bubbles of air are introduced in the grit chamber to reduce sedimentation of organic material with moderate settling velocity. These bubbles increase the contact area between air and water and thereby the area over which evaporation of volatile substances can occur. A reduction of volatile substances in the water phase can therefore be expected in the grit chamber. However, the main objective of this first mechanical treatment is to remove coarse material and grit that might cause clogging and mechanical abrasion at the plant. Due to the composition and the small volume of the removed material, removal of micropollutants through sorption to particulate material is limited at this first stage of the treatment process.

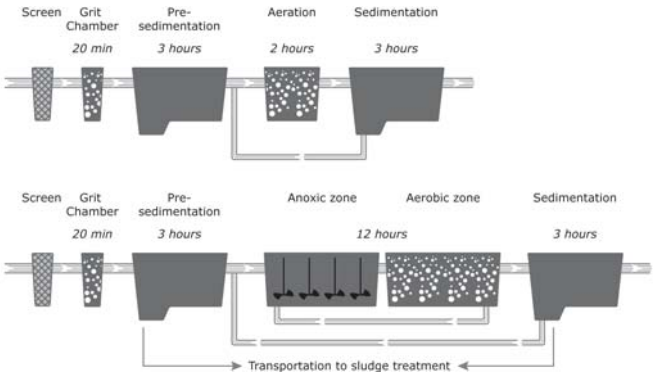
The final step in the mechanical treatment is generally a clarifier, where a significant amount of the particulate organic material and some organically bound nutrients are removed through sedimentation. The removal efficiency

of the clarifier can be enhanced by adding coagulants, typically iron and aluminium salts. These salts precipitate phosphorus and help small suspended particles to aggregate and form larger particles, which are prone to settle. A few medium-sized and a large number of small wastewater plants in Sweden are operated without any further treatment than this mechanical and chemical treatment, whereas most other wastewater treatment plants have additional biological treatment. Micropollutants sorbed to particulate matter are partly removed in the first clarifier, with the coarse fraction settling naturally and the fine fraction settling when complementary chemical treatment is used. The smallest particles have the largest area per volume ratio, which enables high sorption. Whether this high sorption potential of the small particle fraction is important to the overall removal of micropollutants in the primary clarifier is not fully known. Primary clarification and addition of coagulants affect the load of organic material, nutrients and other substances to the subsequent biological treatment and thereby the bacteria composition within it.

At Swedish wastewater treatment plants, the biological processes range from removal of organic material to the most advanced biological phosphorus and nitrogen removal. Phosphorus removal and biological removal of organic material can be seen as mandatory for all Swedish wastewater treatment plants larger than 10 000 pe and for plants larger than 2 000 pe with a freshwater recipient, but there are exceptions. On the other hand, biological nitrogen removal can only be seen as mandatory for Swedish wastewater treatment plants larger than 10 000 pe in coastal areas south of a line from

Norrtälje municipality on the east coast to the Norwegian border on the west coast. There are virtually no plants with extended biological nitrogen removal north of this line. The introduction of extended biological nitrogen removal at wastewater treatment plants has been achieved mainly through regulations, whereas the driving force for introduction of extended biological phosphorus removal has been the possibility to reduce the consumption of chemicals associated with traditional chemical phosphorus removal. Since the introduction of extended biological phosphorus removal is based on a plant initiative, the geographical distribution and plant size distribution are not as clear as they are for plants with extended biological nitrogen removal.

The biological treatment most frequently used in Sweden is the activated sludge process, which is based on floc-forming bacteria that are responsible for the treatment. The process can be operated with and without extended nitrogen removal. Activated sludge treatment with extended nitrogen removal is only found in southern Sweden, while activated sludge treatment without extended nitrogen removal is typically found in northern Sweden and at small plants in southern Sweden. *Figure 4* shows typical process schemes for activated sludge treatment plants with and without extended nitrogen removal along with their expected removal of suspended solids, BOD (indicates the amount of biodegradable substances in the water), phosphorus and nitrogen. As indicated, the volumes and retention times




Reduction of organic material and nutrients at:

Northern plants and Southern plants
<10 000 pe
SS > 90%
BOD > 90%
P > 90%
N ~ 25%

Southern plants
>10 000 pe
SS > 90%
BOD > 90%
P > 95%
N > 70%

**Figure 4**. Typical process schemes for activated sludge treatment plants of various geographical origin and size (Northern plants and Southern plants <10 000 pe above, and Southern plants >10 000 pe below). The tables show the expected removal of suspended solids (SS) and organic matter as BOD together with reduction of phosphorus and nitrogen if the biological treatment is combined with chemical precipitation of phosphorus.

required for the biological treatment are very different for these two plant types. The longer retention time is required to achieve full nitrification and denitrification in the activated sludge process. The retention time of the biological treatment is also important to the reduction of other substances, since it represents the time over which biodegradation can occur. In terms of the rate of biodegradation of micropollutants, the sludge age, which is the average time bacteria are maintained within the biological treatment, has been shown to be important.

An increased sludge age promotes more slow-growing bacteria species, which increases the diversity of the bacteria community and thereby the probability that some bacteria will be able to degrade a specific substance. Activated sludge processes designed solely for removal of organic substances are operated with a short sludge age, while higher sludge ages are required for nitrogen removal. The higher sludge age is required by some slow-growing bacteria species necessary for the nitrogen removal process, and the sludge age required by these bacteria seems to be critical for the removal of some micropollutants.

The activated sludge process ends with a clarifier, from which sludge rich in bacteria is either recirculated or withdrawn as excess sludge. The withdrawal of sludge not only represents a possibility to remove organic

material, nutrients and other substances, but also a possibility to control the performance of the activated sludge process and the sludge production. This control mechanism can for example be used to compensate for some of the annual variations in microbial activity, caused by temperature, through direct adjustments of the sludge concentration in the process. Nowadays, many activated sludge plants are operated at lower sludge concentrations and sludge ages in summer compared to winter.

In contrast to the activated sludge processes, biofilm processes are not based on floc-forming bacteria in suspension but to bacteria attached to a solid matrix. Bacteria attached to the solid surface form a bacteria layer, a biofilm, through which substances required for bacteria growth are transported. The transport resistance and the thickness of the biofilm are therefore important to the growth of bacteria and the reduction of organic substances and nutrients in the water. When the biofilm becomes too thick it starts to peel off. The diversity of the bacterial community in biofilm processes is generally high with many slow-growing bacteria species.

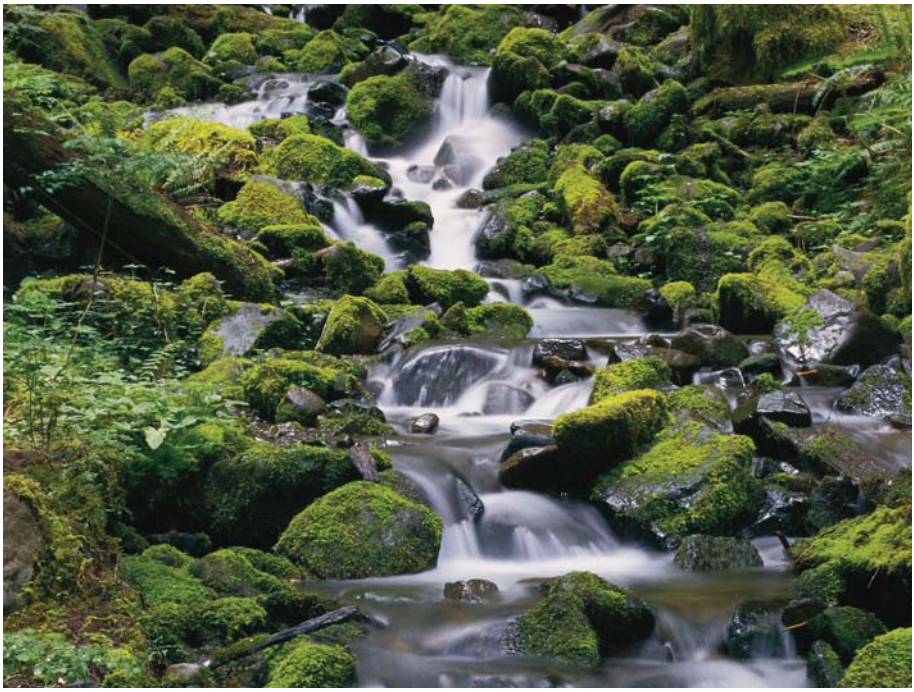
Trickling filters, the most frequently used biofilm technology in Sweden, consist of a bed of biofilm carriers through which wastewater percolates. Trickling filters can constitute the only biological treatment at plants without nitrogen removal requirements, but additional biological treatment is required when nitrogen removal requirements are to be met. Trickling filters for biological removal of organic substances are frequently used at small and medium-sized wastewater treatment plants. The bacteria composition in trickling filters is highly dependent on the load of nutrients and organic material, which has to be kept in mind when the nitrification capacity of the trickling filter is of interest. This is the case for some plants with trickling filters and nitrogen removal requirements. Even though the retention time of the wastewater in the trickling filters tends to be short, large amounts of organic material can be removed. Whether this is the case for more recalcitrant micropollutants is more uncertain.

The use of suspended biofilm carriers at Swedish wastewater treatment plants is increasing. The biofilm carriers applied are small and suspended in water and they can be used for removal of both organic substances and nitrogen. One of the main advantages of processes with suspended biofilm carriers is that high bacteria concentration can be achieved in the reactor. Subsequent separation of sludge from biofilm processes is required and this separation is most often achieved with a clarifier. Addition of coagulants in this treatment step enhances the removal of nutrients, organic substances and other substances sorbed to particles.

In order to further reduce the phosphorus content of the biologically treated wastewater, coagulants are often added, and the sludge produced is most often removed with a final clarifier or a sand filter. At some Swedish wastewater treatment plants, wastewater undergoes further treatment in wetlands and additional filters.

Sludge separated in the clarifiers is dewatered and at most large and medium-sized plants additional treatment exists. The most common sludge handling method is anaerobic digestion, which enables the production of biogas, reduces the volume of sludge and reduces the amount of pathogens in the sludge. Sludge handling is costly for small plants, so sludge from small plants is often transported to larger wastewater treatment plants in the vicinity for further treatment.

Micropollutants sorbed to sludge tend to end up in the sludge handling part of the plant, where further removal can occur. However, the removal of more recalcitrant micropollutants through anaerobic digestion is expected to be limited, which means that some micropollutants will reach the soil when anaerobically digested sludge is used as soil conditioner or fertiliser.



## Pharmaceuticals in the urban wastewater system

Pharmaceuticals for human medication are used throughout society and they tend to end up in the sewer after use, in their original form or as metabolites. Only a few point sources, such as hospitals and care institutions, can be identified. Actions for removal at these sites can reduce the amount of pharmaceuticals in wastewater, but it will not dramatically affect the total pharmaceutical load on wastewater treatment plants. Consequently, the ability of wastewater treatment plants to remove pharmaceuticals is important for the amount of pharmaceuticals that reach Swedish waters.

The removal of pharmaceuticals at wastewater treatment plants depends on the physical, chemical and biological properties of the substance as well as the biochemical processes at the plant, the plant configuration and the operation of the different treatment processes. Since very few pharmaceuticals are volatile, the removal through evaporation is expected to be insignificant for most pharmaceuticals. Some pharmaceuticals adhere strongly to sludge, which is separated from the water phase at several stages of the treatment process. Pharmaceuticals sorbed to sludge, that has been removed from the wastewater treatment end up in the sludge handling part of the plant.

The biological treatment method and the biochemical processes within it are important for the removal of biodegradable pharmaceuticals. Pharmaceuticals cannot be removed biologically in plants operated solely with chemical-mechanical treatment. For activated sludge treatment processes the sludge age has been addressed as an important parameter for the removal of pharmaceuticals, and activated sludge processes with extended nitrogen removal are operated at higher sludge ages than those without extended nitrogen removal. The increased microbial diversity induced by the higher sludge age in activated sludge with extended nitrogen removal increases the probability of degradation of single pharmaceutical substances. This and the prolonged hydraulic retention time in activated sludge processes with extended nitrogen removal are clearly of interest from a Swedish perspective on pharmaceutical removal, because extended nitrogen removal in Sweden is unevenly distributed over the country and among plants of different sizes. The research in MistraPharma on pharmaceutical removal in activated sludge treatment plants is partly related to these differences in sludge age and nitrogen removal. Evaluation of these differences are expected to provide useful information on pharmaceutical removal at existing plants and it might indicate a need for integration of new treatment technologies. A number of new promising treatment methods with sorbents, membranes, ozone and other chemical oxidants are also investigated in MistraPharma.

Current knowledge about the mechanisms and parameters important to the removal of pharmaceuticals in biofilm systems is rather limited and a study on pharmaceutical removal in these systems has therefore been initiated in MistraPharma. For some pharmaceuticals however, removal seems to be less efficient for trickling filters than for activated sludge processes.

Some pharmaceuticals found in the influent of Swedish wastewater treatment plants show limited removal at all existing plants, while other pharmaceuticals show limited removal at some plants or complete removal at most plants. Evaluation of the environmental toxicity of pharmaceuticals found in treated wastewater is important for the identification of the ones that pose a significant risk to the environment. Integration of new suitable methods for the removal of the most problematic pharmaceuticals should then be considered. However, the great variability in removal efficiency between different pharmaceuticals and different groups of treatment plants indicates that pharmaceutical specific and plant specific treatment technologies are needed for the removal of most pharmaceuticals.

## Concluding remarks

Increasing discharge demands have been important to the development of Swedish wastewater treatment for decades and they have often been met through plant specific process changes. As a result of this development, Swedish wastewater treatment comprises a broad spectrum of treatment technologies and some of these differences are known to affect the removal of pharmaceuticals. Reduction of pharmaceuticals is a great challenge for Swedish wastewater treatment, since there is a great variability in the removal efficiency between different pharmaceuticals and different groups of plants. A prioritisation system for pharmaceuticals with the highest environmental impact is therefore needed to target new treatment technologies towards the pharmaceuticals of the highest environmental concern.

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# 9 The Vision – Sustainable Pharmaceutical Management in a Sustainable Society

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## Approaching the dead end

It is not an easy task to identify a new molecule that can be used as medicine for humans. Our knowledge about the molecular basis of several diseases is not yet detailed enough to allow development of more specific therapeutic agents. Hence, improved markers of disease, preferably characterized by causal relationship to their health-threatening manifestations, are looked for, albeit with modest success.

Since it is so difficult to discover and develop a new drug, the R&D costs are rising dramatically in the pharma industry. The industry has developed several strategies to overcome the problem. The first is to direct R&D to products that are sold in vast amounts. The second is to focus on drugs displaying minimal risks of failure during the development. The third is to

conduct extensive marketing operations even before a new product has been authorised.

As a consequence of the first two of these strategies, development of certain groups of drugs has become particularly interesting: antihypertensive, anti-arrhythmic and anticoagulant drugs, pain killers with new action mechanisms, anti-obesity drugs, antidepressants and drugs promoting male sexual capacity. In parallel a number of therapeutic areas have become less attractive: new antibiotics, drugs used in poor countries and drugs which are used by a limited fraction of the population.

The factors mentioned above have yielded an emerging development and use of pharmaceutical products in industrialised countries that are not sustainable. Nor is the shortage of pharmaceuticals in developing countries sustainable.

## Defining sustainability

A sustainable society is characterised by managing economic, environmental and social issues in a long-term sustainable way. Needless to say, a sustainable society must have a health care system that is built on the same foundations, also with respect to use of pharmaceutical products. This implies that the entire flow from development and production to use and disposal of pharmaceuticals must be sustainable. It is necessary to stress that the requirements for sustainability cannot be circumvented by importing products from other countries; the responsibility for using sustainable products does not recognize national borders.

In developed countries the pharmaceutical industries have, during recent years, become increasingly interested in applying "green chemistry" both in their R&D and in the production. It may be assumed that both environmental and economic considerations support this interest. Some pharma companies have even gone public to disseminate information on their progress, e.g. in reduction of solvent use in the manufacturing process. This is a positive trend, in particular since there is an economic driving force supporting the environmental ambitions. Hence, it may be assumed that the pharma industries in developed countries have begun to react to the need for change in sustainable direction. But to date this is no more than a beginning.

In developed countries the governmental control of emission of pollutants from the production is probably rather efficient. In contrast, reports from low cost production countries like India and China indicate very large emissions of hazardous material from their pharma production sites. This may well be a parallel to the emissions from corresponding production sites in developed societies occurring no more than 4-5 decades ago.

A common policy describing the considerations, decisions, components, working plans and surveillance of sustainable development and production of pharmaceuticals has not yet been adopted. There is, however, reason to assume that at least some of the following components should be included:

- All raw materials and all disposables are extracted from nature in a way that does not pose any kind of burden to future generations, neither in terms of hampered access to raw materials or accumulation of rest products
- All staff involved in the production of raw material, disposables and final products are protected from any kind of production related health impairment. They have labour conditions harmonizing with international rules and regulations, and are paid at least according to national minimum rules. Children at school age may not be used in any step of the production
- No emissions from the production causing harm to public health or to the environment may occur
- The R&D and the production covers its own costs, including those required to fulfil the commitments in the paragraphs above. The costs of the products for the health care system do not systematically excavate the society's health care budget

The following step in the flow of pharmaceuticals is its use by patients, for diagnostic purposes, following prescription by a health care professional or following the patient's own purchase over the counter in a pharmacy shop. Is it possible to characterize sustainable use and disposal of pharmaceuticals under these different conditions? Let us first have a look at the pharmaceutical product in its intended activity. It is easy to realise that the ideal sustainable pharmaceutical product (i) should be fully efficient in all patients, according to a dosage that is easy to predict prior to treatment, and (ii) that the therapy should have no adverse effects or negative interactions with other drugs given or with external factors. The balance between positive therapeutic effects and adverse or unwanted effects of pharmaceutical products is, since many years, the most crucial issue of decision in the authorisation for marketing process, and is regulated carefully in a separate legislation. Therefore it will not be further considered here.

Medicines used by patients are, in one way or another, excreted from patients under treatment. Such emissions mainly occur via the urine or faeces, but in some cases via the skin, saliva, expectorate or expired air. These emissions are very important to consider when analysing the sustainability of pharmacological treatment.

To achieve a fully sustainable use of pharmaceuticals all such emissions should either be fully eliminated in the environment without having elicited any negative effects on flora or fauna, or alternatively, be taken care of by technical processing to fully degraded products in the sewage treatment plants or elsewhere. This is not an easy task. The currently available methods for elimination of anthropogenic chemical residues in the sewage treatment plants are reasonably efficient but energy-consuming, and there is considerable reluctance among politicians and other decision-makers to accept the emerging need for additional investments in advanced waste water treatment technologies. But water is a limited resource on our planet, and with increasing population and decreasing fresh water reservoirs globally the need for action to protect our water sources will continue to grow.

Another factor determining the sustainability of pharma use is the disposal of expired or unused medicines. It is well known that such medicines may accumulate in the homes of patients, either because the medicines were not well tolerated, because they are no longer needed, or for other reasons. In the EU it is enforced by law that all member states must arrange take-back systems for expired or unused medicines. The national compliance to adopt this EU law varies between states, as does the compliance of the member state populations to use the take-back systems. No doubt, there is a potential for improvement in order to ensure complete return and destruction of all unused or expired medicines from hospitals, other health care institutions and patients in the European community. This is a very important step on the road towards sustainability.

## Moving towards sustainability

It seems unlikely that the free market driving forces alone would lead society into sustainability. Few enterprises have business targets looking more than 5-10 years ahead. In a competitive market it may be risky to invest in activities paying off too long ahead. The assumed reaction of the stock market is an important compass for any company when making up its business plans, and if a company is not rewarded by the stock market when going "green" or moving towards sustainability the board of the company may be reluctant to further activities in this direction.

A strong market demand, from patients or from health care professionals, for more sustainable pharmaceutical products would probably elicit a powerful stimulus for the producers to speed up their transfer in "green" direction. But most patients, even those realizing the need for societal change towards sustainability, may primarily have their own health in focus when taking medicines. Health care staff prescribing pharmaceutical products may follow the same logics, i.e. to give priority to the health of their current patients instead of considering the possible threat to future generations and the environment. Therefore, the probability is weak that market demands for sustainable pharmaceutical products should become a strong driving force to the producers to go green.

This would tend to allocate the responsibility for transfer towards sustainability to our democratically elected representatives. But politicians are elected for periods seldom lasting longer than 4-6 years, i.e. extremely short when discussing how to achieve long-term sustainability. Furthermore, politicians want to become re-elected and therefore want to launch initiatives that render them attention in the public opinion. In this process dramatic, instantaneous, heart-breaking initiatives always overcome long-term demands requiring intellectual analysis and conception.

New decision mechanisms, possibly in the hands of globally acting international and non-political organisations, might offer a solution. But international organisations have hitherto displayed low potency to pave the way for long-term agreements which do not offer any direct and instantaneous favour to its members. After a catastrophic event or period the readiness to take significant steps towards change seems more at hand, as shown e.g. by the establishment of UN after the end of world war two, or its corresponding organisation following world war one.

Proactivity, i.e. taking action according to the precautionary principle, is always more difficult to implement than reactivity. Nevertheless, to avoid public health and environmental disasters caused by pharmaceutical emissions in the future pro-activity is needed now. It is today we need to take full responsibility for the emissions we cause, instead of trusting that future generations will solve the problem with large pollutions they did not cause, but only inherited. And if the structures to take on the responsibility are not currently at hand, it is up to us to establish these structures now.

## Promising signs in the sky

What has been mentioned above may yield a somewhat pessimistic view as to the abilities of contemporary societies to move towards sustainability, also in the health care and pharmaceutical therapy areas. Some indications that steps are taken in right direction have, however, been around for some years. In fact, these indications have become more frequent recently.

First, several research projects to elucidate the current situation and to propose relevant actions have been granted. The EU was early in supporting such projects; successful examples are POSEIDON and Rempharmawater. In Sweden the current project – MistraPharma – seeks to develop new methods for identification and assessment of environmental effects of

pharmaceutical residues in the aquatic environment, as well as techniques to reduce emissions via waste water treatment. The EU project Knappe focused on compiling the current qualitative and quantitative knowledge on pharma emissions into the aquatic environment, and on proposing means and measures to convert the knowledge into practicable strategies and working plans.

EU has not only recognized the problem of pharma emissions into surface waters by supporting research projects. Thus, in the human medicines directive 2004/27/EG some initial steps were taken to counteract the problem. The most important were, (i) to require a mandatory risk assessment from the producer in the application for marketing for any pharmaceutical substances estimated to exceed a level in surface water of 0.01µg/L, and (ii) to require all member states to establish take-back systems for unused or expired medicines. In the "pharma package", a series of documents adopted by the Commission in December 2008, and currently being processed in the Council and the Parliament a very positive message is found in the "Communication" (COM [2008]666, paragraph 1.3.3). The text reads as follows:

**Addressing the Environmental Impact**
*Pollution of waters and soils with pharmaceutical residues is an emerging environmental problem and also an emerging public health concern. The Commission recognizing these concerns has funded several research projects to assess possible environmental and health impacts of pharmaceuticals. It is now necessary to focus on measures that could reduce the potentially harmful impact of pharmaceuticals on the environment and public health. Areas for further actions include evaluating environmental information on pharmaceuticals collected by the European Medicines Agency and national medicines authorities with a view to integrating this information into the current EU legislative framework.*

*Objective #12: Measures to reduce the potentially* ***harmful impacts of pharmaceuticals on the European environment and public health*** *should be proposed.*

Although only presenting general ideas about future legislative measures to protect European water sources from pharma pollution, this text firmly rivets such pollution as a significant problem within the community. Thereby, its adoption as a communication from the Commission can be regarded as an important policy act in this arena.

In Sweden the Environmental Protection Agency (Naturvårdsverket) has conducted a broad survey of the appearance of pharmaceuticals in a large number of national surface waters. The Swedish Medical Products Agency (Läkemedelsverket) has released a report (December 2009) to the Government in which it is suggested that (i) the GMP (Good Manufacturing

Practice) criteria should be amended to include requirement for an environmental certificate for producers of pharmaceuticals and active ingredients, and (ii) the current definition of risk/benefit balance in the EU legislation on human pharmaceuticals should be amended. The Agency proposes that risk for the environment should be part of this assessment, implying that environmental risk under certain circumstances should create basis for refusal of a marketing application.

During the Swedish presidency of the European Union 2009 an innovative step was taken to stimulate the pharmaceutical producers to develop new antibiotic drugs. Development of new antibiotics has not been a priority for the pharmaceutical producers since such products must be used with restriction (to avoid development of resistance, cf. above). Restricted use hampers the possibility for pay-back to the producers of the R&D costs for the substance. The Swedish Government has instructed the European Commission to prepare a proposal implying that member countries directly cover R&D costs for new antibiotics, aside the normal financing via market sales. Hence, the producer should be guaranteed pay-back even if sales remain low. Such a system for financing of pharmaceutical R&D costs implies a new paradigm, and opens up very promising perspectives not only for development of new antibiotics, but also for e.g. new medicines against third world disease and drugs sold in very small quantities.

What was the reaction from the pharmaceutical producers to such a radical idea? Hitherto, the comments have been precautious but positive. As to date no firm proposal to the financing system has been presented, but there is no doubt that all parties involved are looking forward to the result of the Commission's task with great interest.

The pharmaceutical producers have also, to various extents, begun adopting "green chemistry" techniques both in their R&D and in the production. As mentioned earlier the results of these efforts have been used even in marketing activities. It may be assumed that many more companies than those going public with their "green" ambitions follow the development with great attention.

To summarize, the transformation of pharmaceutical management towards sustainability has been initiated in various arenas, to variable extent and with different degrees of success. In discussing pharmaceutical sustainability one specific aspect must not be forgotten, i.e. the sustainable character of the drug that was never prescribed. There is a trend in the modern society that any symptom, any disease and any malfunction, mental or physical, can and

must be eliminated with a suitable pharmaceutical product. Some patients believe that medical products can completely remove any symptom, without adverse effects. Some prescribing doctors, when meeting such patients, may find signing a prescription the easiest way to bring the consultation to an end.

These trends are no doubt beneficial to the pharmaceutical producers. From a public health perspective the trend can be questioned, since it may allocate the individual health responsibility away from the subject. The health care system must continuously recognize the trend and counteract it whenever necessary. The drug that was never prescribed, the drug that was not necessary to take, is not only part of the solution to sustainable pharmaceutical management. It may also be a benefit for the patient and the environment. As such it is a necessary component in the realisation of the sustainable society.

The opinions and views expressed in this chapter are those of the authors, and may not be representative for those of the collaborators of the Mistra-Pharma project.

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## Ethel Forsberg

*Director General, the Swedish Chemicals Agency*

Ethel Forsberg has been the Director General of the Swedish Chemicals Agency (KemI) since 2001. She has a Master of Science Degree in Landscaping Architecture. Before joining KemI she held positions at several sectors within the Swedish society: as environmental manager in the Federation of Swedish Farmers, senior administrative Officer at the Ministry of the Environment, Manager of Food Safety, Environment and Consumer Affairs at COOP (Head office of Cooperative Retailers in Sweden) and as Director of Administration of Health, Food and Environment in the City of Stockholm. At present she is on the board of European Chemicals Agency (ECHA).

## Bo Gunnarsson

*Doctor of Medical Science, Executive Office Support Apoteket AB.*
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Bo Gunnarsson is also o pharmacist and has since 1971 been in different positions on the Swedish drug market. He has published about 50 scientific papers regarding medical, pharmaceutical and environmental subjects. In 1999 he becomes the Head of Environmental Department in Apoteket (National Corporation of Swedish Pharmacies) with focus on drugs in the environment.

## Lina Gunnarsson

*Postdoctoral Fellow, the Sahlgrenska Academy at the University of Gothenburg. lina.gunnarsson@fysiologi.gu.se*

Lina Gunnarsson is a molecular biologist by training. She received her PhD in 2009 from the University of Gothenburg on a thesis entitled 'On the use of genomics to assess environmental risks of pharmaceuticals'. Her most important contribution so far to the field is a study published in 2008 on 'Evolutionary conservation of human drug targets in organisms used for environmental risk assessments'.

## Roman Grobic

*Post Doc., Department of Chemistry, Umeå University*
*roman.grabic@chem.umu.se*

Roman Grabic is a researcher within environmental and analytical chemistry. He performs method development of new techniques for LC/MS/MS and LC/LCMS/MS analysis of pharmaceuticals in the environment. He has developed multiresidual methods for analysis of wide range of pharmaceuticals in different matrixes as drinking, surface and waste water or biological fluids.

## Malena Göransson

*Student, Royal Institute of Technology (KTH)*
*malenag@kth.se*

Malena Göransson is a student from the environmental biotechnology programme at KTH doing her Masters thesis project within Mistra-Pharma. The aim of the project is to make a systematic analysis of the sensitivity of standard vs. non-standard tests for environmental risk assessment of pharmaceuticals.

## Joakim Larsson

*Associate Professor, the Sahlgrenska Academy at the University of Gothenburg. joakim.larsson@fysiologi.gu.se*

Leading research on drugs in the environment since 1998, now merging toxicogenomics with ecology and pharmacology. Contributed to identifying ethinylestradiol as drug of environmental concern in the 1990ies. Recently identified production sites as a source for drugs to the environment. Project leader in the MistraPharma programme.

## Richard H Lindberg

*Post Doc., Department of Chemistry, Umeå University*
*richard.lindberg@chem.umu.se*

Richard Lindberg is a researcher within environmental chemistry. He performs research about the fate of pharmaceuticals in sewage water treatment plants and in the environment. He has developed several novel analytical methods for the determination of pharmaceuticals in various matrices including sewage sludge.

## Linda Molander

*PhD student, Royal Institute of Technology (KTH).*
*linda.molander@abe.kth.se*

In her master thesis, which was conducted within MistraPharma, she studied the risk classifications and the criteria for selection of ecotoxicity data according to the Swedish environmental classification system for pharmaceuticals.Linda is now a PhD student and her current research project concerns health and environmental risks associated with hazardous chemicals in articles, with an emphasis on evaluating risk reduction strategies.

## Christina Rudén

*Professor, Royal Institute of Technology (KTH), Stockholm, Sweden.*
*cr@infra.kth.se*

Toxicologist specialising in regulatory aspects of toxicology and the risk assessment process. In particular how scientific data are generated and used for risk assessment purposes, and how scientific uncertainty is handled in the process.

The programme director of the Swedish research initiative, MistraPharma.

## Mats Tysklind

*Professor in Environmental Chemistry, Umeå University.*
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Research on the environmental behaviour of anthropogenic compounds, such as persistent organic pollutants and pharmaceuticals. His work includes e.g. studies of transport and fate processes and development of analytical techniques. The interactions between inherent physico-chemical properties and environmental matrices, including development of QSARs, are of special interest. Project leader in the MistraPharma programme.

## Åke Wennmalm

*Former Environmental Director, Stockholm County Council.*
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Physician and cardiovascular researcher. In collaboration with Bo Gunnarsson, Apoteket AB, initiated the Swedish work to lift the issue of pharmaceuticals in the environment in the EU, among pharmaceutical producers and in the health care system. Participated in the development of the environmental classification system for pharmaceuticals.

## Marlene Ågerstrand

*PhD-student, Royal Institute of Technology (KTH)*
*maa2@kth.se*

Marlenes work concerns regulatory aspects of ecotoxicology, with focus on risk assessment and risk management. Currently she is conducting an evaluation of the Swedish environmental classification system for pharmaceuticals. The evaluation concerns driving forces behind the implementation of the system, its scientific basis, actual workings, and efficiency towards reducing environmental risks.

## Christina Åkerman

*M.D., Ph.D., Executive MBA. Director General at the Medical Products Agency, Sweden.*

Since September 1, 2008 Dr Åkerman's position is Director General at the Medical Products Agency, Sweden. Prior to this she had the position as Working Chairman of the board P.U.L.S. AB (Partners for Development Investments in Life Science Inc.) and before that as Executive Vice President Technology & Product Development, Orexo AB.

Prior to joining Orexo, Dr Åkerman held the position as Marketing Company President of AstraZeneca in the Philippines. Since 1995, she has held senior positions as Project Manager, Medical Director and Marketing Director at Hässle Pharmaceuticals and AstraZeneca Sweden.

## Photo credits

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The MistraPharma research programme aims at identifying human pharmaceuticals that may pose a significant risk to organisms in the aquatic environment, recommend additional waste water treatment technologies and provide knowledge that could contribute to an improved European legislation in this field. The programme is financed by the Swedish Foundation for Strategic Research (Mistra).

Last year MistraPharma published a book entitled ”A Healthy Future – Pharmaceuticals in a Sustainable Society”. That volume summarized the current knowledge of the problems that surround pharamaceuticals and sustainable development.

The book you now hold in your hands gives an overview of the achievements obtained during the first two years of research within MistraPharma. It also contains a global outlook and visions for the future. We hope that you will find it interesting.

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