THESIS ON CHEMISTRY AND CHEMICAL ENGINEERING G29

(Eco)toxicological Information on REACH-Relevant Chemicals: Contribution of Alternative Methods to *in vivo* Approaches

MARILIIS SIHTMÄE

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TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Chemical and Materials Technology
Department of Chemical Engineering

NATIONAL INSTITUTE OF CHEMICAL PHYSICS AND BIOPHYSICS
Laboratory of Molecular Genetics

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Supervisors: Dr. Anne Kahru, leading research scientist, Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Estonia

Prof. Rein Munter, Department of Chemical Engineering, Tallinn University of Technology, Estonia

Opponents: Prof. Dr. med. Horst Spielmann, Faculty of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Germany

Associate Prof. Karin Reinhold, Department of Business Administration, Tallinn University of Technology, Estonia

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

Mariliis Sihtmäe

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MARILIIS SIHTMÄE

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to by their Roman numerals in the text.

I **Sihtmäe, M.**, Dubourguier, H.C., Kahru, A. 2009. Toxicological information on chemicals published in the Russian language: Contribution to REACH and 3Rs. *Toxicology* 262, 27 - 37.

II **Sihtmäe, M.**, Blinova, I., Aruoja, V., Dubourguier, H-C., Legrand, N., Kahru, A. 2010. E-SovTox: An online database of the main publicly-available sources of toxicity data concerning REACH-relevant chemicals published in the Russian language. *Alternatives to Laboratory Animals (ATLA)* 38, 297 - 301.

III **Sihtmäe, M.**, Mortimer, M., Kahru, A., Blinova, I. 2010. Toxicity of five anilines to crustaceans, protozoa and bacteria. *Journal of the Serbian Chemical Society* 75, 1291 - 1302.

IV Aruoja, V., **Sihtmäe, M.**, Kahru, A., Dubourguier, H-C. 2011. Toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata* and bacteria *Vibrio fischeri*: comparison with published data and QSARs. *Chemosphere* 84, 1310-1320.

Other publications:

Kurvet, I., Ivask, A., Bondarenko, O., **Sihtmäe, M.**, Kahru, A. 2011. LuxCDABE - transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: comparison with naturally luminous *Vibrio fischeri*. *Sensors* 11, 7865-7878. Online available at: http://www.mdpi.com/1424-8220/11/8/7865/

AUTHOR`S CONTRIBUTION TO PUBLICATIONS

I The author was responsible for the data retrieval from the literature and databases, analysed the obtained results and wrote the article in co-operation with her colleagues. The results were also presented by the author at the SETAC Europe 18th Annual Meeting, Warsaw, in 2008.

II The author was responsible for composing and managing of E-SovTox database described in the paper and participated in writing the article. The results were also presented by the author at the 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome, in 2009 and at the conference of Linz 2010-EUSAAT 2010-ESTIV 2010, Linz, Austria, in 2010 *(The ESTIV and Elsevier Young Scientist Award for the best poster*

presentation: A web based-database on the main publicly available sources of toxicity data published in Russian language).

III The author performed the testing with naturally luminescent bacteria *Vibrio fischeri*, calculations using ECOSAR program, data analysis and writing of the manuscript in cooperation with other authors.

IV The author performed the toxicity testing with naturally luminescent bacteria *Vibrio fischeri,* interpreted the respective data and participated in writing the article. The results were also presented by the author at the NATO SfP 982590 project workshop, Dubrovnik, in 2010.

INTRODUCTION

The modern society is entirely dependent on ever increasing use of chemicals for the production of food, energy, medicines, textiles etc. However, if not properly used or assessed for hazard, chemicals can have negative impacts on human health and the environment. Therefore, their safety has to be carefully studied. For regulating industrial chemicals in EU, a new chemical policy REACH (**R**egistration, **E**valuation, **A**uthorisation and restriction of **Ch**emicals) came into force in 2007. REACH requires that all substances on the European market, which are manufactured or imported in quantities of 1 tonne or more per year have to be evaluated for hazardous effects to humans and environment. One of the main reasons behind REACH was a large number of chemicals manufactured and placed on the market in Europe for many years in substantial amounts without sufficient information on their potential hazard to human health and the environment. According to REACH these chemicals have to be evaluated for their hazard during a relatively short time period, by the year 2018.

Traditionally, toxicity testing is conducted on vertebrate animals. According to Abbott (2005) each chemical that goes through the multiple tests required for the registration can use up to 5 000 laboratory animals. However, new approaches to obtain reliable data on safety of chemicals have been developed which are remarkably faster, less expensive, and more ethical than experiments on animals. These new approaches/techniques can significantly reduce the need for the *in vivo* tests with vertebrate animals. Nowadays, 3R's (**R**eduction, **R**eplacement, **R**efinement) strategy introduced by Russell and Burch in 1959 in the book 'The principles of humane experimental technique' has become a worldwide acknowledged and an EU-level prioritized strategy for the reduction of the use of laboratory animals in fundamental studies as well as in the legislature-driven research. REACH should also promote the development of alternative methods for the assessment of hazards of substances. To further support the 3R's policy, **I**ntegrated **T**esting **S**trategies, ITS (combination of the available existing toxicity data, *in silico*, *in vitro, in vivo etc* approaches) have been envisaged.

The work of the current thesis was performed in the frame of the EU FP6 Integrated Project OSIRIS (Optimized Strategies for Risk assessment of industrial chemicals through integration of non-test and test information, 2007-2011). The aim of the study was to contribute to the development of integrated test strategies for REACH by: i) collecting the existing publicly available toxicity data for REACH-relevant chemicals from Russian language sources; ii) experimental determination of ecotoxicity of 58 congeneric anilines and phenols with bioluminescent bacteria *Vibrio fischeri* in the high-throughput Flash Assay format and iii) comparison of the experimental toxicity data across species as well as for QSAR predictions.

ABBREVIATIONS

CAS	Chemical Abstracts Service (Columbus, Ohio, USA); www.cas.org
CEFIC	The European Chemical Industry Council (Brussels, Belgium); www.cefic.org/
CHEMLIST®	Regulated Chemicals Listing, produced by CAS, is an electronic collection of thousands of chemical substances that are regulated in key markets across the globe
CLP	Classification, Labelling and Packaging
CSA	Chemical Safety Assessment
CSR	Chemical Safety Report
EC	European Commission
EC_{50}	The median effective concentration of the toxicant that induces a designated effect in 50% of the test organisms after a specified exposure time
ECHA	European Chemicals Agency (Helsinki, Finland); www.echa.europa.eu/
ECOSAR	ECOlogical Structure Activity Relationships; http://www.epa.gov/oppt/newchems/tools/21ecosar.htm
ECOTOX	ECOTOXicology database; http://cfpub.epa.gov/ecotox/
ECVAM	European Centre for the Validation of Alternative Methods (Ispra, Italy); http://ecvam.jrc.it/
EINECS	European Inventory of Existing Commercial Chemical Substances (substances that were commercially available in the EU from January 1st 1971 to September 18th 1981)
ELINCS	European List of Notified Chemical Substances (substances that became commercially available in the EU after September 18th 1981)
EU	European Union
EU27	The 27 member countries of the European Union: Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovak Republic, Slovenia, Spain, Sweden, United Kingdom.
GHS	Globally Harmonized System
GLP	Good Laboratory Practice
HPVC	High Production Volume Chemicals. Chemicals placed on the EU market in volumes exceeding 1000 tonnes per year per manufacturer or importer.
INH	Inhibition
ITS	Integrated Testing Strategies

IUCLID	International Uniform Chemical Information Database; http://esis.jrc.ec.europa.eu/index.php?PGM=dat
JRC	Joint Research Centre (Ispra, Italy); http://ec.europa.eu/dgs/jrc/index.cfm
K_{ow}	Octanol-water partition coefficient
LD_{50}	The median lethal dose of the toxicant that induces death in 50% of the test organisms after a specified exposure time
LDLo	Lethal Dose Low, the minimum amount of a chemical which tests have shown will be lethal to a specified type of animal
LOAEL	Lowest Observed Adverse Effect Level
MAC	Maximum Allowable Concentration
OECD	Organisation for Economic Co-operation and Development (Paris, France); www.oecd.org/
OSIRIS	Optimized Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information (EU 6th Framework Integrated Project; contract no. GOCE-CT-2007-037017); www.osiris-reach.eu/
(Q)SAR	(Quantitative) Structure Activity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SciFinder	CAS database of chemical and bibliographic information
SDS	Safety Data Sheet
TOXNET	TOXicology Data NETwork; http://toxnet.nlm.nih.gov/
USA	The United States of America
USSR	The Union of Soviet Socialist Republics

TERMS

Alternative test - alternative techniques that can provide the same level of information as current animal tests, but which use fewer animals, cause less suffering or avoid the use of animals completely. Such methods, as they become available, must be considered wherever possible for hazard characterisation and consequent classification and labelling for intrinsic hazards and chemical safety assessment.

Article is an object which during production is given a special shape, surface or design which determines its function to a greater degree than does its chemical composition (e.g. manufactured goods such as cars, textiles, electronic chips).

Chemical Abstracts Service (CAS) is a division of the American Chemical Society and is the world's leading source of chemical information. CAS is located in Columbus, Ohio, USA. www.cas.org/

Chemical Safety Assessment (CSA) is the process aimed at determining the risk posed by a substance and, as part of the exposure assessment, develop exposure scenarios including risk management measures to control the risks. It is carried out for all registered substances manufactured or imported at 10 tonnes per year or greater.

Chemical Safety Report (CSR) documents the chemical safety assessment (CSA).

European Chemicals Agency (ECHA) is an agency of the EU, which manages the technical, scientific and administrative aspects of REACH Regulation. It is the driving force among regulatory authorities in implementing the EU's groundbreaking chemical legislation for the benefit of human health and the environment as well as for innovation and competitiveness. ECHA was established in June 1, 2007 and is located in Helsinki, Finland. www.echa.europa.eu/

Exposure-based waiving is one of the elements in integrated testing strategies, which is used in situations where human or environmental exposure to chemical substances is absent or so low that additional information on effects will not lead to improvement of risk management and derogation (omission) of standard testing requirements can be justified.

Globally Harmonised System (GHS) of classification and labelling of chemicals is an internationally agreed system set to replace the various different classification and labelling standards used in different countries. It is contained within the UN GHS Document (referred to as 'Purple Book'), which is not a formal treaty but a non-legally binding international agreement that provides countries with a regulatory framework to develop or modify existing programmes. Countries (or trading blocks) must create local or national legislation to implement the GHS. Within Europe, for example, Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging of Hazardous Substances and Mixtures (CLP) has been developed.

Good Laboratory Practice (GLP) is a quality system concerned with the organizational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported.
Grouping of chemicals is the development of chemical categories or analogue approach. A chemical category is a group of chemicals whose physico-chemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristic). In principle, more members are generally present in a chemical category, enabling the detection of trends across endpoints. As the number of possible chemicals being grouped into a category increases, the potential for developing hypotheses for specific endpoints and making generalisations about the trends within the category will also increase, and hence increase the robustness of the evaluation. The term 'analogue approach' is used when the grouping is based on a very limited number of chemicals, where trends in properties are not apparent.
***In silico* test** is a method performed on computer or *via* computer simulation.
***In vitro* test** literally stands for 'in glass' and refers to the technique of performing a given experiment in a test tube, or, generally, in a controlled test conditions outside a living organism. Tests with invertebrate animals such as protozoa, daphnids and nematodes, but also with bacteria and plants, e.g., ecotoxicological tests, are also often considered as *in vitro* tests.
***In vivo* test** is a test conducted within a living organism.
Non phase-in substance is a 'new' chemical/substance, i.e., has not previously been placed on the EU market (is newly regulated under REACH), falls outside the transitional arrangement and requires a registration before manufacture, import or placing on the EU market.
Phase-in substance is an 'old'/existing chemical/substance, i.e., already produced or on the market before REACH came into force and is covered by/subject to transitional arrangements (if pre-registered).
QSARs and SARs (Q(SAR)) are theoretical models that can be used to predict in a quantitative or qualitative manner the physicochemical, biological (e.g. (eco)toxicological) and environmental fate properties of compounds from knowledge of their chemical structure. A SAR is a qualitative relationship that relates a (sub)structure to the presence or absence of a property or activity of interest. A QSAR is a mathematical model relating one or more quantitative parameters, which are derived from the chemical structure, to a quantitative measure of a property or activity.
Read-across is a method of filling in data gaps for a substance by using surrogate data from another substance. Read-across can be between two substances or through a group or category of chemicals. The groups are selected on the assumption that the properties of a series of chemicals with common structural features will show similar trends in their physico-chemical properties and in their toxicological effects or environmental fate properties.

Safety data sheet (SDS) is the main tool used in industry for communicating information on the hazard of dangerous substances and preparations through the supply chain.
The European Chemical Industry Council (CEFIC) is the main European trade association for the chemical industry. It was founded in 1959, and its history follows through the creation of the European Union. Its headquarters are located in Brussels, Belgium. www.cefic.org/
Threshold of Toxicological Concern is a concept that refers to the establishment of a human exposure threshold values for all chemicals, or groups of chemicals, below which there should be no appreciable risk to human health.
Validated test is a test for which its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose.
Validation is the process by which the reliability and relevance of a test method are evaluated for the purpose of supporting a specific use.
Verhaar classes are the most common method to group chemicals according to the mode of action. Verhaar scheme distinguishes four classes based on structural features of the molecules: class 1 – inert chemicals or non-polar narcotics; class 2 – less inert chemicals or polar narcotics; class 3 – reactive chemicals; and class 4 – specifically acting chemicals (Verhaar et al., 1992).
Weight of evidence is an evidence based approach, which involves an assessment of the relative values/weights of different pieces of the available information that have been retrieved and gathered in the chemical safety assessment. To this end, a value needs to be assigned to each piece of information. These weights/values can be assigned either in an objective way by using a formalized procedure or by using expert judgment. The weight given to the available evidence will be influenced by factors such as the quality of the data, consistency of results, nature and severity of effects, relevance of the information for the given regulatory endpoint, leading to the assumption/conclusion that a substance has or has not a particular dangerous property, while the information from each single source alone is regarded insufficient to support this notion.
White Paper on the Strategy for a future Chemicals Policy was adopted by the European Commission on the 13th of February, 2001. The White Paper proposed to establish a central entity for the administration of the REACH system and the provision of technical and scientific support.

1. LITERATURE REVIEW

1.1. Global chemical industry

The progress that has accompanied the industrial revolution and caused tremendous changes in transportation, agro- and medical industries, just to name a few areas, are largely due to the progress in chemical industry and chemical engineering. Currently, chemical industry is one of the largest industrial sectors in the world. It is also one of the most diverse and the most regulated of all industries (OECD, 2001). Global sales of the chemical industry in 2009 were valued at €1871 billion. The chemical industry in Europe is the third largest and one of the most competitive industrial sectors, accounting for around 30% of the total chemical production in the world. In 2009 EU produced €449 billion worth of chemicals (Fig. 1.1). Taken together, the European Union, Asia and North American Free Trade Area account for 89.7% of the world's turnover in terms of chemical sales. Chemical industry includes all the companies that use raw materials to produce chemical substances and all the companies down the line that alter or blend these substances. More than one third of the world's top thirty chemical companies have their headquarters in the EU. The largest European producers of chemicals are Germany, France, Italy and United Kingdom. Together, these four countries account for 60% of the EU chemical sales, valued at €269 billion in 2009 (CEFIC, 2010; Fig. 1.1).

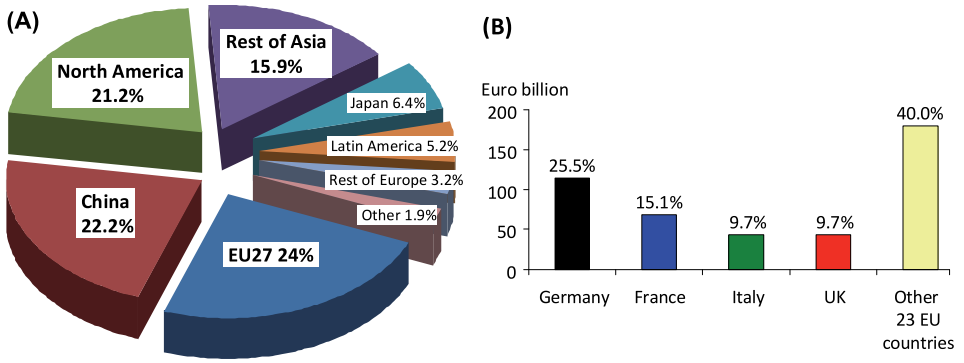

Figure 1.1. Geographic breakdown of the world chemical sales in 2009 (A) and four top chemical-producing countries in the EU (B).
Based on the data from the report of CEFIC, 2010.

Around 30 000 chemical companies in the EU employ a total staff of about 1.2 million people. Another three million employees work in the sectors using the output of the chemical industry and thus depend on its competitiveness (EC's Directorate-General for Trade, 2010). The output from the EU chemical industry covers three wide ranges of products: base chemicals, specialty chemicals and

consumer chemicals. **Base chemicals** cover petrochemicals and derivatives and basic inorganics. They are produced in large volumes and sold within the chemical industry itself or to other industries and comprised around 60% of the total output of EU chemical industry in 2009. **Specialty chemicals** cover the auxiliaries for industry, paints and inks, crop protection, and dyes and pigments. These chemicals are produced in small volumes but nevertheless represented 26% of total EU chemical sales in 2009. **Consumer chemicals** such as soaps and detergents, perfumes and cosmetics are sold to final consumers. Consumer chemicals represented 14% of total EU chemical sales in 2009 (CEFIC, 2010).

1.1.1. Chemical industry in Estonia

With only 1.3 million inhabitants Estonia comprises one of the smallest populations in the EU. From 1940 to 1991 it was occupied by Soviet Union. In August 1991 Estonia regained its independence and in 2004 became a member of the EU and the North Atlantic Treaty Organization (NATO). Historically, Estonia has been an agricultural country and thus chemical industry forms small proportion of Estonian industry: its contribution to the country's gross domestic product (GDP) is relatively low (0.7% in 2008). However, the productivity per worker in chemical industry is the highest compared to other sectors. Currently, the Federation of Estonian Chemical Industries (FECI), which is a non-profit organization representing Estonian chemical industry, has about 50 members. Estonian chemical industry is characterized by strong territorial concentration, as more than half of the chemical industry is located in the north-eastern part of Estonia (Viru Keemia Grupp AS, Nitrofert AS, Kiviõli Keemiatööstuse OÜ, Eastman Specialties AS, Molycorp Silmet AS etc). The most important chemical sectors are the oil shale industry and the production of rare metals, rare earth metals and their oxides. Up to 85% of the production of chemical industry is exported into the member states of the EU. The main export articles are shale oil and –phenols, benzoic acid, sodium benzoate and plasticizers, rare earth metals and their oxides, the sealants and construction adhesives, paints, varnishes and other finishing materials. Export volumes of consumer chemicals are more modest, but Estonia has a long-term experience in producing cosmetics and household chemicals (FECI, 2010).

1.2. Global chemical policy

Regulation has a significant impact on the chemical industry. The quality of legislation, correct implementation and proper enforcement are not only of high significance for the achievement of health and environmental objectives, but also for the competitiveness of the chemical industry. Various countries and regions (e.g. Canada, Australia, Japan, China, Korea, Russia, USA, EU) of the world have established their own management systems for chemicals. However, the common objective is the control of hazardous chemicals and protection of

humans and the environment. Over the last decade, many regions have changed or are going to change its chemical legislation according to the evolving scientific information, testing capabilities, and public concern that chemicals in commerce require more attention (REACH in EU; Chemicals Management Plan in Canada; proposed 'Safe Chemicals Act' in USA).

Currently, the Chemical Abstracts Service (CAS) Registry, which is the most authoritative collection of disclosed chemical substance information, covers more than 63 million organic and inorganic substances identified from the scientific literature from 1957 to the present (as of September 5, 2011), with additional substances going back to the early 1900`s (CAS, 2011). According to CHEMLIST® produced by CAS there are around 300 000 chemical substances that are regulated in key markets across the globe. In the EU more than 100 000 chemicals are registered with 100 204 existing substances in EINECS (EC, 1990) and 5 292 new substances in ELINCS (Baraibar et al., 2009). It is uncertain how many synthetic chemicals are on the market and how many of those people are exposed to, but estimates vary from 30 000 to 100 000 (CEC, 2001; Schörling, 2004; Rovida and Hartung, 2009). Most of the substances produced by the chemical industry eventually end up in the environment and, *via* the environment, in humans. Thus, various industrial chemicals can be found in water, soil, air, plants, animals and humans. However, reliable toxicity data exist for only a small proportion of these chemicals. Existing (EINECS) substances account for 99% of the chemicals on the European market (Vogelgesang, 2002). However, the knowledge on the use, fate and (eco)toxicological properties of these substances remains insufficient, e.g. **limited or no data were available for 86% of high production volume chemicals** (HPVCs) by 1999 (Allanou et al., 1999).

1.3. Legislation on industrial chemicals in the EU

In EU the legally binding frameworks for the control of chemicals date back as far as to the 1960s (Harremoës et al., 2001; van Leeuwen et al., 2007). In 1967 the European Community adopted Council Directive 67/548/EEC on the classification, packaging and labelling of dangerous substances (EC, 1967). Legislation for dealing with dangerous preparations was introduced in 1988 with the Directive 88/379/EEC. Gradually the legislation was established to different uses of chemicals (e.g. plant protection products, human and veterinary drugs, food and feed additives, cosmetics, fertilisers; explosives, etc). In 1990s the need for a review of the legislation on industrial chemicals was recognized as there was a general lack of knowledge on properties and uses of most chemicals on the market (Allanou et al., 1999), the burden of proof laid on the public authorities and the identification and management of risks was problematic (Bodar et al., 2005).

Currently, the main regulatory challenges are the implementation of REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) (EC,

2006) and CLP (Classification, Labelling and Packaging) (EC, 2008a) Regulation.

1.3.1. REACH Regulation (EC) No. 1907/2006

In 2001 European Commission issued a White Paper (CEC, 2001) that proposed a major new policy called the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), which entered into force on June 1, 2007 (EC, 2006). REACH replaced approximately 40 different regulations with a single, streamlined and improved regulation and created the European Chemicals Agency (ECHA) situated in Helsinki, Finland. REACH has been designed not to overlap or conflict with the rest of chemical legislation. The main changes in REACH compared to previous legislation were that it created a single coherent system for all industrial chemicals (i.e. new and existing substances) and transferred the burden of proof for demonstrating the safe use of chemicals from EU Member State authorities to the chemical industry (CEC, 2001).

The main aims of REACH are to:

- improve the protection of the environment and human health from the risks that can be posed by chemicals;
- promote non-animal testing (alternative methods) for the assessment of hazards of substances;
- enhance the competitiveness of the EU chemical industry, a key sector for the economy of the EU;
- ensure the free circulation of chemical substances in the EU.

1.3.1.1. REACH: implications to Estonian chemical industry

As for all EU countries REACH Regulation poses a major challenge also for the Estonian chemical industry. It has been suggested that the chemical industry in Estonia will be affected by REACH at a greater extent than some other EU countries because the sector of the specialty chemicals represents a large part of Estonian chemical industry (Angerer et al., 2007). In Estonia the competent authority responsible for REACH is the Health Board (www.terviseamet.ee).

1.3.1.2. Registration of chemicals under REACH

The scope of REACH is broad. In principle it applies to all chemicals, which are manufactured, placed on the market (imported) or used as substances on their own, in preparations or in articles. The Registration is only one aspect of REACH, Evaluation, Authorisation and Restriction being the complementary ones. A major component of REACH is the requirement for manufacturers or importers to register the substances produced or imported above 1 tonne per company per year with the European Chemicals Agency (ECHA). REACH provisions are being phased-in over 11 years and will cover approximately

30 000 – 100 000 substances in total (Pedersen et al., 2003; Rovida and Hartung, 2009). Regulation foresees three registration phases, the first one ended on November 30, 2010, the two following ones are planned to end in 2013 and 2018, respectively (Fig. 1.2). Companies not registering their substances within this time-frame will no longer be able to manufacture or supply these chemicals legally, i.e. 'no data, no market'.

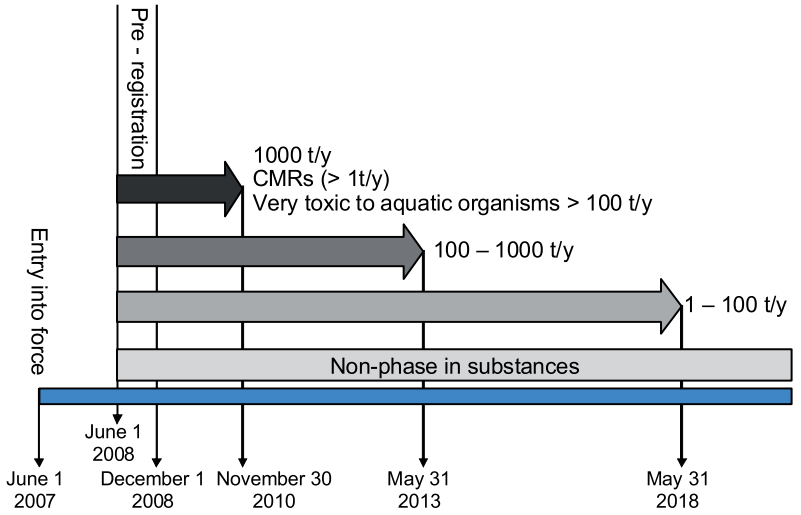

Figure 1.2. The overall time-schedule for registration under REACH. The regulation distinguishes between 'phase-in' (old/existing) and 'non-phase-in' (new) substances (see Terms). 'Non-phase-in' substances do not benefit from the transitional regime provided for 'phase-in' substances under REACH and therefore have to be registered before manufacture or import starts. Priority has been given to the substances manufactured/imported in high volumes and to the substances of high concern e.g. carcinogenic, mutagenic, reprotoxic substances (CMRs) and substances very toxic to aquatic organisms. *Adapted from the guidance document of ECHA, 2011a.*

Registration information requirements depend on the tonnage of the substance (REACH Annexes VII to X). The standard information for all substances includes:

- the identification of the substance;
- information on the physicochemical properties;
- **toxicological information**;
- **ecotoxicological information**;
- information on manufacture and use(s);
- information on human and environmental exposure;
- the classification and labelling;
- guidance on safe use.

One of the most important aspects from the above mentioned list is the identification of the (hazardous) properties (physicochemical, toxicological and

ecotoxicological information) of the chemical substances. However, the determination of the toxicity and ecotoxicity is very expensive and time-consuming as it involves also the testing of the substances on vertebrate animals. (Eco)toxicity studies are used to evaluate the potential of a chemical substance to cause damage to humans and/or the environment and need to be conducted according to the principles of Good Laboratory Practice, GLP (OECD, 1998) and to the test guidelines published in the Test Methods Regulation 440/2008/EC (EC, 2008b).

1.3.1.3. Costs and benefits

Costs and benefits of implementing REACH have been widely discussed ever since the respective White Paper was introduced in 2001. Many reports have been composed by the Commission as well as by the Member States and industry to estimate the costs (both financially and in terms of the number of animals used for toxicity testing) and the benefits (both in terms of human and environmental health) of the regulation. A comprehensive overview of the first impact studies was published in October 2004 as a summary report of 36 impact assessments (Witmond et al., 2004). The estimates of the associated costs varied greatly, from €2 billion to hundreds of billions. The EU Commission estimated that REACH would cost the EU chemical industry around €2.3 billion (with a maximum total cost for industry €5.2 billion) and about 3 million vertebrate test animals (mammals, birds and fish) but would yield health benefits of at least €50 billion (CEC, 2003, van der Jagt et al., 2004), whereas the industry claimed that the costs would be much higher. Further studies went on and in June 2005 a meeting of the Competitiveness Council concluded that with 50 studies sufficient work had been done on the impact assessments of REACH (Competitiveness Council, 2005). In 2005 the European Environment Bureau (EEB) and the World Wildlife Fund (WWF) published a critical analysis of the impact assessments of REACH (EEB and WWF, 2005) and pointed out the deficiencies of main impact studies. The recent study made by Rovida and Hartung in 2009 suggested that REACH would require 54 million vertebrate animals and cost €9.5 billion over a decade. This is about 20 times more animals and 4 times more money compared to EU official estimates. ECHA however, has emphasized that the above mentioned study overestimates the impact of REACH on animal testing and the real figures are more likely to be the ones originally provided by EC (ECHA press release, 2009). Thus, despite the number of studies available it is still difficult to give a reliable estimate of the costs and benefits of REACH.

1.3.2. Classification, Labelling and Packaging Regulation (EC) No. 1272/2008

REACH is strongly linked to the EC Regulation No. 1272/2008 on the classification, labelling and packaging (CLP) of substances and mixtures (EC,

2008a), which entered into force on January 20, 2009. It complements REACH and is based on the United Nations' Globally Harmonized System (GHS), which goal is to harmonize worldwide hazard classification and labelling systems to promote the safe use of chemicals and facilitate international trade of chemicals. Chemicals placed on the European market must be classified, labelled and packaged according to the CLP Regulation. It aims to determine whether a substance or mixture displays properties that lead to classification as 'hazardous'.

CLP Regulation replaces two previous directives (Dangerous Substances Directive; EC, 1967 and Dangerous Preparations Directive; EC, 1999) in a stepwise approach by the year 2015. The concept of CLP is the same as for the previous directives: it deals with classification, hazard communication through labelling and packaging of hazardous chemicals. The main differences are for example in terminology (e.g. 'preparation', 'dangerous', 'category of danger', 'risk and safety phrase' are now replaced with 'mixture', 'hazardous', 'hazard class', and, 'hazard and precautionary statement', respectively), classification criteria (e.g. hazard classes), labelling elements (e.g. pictograms) and classification procedure (e.g. notification of the classification and labelling of substances to the Classification & Labelling Inventory) (ECHA, 2009a). It is the task of industry to establish the hazards of chemicals before they are placed on the market and to classify them in line with the identified hazards. The classification describes the potential of substances to cause harm to human beings or the environment. The data on the label has to inform all those who handle the chemical about its hazards. In case of hazardous substances supplier must also provide a Safety Data Sheet (SDS) to its industrial customers in the supply chain. Companies must notify the classification and labelling of substances to the Classification & Labelling Inventory, which will be established and maintained by ECHA (the public version of the Inventory is expected to be available by the end of 2011). The inventory is a database that will contain basic classification and labelling information on notified and registered substances received from manufacturers and importers. The first notification deadline was January 3, 2011 and concerned all substances, which were on the market before December 1, 2010. By this date, ECHA had received 3.1 million notifications of 107 067 substances for the Classification and Labelling Inventory (ECHA press release, 2011). For substances placed on the market after December 1, 2010, companies must submit the notification to the Classification & Labelling Inventory within one month after placing the substance on the market.

1.4. Regulatory toxicology and safety assessment

Toxicology dates back to the earliest humans, who used animal venoms and plant extracts for hunting, warfare, and assassination (about 1500 B.C) (Gallo, 2001). Various specialized subdisciplines, e.g. food toxicology, forensic toxicology, clinical toxicology, ecotoxicology, regulatory toxicology etc, have been developed over time. The aim of regulatory toxicology is to protect public

health by regulating exposure to potentially harmful chemicals (Beck et al., 2001). Regulatory toxicology encompasses the collection, processing and evaluation of epidemiological as well as experimental toxicology data to permit toxicologically based decisions on the protection of health against harmful effects of chemical substances. It deals also with concepts for risk assessment and management of substances with potentially toxic properties. Furthermore, regulatory toxicology supports the development of standard protocols and new testing methods in order to continuously improve the scientific basis for decision-making processes (Schwenk et al., 2002). Laws and regulations provide the framework for organized efforts to prevent toxic outcomes and to protect humans and the environment from hazardous effects of chemicals. Sanctions are necessary to prevent those without social conscience from deliberately exposing their fellow citizens to risks from toxic hazards. The development of the legislation requires a good interaction between and corresponding input from different stakeholders, e.g. scientists, society, private and public sector.

1.4.1. Risk assessment as a central instrument in the control of chemicals under REACH Regulation

'Human risk assessment' as a decision making instrument was introduced about thirty years ago and can be defined as the systematic scientific characterization of potential adverse health effects resulting from human exposures to hazardous agents or situations (NRC, 1983). 'Environmental risk assessment' is a wider term and covers the risk to the ecosystem, including humans, exposed or impacted *via* air, water and land (EHSC, 2008).

Risk assessment is a central theme in chemical control under REACH, either explicitly or implicitly. It is conducted as a part of the chemical safety assessment (CSA) in accordance with Article 14 of REACH. CSA is required for all registered substances manufactured or imported in quantities of 10 tonnes or more per year. It is the main endpoint for data assessment under REACH in which hazard and exposure data are considered together to assess the risk of a substance. Overall process related to information requirements and chemical safety assessment under REACH is shown in Fig. 1.3. The CSA starts with the collection of all available physicochemical, environmental fate, toxicological and ecotoxicological information that is relevant and available. When as a result of the hazard assessment it is found that a substance meets the criteria for classification as hazardous, an exposure assessment (consists of the development of exposure scenarios and the related exposure estimation) is required. The iterative CSA process of hazard assessment, exposure assessment and risk characterization ends when the information requirements for intrinsic properties are fulfilled and risks are shown to be controlled for all exposures and all exposure scenarios. CSA is documented in a chemical safety report (CSR) and communicated to downstream users of the substance *via* safety data sheet (SDS) (ECHA, 2008a).

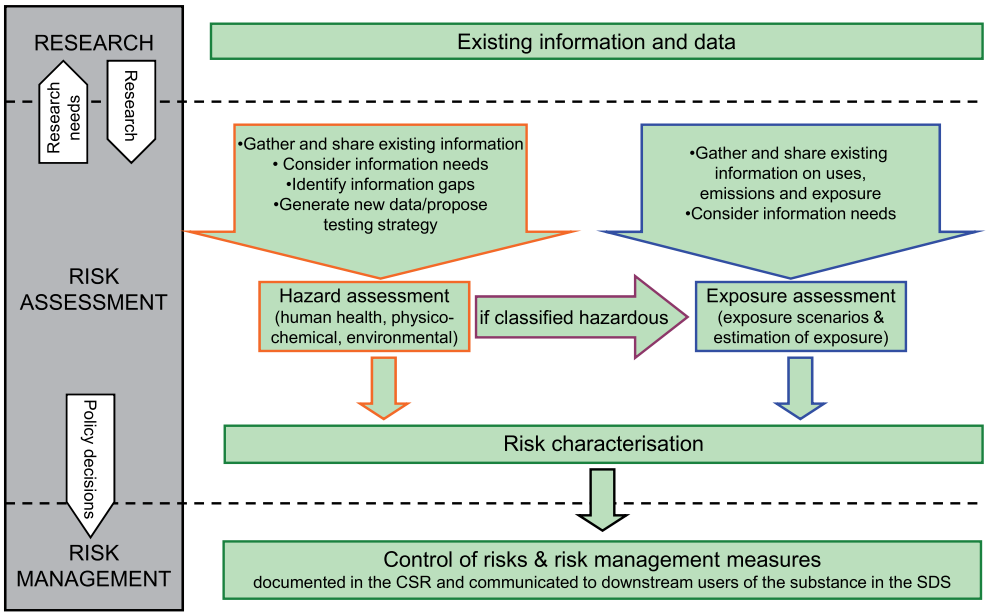

Figure 1.3. Overall process related to information requirements and chemical safety assessment under REACH. Also the connections between the research and risk assessment process are shown, indicating that the risk assessment process drives new research, and new research findings modify the risk assessment outcomes. *Adapted from ECHA, 2008a and Faustman and Omenn, 2001.*

1.4.2. *In vivo* testing in safety assessment

As mentioned above, safety testing of chemicals has to be carried out before they can be marketed. Knowledge of toxicity is primarily obtained from *in vivo* methods (experimental studies on animals), *in vitro* methods (studies using e.g, human cells, invertebrate animals, microbes, plants) and epidemiological studies of exposed humans (e.g. occupational exposures).

In regulatory toxicology, decision-making about hazards and risks requires data that include toxicological test results. A broad range of animal tests has been developed to predict possible adverse effects of chemical substances to humans (EC, 2008b). Researchers and regulatory agencies develop and adopt these test methods or strategies to ensure that toxicological data are scientifically sound, consistent, and usable in the risk assessment process. Traditionally, safety evaluation is conducted in specialized laboratories using laboratory animals (*in vivo* testing). In fact, almost all of the products used and consumed by humans have to be tested on animals (Timbrell, 2002; EC, 2010a). In broader terms, the modern animal experimentation began in 17th century in England and France (Olsson et al., 2003). More specifically, the history of testing the chemical products on animals traces back to 1930s. Two first and most common *in vivo* test used were the acute oral toxicity test (LD_{50} test; Trevan, 1927) and the acute

eye irritation test (Draize Test; Draize et al., 1944). Since 2001 the LD_{50} test has been deleted in OECD and EU test guidelines (Schlede et al., 2005) because of the cruelty to animals and the lack of validity/sensitivity of the test to humans. It is replaced by the other *in vivo* alternatives (e.g. fixed dose procedure, OECD, 2002a; acute toxic class method, OECD, 2002b; up-and-down procedure, OECD, 2008), which use fewer animals. Also, several *in vitro* alternative methods have been developed to the Draize Test (e.g. isolated chicken eye test, OECD, 2009a; bovine cornea opacity test, OECD, 2009b; cytosensor microphysiometer test, OECD, 2010).

As an example of the numbers of animals used in some experiments performed by former Soviet Union toxicologists, 180 white rats (180–240 g), 130 white mice (18–24 g) and 40 rabbits (2–2.5 kg), were used to derive a maximum allowable concentration (MAC) limits for benzonitrile in the working area air (Agaev, 1977).

The estimation of the numbers of animals used in toxicity testing worldwide is difficult as many countries do not provide statistics on it (according to the British Union for the Abolition of Vivisection 79% of countries don't appear to publish the number of animals they use). Based on available data, it is estimated that around 115 million vertebrate animals are used worldwide annually (Taylor et al., 2008). According to statistics published by the EC 12 million vertebrate animals (regulatory toxicity testing comprises about 10% of all animals use) were used in experiments in the 27 Member States of EU in 2008 (EC, 2010a). Mice (59%) and rats (17%) were by far the most used species (Table 1.1).

Table 1.1. Percentages of different groups of vertebrate animals used for experimental purposes in EU in 2008 (EC, 2010a).

Group	Percentage used, %
Mice	59.3
Rats	17.7
Guinea pigs	1.84
Other rodents	0.6
Rabbits	2.78
Cold-blooded animals	9.62
Birds	6.38
Artio+Perissodactyla	1.39
Carnivores	0.26
Prosimians+monkeys+apes	0.08
Other Mammals	0.05

The largest percentage (almost 45%) of animals in toxicological and other safety evaluations is used in acute and sub-acute toxicity tests. Together with the animals used for sub-chronic and chronic toxicity testing, the percentage of animals used in short and long term systemic toxicity testing accounts for 55% in this area (Fig. 1.4).

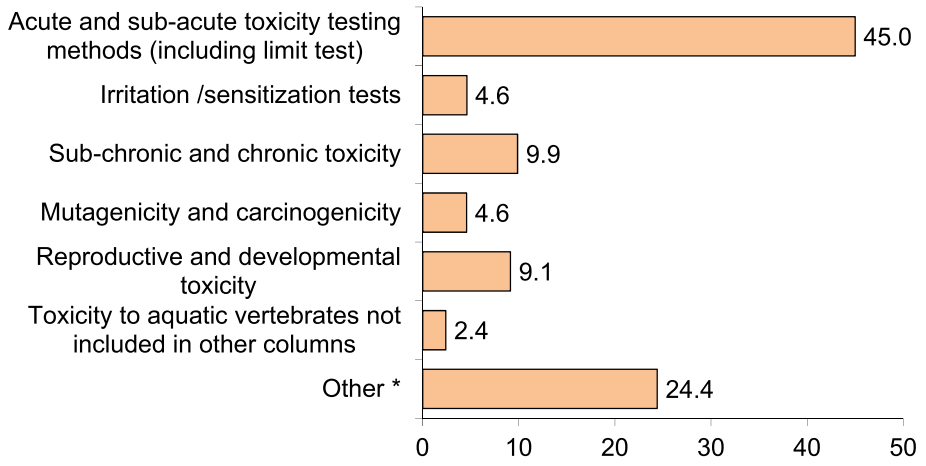

Figure 1.4. Percentages of animals used in different toxicity tests for toxicological and other* safety evaluation. *Adopted from the report of EC, 2010a.*
** 'other' designates the tests for biological screening for pharmaceutical, healthcare and veterinary products. It includes neurotoxicity, toxicokinetics, testing of biological evaluation of medical devices; intracutaneous testing of reactivity in rabbits, studies into the penetration of nanoparticles through tissue and their biocompatibility, studies into the evaluation of sensitization potential of dyestuffs used in the textile industry and pharmacological studies included in safety tests.*

It is obvious that implementation of REACH will result in an increased use of laboratory animals for the next decade. Latest statistics of scientific procedures on living animals in Great Britain in 2010 showed that the level of animal experimentation continues to rise. Interestingly, experiments on genetically modified (GM) animals are now accounting for about 50% of all the procedures (Hudson, 2011). The exposure of laboratory animals to hazardous chemicals cause pain, distress and suffering to animals (Russel and Burch, 1959; Stokes, 2002; Spielmann, 2002; Nuffield Council on Bioethics, 2005). In addition, animal experiments are time-consuming, problematic in economic terms and from an ethical point of view. Animal experiments are conducted in order to extrapolate the toxicity data for humans. However, it remains problematic how relevant these data are to humans (Garattini, 1985; Voisin et al., 1990) and it will sometimes be appropriate to ask whether the resources a study consumes could have been used more effectively (Spielmann, 2009; Holmes et al, 2010).

1.4.3. Alternatives to the animal testing in safety assessment

In the context of the laboratory animal use, alternative methods include all procedures that can refine, reduce or replace the animal use for toxicity testing. The concept of the 3R's (reduction, refinement and replacement) was introduced

by Russell and Burch already in 1959. It took about 20 years until the scientific institutions and the public became increasingly concerned about the use of animals in toxicity testing. In EU, the concept of 3R's was adopted as a law in 1986, Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes (EC, 1986). In 2010, it was updated and replaced by the Directive 2010/63/EU (EU, 2010). The directive will take full effect from January 1, 2013. The aim of the new directive is to strengthen legislation and improve the welfare of those animals still needed to be used as well as to firmly anchor the principle of the 3R's in EU legislation. Over the last thirty years there has been a considerable effort to develop and validate alternative (*in vitro*) test systems to animal testing with the aim of increasing their use in regulatory area (Carere et al., 2002). In recent years non-animal testing has gained higher profile in toxicology due to the convergence of scientific, ethical/animal welfare, financial and legislative imperatives. Toxicity testing in the 21st century is at a pivotal juncture where future toxicity testing is envisioned without animal experimentation and relies mainly on understanding 'toxicity pathways'—the cellular response pathways that can result in adverse health effects when sufficiently perturbed (NRC, 2007; Hartung, 2009; Berg et al., 2011, Hartung, 2011).

According to Worth (2004) the two major alternatives to animal testing in addition to physicochemical methods are:

1. *in silico* methods – computer-based models, e.g. (Quantitative) Structure Activity Relationships, (Q)SARs (section 1.4.3.1);
2. *in vitro* methods, in which biological effects are observed in cell cultures, tissues, organs (section 1.4.3.2)

In EU the two key legislations reinforcing the development of alternative methods are REACH and the 7th amendment of the Cosmetics Directive 76/768/EEC (the directive prohibits to put animal-tested cosmetics on the market in Europe after 2013). Within the legal text of REACH, it is emphasized that the vertebrate test data has to be shared to avoid repeating or duplicating animal experiments. In addition, in order to minimize the number of animal tests, REACH also provides a number of possibilities to adapt the testing requirements and use existing data and alternative assessment approaches instead, for example:

Article 13 ... *information shall be generated whenever possible by means other than vertebrate animal tests, through the use of alternative methods, for example, in vitro methods or qualitative or quantitative structure-activity relationship models or from information from structurally related substances (grouping or read-across) ...*

Article 25 ... *In order to avoid animal testing, testing on vertebrate animals for the purposes of this Regulation shall be undertaken only as a last resort. It is also necessary to take measures limiting duplication of other tests. ...*

The most likely scenario for REACH according to the EU Joint Research Centre (JRC) is that the use of alternative methods could save 1.3 million test

animals (van der Jagt et al., 2004). If no reduction measures are taken into account, testing under REACH would require about 4 150 test animals per one chemical, which is produced at above 1 000 tonnes per year, and about 20 animals for each chemical produced at between 1 and 10 tonne per year (Schoeters, 2010). Thus, the replacement of animal tests with a modern, non-animal testing strategy would lead to a number of improvements. This would both encourage innovation and benefit all stakeholders concerned with human safety, environmental protection, animal welfare and consumer confidence. The number of new substances and products entering our lives is growing, but the number of tools to assess their safety is practically not increasing (Hartung, 2011). The EU Commission supports the idea of alternative testing methods, but the development and validation of these methods is a slow process (Spielmann and Liebsch, 2001; Schaafsma et al., 2009; Hartung, 2011), and many are unlikely to be in place in time for the implementation of REACH (Rovida and Hartung, 2009) or the 7th amendment of the Cosmetics Directive 76/768/EEC (Adler et al., 2011). However, more effort should be made by the EC to promote the use of alternative non-animal methods and more precise guidelines should be developed on how to implement these methods in the regulatory purposes (Balls and Clothier, 2010; Spielmann, 2010). Currently, the main emphasis in the development of alternative non-animal methods is on five toxicological areas, i.e. toxicokinetics, repeated dose toxicity, carcinogenicity, skin sensitisation, and reproductive toxicity, and on more extensive use of already developed alternative methods (van der Jagt et al., 2004; EC, 2010b). Regarding the alternative (non-animal) methods for cosmetics testing it was recently confirmed that it will take at least another 7–9 years for the replacement of the current *in vivo* animal tests used for skin sensitisation. For toxicokinetics the timeframe was 5–7 years to develop the models for predicting the lung absorption and renal/biliary excretion, and even longer to integrate the methods to fully replace the animal toxicokinetic models. For the systemic toxicological endpoints of repeated dose toxicity, carcinogenicity and reproductive toxicity, the time horizon for full replacement could not be estimated (Adler et al., 2011). Thus, for both economical and ethical reasons, there is a great demand for new non-animal tests on these five endpoints. One of the most recent achievements are in the field of reproductive toxicity where improvements have been made in the Embryonic Stem cell Test (EST) to predict embryotoxicity *in vitro* (Seiler and Spielmann, 2011).

Nevertheless, a number of successfully validated alternative tests have already been accepted and the harmonization of test guidelines at the international level has significantly reduced testing in animals (Spielmann, 2002; Worth and Balls, 2002). A comprehensive list of the current status of alternative tests in EU can be found on The European Centre for the Validation of Alternative Methods (ECVAM) website. They have established a comprehensive information system, the DataBase Service on ALternative Methods (DB-ALM, http://ecvam-dbalm.jrc.ec.europa.eu/) that provides detailed information on

various aspects of animal alternatives (e.g. summaries of method descriptions and protocols for their performance, details on validation studies, persons and institutions active in the field of alternative methods, etc.) with focus on toxicology assessments of chemicals. The current international state-of-play in the validation and acceptance of alternative methods during 1998-2010 is reviewed in ECVAMS's most recent review (Zuang et al., 2010). Another website dedicated to advancing non-animal methods for the toxicity testing through online discussion and information exchange is AltTox.org (http://alttox.org/) (Stephens and Ward, 2010).

1.4.3.1. *In silico* methods

The term *in silico* is used to express 'performed on computer or *via* computer simulation'. The phrase was coined in 1989 as an analogy to the Latin phrases *in vivo* and *in vitro* which are commonly used in biology and refer to the experiments conducted in living organisms and outside of living organisms, respectively (Wikipedia, http://en.wikipedia.org/wiki/In_silico). Hartung and Hoffmann (2009) have defined *in silico* methodologies in toxicology as *"anything we can do with a computer in toxicology, and there are few tests that would not fall into this category, as most make use of computer-based planning and/or analysis."* Altogether they identified nine types of *in silico* approaches (Q)SARs as the most prominent technique.

In silico methods are extensively used for predicting toxicity worldwide e.g. in the USA, Canada, Japan and Australia. In EU, these methods have recently become increasingly important due to REACH and the Cosmetics Directive. In assessing the risk that a chemical may pose to human health or to the environment, focus is now being directed towards exploitation of *in silico* methods to replace *in vivo* or *in vitro* techniques (Cronin and Madden 2010).

Within REACH *in silico* methods are for example (Q)SARs, grouping of chemicals, read-across and expert systems (diverse group of models consisting of combinations of (Q)SARs and databases) (ECHA, 2008b). The organization which coordinates the framework of computational toxicology and modelling in EU is the Joint Research Centre (JRC). To promote the availability of reliable computer-based estimation methods for use in the regulatory assessment of chemicals they have developed a range of freely available software tools, which were reviewed by Pavan and Worth in 2008 and are accessible from the JRC webpage (http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology):

- JRC QSAR Model Database, an online database which hosts structured and peer-reviewed information on QSAR Models;
- Toxtree, a software tool which predicts mechanisms of action or toxicological effects by applying decision-tree approaches;
- DART, (Decision Analysis by Ranking Techniques) software tool designed for the ranking of chemicals according to their environmental and toxicological concern;

- Toxmatch, an application for grouping chemicals on the basis of chemical similarity. It is designed to facilitate the formation of chemical categories and the application of read-across.
- Stat4tox, a software tool which carries out concentration-response analysis for *in vitro* experiments.

The list of available QSARs for the purposes of REACH can be found from the JRC QSAR Model Database (http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/doc/JRC_QMRFs_published.xls; Appendix 1 of the current thesis). The OECD Principles for validation of QSAR models (OECD, 2004) are indispensable for the assessment of the validation status and its regulatory applicability. Notably, the Estonian company MolCode AS (www.molcode.com), is one of the leading providers of REACH compliant QSAR testing. They have one of the largest database of JRC approved QSAR models, validated in accordance with the OECD principles. Currently they have developed >30 QSAR models covering different toxicity endpoints (e.g. acute toxicity, eye irritation, biodegradation, etc.; Appendix I of the current thesis).

In addition to the above mentioned EC sources a free online resource, QSAR World (http://www.qsarworld.com/index.php), targeted to professionals, researchers and students, has been developed for QSAR modelling and contains extensive lists of freely downloadable datasets, databases and programs.

1.4.3.2. *In vitro* methods

The term *'in vitro'* literally stands for 'in glass' and refers to the technique of performing a given experiment in a test tube. Most often, *in vitro* tests in biology are considered as tests with animal (or human) cell cultures. However, this definition is currently broader and tests with invertebrate animals such as protozoa, algae, daphnids and nematodes, but also with bacteria and plants, i.e., ecotoxicological tests, are also considered as *in vitro* tests (Isomaa and Lilius, 1995).

Over the last 30 years, alternative, non-animal test systems (mainly cell cultures) have been introduced to supplement and, in some cases, replace toxicity tests using animals (Carere et al., 2002), contributing to the 3R's concept. In addition, several international projects (e.g. MEIC, 1989-1996; ACuteTox, 2005-2010; ReProTect, 2004-2009; Sens-it-iv, 2005-2010; carcinoGENOMICS, 2006-2011; Predict-IV, 2008-2013; COLIPA-DG RTD Joint Research Initiative, 2009; OSIRIS, 2007-2011) aimed to develop alternative methods or validate the predictability of *in vitro* tests have been launched in recent years. For example, the aim of the MEIC (**M**ulticenter **E**valuation of ***In Vitro*** **C**ytotoxicity) programme, organised by the Scandinavian Society of Cell Toxicology (Bondesson et al., 1989; Ekwall et al., 1989), was to evaluate the relevance and reliability of a wide variety of *in vitro* tests for predicting general toxicity in more complex biological systems. In addition to animal cell cultures (comprising majority of the assays) also several

ecotoxicological test systems such as luminescent bacteria were used in MEIC project (Kahru and Borchardt, 1994). The results from the MEIC programme showed a good correlation (R^2=0.7) between *in vitro* basal cytotoxicity data and human lethal blood concentrations (Ekwall, 1999). As a follow-up of the MEIC Programme, the FP6 ACuteTox project was launched with the overall aim to develop and pre-validate a simple and robust *in vitro* testing strategy for prediction of human acute systemic toxicity, which could replace the animal acute toxicity tests for regulatory purposes (Clemedson et al., 2007). While the number of reference chemicals in MEIC project was 50, in the course of the AcuteTox project 97 reference chemicals (50% of which were drugs and 30% industrial chemicals, 20% others) were tested in approximately 100 *in vitro* assays, including models for general acute cytotoxicity, metabolism-mediated toxicity, biokinetics, haemato-, immuno-, neuro-, nephro-, and hepatotoxicity. In parallel, human and animal *in vivo* (LD_{50}) data for these substances were collected from published literature. The data generated in the project are stored in a novel internet-based database AcutoxBase (Kinsner-Ovaskainen et al., 2009) developed within the project, and have been used to assess the intralaboratory variability, the preliminary predictive capacity and in some cases also the interlaboratory variability of each *in vitro* assay. An in depth statistical analysis of the large dataset generated in this project resulted in a list of 8 *in vitro* and *in silico* methods, which resulted to be the most promising for inclusion in the proposal of potential testing strategies. Protocols of all these methods will be available to the public as INVITTOX protocols, through the ECVAM database on alternative methods (DB-ALM).

1.4.3.3. The use of existing toxicity data

REACH Annexes VII – X state:
"Before new tests are carried out ... ***all available*** *in vitro data, in vivo data, historical human data, data from valid (Q)SARs and data from structurally related substances (read-across approach) shall be assessed first."*

The existing (eco)toxicity data can be found in several easily accessible electronic databases. The ECOTOX database, created and maintained by the U.S. Environmental Protection Agency (EPA), is one of the most comprehensive collections of toxicity data for aquatic and terrestrial species. The IUCLID Chemical Data Sheets Information System provides data on High Production Volume Chemicals (HPVC) reported by the European industry. This EU HPVC list contains toxicological data on 2465 chemicals (Allanou et al., 1999). However, these and other relevant databases such as the International Programme on Chemical Safety (IPCS) INCHEM and TOXNET still represent only a small fraction of chemicals requiring toxicity assessment. Besides these there are many smaller and not so well-known databases which could also be useful for toxicological information retrieval. However, as the amount of information is rapidly increasing it is quite difficult to orientate and to find the necessary data. Furthermore, for some of the old but useful data there is limited

electronic access (low level of digitalization) and in some areas one faces language barrier (papers **I**, **II**). Several attempts have been made to integrate the existing toxicological information from different databases and projects (section 1.4.3.4.1.). The most recent one is the creation of eChemPortal (http://www.echemportal.org/), the Global Portal to Information on Chemical Substances developed by the OECD. It provides free public access and direct links to collections of chemical information (e.g. physico-chemical and toxicological data), which is submitted to government chemical review programmes at national, regional and international levels. Currently, 24 data sources have been included in eChemPortal. The list of data sources is available from the eChemPortal webpage (Appendix 2 of the current thesis).

1.4.3.4. Integrated Testing Strategies (ITS)

Integrated (or sometimes also referred to as Intelligent) Testing Strategies (ITSs) originate from the mid-1990s, from the research initiatives on minimizing animal use in toxicity testing (Barratt et al., 1995). The concept of integrated testing strategy has been initially defined by Blaauboer et al. (1999) as follows:
"An integrated testing strategy is any approach to the evaluation of toxicity which serves to reduce, refine or replace an existing animal procedure, and which is based on the use of two or more of the following: physicochemical data, in vitro data, human data (for example, epidemiological, clinical case reports), animal data (where unavoidable), computational methods (such as quantitative structure-activity relationships (QSAR)) and biokinetic models."

The focus of ITS is especially on the development of strategies based on the tests at cellular level (*in vitro*) and mathematical methods (*in silico*). The mathematical methods are needed for the assessment of exposure and the relation between effects and chemical structure. Some tests with experimental animals (*in vivo*) will also remain necessary. Integration of multiple approaches and methods has a greater potential to provide relevant data for a weight of evidence approach in the safety evaluation and risk assessment than individual tests, even if they have high predictive capacity (Hoffmann and Hartung, 2006). In this way, all possible available information on a substance can be optimally used and further testing will only be required where essential information is lacking (Bradbury et al., 2004). ITSs were developed and used for regulatory purposes already before REACH. However, REACH has led to a considerable expansion of systematic research on ITS development for a broad range of toxicological endpoints. Under REACH, ITSs are assumed to speed up hazard and risk assessment of chemicals, while reducing testing costs and animal use (Grindon et al., 2006; Ahlers et al., 2008; Lilienblum et al., 2008). It has been estimated that applying ITS could reduce the need for testing by up to 70% resulting in significant savings in testing costs and use of animals (van der Jagt et al., 2004). Recently, several ITSs for environmental and human health endpoints have been proposed in the literature (Jaworksa et al., 2010), many of

them were suggested by the regulatory bodies such as ECHA (ECHA, 2008c,d,e).

1.4.3.4.1. EU FP6 Project OSIRIS: contribution to REACH

Within the EU 6th Framework Integrated Project OSIRIS (Optimized Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information, http://www.osiris-reach.eu/), 2007-2011, involving altogether 31 partners from 14 EU countries and with the total cost of 10 million Eur the overall aim was to develop integrated testing strategies (ITS) fit for REACH that enable to significantly increase the use of non-testing information for regulatory decision making, and thus to minimise the need for animal testing (Appendix 3 of the current thesis).

By the time OSIRIS project started, the concept of ITS was already widely acknowledged as the most efficient approach towards the aims of REACH. However, concerted action and intensive efforts were needed to translate the concept into a workable, consensually acceptable, and scientifically sound strategy (JRC, 2005; Combes and Balls 2005). OSIRIS was designed to meet this challenge by developing integrated testing strategies fit for regulatory purposes that fulfil both safety criteria as well as animal welfare goals, and that are practicable both from a technical and an economic perspective. The basic idea was to obtain the information needed for carrying out hazard and risk assessments for large numbers of substances by integrating multiple methods and approaches. The realistic aim was not to abolish all animal tests under REACH, but to reduce them to the minimum level needed from a risk perspective. The starting point of OSIRIS was the concept of 'Intelligent Testing Strategies' for regulatory endpoints outlined by Joint Research Centre (JRC, 2005). Six components (*in vitro* tests, optimized *in vivo* tests, (Q)SARs and *in silico* methods, read-across and chemical categories, threshold of toxicological concern and exposure-based waiving) of ITS proposed by JRC served as cross-cutting themes to feed the decision-theory framework to be developed, possibly augmented by additional ITS components evolving during the OSIRIS project. Accordingly, the task of OSIRIS was to undertake distinct research into the scientific scope and regulatory applicability of these components. Within OSIRIS, a large number of compound-related data sets were built. The data sets cover physicochemical, toxicological and ecotoxicological data within the chemical domain of REACH for risk-targeted prioritization. All these data are organized in a central OSIRIS database. Technically, the OSIRIS database system is a special edition derived from an ongoing development of the QSAR software system ChemProp (Chemical Properties Estimation Software System). (Eco)toxicological data collection started with integrating the existing databases (e.g. Tox-2, REPDOSE, Breath, ISSCAN, CAESAR, TOX-1, CAESAR, CEFIC LRI Gold Standard) provided by OSIRIS partners covering a wide variety of REACH-relevant endpoints. In addition, specific efforts were undertaken to

investigate the Russian literature as a novel data source for toxicity data (papers **I**, **II**).

The full list of OSIRIS methods and models are outlined in the OSIRIS flyer (Appendix 3 of the current thesis). The methods and ITS developed are implemented in the OSIRIS Webtool (http://osiris.simpple.com). When using the Webtool users may add their own additional data to that available in OSIRIS and the Web tool. These data can be taken into account in the testing strategy. As an outcome the ITS tool combines all available testing and non-testing data and conclude if there is sufficient information for Classification & Labelling and Risk Assessment. In the case of data gaps, the ITS tool proposes the most appropriate method to acquire the missing information. Ideally, with regard to the 3R's principle non-testing methods such as *in vitro* assays and QSAR methods are preferred for this purpose. OSIRIS Webtool will be made freely available for end-users from industry, regulatory authorities and academia.

In conclusion, in the frame of OSIRIS an extensive research has been conducted on ITS development and improvements have been made to incorporate all relevant information for updating and reducing uncertainty across testing stages, and for handling conditionally dependent evidence (Vonk et al., 2009; Jaworksa et al., 2010; Jaworska and Hoffmann, 2010).

1.4.3.5. The use of alternatives to testing on animals for REACH

In order to have an overview of the current situation in Europe related to substances with hazardous properties and companies using such substances, the results from the pre-registration phase (June 1–December 1, 2008) and the registration dossiers that were submitted between June 1, 2008 and February 28, 2011 were analysed and the results are commented below. The objective of pre-registration was to facilitate sharing of data between registrants, where possible, in order to reduce unnecessary testing, especially on vertebrate animals, and to decrease costs for the industry (ECHA, 2011a). During the six months of the pre-registration period more than 65 000 companies submitted more than 2 750 000 pre-registration dossiers, which covered nearly 150 000 different substances (ECHA, 2009b). These numbers represent the starting point of the REACH process. Between 2008 and February 2011 ECHA has received 24 560 registration dossiers for 3 308 phase-in and 1 347 non-phase-in substances (at all tonnages). Testing proposals were made in 574 dossiers covering a total of 1 175 tests of which 711 were vertebrate animal studies (Fig. 1.5).

ECHA`s analysis shows that in total 1 491 new *in vitro* studies and 1 849 new animal studies have been conducted since REACH entered into force. Although the animal experiments cannot be conducted without permission, 107 studies on animals were found to be conducted in the absence of the respective proposal (ECHA, 2011b).

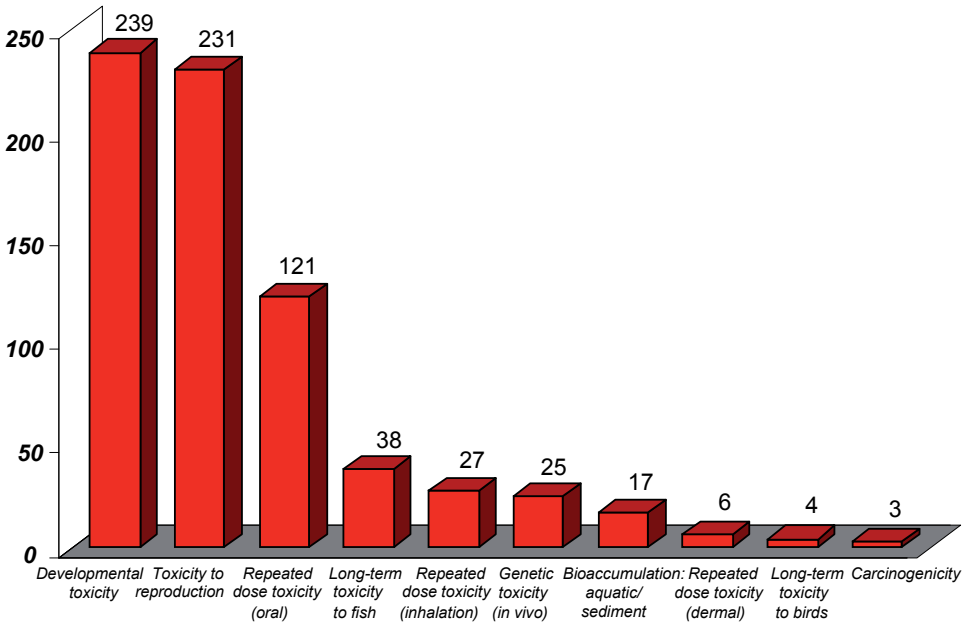

Figure 1.5. Number of proposals submitted to ECHA on testing using vertebrate animals, according to the 24 560 registration dossiers submitted by registrants from June 1 until 28 February 2011. *Based on data from ECHA, 2011b.*

In the context of this thesis it is of interest to discuss the use of alternatives to testing on animals by industry. For this purpose data from the ECHA`s latest report (ECHA, 2011b) regarding Endpoint Study Record (ESR) approach is used. It provides an overall picture on data availability and the relative proportions of the principal options used by registrants to fill the information requirements per toxicity endpoint. These options have been categorised as follows: 'testing proposals', 'experimental studies' (*in vitro* and *in vivo* studies are treated separately if applicable) and alternative methods such as 'read-across', 'to omit the study' (the submission of the required data by choosing the appropriate option from those available from 'data waiving', these options are to be used to indicate when testing does not appear to be: scientifically necessary; technically not possible; or not necessary based on low exposure considerations), 'weight of evidence' (covers various combinations of old experimental data, literature information and read-across possibilities and it is assumed that it does not contain new animal tests performed for the registered substance) and '(Q)SAR studies'. The above described options have been used for the graphical illustration of the findings of registration dossiers for phase-in substances at or above 1 000 tonnes *per annum* (Fig. 1.6).

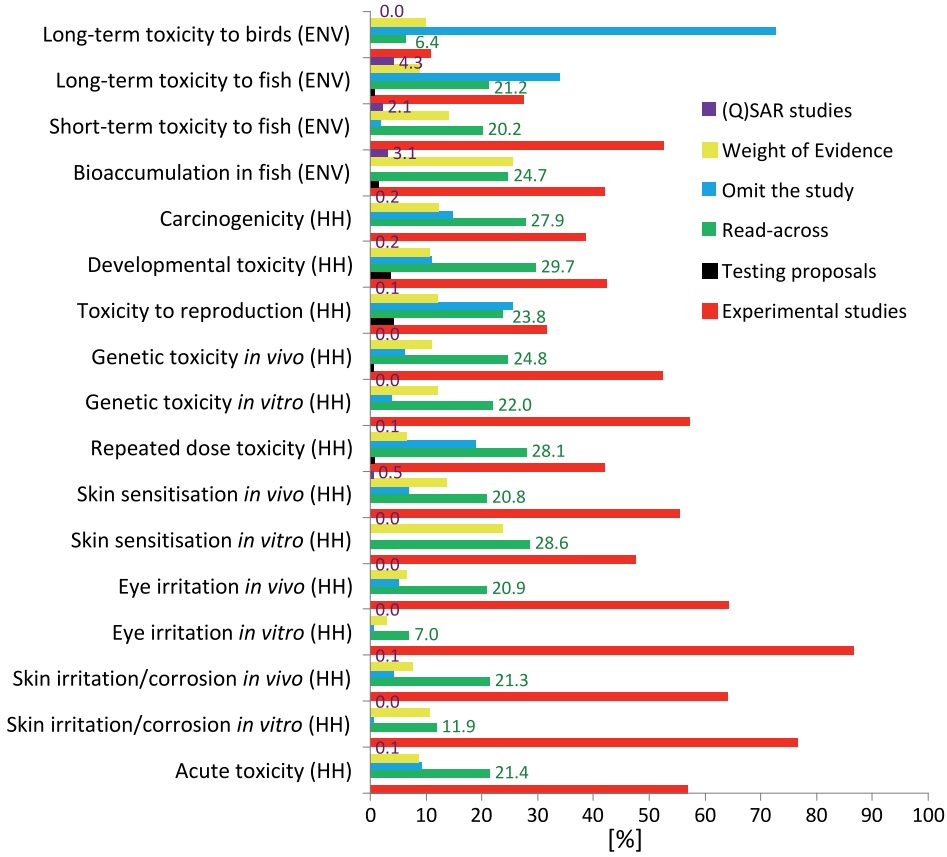

Figure 1.6. The use of alternatives to testing on animals for REACH according to the analysis of endpoint study records submitted for the 1 504 dossiers for a given endpoint for phase-in substances at or above 1 000 tonnes per annum. ENV – environmental endpoint; HH – human health endpoint. *Based on the data from the report of ECHA, 2011b.*

It can be seen from Fig. 1.6 that registrants used data from experimental studies as the main source of information to meet the information requirements under REACH. Notably, from the alternative approaches **read-across** was the most widely used method. It was used for all human health endpoints in about 20% and for developmental, carcinogenicity and repeated dose toxicity up to 30%. At the same time (Q)SARs were used only in about 1% of cases.

1.5. Bacteria – the unicellular organisms for the cost-effective *in vitro* toxicity screening

Bacteria are the simplest systems for toxicological studies on the whole-cell level. In addition, in case of bacteria the cell also means a whole organism with all of its functional complexities. Microbiologist Rex Burch, one of the founders

of 3R's concept has stated already more than 50 years ago (Russel and Burch, 1959): '*The uses of microorganisms in some other contexts have yet to be explored*'. Indeed, bacteria are attractive alternatives to higher organisms in toxicity testing: they are easy to grow in laboratory conditions, they can be stored freeze-dried, tested in small volumes in high numbers (>10^6 organisms per ml) enabling high-throughput analysis. One of the advantages of bacterial assays is their high statistical significance since the observed response is produced by a large number of cells. In addition, bacteria possess several attributes that support their use for toxicity testing: the small size (i.e., high surface to volume ratio), the relatively simple morphology (for example the lack of mitochondria), providing many target sites at or near the cytoplasmic membrane (Kahru, 1993). For chemicals with a toxic action common for different types of organisms, such as interference with cellular membranes, bacteria should be the most suitable test system for rapid screening of toxic potency. In addition, bacteria can be easily genetically modified that allows tailoring of new organisms for targeted testing. The most well-known bacterial *in vitro* test is the Ames assay (OECD, 1997) with *Salmonella typhimurium* (Claxton et al., 2010), which may predict genotoxic effects of chemicals also to higher organisms (e.g., humans).

In the frame of the current thesis the focus was set on the test system using naturally luminescent gram-negative bacteria *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum* and recently re-classified as *Aliivibrio fischeri*) (Urbanczyk et al., 2007). Luminescent bacteria are predominantly found in the marine environment, but freshwater and terrestrial species are also known. They are free-living, or as symbionts in light organs of fish (Hastings et al., 1987). *V. fischeri* is probaby the most widely used bacterium for (eco)toxicological studies (Kaiser and Devillers, 1994) but also remarkably often used for the development of QSARs (Cronin and Schultz, 1997). Naturally luminescent bacteria have been used for toxicity testing since 1979, when the first commercialized bacterial bioluminescence inhibition assay, Microtox™ test, involving the *V. fischeri* strain NRRL B-11177 was introduced (Bulich and Isenberg, 1981). Currently, the acute toxicity test with this specific strain is commercially available also under other trademarks (e.g., ToxAlert™, LUMIStox™, BioTox™) (Jennings et al., 2001). The standards for *V. fischeri* luminescence inhibition toxicity assays (ISO, 1998; ASTM, 2009) for aqueous samples have been developed and recently also a kinetic format of the *V. fischeri* bioluminescence inhibition assay for sediments, solids and coloured samples (Lappalainen et al., 1999; Põllumaa et al., 2000) has been standardized (ISO, 2010). The latter test format of *V. fischeri* (Flash Assay) has also been used for high-throughput screening of the toxicity of metal oxide as well as organic nanoparticles that are 'problematic' test substances due to the color/turbidity/insolubility/aggregation of primary particles (Mortimer et al., 2008). There are toxicity data for more than 1 000 chemicals available for *V. fischeri* (Kaiser and Devillers, 1994) that, as noted above, can be used for QSARs (Cronin and Schultz, 1997). Notably, the *V. fischeri*

bioluminescence inhibition assay has been most widely used for ecotoxicological studies (Wolska et al., 2007) as bacteria are ubiquitous organisms in soil and water and important link in the terrestrial and aquatic food-webs as decomposers. The genes encoding luminescence in naturally luminous bacteria (e.g., *V. fischeri*, *Photorhabdus luminescens*) have been inserted also to other common bacteria such as *Escherichia coli* and used as easily detectable acute toxicity reporters for toxicity screening of chemicals (Kurvet et al., 2011). In the study of Kurvet et al. two different recombinant luminous *E. coli* strains were used (pSLlux and pDNlux) and data obtained with both constructs significantly correlated with *V. fischeri* results.

In *V. fischeri*, luxCDABE genes are responsible for their bioluminescent reaction. LuxCDE genes encode a fatty acid reductase complex involved in synthesis of the long chain aliphatic aldehyde (RCHO) substrate for the luminescence reaction catalysed by the luciferase LuxAB subunits (Meighen, 1991). Bacterial luciferase enzymes mediate the oxidation of reduced flavin mononucleotide ($FMNH_2$) and long chain fatty acid aldehyde (RCHO) by molecular oxygen (O_2) to produce bioluminescence (blue-green light emission) with a maximum intensity at about 490 nm. The overall reaction can be summarized as:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + light$$

For the regeneration of $FMNH_2$ cellular NADH is needed and due to that the bioluminescence of the bacteria is intrinsically tied to their central metabolism. Thus, any damage of cellular metabolism caused by the toxicity of a sample could be monitored by measuring the change in light output of bacteria, the degree of toxicity being proportional to the light loss (Bulich and Isenberg, 1981; Hastings, 1978).

Light production is a measure of the overall 'well-being' of the bacteria. The concentration of the chemical, which causes a 50% reduction in light (INH%=50) after a certain exposure time (usually 5, 15 or 30 minutes) is designated as respective EC_{50}. The bioluminescence inhibition assay offers a rapid, simple and sensitive method to test a wide spectrum of chemical substances (Kahru, 1993; Kahru and Borchardt, 1994; Loibner et al., 2004; Mortimer et al., 2008) and environmental samples including wastewater, solid waste, soil and sludge extracts (Lapa et al., 2002; Wang et al., 2002; Manusadzianas et al., 2003; Põllumaa et al., 2004). A number of comparisons of the *V. fischeri* test (Microtox^{TM}) with other standardized bioassays have been made (Kaiser, 1998). Using 47 MEIC reference chemicals, it has been shown that the 5-min EC_{50} values of *Photobacterium phosphoreum* for the chemicals correlated with data from the literature: octanol/water partition coefficients, acute toxicity data for daphnids, fish, animal and human cell line, rodents, dog and man. The log-log correlation coefficients (R^2) ranged between 0.20-0.79, depending on the data compared (Kahru, 2006). Thus, natural luminous bacteria *V. fischeri* have already proven their potential in toxicity testing.

AIMS OF THE STUDY

For the implementation of EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation, the hazard of a large group of chemicals (around 100 000) needs to be assessed in a relatively short time period. This is an enormous challenge to the European chemical industry and thus needs strong support and assistance from the scientists in providing (eco)toxicological information beneficial for the development of intelligent testing strategies (ITS), e.g. by exploring existing *in vivo* toxicity data and using Quantitative-Structure-Activity-Relationship (QSAR) approaches. The application of ITS facilitates the use of alternative testing information for regulatory decision making, thus minimizing the need for animal testing according to the 3R's strategy (**R**eduction, **R**eplacement, **R**efinement).

The aim of the thesis was to contribute to the development of integrated testing strategies by:

- mapping, collecting, and critically analysing the existing publicly available toxicity data for REACH-relevant chemicals from Russian language data sources (scientific publications, databases, etc) (paper **I**);
- creating an open-access online web-database of chemical toxicity data collected from publicly available Russian language data sources (paper **II**);
- experimental determination of the toxicity of 58 congeneric substituted anilines and phenols relevant for REACH and QSARs using the bioluminescence inhibition assay of the naturally luminescent bacteria *Vibrio fischeri* in the high-throughput Flash Assay format (papers **III, IV**);
- critical analysis of the experimentally obtained data on *Vibrio fischeri* toxicity and comparison with toxicity data to other aquatic organisms determined in our laboratory using the same set of chemicals (papers **III, IV**):
    - comparison of the toxicity of five selected anilines to two crustacean species *(Daphnia magna, Thamnocephalus platyurus)* and protozoa *Tetrahymena thermophila* (paper **III**);
    - using the set of 58 anilines and phenols to compare their toxicity to bacteria *V. fischeri* and algae *P. subcapitata* (paper **IV**)*;*
    - using data on 58 anilines and phenols to evaluate the predictive power of *V. fischeri* toxicity data for ranking of the chemicals to different hazard classes and (paper (**IV**);
    - evaluation of the use of the obtained *V. fischeri* toxicity data on 58 anilines and phenols for the hydrophobicity-based QSARs (paper **IV**).

2. MATERIALS AND METHODS

2.1. Mapping, collecting, and analysing the toxicity data of chemicals from Russian language sources

The mapping of the landscape of toxicological data sources in Russian language is described in the review article (paper **I**) that covers the following sub-areas:

- toxicological research in Russia and former Soviet Union;
- the current key actors in toxicological research in Russia and former Soviet Union (specifically in Estonia);
- bibliometrical analysis of toxicological information published in the Russian language;
- main resources (e.g. libraries, institutions, websites, web-based databases, etc) for collecting toxicological and ecotoxicological documents in Russian language;
- main documents containing toxicological data published in the Russian language;
- impact factors of Russian scientific journals;
- comparison of the Russian database "Hazardous Substances" with analogous European and American databases.

The search was performed in Estonian but also in Russian libraries as well as on websites. The types of information sources searched included books, journal articles, electronic databases and PhD dissertations. Altogether around 1 000 items were analysed (Fig. 2.1).

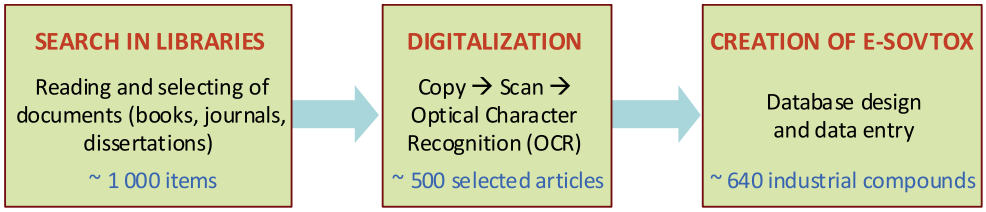

Figure 2.1. The schematic description of the creation the E-SovTox database.

2.2. Creating an open-access online database E-SovTox

Before the creation of E-SovTox database, different Western databases were analysed. It was found that some articles or abstracts of Russian scientific papers were available in English in a few public access Western databases. Examination of different databases was useful for elaboration of the final database format of the E-SovTox. Following literature analysis, main documents containing toxicological data published in the Russian language were selected for the

database. Literature examined dated back to the 1950s. The information included in the E-SovTox database derived mainly from scientific peer-reviewed journals published during the Soviet Union era and original papers were linked directly to the toxicity data. The printed text was scanned and selected parts of each scanned article were digitalized by ABBYY FineReader 9.0 Professional Edition Optical Character Recognition System to convert scanned versions of paper documents to editable and searchable PDF-documents. Criteria for the choice of the chemicals/endpoints for the database were based on the requirements of REACH but also 3R's strategy. Thus, the main emphasis was put on collecting *in vivo* toxicity data focusing on the long-term toxicity (most rare information and most 'expensive' research for REACH in terms of both cost and lives of experimental animals, van der Jagt et al., 2004).

The criteria for selecting the toxicity endpoints were as follows:
- relevance to REACH (e.g. animal usage, cost etc);
- use of animals (number of animals per test);
- cost of the endpoint/test;
- potential for savings;
- requirement for modelling.

Toxicity endpoints/tests considered were as follows:
- two-generation reproductive toxicity;
- developmental toxicity;
- mutagenicity;
- carcinogenicity;
- skin sensitisation;
- long-term fish toxicity;
- accumulation in aquatic species;
- short-term repeated dose toxicity;
- sub-chronic toxicity;
- long-term repeated dose toxicity;
- short-term fish toxicity;
- acute inhalation toxicity;
- acute oral toxicity;
- *in vivo* eye irritation;
- *in vivo* skin irritation;
- long-term bird toxicity.

The identification of chemicals was carried out using different databases containing chemical information (PubChem, TOXNET, IPCS INCHEM, ESIS, ECOTOX Database, SciFinder, Merck Index). The whole process of the creation of the E-SovTox database is schematically described in Fig. 2.1.

Characterisation of the E-SovTox Database
— name of the Database: E-SovTox;
— web address: http://kbfi-databases.eu/;
— fee: free after registration;
— year of availability of the demo-version: 2010;
— hardware and software required: any computer with Internet capabilities;
— programming language: HTML/PHP/MySQL;
— programmed by: Protopro-websites OÜ (www. dynameeks.com)

The database is hosted by the Laboratory of Molecular Genetics (MGL), National Institute of Chemical Physics and Biophysics, Tallinn, Estonia. The MGL is in charge of data collection and editing, database maintenance and dissemination. The scientific leader of the MGL either acts as or nominates the administrator who grants user access.

2.3. Congeneric set of 58 substituted anilines and phenols for the toxicological and QSAR analysis

The analogous sets of substituted anilines and phenols (Fig. 2.2, Table 2.1), based on their industrial and commercial importance (sections 1.1, 3.2), were selected for the experimental toxicity testing. The 58 anilines and phenols studied for toxicity were ≥95% pure and purchased from Sigma-Aldrich (Schnelldorf, Germany), Acros Organics (Geel, Belgium), TCI Europe (Antwerp, Belgium), Merck (Schuchardt, Germany) and Fluka (Buchs, Switzerland) (Table 1 in paper **IV**).

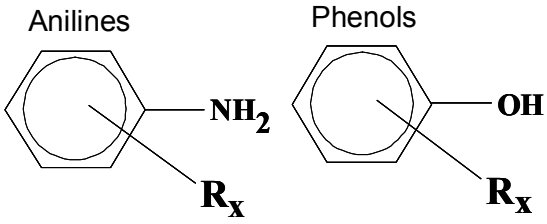

Figure 2.2. General formula of the studied compounds.
R stands for -Cl, $-CH_3$ or $-CH_2-CH_3$, x is 1, 2 or 3.

Table 2.1. Congeneric sets of 58 selected anilines and phenols chosen for the current study (paper **IV**).

Phenols	Abbreviation	Anilines	Abbreviation
Phenol	P	Aniline	A
2-chlorophenol	2-CP	2-chloroaniline	2-CA
3-chlorophenol	3-CP	3-chloroaniline	3-CA
4-chlorophenol	4-CP	4-chloroaniline	4-CA
2,3-dichlorophenol	2,3-DCP	2,3-dichloroaniline	2,3-DCA
2,4-dichlorophenol	2,4-DCP	2,4-dichloroaniline	2,4-DCA
2,5-dichlorophenol	2,5-DCP	2,5-dichloroaniline	2,5-DCA
2,6-dichlorophenol	2,6-DCP	2,6-dichloroaniline	2,6-DCA
3,4-dichlorophenol	3,4-DCP	3,4-dichloroaniline	3,4-DCA
3,5-dichlorophenol	3,5-DCP	3,5-dichloroaniline	3,5-DCA
2,3,4-trichlorophenol	2,3,4-TCP	2,3,4-trichloroaniline	2,3,4-TCA
2,3,5-trichlorophenol	2,3,5-TCP	-	-
2,3,6-trichlorophenol	2,3,6-TCP	-	-
2,4,5-trichlorophenol	2,4,5-TCP	2,4,5-trichloroaniline	2,4,5-TCA
2,4,6-trichlorophenol	2,4,6-TCP	2,4,6-trichloroaniline	
3,4,5-trichlorophenol	3,4,5-TCP	3,4,5-trichloroaniline	3,4,5-TCA
2-methylphenol	2-MP	2-methylaniline	2-MA
3-methylphenol	3-MP	3-methylaniline	3-MA
4-methylphenol	4-MP	4-methylaniline	4-MA
2,3-dimethylphenol	2,3-DMP	2,3-dimethylaniline	2,3-DMA
2,4-dimethylphenol	2,4-DMP	2,4-dimethylaniline	2,4-DMA
2,5-dimethylphenol	2,5-DMP	2,5-dimethylaniline	2,5-DMA
2,6-dimethylphenol	2,6-DMP	2,6-dimethylaniline	2,6-DMA
3,4-dimethylphenol	3,4-DMP	3,4-dimethylaniline	3,4-DMA
3,5-dimethylphenol	3,5-DMP	3,5-dimethylaniline	3,5-DMA
2,3,5-trimethylphenol	2,3,5-TMP	-	-
2,3,6-trimethylphenol	2,3,6-TMP	-	-
2,4,6-trimethylphenol	2,4,6-TMP	2,4,6-trimethylaniline	2,4,6-TMA
2-ethylphenol	2-EP	2-ethylaniline	2-EA
3-ethylphenol	3-EP	3-ethylaniline	3-EA
4-ethylphenol	4-EP	4-ethylaniline	4-EA
2,6-diethylphenol	2,6-DEP	2,6-diethylaniline	2,6-DEA

2.4. Toxicity testing with *Vibrio fischeri* – kinetic luminescent bacteria test (modified Flash Assay)

Toxicity tests were conducted with the bioluminescent bacterium *Vibrio fischeri* strain NRRL B-11177. The bacterial suspension used for the toxicity measurements was prepared from freeze-dried bacteria originating from

V. fischeri reagent (Aboatox, Turku, Finland; papers **III, IV**). The kinetic luminescent bacteria test using the bacterium *V. fischeri* (acute test, exposure times of 30 s, 15 and 30 min) was performed at room-temperature (~ 20° C) in 96-well polypropylene white microplates (Greiner Bio-One, Frickenhausen, Germany) using the modified Flash Assay protocol (ISO, 2010). Briefly, 100 µl of test solution in 2% NaCl was pipetted into each well, which was supplemented with 100 µl of bacterial suspension by automatic dispensing in the Microplate Luminometer Orion II (Berthold Detection Systems, Pforzheim, Germany; Fig. 2.3) testing chamber. The luminescence was recorded during the first 30 seconds (Fig. 2.3) after the dispensing of the bacterial suspension in each well, after 15- and 30-minute incubation the light output was recorded again. All the chemicals were tested on three different days, in 5-7 dilutions for each chemical, each dilution in two replicates. Eight controls, both negative (2% NaCl) as well as positive (3,5-dichlorophenol) control were always included in each run. The inhibition of bacterial bioluminescence by the tested compounds was calculated as a percentage of the unaffected control (2% NaCl) at 30 s, 15 min and 30 min.

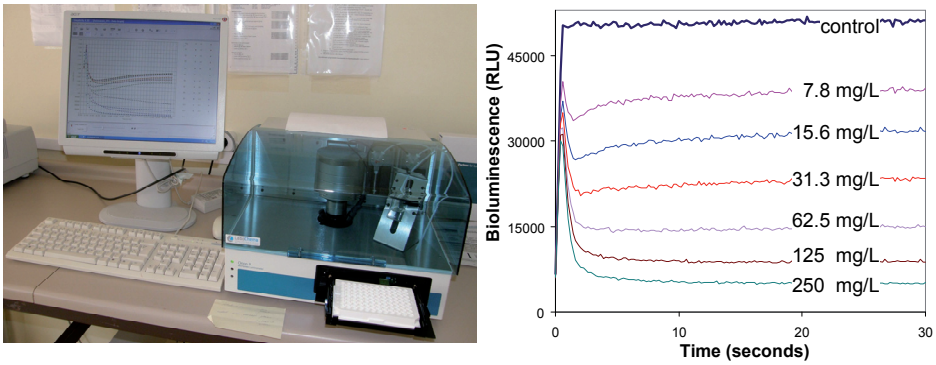

Figure 2.3. Microplate luminometer Orion II (left panel) and the kinetic dose-effect curves of luminescence of *Vibrio fischeri* exposed during 30 seconds to different concentrations of 2-chloroaniline (right panel). RLU – relative light units; control – 2% NaCl.

Inhibition of the luminescence (INH%) of *Vibrio fischeri* by a certain concentration/dilution of chemical was calculated as follows:

$$INH\% = 100 - \left(\frac{IT_t * 100}{IC_t} \right) \quad (1)$$

where

IT_t – luminescence of bacteria exposed to certain concentration of chemicals after certain time of exposure (t=30 s, 15 min, 30 min);

IC_t – luminescence of bacteria in the control solution (2% NaCl) after certain time of incubation (t=30 s, 15 min, 30 min).

30-s, 15-min and 30-min EC_{50} values (the concentration of chemical which reduces the luminescence of bacteria by 50% after contact time of 30-s, 15-min or 30-min, respectively) were determined from concentration *versus* INH% curves. The concentration-effect curves used for the 30-s, 15-min and 30-min EC_{50} calculations were fitted with REGTOX software for Microsoft Excel™ using the log-normal model (Vindimian, 2005).

Vibrio fischeri toxicity data (papers **III, IV**) were compared with toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata,* obtained in our laboratory using the same set of chemicals (section 2.3), as well as to toxicity of five anilines (aniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline, 3,5-dichloroaniline) to multitrophic aquatic test battery, i.e. crustaceans *Daphnia magna and Thamnocephalus platyurus,* and protozoa *Tetrahymena thermophila.*

3. RESULTS AND DISCUSSION

As described in section 1.3.1, according to REACH the toxicological properties of thousands of industrial chemicals need to be assessed in the near future (by the year 2018). Thus, implementation of this regulation should be based on the broad use of alternative approaches, e.g. the use of all available existing information, alternative methods alone or in combination as integrated testing strategies (ITS), and animal testing should be performed only as a last resort (section 1.4.3). However, the results obtained after the first REACH registration deadline (the dossiers submitted to ECHA by November 30, 2010; ECHA, 2011b) show that the use of alternative methods (section 1.4.3.5), especially regarding (Q)SARs, is remarkably lower than expected. Experience from the US Challenge Program for the HPVCs has shown that 35% (for environmental data) or 44% (for human health endpoints) of the data needed was estimated by the use of QSARs and read-across methods (Bradbury et al., 2004), indicating that application of QSARs at much higher rate is feasible. More importantly, as the assessment of the toxicology of industrial chemicals and the promotion of alternative methods do not end with REACH further harmonization of and a greater focus on non-animal methods/approaches is urgently needed.

3.1. Toxicity data published in Russian in the former Soviet Union (paper I, II)

3.1.1. Availability and visibility of the Russian data sources

REACH requests the use of all available existing information as a first step in the evaluation of the toxicological properties of substances. The system for publication and dissemination of chemical information in the USSR was highly developed (Allcock, 1980; Kurlyandskiy and Sidorov, 2003; paper **I**). Soviet and Russian toxicologists have studied thousands of chemical substances for their toxic properties, in order to provide scientifically justified values for the maximum allowable concentration (MAC) limits for chemicals in working/occupational environments, ambient air, drinking water, food, natural waters and soil. These data were, and still are, published in peer-review journals and other periodical issues (Allcock, 1980; Kurlyandskiy and Sidorov, 2003; paper **I**). Most of these publications from the Soviet Union era are in Russian. At the same time, Estonian toxicologists, for example, have also been publishing in international journals, e.g. data on the toxicological properties of various oil-shale chemicals (Kahn, 1979; Veldre and Jänes, 1979) as well as on occupational health issues. In a paper entitled 'Research Results of Soviet Scientists in Some Problems of Occupational Medicine: Review of the Years 1981–1984', Kahn stated: "*We have set MAC values for more than 800 chemical substances… the research has become more complicated because the traditional*

toxicological experiments must now be supplemented by studies of various other biological effects, such as the sensitisation of the organism and mutagenic, teratogenic, carcinogenic and other effects…" (Kahn, 1985).

Numerous available Internet resources provide guidance on alternatives to animal testing. However, Russian scientific literature is poorly retrievable by commonly used search engines (Table 1 in paper **I**) and valuable toxicological data published in the Russian language have remained non-cited in recent scientific papers (Felsot, 2002; Hakkinen and Green, 2002; Poppenga and Spoo, 2002; Russom, 2002; Winter, 2002; Wolfgang and Johnson, 2002; Young, 2002; Junghans et al., 2004, de Marcellus, 2003; Aggrawal, 2005) except when these were the only information sources available concerning a particular compound (Polifka and Faustman, 2002). The access to Russian information is limited due to the language barrier and low level of digitalization of the respective journals and books (paper **I**).

The bibliometrical analysis of the toxicological scientific papers using the Scopus® database showed that the Russian toxicological literature was less cited than the literature published in Northern USA or Western Europe, reflected also by lower H index values (Fig. 1, Fig. 2 and Table 2 in paper **I**). Updated bibliometrical analysis of the toxicological scientific papers in the subject area 'Pharmacology, Toxicology and Pharmaceutics' was performed in August, 2011 using the SCImago Research Group search engine in the Scopus® database. As seen from the Fig. 3.1, 4 years after the publication the percentage of non-cited documents for Western countries is low (less than 20%) compared to Russian papers (40-60% not cited).

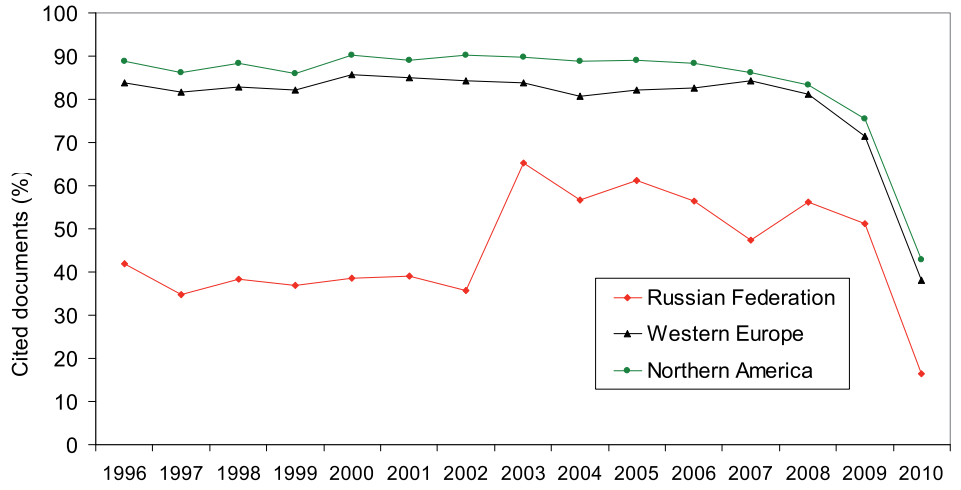

Figure 3.1. Percentage of cited documents of Russian, Northern American and Western European scientists in the subject area 'Pharmacology, Toxicology and Pharmaceutics' of the SCImago classification. *Source: Scopus®, analysis was performed in August, 2011. Available at: http://www.scimagojr.com. Updated version of the Fig.1 in paper **I**.*

One of the aims of OSIRIS project was to fill in this gap and collect the information on toxicological data published in Russian available in various data sources. Taking up the task was possible due to our knowledge of the Russian language and the access to the corresponding literature archived in Estonian libraries during the last century. Multilanguage training of Estonians was common even during the Soviet time: schools and universities taught in Estonian but Russian was learned for 11 years and English, German or French for 6 years in school by every child. Therefore, one of our aims (paper **I**) was to upgrade the information concerning the sources of toxicological literature published in the Russian language and to contribute to filling in the gap, focusing on mapping of the main publicly available sources of toxicological information. The sources included mainly web-sourced, peer-reviewed articles and books, the URLs are listed as Supplementary material in Table S1 in paper **I** (see also scheme in Fig. 2.1). This approach contributes to the 3R's strategy, a worldwide acknowledged and EU-level prioritized strategy for the reduction of the use of laboratory animals in scientific studies.

3.1.2. E-SovTox database

After literature analysis the main sources containing toxicological data published in the Russian language were identified for the creation of the database E-SovTox (Fig. 3.2; paper **II**).

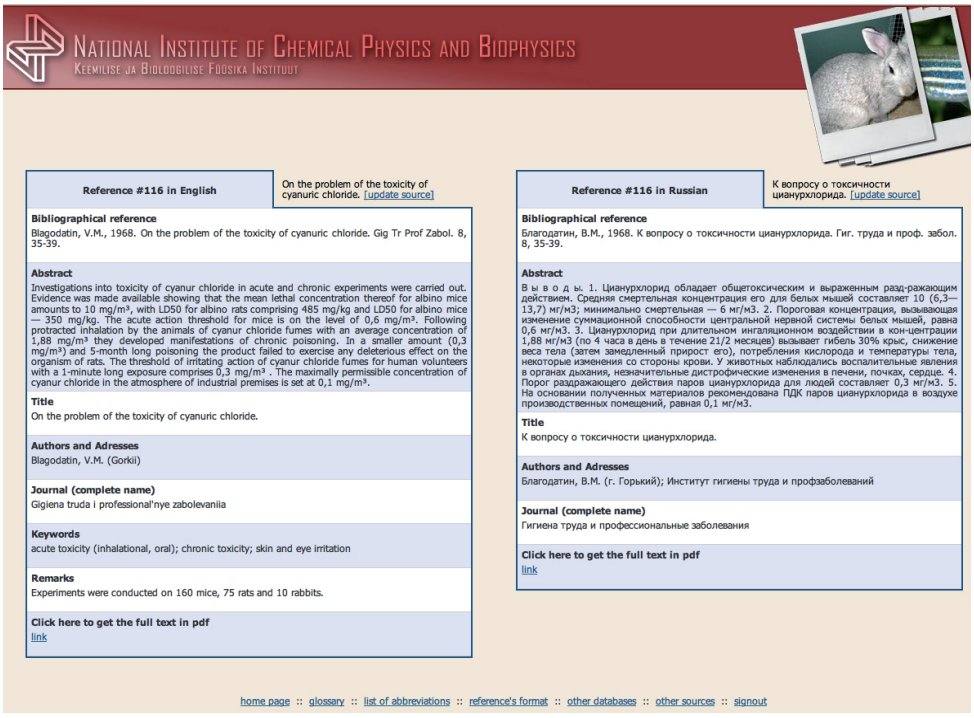

Figure 3.2. Screen view of an E-SovTox literature-reference page.

Main journals and proceedings containing toxicological data published in the Russian language are:

1. Mammalian toxicity data:

- 'Industrial Hygiene and Occupational Diseases'[1] (Gigiena Truda i Professional'nye Zabolevaniia, *Гигиена труда и профессиональные заболевания*). Published during the period 1957 – 1992.
- 'Hygiene and Sanitary'[1] (Gigiena i Sanitariia, *Гигиена и санитария*). Published since 1936 till present.
- 'Pharmacology and Toxicology' (Farmakologiia i Toksikologiia, *Фармакология и токсикология*). Published during the period 1939 – 1991.

2. Ecotoxicity data:

- 'Hydrobiological Journal' (*Гидробиологический журнал*). Published since 1965 till present.
- 'Inland Water Biology' (*Биология внутренних вод*). Published since 1995 till present.
- Annual proceedings of Latvian Institute of Biology 'Experimental Water Toxicology'[1] («*Экспериментальная водная токсикология*»: сборник статей/Академия наук Латвийской ССР; Институт биологии Рига). Published during 1970-1991.

The bibliometric analysis in PubMed showed that from the scientific journals published during the Soviet Union era the journal 'Industrial Hygiene and Occupational Diseases' *(Gigiena Truda i Professional'nye Zabolevaniia)* (Fig. 3.3) was the most representative for the toxicological studies (Table 3 in paper **I**). Therefore, this journal was selected as a starting point in the collection of the toxicity data and currently it is the main information source in the E-SovTox database.

Figure 3.3. Cover of the scientific journal Industrial Hygiene and Occupational Diseases *(Gigiena Truda i Professional'nye Zabolevaniia)*.

[1] Free translation

The above mentioned journal contains acute and chronic toxicity data for numerous industrial chemicals tested on several different groups of animals, e.g. rats, mice, guinea-pigs and rabbits. The main goal of the toxicity studies published in the retrieved articles was to derive the maximum allowable concentration limit (MAC) values for industrial chemicals in the occupational health settings in the former Soviet Union. Thus, articles featured in the database include mostly data on LD_{50} values, skin and eye irritation, skin sensitisation and cumulative properties of various industrial chemicals. Concerning test organisms used for the toxicity testing in papers featured in the E-SovTox database, most toxicity data were obtained on rodents (Fig. 3.4). Currently, the E-SovTox database contains toxicity data selected from more than 500 papers covering more than 600 chemicals (paper **II**).

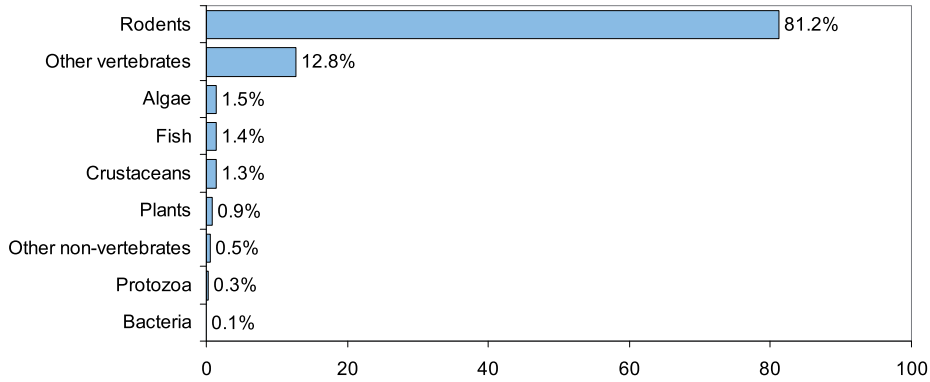

Figure 3.4. Characterisation of the toxicity data included in E-SovTox database (http://kbfi-databases.eu/database/): test organisms used (% of total data).

The E-SovTox database can be used as a decision-support tool by researchers and regulators for the hazard assessment of chemical substances. Currently, E-SovTox has registered users from different regions, e.g. Germany, Netherlands, Russia, Sweden, USA, UK, Finland, etc. The examples of the purpose and activity of current users of E-SovTox are summarized in Table 3.1.

Table 3.1. The purpose and activity of current users of E-SovTox as stated in their application forms for registration.

Purposes	Activities
• complete overview of published data and fill data gaps, purchase data copy rights, data for hazard assessment • scientific interest • to verify the quality and integrity of data for possible QSAR modelling • risk assessment • search for toxicological data to be used	• regulatory toxicology, risk assessment. • structure-activity relationships • data search and assembly for QSAR modelling • *in vitro* toxicology; REACH • animal protection • risk and safety assessment of

Purposes	Activities
for testing proposals commenting under REACH • compile data that could be used in risk and safety assessment • REACH, public consultation on testing proposals • accessing data from Russian journals cited in compiled health and safety reports such as RTECS or IUCLID, SIDS, etc • experimental investigations of new potential drugs • literature review • getting access to publication that might be of use in the evaluation work	chemical products • library and information management services for the toxicology department • ophthalmology, pharmacology • toxicity database development • evaluation, toxicology

Notes: RTECS - Registry of Toxic Effects of Chemical Substances; IUCLID - International Uniform Chemical Information Database; SIDS - Screening Information Data Set

3.1.3. Comparison of the E-SovTox data with the European and American databases

Toxicity data from Russian language data sources in E-SovTox were compared with the toxicity data from analogous European and American databases. Comparison was made between acute toxicity data available for the anilines and phenols from the set of 58 chemicals (section 2.3) in the E-SovTox database and the same type of toxicity data were searched comparatively from US TOXNET (http://toxnet.nlm.nih.gov/), IUCLID Chemical Data Sheets Information System (http://esis.jrc.ec.europa.eu/index.php?PGM=dat) and The Merck Index v 14.0 database (Table 3.2). Altogether acute toxicity data for 17 out of 58 chemicals were found from Russian papers. For these 17 chemicals data were searched also in the European and American databases. US TOXNET covers the scientific literature in toxicology including literature in Russian published in the former socialist countries (paper **I**). US TOXNET covered also majority of toxicity data from Russian literature for 17 chemicals indicated in Table 3.2. IUCLID Chemical Data Sheets Information System did not refer to the original literature, instead the source of toxicity data was indicated as the name of the company which had submitted the data to the system. However, it can be assumed that it includes also toxicity data from Russian data sources as in some cases the data are comparable with the data from E-SovTox (e.g. 2-CA; 3,4-DCA; 2-MP; 3-MP; 4-MP; 2,6-DMP; 3,5-DMP). In the Merck Index original literature references were included but no Russian data were available for the 17 chemicals under examination. It can be seen from Table 3.2 that there were no toxicity data in the western or US data sources for many substances and endpoints compared to the data available in Russian data sources.

Table 3.2. Comparison of the toxicity data for 17 anilines and phenols: Russian toxicity data from E-SovTox database *versus* data from the selected European and American databases. *Abbreviations for chemical names are explained in Table 2.1 (section 2.3.)*

		Toxicity data (LD_{50} or LC_{50}, mg/kg or mg/m^3)			
Chemical	Test organism (administration route)	E-SovTox	TOXNET (Russian toxicity data are excluded)	IUCLID	Merck Index
Aniline	rats (oral)	550 (470-680)	250	200-2000 572	440
	mice (oral)	690 (650-870) 464 (375-576)	n.d.	n.d.	n.d.
	cats (dermal)	254 (166-342)	100*	n.d.	n.d.
2-CA	mice (oral)	256 (188-348)	n.d.	256	n.d.
	cats (dermal)	222 (142-302)	310*	222	n.d.
3-CA	rats (females) (oral)	1034	n.d.	n.d.	n.d.
	rats (male) (oral)	880	n.d.	n.d.	n.d.
	mice (oral)	334 (269-414) 1100	n.d.	n.d.	n.d.
	cats (dermal)	223 (169-277)	125*	n.d.	n.d.
	guinea pigs (oral)	750	n.d.	n.d.	n.d.
4-CA	rats (females) (oral)	370	n.d.	n.d.	310
	rats (male) (oral)	300	n.d.	n.d.	n.d.
	mice (oral)	228 (198-262) 400	100	n.d.	n.d.
	cats (dermal)	239 (167-311)	125*	n.d.	n.d.
	guinea pigs (oral)	350	n.d.	n.d.	n.d.
2,5-DCA	rats (oral)	3000	1600	n.d.	n.d.
	mice (oral)	2500	1600	n.d.	n.d.
	guinea pigs (oral)	3750	n.d.	n.d.	n.d.
	rabbits (oral)	3750	n.d.	n.d.	n.d.
3,4-DCA	rats (oral)	700	545	570 530 880 648 545 570	n.d.
	mice (oral)	1000 740 (587-933)	n.d.	740 470 510	n.d.
	cats (dermal)	700 (590-810)	n.d.	700	n.d.
	guinea pigs (oral)	675	n.d.	675	n.d.
	rabbits (oral)	675	n.d.	675	n.d.
2,4,6-TMA	mice (oral)	516	n.d.	n.d.	n.d.
	mammal (species not specified) (oral)	372	506**	n.d.	n.d.
Phenol	mice (oral)	436 (311-610)	n.d.	282 300	n.d.
4-CP	rats (oral)	625	670	670 500	670
	mice (oral)	605	n.d.	1373-1422	n.d.

(Continued on the next page)

Chemical	Test organism (administration route)	Toxicity data (LD_{50} or LC_{50}, mg/kg or mg/m^3) E-SovTox	TOXNET (Russian toxicity data are excluded)	IUCLID	Merck Index
2-MP	rat (oral)	1470	121	121 1350 1470 360	1350
	mice (oral)	344 (270-436)	n.d.	344	n.d.
	mice (inhalation)	179	n.d.	178 179	n.d.
	rats (dermal)	620 (370-1110)	65*	1000 620	n.d.
3-MP	rats (oral)	2010 (1240-3200)	242	242 2020 2010 520 1454	2020
	mice (oral)	828 (695-985) 600***	n.d.	828 561 861	n.d.
	rats (dermal)	1100 (800-1400)	900*	1100	n.d.
4-MP	rats (oral)	1460 (1260-1670) 1400	207	1800 207 1460	1800
	mice (oral)	344 (266-443) 440***	160***	344	n.d.
	rats (dermal)	750 (510-1100)	500*	750	n.d.
2,4-DMP	rats (oral)	3200 (2780-3680)	n.d.	n.d.	n.d.
	mice (oral)	809 (724-914)	n.d.	n.d.	n.d.
	rats (inhalation)	> 30	n.d.	n.d.	n.d.
	rats (dermal)	1040 (630-1716)	n.d.	n.d.	n.d.
2,5-DMP	rats (oral)	444	730***	n.d.	n.d.
	mice (oral)	1140 (797-1530)	383	n.d.	n.d.
2,6-DMP	rats (oral)	296 1750 (1420-2150) 406 (329-600)	n.d.	296	n.d.
	mice (oral)	980 (823-1166) 450 (300-600)	n.d.	n.d.	n.d.
	rats (dermal)	2325 (1160-4650)	n.d.	n.d.	n.d.
	mice (dermal)	920 (575-1472)	n.d.	n.d.	n.d.
	mice (intraperitoneal)	271 (206-356)	150	150	n.d.
3,4-DMP	rats (oral)	1620 727	n.d.	n.d.	n.d.
	mice (oral)	948 (658-1365)	400	n.d.	n.d.
3,5-DMP	rats (oral)	2250 (1600-3150) 1915 608	n.d.	3620 608 600-1000	n.d.
	mice (oral)	836 (773-906)	477	477	n.d.
	rat (inhalation)	> 4	n.d.	n.d.	n.d.

Notes: n.d. - no data available; * LDLo (lowest published lethal dose, subcutaneous); ** rat LD_{50} value, administration route not reported; *** administration route not reported.

3.1.4. Evaluation of available information from Russian language data sources

Under REACH toxicological information should be evaluated for its completeness (whether the available data meet the criteria for the information required under REACH) and quality (relevance, reliability and adequacy; ECHA, 2011c). **Relevance** is the extent to which data and tests are appropriate for a particular hazard identification or risk characterization. **Reliability** is the inherent quality of a test report or a publication related to preferably standardized methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings. The Klimisch code is used for scoring the reliability of the data (Klimisch et al., 1997). Four reliability categories are distinguished:

1. reliable without restrictions;
2. reliable with restrictions;
3. not reliable;
4. not assignable.

Adequacy is the usefulness of the data for hazard and risk assessment purposes, in other words, adherence to EU or international standardised methods. Test data derived with other methods may also be considered adequate for use under REACH when conditions described in REACH, Annex XI 1.1 are met, e.g. (i) adequacy for the purpose of classification and labelling and/or risk assessment; (ii) sufficient documentation is provided to assess the adequacy of the study; and (iii) the data are valid for the endpoint being investigated and the study is performed using an acceptable level of quality assurance.

Toxicity data collected for the E-SovTox from Russian literature dated back to the 1950s. Toxicity tests during this period were made according to the USSR and Russian standards and methods (Sanotski, 1970; Ministry of Natural Resources of the Russian Federation, 2001) that differ from currently valid test guidelines in EU. This made the interpretation of the test results difficult and in some cases even the identification of the tested substance was problematic. Nevertheless, in the Russian language scientific papers citation to test guidelines were usually presented in the list of references and can be therefore further explored if needed. It should be noted that the Russian articles published in Soviet time did not contain CAS numbers. Usually, molecular weight of the chemical was stated but formula and/or structure were not always included and thus identification of compounds was not always straightforward. In addition, it cannot be unambiguously concluded under which Klimisch category toxicity data from Russian data sources belong, and it has to be decided on a case by case basis. However, despite of some of the drawbacks these data can be still used for REACH, even if not always classified as high-quality data.

In conclusion, one of the main advantages of E-SovTox database is that pdf-s of original papers are linked directly to the toxicity data which is not the case for most of the databases and is crucial for Russian papers as they are apparently of

limited access. Thus, users can read more detailed information and decide by themselves to what extent the information can be used.

It is important to note that the mapping, collecting and critically analysing the toxicity data of chemicals from the literature published in Russian in the former Soviet Union and Russia (paper **I**) and creation of an open-access database E-SovTox (paper **II**) has been considered one of the novel aspect in the frame of OSIRIS project. The database is highlighted as one of the main accomplishments in FP6 project OSIRIS in terms of data collation in OSIRIS flyer (Appendix 3 of the current thesis).

3.2. *In vitro* experimental data on 58 congeneric anilines and phenols (paper III, IV)

3.2.1. Design of the study

Anilines and phenols are compounds of considerable industrial and commercial importance, which makes them important environmental pollutants (Keith and Telliard, 1979; Woo and Lai, 2004). In addition, these chemicals are important also for Estonian oil-shale-based chemical industry (section 1.1). However, for many substituted anilines and phenols there are no ecotoxicity data available (Table S3 in paper **IV**). All the 58 selected anilines and phenols have been pre-registered under REACH referring to European Union production or import quantities of 1 tonne or more per year.

One of the aims of the FP6 Integrated Project OSIRIS, under which the work of the current thesis was performed, was to investigate the interspecies relationships based upon toxicogenomics using aniline and 3 'increasingly' chlorinated anilines (aniline; 4-CA; 3,5-DCA and 2,3,4-TCA) as model compounds. Thus, in the current study (paper **III**) we chose also a set of chlorinated anilines (2-CA; 3-CA; 4-CA and 3,5-DCA) in addition to aniline to investigate the relationship between the chemical structure and toxicity in a multitrophic test battery comprised of bacteria *Vibrio fischeri*, a ciliated protozoan *Tetrahymena thermophila* and two crustaceans (*Daphnia magna* and *Thamnocephalus platyurus*). The main focus was to study how the toxicity of anilines correlates with the degree of chlorosubstitution and to compare the sensitivity of different test species, i.e. unicellular (bacteria and protozoa) *vs* multicellular organisms (crustaceans) as well as prokaryotes *vs* eukaryotes. Also, the experimental toxicity data on *D. magna* were compared with the calculated toxicity values using ECOSAR in order to assess the prediction accuracy of the ECOSAR programme for chlorinated aniline toxicity (paper **III**). In the following set of experiments, the list of chemicals was extended to 58 chloro-, methyl- or ethyl-substituted anilines and phenols (section 2.3) to further examine whether the bacterial toxicity data correlate with algal data, considering that both are unicellular organisms but bacteria are prokaryotes and algae eukaryotes. The toxicity of the 58 chemicals was determined to bacteria *Vibrio fischeri* using the

high-throughput Flash Assay format and to algae *Pseudokirchneriella subcapitata* using the growth inhibition test (OECD, 2011) (paper **IV**). The set of selected chemicals was a good representative group of structure analogues, which fits well also into a QSAR context. According to the tentative mechanism of action all chosen chemicals belong to Verhaar class 2 (Verhaar et al., 1992) i.e. causing polar narcosis. Narcosis (i.e. non-polar and polar narcosis) is the least specific and the least reactive mode of toxic action but it is still important in ecotoxicology since approximately 70% of all industrial organic chemicals are estimated to act *via* narcosis in the acute exposures (1–14 days) (Bradbury et al., 2003). Narcotic effects are estimated by the ability of a compound to interact with cellular membranes. The toxic potency of these chemicals correlates strongly with their hydrophobicity and may be modelled using K_{ow} as a descriptor (Veith et al., 1990; Verhaar et al., 1992). Bacteria *V. fischeri* have been extensively used in QSAR modelling (Cronin and Schultz, 1997). The algal growth inhibition test (OECD, 2011) is one of the regulatory tests in ecotoxicology but still there is a severe shortage of algal toxicity data for REACH-relevant chemicals (paper **IV**). Experimentally determined EC_{50} values derived from the *V. fischeri* 15-min luminescence inhibition test as well as from the *P. subcapitata* 72-h growth inhibition test for the 28 anilines and 30 phenols are presented in paper **IV** (Table 2). In case of bacteria there were toxicity data available in the literature for the majority of selected anilines and phenols. However, there was a broad variation in the toxicity values reported for the same substances in different publications (section 3.2.2.1) and this can quite often be the limiting factor in the development of QSARs.

3.2.2. *Vibrio fischeri* luminescence inhibition assay for the analysis of the toxicity of chemicals

For the majority of the chemicals (including anilines and phenols studied in the current thesis) the modified Flash Assay (kinetic bioluminescence inhibition test) is a very rapid and sensitive method to evaluate the toxicity of a chemical: if the chemical is toxic, the effect on bacterial bioluminescence is noticeable already in 30 seconds of exposure. For heavy metals, however, the toxic effect is not reached in seconds but in about 30 minutes (Mortimer et al., 2008). Flash Assay is a modification of the conventional photobacterial bioluminescence inhibition assay (Microtox) that can be performed also in microplate luminometers allowing high throughput format. In both assays the decrease of bacterial luminescence upon exposure to chemicals is dose-wise related to their toxic effects and results mostly from the damage to the bacterial membranes.

3.2.2.1. Comparison of the Microtox and Flash Assay test formats

In the Microtox assay the testing is performed in cuvettes. First the test bacteria are pipetted into the cuvette, the luminescence at time zero is measured and then the test chemical is added to the cuvette containing photobacterial suspension.

After certain exposure time (usually 15 or 30 minutes) the luminescence is measured again and compared with the luminescence at time zero. Differently from the Microtox test format, in the Flash Assay mode the photobacterial suspension is added to the test chemical solution and the kinetics of the bioluminescence is continuously followed during 30-seconds and then at certain time intervals, usually at 30 minutes and 60 minutes. We observed that the toxic effects of anilines and phenols analysed was evident already during first seconds of the exposure. Interestingly, the decrease of luminescence of bacteria compared to the control was most remarkable at time scale from zero to 5-seconds and after that the bioluminescence started to increase (Fig. 3.5).

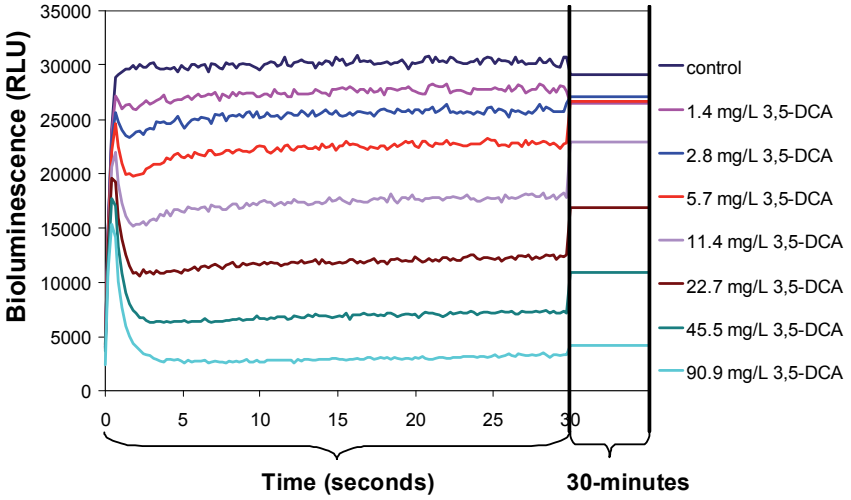

Figure 3.5. Kinetics of the bioluminescence of *Vibrio fischeri* during the exposure to different concentrations of 3,5-dichloroaniline (3,5-DCA) in the Flash Assay at 20 °C. The whole kinetic profile is shown for the first 30 seconds of the exposure and then for 30 minutes (a single point value). 30-s EC_{50} = 15.1 mg/L and 30-min EC_{50} = 41.1 mg/L. RLU – relative light units; control – 2% NaCl

Another technical aspect of bacterial bioluminescence inhibition assays run on microplate luminometer is that conventional Microtox test has to be performed at 15°C (optimal temperature for marine bacteria *Vibrio fischeri*) but this temperature is not compatible with the conventional plate luminometers. Thus, the assay was performed at 20°C. However, at 15°C the bacteria *V. fischeri* were more sensitive to the tested chemicals than at 20°C. Our study on the toxicities of anilines and phenols to *V. fischeri* at different temperatures showed 2-fold decrease in the toxicity at 20°C compared to 15°C in all incubation time-points (5, 15 and 30 min; data not shown).

In order to compare the experimental data to previously existing data for the studied anilines and phenols (section 2.3) relevant values were collected from the literature (Table S1 in paper **IV**). For the conventional *V. fischeri* bioluminescence inhibition assay (Microtox) there were data for 54 substances with 15-min exposure time at 15°C (Table S3 in paper **IV**). However, the EC_{50}

values available in the literature for *V. fischeri* varied considerably (up to 40 times), which is illustrated in Fig. 3.6.

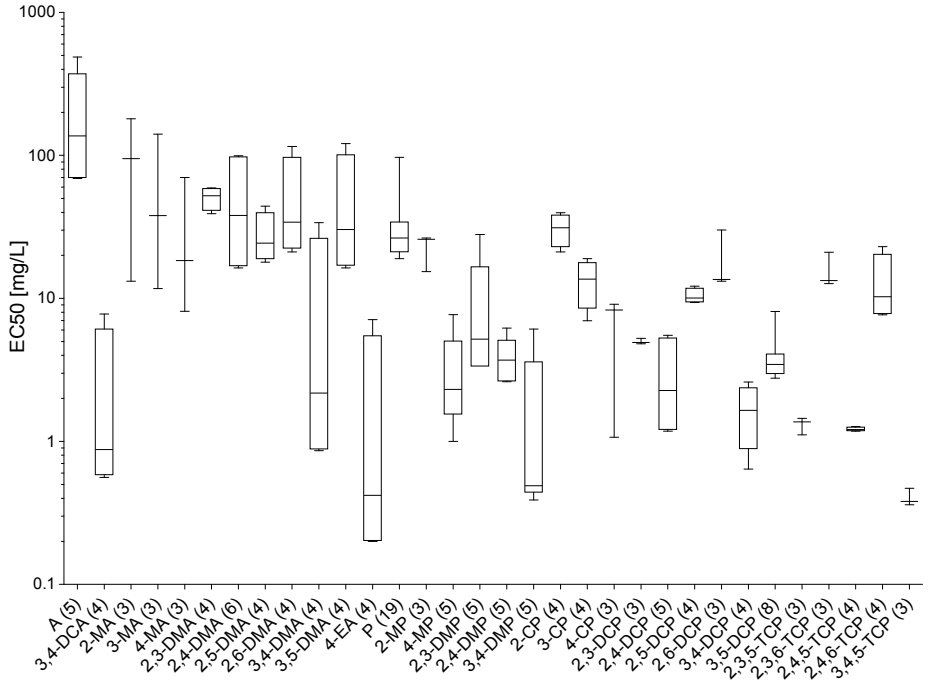

Figure 3.6. Variation of *V. fischeri* toxicity data, EC_{50} (mg/L), for 32 anilines and phenols (exposure time 15 and 30 min, 15°C, median values) from literature (Kaiser and Devillers, 1994; Rozkov et al., 1999; Kahru, et al., 2000; Osano et al., 2001) on a boxplot (numbers in brackets are the toxicity data available for a respective chemical). Note the logarithmic y-scale. Abbreviations for chemical names are explained in Table 2.1 (section 2.3).

Despite of high variability the median values of the published toxicity data from the conventional *V. fischeri* bioluminescence inhibition assay (Microtox, 15°C) correlated well with our experimental EC_{50} values from the Flash Assay at 20°C (log–log $R^2 = 0.74$, n = 54, $p < 0.01$ (Fig. 2C in paper **VI**).

3.2.2.2. Toxicity of the 58 anilines and phenols analysed in the *Vibrio fischeri* Flash Assay

In general, the toxicity of the studied 58 compounds was dependent on the type (chloro-, methyl-, ethyl-), number (mono-, di-, tri-) and position (ortho-, meta-, para-) of the substituents. The chloro-substituted molecules were generally more toxic than alkyl-substituted ones. The substituent in the para-position tended to increase the toxicity whereas the ortho-substituted congeners decreased the toxicity. The lower toxicity of ortho-substituted phenols has also been observed in other studies (Beltrame et al., 1984; Escher 1996; Argese et al., 1999) but

according to our knowledge has not been described for anilines. In addition, phenols were more toxic than anilines in the *V. fischeri* Flash Assay (paper **IV**). Interestingly, for most of the tested chemicals 30-min toxicity to *V. fischeri* was 1.3 – 4.6 fold lower than the toxicity at 30-s of exposure. Thus, it could be assumed that *V. fischeri* was rapidly adapting to the (sub)toxic effect of anilines and phenols. One of the known rapid adaption mechanisms that protect the cells against the presence of toxic organic compounds in some *Vibrios* is the fatty acid *cis-trans* isomerization reaction (Denich et al., 2003; Zhang and Rock, 2008). This short-term *in situ* mechanism is in terms of metabolic energy most efficient as it is post-synthesis modification process where existing lipids are used as the substrate and *de novo* lipid synthesis is not required. Given the time scale of changes in toxicity that we observed in our experiments (up to 30 min), *in situ* cis-trans isomerization of the membrane fatty acids might be the mechanism for the recovery. However, as in case of trichlorophenols and 3,5-dichlorophenol the toxicity (30 s *versus* 30 min) increased instead of decreasing, the reason for the 'luminescence recovery' phenomenon observed for most of the chemicals studied by us remains to be elucidated. It could be assumed that the toxicity of chemicals to *V. fischeri* depended on other characteristics in addition to $\log K_{ow}$ (section 3.3). However, despite of these discrepancies the 30-s and 30-min EC_{50} values correlated well (R^2=0.90; Fig. 3.7).

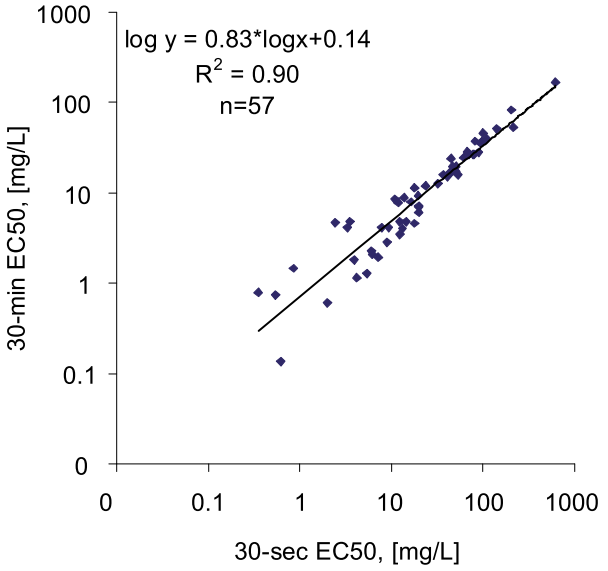

Figure 3.7. Effect of the exposure time on the toxic effect (inhibition of the luminescence) for 57 anilines and phenols (section 2.3) of *Vibrio fischeri*: Flash Assay data, 20°C, 30-s *versus* 30-min (data not shown). Note the logarithmic scale of the axis. 15-min EC_{50} values are presented in Table 2 of paper **IV**.

3.2.3. Comparison of the toxicity data of anilines and phenols to different aquatic species

3.2.3.1. Toxicity of five anilines to crustaceans, protozoa and bacteria

The toxicity of aniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline and 3,5-dichloroaniline toward different aquatic test species belonging to different trophic levels was investigated to establish the relationship between chemical structure. A multitrophic test battery comprising of one decomposer (bacteria *Vibrio fischeri*) and three consumers (crustaceans *Daphnia magna* and *Thamnocephalus platyurus*, protozoa *Tetrahymena thermophila*) was used. Our results showed that in the test conditions used, no obvious tendency identical for all tested species between the chemical structure of the anilines (the degree of chloro-substitution and the position of the chloro-substituents) and the respective toxicity was found. In case of protozoa, the toxicity of anilines depended on the position of chloro-substituents and increased in accordance with the degree of chlorosubstitution. Thus, aniline was about 12-fold less toxic than 3,5-dichloroaniline. Aniline was also approximately 10-times less toxic than the substituted anilines to the bacteria *V. fischeri* (403 mg/L *vs*. 13–59 mg/L) (Table III in paper **III**). For both crustaceans, however, it was difficult to reveal a distinct relationship between the chemical structure of the tested compound and toxic effect. Still, aniline was slightly more toxic than the chlorinated anilines studied. Moreover, the bacteria and protozoa were much less sensitive towards the anilines than the crustaceans (especially *D. magna*): EC_{50} values 13–403 mg/L *versus* 0.13–15.2 mg/L, respectively (Table III in paper **III**). These data are in agreement with the data of Abe et al. (2001) and Dom et al. (2010) who also showed that *D. magna* is highly sensitive to anilines. Also, it has been demonstrated that aniline and 4-chloroaniline are more toxic than the di- and trichlorosubstituted anilines (Abe et al., 2001; Ramos et al., 2002; Dom et al., 2010). However, an opposite trend i.e. decreasing toxicity with increasing chlorosubstitution has been recently observed in bacteria (*E. coli*), soil invertebrates (*F. candida*) and fish (*D. rerio*) (Dom et al., 2010; Kurvet et al., 2011; Janssens et al., 2011)

Thus, toxicity of anilines showed large interspecies variations, demonstrating that in different organisms the toxicity mechanisms for certain chemicals may differ and the extrapolation of toxicity data from one species to another could lead to incorrect deductions.

3.2.3.2. Comparison of toxicity data: *Vibro fischeri versus Pseudokirchneriella subcapitata*

In case of extended list of chemicals (section 2.3; paper **IV**), the data from *V. fischeri* acute test was compared with regulatory chronic bioassay data for *P. subcapitata* (72-h growth inhibition assay; OECD, 2011). EC_{50} values for the majority of the tested compounds for both, bacteria and algae, were between

1 and 100 mg/L (Fig. 3.8, Table 2 in paper **IV**). Comparing the hazard ranking on the basis of the obtained results of the selected 58 anilines and phenols it appeared that the classification would overlap for 59% of substances (for 34 out of the 58 tested substances) (Fig. 3.8, Fig. S1 in paper **IV**). This suggests that *V. fischeri* toxicity data may be useful for environmental toxicity screening. In paper **IV** the comparison of the hazard classification was made according to Dangerous Substances Directive which will be replaced by year 2015. Within CLP Regulation, which will replace the current directive, some changes compared to the previous system have been introduced (e.g. the hazard categories for acute and chronic aquatic toxicity, pictograms, hazard and precautionary statements, etc.; Table 3.3). However, as the concentration limits to the aquatic hazard classification have not been changed, the conclusion from the paper **IV** remained the same.

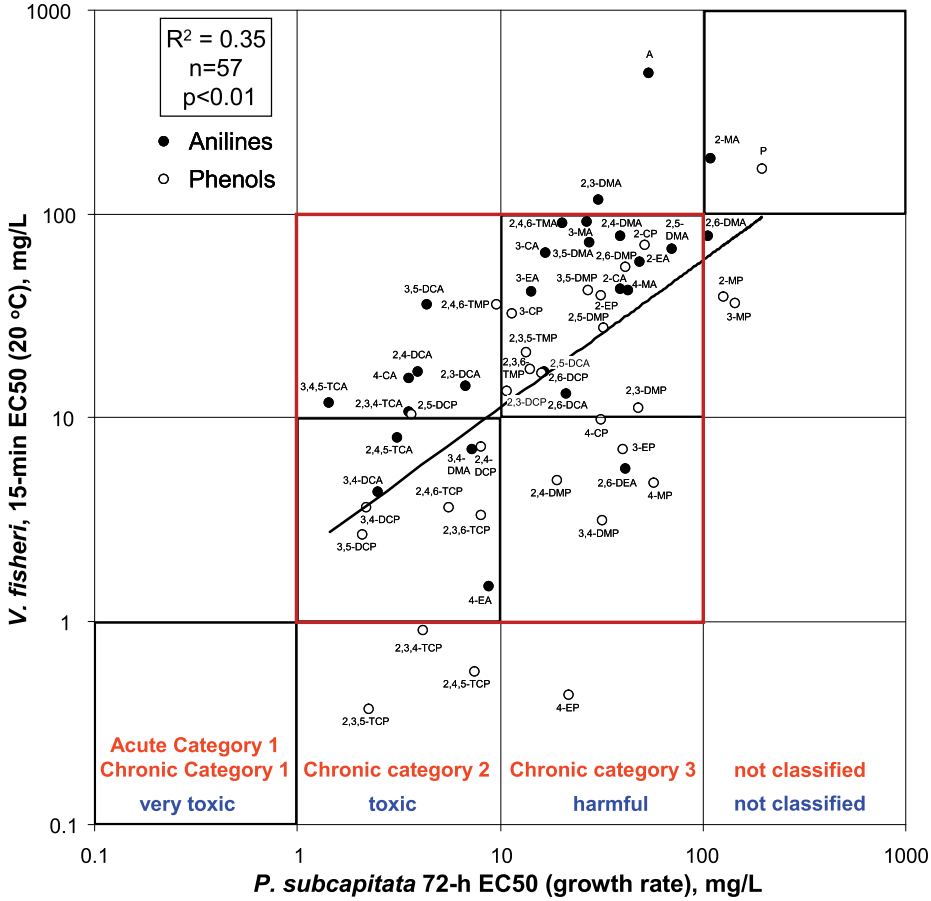

Figure 3.8. Comparison of the toxicity of substituted anilines (filled symbols) and phenols (open symbols) to *Pseudokircheneriella subcapitata* (72-h EC_{50}, mg/L) with *Vibrio fischeri* (15-min EC_{50}, mg/L). Classification is based on the criteria for hazards to the aquatic environment (classification categories in CLP Regulation are in red and previous legislation in blue). *Figure is modified from Fig. S1 in paper **IV**.*

As an example, according to the classification criteria for the aquatic organisms (CLP Regulation, Part 4: Environmental hazards; EC, 2008a), 2,4-dichlorophenol is hazardous to the aquatic environment, and would be classified as toxic to Chronic Category 2 (72-h EC_{50} for algae is 8.13 mg/L, i.e. EC_{50} is > 1 and ≤ 10 mg/L). Label elements that should be used are demonstrated in Table 3.3.

Table 3.3. Label elements that should be used for 2,4-dichlorophenol according to the criteria for hazardous to the aquatic environment classified as toxic (Chronic Category 2).

Pictogram:	
Hazard Statement:	H411: Toxic to aquatic life with long lasting effects
Precautionary Statement Prevention:	P273: Avoid release to the environment
Precautionary Statement Response:	P391: Collect spillage
Precautionary Statement Disposal:	P501: Dispose of contents/container to …

3.3. Experimental toxicity data compared to QSAR-predicted values (paper III, IV)

The QSAR models are one of the few options for filling data gaps in situations where experimental data are not available. The application of QSAR approaches is likely to increase substantially due to REACH (van der Jagt et al., 2004; van Leeuwen et al., 2009). However, the availability of QSARs is directly related to the availability of relevant and consistent toxicity data. Bibliometric analysis (Fig. 3.9) shows that currently most of the QSARs for ecotoxicology have been developed using toxicity data on fish (34%) and protozoa *Tetrahymena* (31%), followed by *Daphnia* (16%) and the bacterium *V. fischeri* (8.4%). Relatively few QSARs (10.8% of the total) have been developed on algal data. This is somewhat surprising, given that algal assay is obligatory for the registration of the chemicals under REACH. Thus, seemingly, QSARs are developed upon availability of the published toxicity data even if the toxicity test used for creation of these data is not a regulatory one such as photobacterial luminescence inhibition assays as well as protozoan growth inhibition test (Kaiser and Devillers, 1994; Dimitrov et al., 2003).

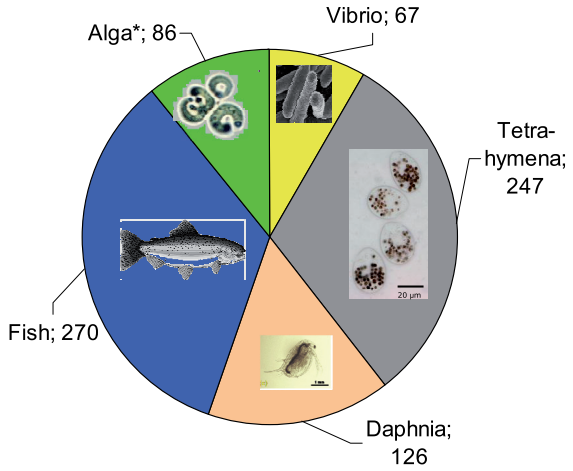

Figure 3.9. Bibliometry of peer-reviewed papers published on QSARs according to ISI Web of Science for years 1991-2011, search was made on April 29, 2011. The area sizes correspond to the amount of papers for certain organism/organism group. Total number of papers on QSARs: 796. *Adapted from Table S2 in paper **IV**.*

However, the existing QSAR models, including those proposed or developed for regulatory purposes (e.g., US EPA ECOSAR, Danish (Q)SAR Database) still require improvement (Reuschenbach et al., 2008). This was demonstrated in paper **III**, where a comparison of experimental results with the predicted toxicity values for *D. magna* obtained with the ECOSAR model (experimentally obtained octanol-water partitioning coefficient, K_{ow}, values were used for the calculations; Table I in paper **III**) showed that the predictive power of the ECOSAR model in case of anilines was limited. The ECOSAR model underestimated the toxicity of aniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline and 3,5-dichloroaniline by almost one order of magnitude (Fig. 1 in paper **III**). Similar results have been recently published by Dom et al. (2010; 2011) where it was illustrated that ECOSAR program was not adequate to predict the acute and chronic toxicity of the chlorinated anilines to *D. magna*.

The analysis of the experimental results with 58 chemicals (section 2.3) showed that the toxicity of the tested anilines and phenols to *V. fischeri* was not well explained by hydrophobicity (Fig. 3C in paper **IV**) suggesting that toxicity of these chemicals to *V. fischeri* depends on other characteristics in addition to $\log K_{ow}$. Also, the comparison of data with QSARs performed on other class 2 chemicals showed that the best fit was observed for toxicity data obtained at 20°C (Zhao et al., 1998; Fig. 3 in paper **IV**). The above described effect suggests that testing temperature should be considered as a relevant factor when comparing *V. fischeri* toxicity data. Thus, consistent experimental toxicity data are crucial for the successful development and application of QSAR models.

4. MAIN RESULTS AND CONCLUSIONS

The main outcomes of this thesis are as follows:

1. We showed that the access to the toxicological information on chemicals published in the Russian language is limited. In order to fill this gap, the criteria for mapping, collecting, and analysing the existing publicly available toxicity data from Russian language data sources were developed. The focus was set on several parameters (e.g., relevance for REACH, type of the toxicity test, cost, number of experimental animals used in the assays) and according to these criteria Russian scientific publications, databases and internet-based sources were explored yielding important toxicological data for scientists not familiar with Russian language (papers **I** and **II**).
2. The above mentioned data were critically analysed and selected to create an open-access online web-database including toxicity data of chemicals from publicly available Russian language data sources. Currently, the E-SovTox database contains 500 papers (with parallel availability of abstracts in Russian and in English) digitalized by OCR-technique covering more than 600 chemicals (www.http://kbfi-databases.eu/) (paper **II**). This database is highlighted as one of the main developments in FP6 project OSIRIS (Appendix 3 of this thesis).
3. Analysis of the toxicity of five anilines (aniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline and 3,5-dichloroaniline) in a multitrophic aquatic test battery showed that toxicity of these anilines to bacteria (prokaryotic unicellular organisms, decomposers) had very different pattern from the toxicity to crustaceans (eukaryotic organisms, consumers) showing that in different organisms toxicity mechanisms for certain chemicals may differ and thus extrapolation from one species may lead to under- or overestimation of toxic effects (paper **III**).
4. A set of 58 congeneric substituted anilines and phenols was chosen bearing in mind the relevance for REACH and applicability for QSARs, and experimentally analysed for the toxicity using naturally luminescent bacteria *V. fischeri* bioluminescence inhibition assay in the high-throughput Flash Assay format. This study provided homogenous high quality training set of toxicity data for further QSAR analysis and also showed that the 15-minute Flash Assay is a valuable tool in high-throughput toxicity screening as the data obtained in this assay (i) correlated well with the data of conventional *V. fisheri* Microtox test and (ii) classified 59% of the chemicals (34 out of 58) in the same hazard classes as 72 hour algal growth inhibition assay (OECD, 2011) suggesting that *V. fischeri* Flash Assay may be useful for ecotoxicity ranking as a screening test (paper **IV**).

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Руководство по определению методом биотестирования токсичности вод, донных отложений, загрязняющих веществ и буровых растворов.

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ABSTRACT

For the implementation of EU Regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) the hazard to the human health and environment of around 100 000 chemicals needs to be assessed during this decade. This is an enormous challenge to EU chemical industry and needs strong support and assistance from the scientists in providing information on toxicity of chemicals. Intelligent testing strategies (ITS) e.g., employing existing *in vivo* toxicity data as well as using Quantitative-Structure-Activity-Relationship (QSAR) approaches, are needed to increase the use of non-testing information for regulatory decision making, thus minimizing animal testing according to the 3R's strategy (Reduction, Replacement, Refinement).

In this thesis we showed that there is a large amount of toxicological information on industrial chemicals available in Russian language data sources that can support the implementation of REACH. However, the access to this information is limited due to the language barrier and poor digitalization of this information. To fill this gap, the respective data from publicly available Russian language data sources were mapped, critically evaluated and selected to create an open-access online web-database E-SovTox, which includes mainly *in vivo* toxicity data obtained on animals. E-SovTox contains information on 500 papers digitalized by OCR-technique on >600 chemicals (www.http://kbfi-databases.eu/). E-SovTox was highlighted as one of the main developments in FP6 project OSIRIS.

To support the development of QSAR approaches for ecotoxicological endpoints relevant to REACH, the relationship between the chemical structure and toxicity of anilines and phenols was analysed. Firstly, this study focused on the analysis of 5 (chloro)anilines: aniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline and 3,5-dichloroaniline. The use of a multitrophic aquatic test battery showed that toxicity of these anilines to bacteria (prokaryotic unicellular organisms, decomposers) had very different toxicity pattern from crustaceans (eukaryotic organisms, consumers). Thus, cross-species extrapolation may lead to under- or overestimation of toxic effects. In the second part of the ecotoxicological study structure-toxicity relationship analysis of 58 congeneric substituted anilines and phenols was conducted. The toxicity was measured using bioluminescent bacteria *Vibrio fischeri* applying Flash Assay (15-min kinetic luminescence inhibition test). As a result, a homogenous high quality training set of toxicity data for further QSAR analysis was obtained. The *V. fischeri* toxicity data were compared to 72-h algal growth inhibition data (a regulatory test within REACH). The comparison showed that the bacterial assay classified 59% of the chemicals (34 out of 58) in the same hazard classes as the algal test. Thus, a high-throughput *V. fischeri* Flash Assay may prove useful as a screening tool for ecotoxicity ranking of chemicals.

KOKKUVÕTE

ELi kemikaaliohutuse poliitika, REACH-määruse (*Registration, Evaluation, Authorisation and Restriction of Chemicals*) raames tuleb sel kümnendil hinnata umbes 100 000 kemikaali mõju inimese tervisele ja keskkonnale. See on suureks väljakutseks ja vastutuseks nii tööstusele, teadlastele kui ühiskonnale. REACHi prioriteediks on viia ka loomkatsete rakendamine miinimumini, kasutades ainete omaduste ja toksilise mõju hindamiseks eelkõige kättesaadavaid olemasolevaid andmeid ja edendades alternatiivsete meetodite (nt kvantitatiivsed struktuur-aktiivsussõltuvused (QSAR) ja *in vitro* meetodid) väljatöötamist ja kasutamist.

Käesolevas töös näidati, et nõukogudeaegses venekeelses kirjanduses on olemas suur hulk andmeid erinevate kemikaalide toksilisuse kohta, mis võivad toetada REACHi rakendamist. Keelebarjääri ja vastavate dokumentide madala digitaliseerimistaseme tõttu ei ole need andmed reeglina mujal maailmas kättesaadavad. Selle puudujäägi kõrvaldamiseks kaardistasime venekeelses teaduskirjanduses publitseeritud kemikaalide toksikoloogilised andmed, analüüsisime neid ja koostasime veebipõhise andmebaasi E-SovTox (http://kbfi-databases.eu/database/). Andmebaas sisaldab peamiselt loomkatsetes saadud toksilisuse andmeid ja hõlmab praegu >500 digitaliseeritud teadusartiklit ning toksikoloogilisi andmeid >600 kemikaali kohta. Antud töö viidi läbi EU 6. raamprogrammi integreeritud projekti OSIRIS raames ja E-SovTox andmebaas märgiti ära kui projekti OSIRIS üks põhitulemustest.

Toetamaks QSAR-lähenemisviiside kasutamist kemikaalide ökotoksikoloogiliste omaduste hindamisel analüüsiti antud töös aniliinide ja fenoolide keemilise struktuuri ja toksilisuse omavahelist sõltuvust. Esimese osana uuriti viit (kloro)aniliini (aniliin, 2-kloroaniliin, 3-kloroaniliin, 4-kloroaniliin ja 3,5-dikloroaniliin), kasutades selleks selgrootutest organismidest koosnevat multitroofset biotestide patareid. Saadud tulemused näitasid, et aniliinide toksilisus bakteritele (prokarüootsed ainuraksed organismid, lagundajad) ja kirpvähilisetele (eukarüootsed organismid, tarbijad) oli väga erinev ja viitas asjaolule, et tulemuste ekstrapoleerimine ühelt liigilt teisele võib aine toksilist toimet ala-või ülehinnata. Teise osana uuriti struktuurist tulenevat ökotoksikoloogilist toimet 58 aniliini ja fenooli näitel. Kemikaalide toksilisuse määramiseks kasutati bakteri *Vibrio fischeri* bioluminestsentsi inhibeerimise testi (15-min kineetiline Flash-test). Tulemusena saadi homogeenne andmekogum edasiseks QSAR analüüsiks. Saadud *V. fischeri* toksilisuse andmeid võrreldi 72-h vetikate kasvu inhibitsiooni testandmetega (regulatoorne test REACHi määruses). Võrdlus näitas, et bakteritestiga saadud andmete alusel klassifitseerus 59% kemikaalidest (34 kemikaali 58-st) samasse ohuklassi kui vetikatestiga saadud andmete põhjal. Seega saab *V. fischeri* Flash-testi tulemusi edukalt kasutada ainete ökotoksikoloogilise toime esmaseks hindamiseks.

PAPER I

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Review

Toxicological information on chemicals published in the Russian language: Contribution to REACH and 3Rs

Mariliis Sihtmäe, Henri-Charles Dubourguier, Anne Kahru*

National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn 12618, Estonia

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ABSTRACT

This review is reporting on the current situation of publicly available toxicological and ecotoxicological information on chemicals published in Russian language in various libraries, databases as well as in the Internet. This information can be beneficial for the new EU chemical policy REACH and for the development of intelligent testing strategies (involving also QSAR and QAAR) that enable a significant increase in the use of non-testing information for regulatory decision making, thus minimizing the need for animal testing according to the 3R's strategy. Currently, the access to this information is limited due to the language barrier and low level of digitalization of respective journals and books. Fortunately, on-line translation services are overcoming language barriers already now.

Contents

* Corresponding author.
E-mail address: anne.kahru@kbfi.ee (A. Kahru).

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1. Introduction

When the USSR launched the world's first satellite in 1957, the US government searched for explanations. One reason was that Russian scientists were using Western scientific literature whereas Western scientists were unable to handle the Russian language, leading to a very low citation frequency and citation impact of most Soviet published materials compared to analogous Western journals (Garfield, 1972).

Russia is the largest country in the world, covering more than 17 million square kilometers and is home to 142 million people. Russia had worldwide power and influence starting from the time of the Russian Empire under Peter the First, through the period of the Soviet Union and currently as the Russian Federation. Russians have a long tradition of excellence in most aspects of the arts and sciences. Some Russian universities are world-famous, such as the State Universities of Moscow, St. Petersburg and Novosibirsk. In the field of the natural sciences, Russia is recognized from the contributions of famous scientists, including Mikhail Lomonosov (1711–1765), who made an important contribution not only to literature and education, but also to science, particularly to chemistry and physics. G. S. Kirchhoff (1764–1833) discovered, in 1812, the conversion of starch into glucose by acidic hydrolysis. Dmitri Mendeleev (1834–1907) was the creator of the periodic table of elements. Nikolay Zelinsky (1861–1953) was one of the founders of the theory on organic catalysis. In 1904 Ivan Pavlov (1849–1936) received the Nobel Prize in Physiology and Medicine for his pioneering studies on the physiology of digestion and conditioned reflexes. Sergei Winogradsky (1856–1953) pioneered the cycle of life concept and discovered the biological process of nitrification, the first known form of chemoautotrophy. More recently, Mikhail Mashkovsky (1908–2002) was a famous pharmacologist and worked as an expert for the World Health Organization (WHO) on quality control of drugs. In addition, scientists from Russia and the former Soviet Union have devoted numerous studies to the toxicology of different compounds.

During the years of the Cold War Soviet scientists were also involved in research related to properties of chemical and biological warfare (see below). In the area of civil research, Soviet toxicologists were active in creating various hygienic and occupational standards for (chemical) industries.

Numerous available Internet resources provide guidance and other information on *in vitro* and other alternatives to animal testing (Hakkinen and Green, 2002; De Marcellus, 2003; Aggrawal, 2005). Despite that, valuable toxicological data published in the Russian language have remained non-cited in recent scientific papers (Hakkinen and Green, 2002; Schwela and Hakkinen, 2004; Poppenga and Spoo, 2002; Junghans et al., 2004; Young, 2002; Russom, 2002; Winter, 2002; Wolfgang and Johnson, 2002; Felsot, 2002; Gilardi and Fubini, 2005) except when these were the only information sources available concerning a particular compound (Polifka and Faustman, 2002). Within the frame of the EC FP6 Integrated Project OSIRIS, the Estonian partner (research group of Dr. A. Kahru) proposed to collect those materials due to their knowledge of the Russian language and their access to the corresponding literature archived in Estonian libraries during the last century. Multilanguage training of Estonians was common even during the Soviet time: schools and universities taught in Estonian but Russian was learned for 11 years and English, German or French for 6 years in school by every child.

Therefore, the aim of this paper is to upgrade the information concerning the sources of toxicological literature published in the Russian language and to contribute to filling the gap, focusing on mapping of the main publicly available sources of toxicological information. Although a number of other republics of the former USSR, such as Ukraine, Belarus or Lithuania are also active in toxicology (see Supplementary material), this review focus only on Russian and Estonian resources. It concerns mainly web-sourced, peer-reviewed articles and books (the URLs are listed as additional material in Table S1). This review contributes to the 3R strategy (reduction, replacement and refinement), a worldwide acknowledged and EU-level prioritized strategy for the reduction of the use of laboratory animals in scientific studies.

2. Toxicological research in Russia and chemical weapons

A brief but important overview of the history of toxicology as well as on current research activities and education in toxicology in Russia (including a list of national bodies and research institutes, laws, regulatory documents and databases in toxicology) has been published in the Elsevier journal "Toxicology" by Kurlyandskiy and Sidorov (2003). However, according to ScienceDirect as well as to the ISI Web of Science, this paper has not been cited yet.

An interesting aspect of toxicology in the Soviet Union, analogous to the situation in the US and other major Western countries, was the close dependence of its development on military applications, particularly concerning chemical weapons (Kobyakov and Orlov, 2005). The history of chemical and biological warfare agents has been recently reviewed by Szinicz (2005) For a few decades now, discussions have taken place at the highest levels between Russia and Western or World organizations for redirecting Russian toxicologists towards civil scientific activities. In 1997, the Russian Federation ratified the

Chemical Weapons Convention (CWC, 1993) and created, in 1999, the Russian Munitions Agency. With the help of the European Commission (http://ec.europa.eu/research/nis/en/istc.html), the International Science and Technology Centre (ISTC) in Moscow and the Science and Technology Centre of the Ukraine (STCU) in Kiev were established in 1992 and 1993, respectively. Since then, the majority of projects were and still are funded through these two research centers to redirect Russian and NIS (Newly Independent States) scientists formerly working on chemical and biological weapons to peaceful scientific activities as well as to support fundamental and applied research. Since 1998 the international series of conferences – Chemical Weapons Demilitarization (CWD) Conferences – have been held annually in order to address and provide potential technical and practical solutions to the key problems associated with chemical weapons disposal (http://www.dstl.gov.uk/conferences/cwd). In addition, the special program "The Strengthening the Global Partnership project" (Center for Strategic and International Studies, 2002) was adopted by the leaders of the Group of Eight industrialized countries (G8) at the Kananaskis summit in Canada in June 2002 (Kobyakov and Orlov, 2005). This program aimed at dismantling excess weapons and weapon-related infrastructure left in Russia and other former Soviet States since the end of the Cold War. In order to counter the risk of proliferation the West has taken a number of initiatives to increase financial support to Russia. The US focus has been on threat reduction and the EU is aiming more at civil R&D (Roffey et al., 2003). For example, in 1991, a US Cooperative Threat Reduction Program (CTR) was launched together with Russia, also known as the Nunn-Lugar CTR Program with a goal to lessen the threat posed by weapons of mass destruction, to deactivate and destroy these weapons, and to help the scientists formerly engaged in the production of such weapons to start working for peace (http://nunn-lugar.com). Since 1992, the European Commission and member states (European Communities, 2000–2005) have also intensively developed cooperation with various NIS and Russia.

3. The current key actors in toxicological research in Russia and in Estonia

As mentioned above, national bodies and major research institutes dealing with issues of toxicology and related disciplines are listed in the review of Kurlyandskiy and Sidorov (2003) and thus will not be repeated here. In addition to governmental organizations, an All-Russian Public Organization of Toxicology (Russian Society of Toxicology) was established in 1996. This organization succeeds the former All-Union Society of Toxicologists, which existed at the time of the USSR. The Russian Society of Toxicology has about 300 members (Kurlyandskiy and Sidorov, 2003).

Within the framework of former cooperation programs, several Russian institutes were engaged in cooperation with the US and Europe concerning toxicological research:

- Institute of Physiologically Active Compounds (IPAC), Russian Academy of Sciences, Chernogolovka, http://www.ipac.ac.ru
- The Institute of Toxicology, St. Petersburg, http://toxicology.ru
- Research Institute of Hygiene, Occupational Pathology and Human Ecology (RIHOPHE), St. Petersburg
- Research Center for Applied Microbiology and Biotechnology (SRCAMB), Obolensk, Moscow region, http://www.obolensk.org
- Research Center for Toxicology and Hygienic Regulation of Biopreparations (RCTandHRB), Federal Medico-Biological Agency, Serpukhov (Moscow region), http://www.toxicbio.ru
- Research Institute of Hygiene, Toxicology, and Occupational Pathology (RIHTOP), Volgograd, http://www.rihtop.ru

A non-profit partnership "Orchemed" (Organic Chemistry and Medicine) was also created as a consortium of research institutes of the Russian Academy of Sciences. This consortium is collaborating with almost all the major Western industrial groups producing chemicals and pharmaceuticals. For commercial activities, a joint scientific center TRUST (Testing and Revalidation of Unique Substances and Technologies) has been funded in Chernogolovka. Both together provide certified preclinical trials including animal experiments (Bachurin, 2007). One of the main foci of current toxicological research in Russia is the harmonization of Russian chemical legislation with the EU new chemical management policy REACH, as well as the Globally Harmonized system of Classification and Labeling of Chemicals (GHS). The "Chemicals management policy of the Russian Federation" has recently been summarized (Eco-Accord, 2006). The Russian Chemists Union (RCU), together with the Russian Industry and Enterprise Union, decided to organize a permanent working commission of experts. In addition, a national REACH Centre was established in connection with the RCU (Kinnunen, 2008). This REACH center organizes informative seminars and publishes recommendations for methodologies. It has also created an information system on IUCLID 5, computer software which plays a central role in the IT environments of all organizations that have to cope with the data submission requirements of REACH. Finally, a Committee "REACH" of the All-Russian Organization for Quality is promoting REACH directive and supports Russian exporters (http://www.reach.ru).

To give also a short overview of Estonian scientists contributing to toxicology within the Soviet Union and after that period, one should know that Estonia is a country of 1.3 million people and the history of industrial toxicology in Estonia dates back to the beginning of 1950s. The first experiments on industrial toxicology in Estonia were started in 1951 at the Institute of Experimental and Clinical Medicine and were related to the investigations of Estonian oil-shale, oil-shale phenols and different derivatives such as adhesives, resins, mastics, softeners and solvents (Loit and Jänes, 1984). Most of these oil-shale derivatives are now defined as new chemicals and are listed in the European List of Notified Chemical Substances (ELINCS) (European Communities, 2006). During the Soviet period (about 50 years), Estonian researchers were mainly publishing in the Russian language. Currently, the search in the web-database of the Estonian Research Portal ETIS (http://www.etis.ee) using the keyword "toxicol" yields 138 scientific papers and proceedings published by Estonian scientists since 1977. At present, toxicological research in Estonia is made mainly in the groups lead by Prof. A. Zharkovsky (Tartu University), Prof. M. Karelson (Tallinn University of Technology) and Dr. A. Kahru (National Institute of Chemical Physics and Biophysics, Tallinn). The Estonian Society of Toxicology (ETS; http://www.kbfi.ee/ets) was created in 1997 and has 50 members. ETS is a member of EUROTOX (Federation of European Toxicologists and European Societies of Toxicology) and IUTOX (International Union of Toxicology).

4. Bibliometrical analysis of toxicological information published in the Russian language

In 1972, the first analysis of citations of and by Russian journals was published by Garfield (1974). The citations analyzed were extracted from the Science Citation Index (SCI) data bank. This analysis showed that many highly cited articles by Soviet scientists have been published in foreign or international journals. The most highly cited Russian journal articles published during 1961–1972 were almost exclusively in physics and mathematical physics (Garfield, 1975). It was pointed out already in 1975 that the citation life of Soviet literature by Western scientists was unfairly shortened at the outset by an overlong gestation period due to the translation from Russian to English (Garfield, 1976). The analysis of the 1000

Table 1
Scientific papers published in Russian and in English in toxicology and ecotoxicology (number of hits obtained by search in various databases).

Database	Keywords	Russian language	English language	All languages	% Russian[a]
Google books	Toxicology[b]	3590	51100	52400	6.56
	Ecotoxicology[c]	28	2142	2147	1.29
ISI Web of Science	Toxicology	71	97244	>100000	0.07
	Ecotoxicology	4	2528	2641	0.16
ISI Web of knowledge	Toxicology	88	97 773	>100000	0.09
	Ecotoxicology	13	25277	25797	0.05
Science Direct	Toxicology	n.a.	n.a.	99251	n.a.
	Ecotoxicology	n.a.	n.a.	1265	n.a.
PubMed[d]	Toxicology	59	4731	5025	1.23
	Ecotoxicology	0	3	3	0.00
Scopus	Toxicology	10748	489865	578737	2.15
	Ecotoxicology	36	10263	11014	0.35

n.a. – not applicable due to the search engine used.
[a] Calculated on the total of Russian and English published papers.
[b] Keywords: toxic OR toxicology OR toxicity.
[c] Keywords: ecotoxic OR ecotoxicology OR ecotoxicity.
[d] Covers 327 journals in Russian.

most cited articles in the period 1961–1982 in the SCI showed that the vast majority of these papers were published in English (976) and only four were written in French and two in Russian (Garfield, 1986). During 1973–1988, physics dominated Soviet science, followed by chemistry and life sciences. Indeed, the word "toxicology" was absent in all previously cited documents. Interestingly, only 35 of the top 100 Soviet scientists belonged to the USSR Academy of Sciences (Garfield, 1990).

To obtain numerical data on toxicological scientific papers published in Russian, an initial bibliometrical search was performed in October 2008 with several commonly used search engines using the same keywords (Table 1). The results varied depending on the search engine used but the number of documents for "toxicology" was always greater than for "ecotoxicology" (Table 1). In addition, according to this search Russian papers represented always less than 1% of the scientific literature and less than 7% of the books. Hence, Russian scientific literature is poorly visible by commonly used search engines. However, this "low visibility" could be partially due to the choice of the keywords, as in Russian language literature the word "toxic" was not often used. Instead, terms such as "negative effect" or "harmful" were applied.

The bibliometrical analysis of the toxicological scientific papers in two subject areas was performed in October, 2008 using the SCImago Research Group search engine in the Scopus® database. The data showed that the Russian and Estonian toxicological literature were both cited less than the US or French literature, reflected also by lower H indexes (Table 2).

During 1997–2007, toxicological scientific papers contributed 18.6% in North America, 15.5 % in Europe and 20.1 % in Estonia to the subject area "Pharmacology, Toxicology and Pharmaceutics" of the SCImago classification. By contrast, less than 7% were contributed by Russia. In addition, 4 years after their publication, the percentage of non-cited documents is low (less than 20%) for Western countries (including Estonia) contrary to Russian papers (almost 60% not cited) (Fig. 1).

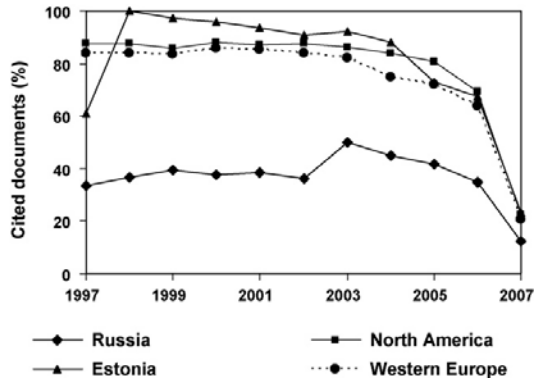

Fig. 1. Percentage of cited documents of Russian, Estonian, North American and Western European scientists in the subject area "Pharmacology, Toxicology and Pharmaceutics". Source: Scopus®, 2008. Available at: http://www.scimagojr.com.

This situation is even worse when comparing Russian and Western scientific journals. For the comparison, two high level Western scientific journals ("Toxicology" and "Toxicology Letters") were compared with similar high-rank Russian journals ("Doklady Akademii Nauk" and "Doklady Biological Sciences"). The comparison showed that more than 70% of documents from Western journals were cited compared to less than 20% of papers from Russian journals (Fig. 2A). However, a slight improvement of citation percentages may be observed for the "Doklady Biological Sciences" between 2003 and 2007. For those two Russian journals, the average number of citations per document (Fig. 2 B) is very low (less than 0.27) in contrast to the two Western journals mentioned above (more than 2.5).

Indeed, Van Raan (2008) in his comparison of the performance of the 100 largest European research universities within the period 1997–2004 on the basis of all publications (published in journals covered by the Citation Index) states: "we have left out Lomonosov University of Moscow. As far as number of publications concerns, this university is one of the largest in Europe (about 24000 publications in the covered 8-year period) but the impact is so low that it would have a very outlying position in the ranking".

5. Main resources for collecting toxicological and ecotoxicological documents in Russian language

For the mapping of the resources for collecting documents published in the Russian language, first the web-resources were

Table 2
Selected bibliometrical data on scientific toxicological literature from Russia: comparison with Estonia, US and France. Time-frame: 1996–2007.

Subject area	Pharmacology, toxicology and pharmaceutics			Environmental sciences		
Subject category:	Toxicology			Health, toxicology and mutagenesis		
Country (population, millions inhabitants)	Number of documents	Cites per doc.	H index	Number of documents	Cites per doc.	H index
United States (305)	41640	13.2	173	20233	10.6	113
France (65)	4164	12.8	77	2072	11.2	56
Russian Federation (142)	425	8.8	27	306	7.8	22
Estonia (1.34)	79	7.7	12	60	6.0	9

Source: Scopus®, 2008. Available at: http://www.scimagojr.com.

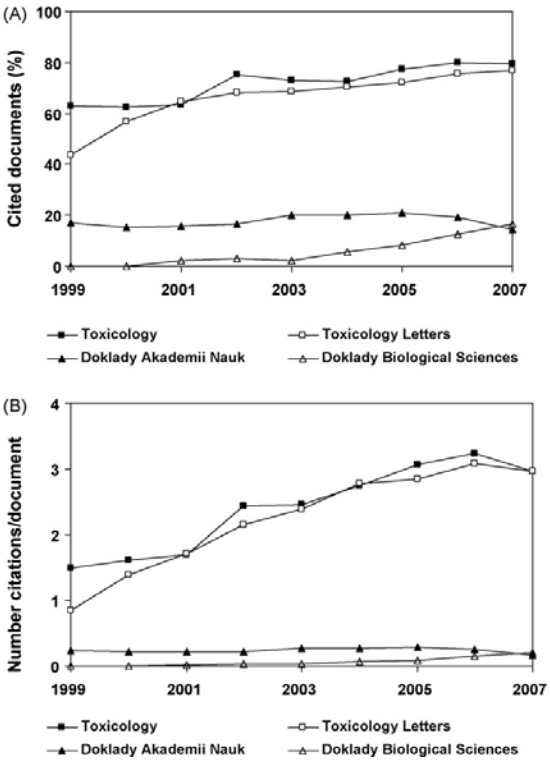

Fig. 2. Comparison of Russian and Western scientific journals by the percentage of documents cited (A) and by the number of citations per documents (B). Time-frame: 1999–2007. Source: Scopus®, 2008. Available at: http://www.scimagojr.com.

searched both in Roman and Cyrillic characters. In addition, the resources of libraries in Estonia and of the State Library of Russia (Moscow) were explored.

5.1. Institutions

5.1.1. Russian Academy of Sciences

The Russian Academy of Sciences (Российская Академия Наук, http://www.ras.ru) consists of the National Academy of Russia and a network of scientific research institutes from across the Russian Federation as well as auxiliary scientific and social units like libraries, publishers and hospitals. Headquartered in Moscow, the Academy is incorporated as a civil, self-governed, non-commercial organization chartered by the Russian Government. It combines the members of the Russian Academy of Sciences and scientists employed by institutions. This institution holds several sources of information (see below).

5.1.2. All-Russian Institute of Scientific and Technical Information (VINITI)

(Всероссийский институт научной и технической информации РАН, ВИНИТИ РАН)

VINITI (http://www2.viniti.ru/) is the leading information centre in Russia and CIS countries supplying scientific and technical information since 1952. During the Soviet era, Referring Journals (Реферативный журнал) of VINITI were the main source of scientific information for Soviet scientists as they covered the contents of the main international journals, providing even the abstracts of papers translated into Russian, and enabled ordering of full copies of original papers. Currently VINITI implements an analytical search in data flows in various natural and technical sciences, receiving information from 130 countries in 66 languages. On that basis, it prepares about one million documents a year, presenting them both as printed publications and computer-readable databases. The unique feature of VINITI is that it collects, creates abstracts and stores all scientific and technical materials which were not published anywhere (deposited manuscripts, dissertations, etc). VINITI can provide printed or electronic copies of these materials. VINITI RAN is one of the largest databases in Russia for natural, exact and technical sciences. It includes the materials of VINITI since 1981 and has more than 26 million documents (periodicals, books, conference proceedings, theses, patents, normative documents, deposited scientific works, etc), 30% of which are from Russian sources.

5.1.3. International Science and Technology Centre (ISTC), Moscow

ISTC (http://www.istc.ru/) is an international organization established by an international agreement in November 1992 to prevent the proliferation of nuclear and other weapons of mass destruction. The ISTC supports innovative projects that are expected to induce business opportunities by launching new commercial joint ventures that link the demands of international markets with the highly qualified scientific talent pool available in Russian and the Commonwealth of Independent States (CIS) institutes. The ISTC is a non-profit organization with a sister organization called the Science and Technology Center of the Ukraine (STCU, http://www.stcu.int/).

5.2. Libraries

Two Russian largest libraries with online catalogues are following:

5.2.1. Russian State Library (RSL), Moscow

From 1925 to 1992, this library was named V.I. Lenin State Library of the USSR and is currently one of the largest libraries in the world with more than 42 million items in 247 languages. Digital library and on-line catalogue are available (http://www.rsl.ru/index.php?f=97).

5.2.2. National Library of Russia (NLR), St. Petersburg

The National Library of Russia in Saint Petersburg holds the most complete collection of publications in Russian. Special attention is traditionally paid to the acquisition of foreign works about Russia and publications in languages of the Russian Federation printed in different countries of the world. Today the National Library of Russia houses more then 34.5 million items, of which 6.2 million items are in foreign languages. Electronic catalogues of books and journals are available in Russian (http://www.nlr.ru/res/cat/) and in English (http://www.nlr.ru/eng/opac/).

5.3. Websites

A well-documented list of web-based resources was previously reviewed by Kurlyandskiy and Sidorov (2003). Additional websites of toxicological and/or ecotoxicological interest are listed below.

5.3.1. Russian section of the Cheminformatics and QSAR Society

In 2006 the Russian section of the QSAR and Modeling Society (http://www.ndsu.edu/qsar_soc/aboutsoc/russia.htm) included members from Moscow, Novosibirsk and other cities of the Russian Federation. The main purpose of the Russian section is the promotion of collaboration between Russian and foreign scientists in the fields of bioinformatics, (Q)SAR, molecular modeling,

and computer-aided drug discovery. Therefore, the Russian section intends to provide its activity in close collaboration with the International Board of the QSAR and Modeling Society.

5.3.2. *Laboratory of Structure-Function Based Drug Design, Department of Bioinformatics, Institute of Biomedical Chemistry RAMS*

This laboratory focuses on bioinformatics and computer design of drugs, particularly on the identification of "structure-activity" and "structure-property" relationships, on the predictions of biological activity, toxicity and the biotransformation of pharmacological substances. Several software and associated databases were developed in the laboratory and are available on the web (http://www.ibmc.msk.ru/en/departments/drug_design/) after registration:

- PASS (PC/Web), for prediction of the biological activity spectrum for a compound on the basis of its structural formula.
- GUSAR (PC) – for the quantitative prediction of physical chemistry properties, biological activity and toxicity.
- PharmaExpert (PC/Web) – to describe the relationship between pharmacological effects and mechanisms of physiologically active substances.
- METAPREDICT (PC) – to predict classes and biotransformation sites of substances.

5.3.3. *FSI "Research and Applied Toxicology Centre (RTIAC)" (*Научно - практический Токсикологический Центр*), Moscow*

The main purpose of this center (http://web.mac.com/sktors/rtiac/index.html) is to improve the quality of medical care in cases of acute chemical poisoning. It provides information regarding the treatment of human poisonings to the general public and health care professionals. This centre has developed an information bank of toxicological data "IPCS INTOKS" for doctors and toxicologists on providing health care for acute chemical poisonings.

5.3.4. *'MAIK Nauka/Interperiodica", Russian Academy of Science, PMG Enterprises Ltd.*

"MAIK Nauka/Interperiodica" (http://www.maik.ru/) is a company that publishes more than 180 academic journals in English in cooperation with Pleiades Publishing Inc. with the support of the Russian Academy of Sciences, and more than 200 journals in Russian in cooperation with Akademizdattsentr Nauka. The system of electronic publication allows the world scientific community to access full-text electronic versions of Russian scientific journals published under the logo of the Russian Academy of Sciences, and provides Russian and foreign scientists with up-to-date world-standard information services. Indeed, MAIK is part of Pleiades Publishing, uniting 10 Russian publishing entities with a combined overall volume of 1000 titles per year. In addition to "MAIK Nauka/Interperiodica", the holding includes the following publishing houses: "Akademkniga" (Chemistry, Biology, Transport, Metallurgy); "Fizmatlit" (Mathematics, Physics, Information Science); "Zoomedlit" (Veterinary Science, Cattle Breeding, Microbiology); "KolosS" (Agriculture and Mechanical Engineering); "Medkniga" (Medicine); and the humanitarian and educational publishing houses "Flinta," "Yurist," "Economist," "Gardariki," and "Akademkniga/Uchebnik". Recently Pleiades Publishing has acquired Allerton Press, which publishes English translations of 45 scientific and technical journals originating from Russia. Both Pleiades Publishing and Springer Science + Business Media entered into a contract, the main project being called "Russian Library of Science" – an online library in English, which became a part of Springerlink (see Section 5.5.4).

5.3.5. *eLIBRARY.RU – Scientific electronic database*

This database is accessible on the website of eLIBRARY.RU after registration (http://www.elibrary.ru/defaultx.asp?lng=EN). It contains more than 27,000 journals of which almost 5100 are Russian. Full texts are available for about 790 Russian journals.

5.4. *Russian web-based toxicological databases*

A search of databases was made through websites of the Ministry of Health of the Russian Federation (http://www.mzsrrf.ru/) and of the Ministry of Natural Resources of the Russian Federation (http://www.mnr.gov.ru/) using various search engines both in English and Russian. The Russian language website of the noncommercial organization of Sciences – Center for the Environment – Risk – Health, has links to foreign toxicological and risk databases (http://www.erh.ru/dbchemicals.php). In addition, two other potentially useful Russian language databases for collecting toxicological data were identified and are described below:

5.4.1. *"ARIPS Hazardous substances" (*Опасные вещества, *http://www.rpohbv.ru/arips/online/)*

This on-line database in Russian language of the Russian Register of Potentially Hazardous Chemical and Biological Substances contains toxicological data on more than 3100 substances handled in the territory of Russia (individual chemical and biological compounds produced and/or imported into Russia). The database includes data on toxicity and hazard to humans and the environment, and also occupational and environmental standards. These data are available after paid registration. Literature references used in this database originate not only from Russian sources but also from various international databases including:

- EU Uniform Chemical Information Database (IUCLID),
- monographs of the International Agency for Research on Cancer (IARC),
- database of the International Labor Organization (ILO),
- International Register of Potentially Toxic Chemicals (IRPTC),
- Registry of Toxic Effects of Chemical Substances (RTECS).

In this database, references are presented as a separate paragraph and are not linked directly with the data and so the origin of data (Russian or foreign) is not always clear. This database will be characterized in more detail below (see Section 8).

5.4.2. *"Chemistry and Toxicology" (*Химия и токсикология, *http://chemister.da.ru/)*

This personal website was created by Mr Ruslan Kiper in 2001 and is constantly updated. It contains well documented information on organic and inorganic synthesis, toxicology and pharmacology. The website is in Russian, although there is a demo version of chemical database in English (http://chemister.da.ru/Database/search-en.dbp). The website contains an impressive collection of data on 5158 chemicals, including toxicity data for 341 chemicals and toxins with literature references) (http://chemister.da.ru/Toxicology/ld50.htm). For 119 highly toxic substances and venoms, there is also information on physico-chemical properties and the chemical formula (http://chemister.da.ru/Toxicology/Toxins/toxins.htm). In addition, the physico-chemical properties and toxicities of 31 chemical warfare substances are documented (http://chemister.da.ru/Toxicology/BOV/bov.htm). Symptoms of poisoning are given for 128 toxic substances and venoms. For 19 chemicals, treatment methods are also given. Moreover, there is a page on laboratory safety and even laws concerning occupational chemical exposure limits.

5.5. Western websites containing Russian data or papers

In few public access Western databases, papers or abstracts in English from Russian scientific papers are available.

5.5.1. US TOXNET and US EPA ECOTOX databases

TOXNET is an US integrated system of toxicology and environmental health databases available free of charge on the web. In turn, several other databases are available for searching via TOXNET. TOXLINE covers the scientific literature in toxicology including literature in Russian published in the former socialist countries (Poland, Czechoslovakia, Hungary and GDR). ChemIDplus provides access to structure and nomenclature information for the identification of chemical substances of over 380,000 chemical records, of which over 289,000 include chemical structures. In addition another US database, the US EPA ECOTOX contains also data from Russian origin.

5.5.2. US PubMed

PubMed is a well-know service of the US National Library of Medicine (NLM) that includes over 18 million citations from MEDLINE and other life science journals for biomedical articles dating back to the 1950s. The search through the PubMed journal database with "russian" as a keyword yielded altogether 342 journals published in former socialist countries (Russia, Poland, Czechoslovakia, etc.) (data not shown). The PubMed journal database contains also English-translated titles of the papers published in Russian and in some cases also abstracts are available. For example, almost 7500 reference titles in English from the journal "Industrial Hygiene and Occupational Diseases" (Гигиена труда и профессиональные заболевания) can be found for 1960–1992. Abstracts in English are partially available only for the period 1989–1992. More information on this Russian journal can be found in the Section 6.2.

5.5.3. French INIST's catalogue of articles and monographs: Article@INIST database

This French on-line database contains about 15 million bibliographic records of documents held in the INIST/CNRS collections starting from 1973 and covering all fields of worldwide research in science, technology, medicine, humanities and social sciences. Freely accessible, Article@INIST provides a direct link to a document copy ordering service. In total 76 Russian journals and almost 140,000 articles published in Russian are indexed in this database.

5.5.4. Springer's "Russian Library of Science", http://www.springer.com/life+sci?SGWID=0-10027-12-129141-0

Springer is one of the largest publishers and distributors of cover-to-cover translations of Russian-language journals, all peer-reviewed and authoritatively translated. The aim of the "Russian Library of Science" is to publish the best articles and papers from research institutes and scientific societies in Russia and the surrounding countries. The "Russian Library of Science" contains more than 200 journals of which 29 are covering various topics of life sciences. The table of contents of the Journals is available online. The recent cooperation agreement between Pleiades Publishing (i.e. Allerton Press) and Springer Science + Business Media, Inc. expands the "Russian Library of Science" (see also Section 5.3.4).

5.6. Estonian libraries and databases

5.6.1. National Library of Estonia, Tallinn

The acquisition of collections in the National Library of Estonia (http://www.nlib.ee/) began with the foundation of the State Library in 1918. Since then, the Library started receiving deposit copies of all Estonian publications. Also, the collections were increased via international book exchange. During the Soviet period, communication with foreign libraries ceased and the collections were dominated by Russian language publications received as deposit copies (that the authors of the current paper also used as information for mapping the toxicological literature sources). Currently, the collections may be searched through the online catalogue "ESTER".

5.6.2. ETIS – Estonian Research Information System

The Estonian Research Information System ETIS (http://www.etis.ee) collects and stores information on research and development institutions, researchers, research projects and various research results. Researchers use also ETIS for submitting applications for grants, for reporting and archiving their documents/projects. In addition, Estonian R&D institutions can submit through ETIS applications and introduce their research results more widely. Research funding organizations use ETIS for evaluating and processing applications and giving feedback. This database allows searches in English and in Estonian by names of scientists and/or institutions, by keywords or by projects.

6. Main documents containing toxicological data published in the Russian language

6.1. Books

The search was performed mainly via card catalogues of the Academic Library of Tallinn University, the former Medical Library of Estonia and electronic catalogues (common electronic catalogue of Estonian libraries ESTER) of different libraries in Estonia, via on-line electronic catalogues of the Russian State Library and the National Library of Russia. Limited search was also made via the e-catalogue of the US National Library of Medicine. One of the most valuable documents in terms of REACH and industrial chemicals is a series of handbooks for chemists, engineers and doctors entitled "Hazardous substances in the industry" (Вредные вещества в промышленности). The first volume of this series was published by Lazarev and Astrachanzev in 1933 and there is a lot of updated editions. Each book of this series is about 600 pages. The first editions of the reference book were entitled as "Hazardous chemical substances in the industry" ("Химически вредные вещества в промышленности"). In 1954 it was renamed as "Hazardous substances in the industry" ("Вредные вещества в промышленности"). These handbooks contained toxicological information on industrial chemicals not only from Russian sources but also from US and Western sources. During the period of 1954 –1964 this handbook was translated and published in Polish, Czech, Romanian and Chinese (Filov, 1987). In Estonian libraries, 18 different editions of this series of handbooks are available (the titles listed as additional material in Table STable S2).

6.2. Journals

The search for scientific journals was carried out mainly via various Russian language internet sites and electronic catalogues of different libraries in Estonia and in Russia, but also by using PubMed databases. As a result, 37 different Russian language peer-reviewed scientific journals published between 1928 and 2007 were identified. Most of these journals are available in Estonian libraries. As no search engines were available for these journals, the journals were partly or fully screened by reading titles, abstracts and in most of the cases, contents. The most important journals among them are:

- "*Doklady Akademii Nauk SSSR*" (Proceedings of the USSR Academy of Sciences, ISSN 0002-3264) was published from 1933 to 1992. Since 1992 it has been continued as "*Doklady Akademii Nauk*" (Rossiĭskaia akademii nauk, Proceed-

ings of the Russian Academy of Sciences, ISSN 0869-5652). Since 2001, several sub-series have been translated into English and are available on-line (http://www.maik.ru/cgi-perl/journals.cgi?lang=eng&action=alphabet#). Main toxicological information can be found in "*Doklady Biochemistry and Biophysics*" (ISSN 1607-6729), "*Doklady Biological Sciences*" (ISSN 0012-4966) and "*Doklady Earth Sciences*" (ISSN 1028-334X).

- "*Hydrobiological Journal*" (Гидробиологический журнал, ISSN 0018-8166 print version), published since 1965 until now by the Institute of Hydrobiology, National Academy of Sciences of Ukraine (http://hydrobiolog.narod.ru/). The journal is simultaneously translated into English and published under the title "Hydrobiological Journal", Begell House, Inc. Pub., USA. Abstracts from 1998 are available in English at http://www.begellhouse.com/journals/38cb2223012b73f2.html. The volumes of the journal published up to 1971 are available in the National Library in Tallinn (Estonia).
- "*Hygiene and Sanitaria*" (Gigiena i sanitaria, Гигиена и санитария, ISSN 0016-9900) has been published since 1922 and covers all aspects of hygiene and sanitary practices. The focus is on hygiene of the environment, communal hygiene, the hygiene of children and teenagers, the hygiene of labor and nutrition, organization and planning of sanitary affairs as well as training of sanitary doctors. The tables of contents are available on http://www.medlit.ru/medrus/gigien.htm. The journal up to 1993 (except 1947) is available in the National Library in Tallinn (Estonia).
- "*Inland Water Biology*" (Биология внутренних вод, ISSN: 1995-0829 (print version) ISSN: 1995-0837 (electronic version) is published since 1995 till now. The journal is published in Russian and in English (http://www.springer.com/life+sci/ecology/journal/12212) under the authority of the Office of Biological Sciences, Russian Academy of Sciences. The journal covers fundamental research on aquatic organisms, from viruses to fish and aquatic mammals.
- "*Modern problems of Toxicology*" (Современные проблемы токсикологии, ISSN 1609-0446 print version, ISSN 1609-0470 on-line version). Since 1998, this journal has been published by the Medved's Institute of Ecohygiene and Toxicology in Kiev, Ukraine. The full content in Ukrainian or Russian is available on the web and the abstracts are translated into English (http://www.medved.kiev.ua/MAG/arhiv.HTM).
- "*Occupational medicine and industrial ecology*" (Meditsina truda i promyshlennaia ekologiia, Медицина труда и промышленная экология, ISSN 1026-9428). This journal was previously named "*Industrial hygiene and occupational diseases*" from 1957 to 1992 (Gigiena truda i professional'nye zabolevaniia, ISSN 0016-9919) and includes papers on theoretical and practical problems of occupational health and diseases, including toxicology. The journal also publishes the description of different test methods and literature reviews from Russia and abroad. Since 2008 the *table of contents is* available online (http://www.niimt.ru/publishing/magaz_contents2008.php?lang=en). The volumes of the journal up to 1992 are also available in the National Library in Tallinn (Estonia).
- "*Pharmacology and Toxicology*" (Farmakologiia i toksikologiia, Фармакология и токсикология; ISSN 0014-8318) was published from 1939 to 1991. Since 1992, the journal continues as "*Experimental and clinical pharmacology*" (Eksperimental'naia i klinicheskaia farmakologiia, Экспериментальная и клиническая фармакология, ISSN 0869-2092). The tables of contents of this journal for 2000–2006 are available on the web (http://ekf.folium.ru/contents.htm). The volumes of the journal up to 1991 are available in the National Library in Tallinn (Estonia), although some volumes are missing (1942–1945, 1950–1956).
- "*Toxicological reviews*" (Токсикологический вестник, ISSN 0869-7922) has been published since 1993 by the Federal State-owned Establishment of Public Health (FSEH) "Russian Register of Potentially Hazardous Chemical and Biological Substances (RRPHCBS)". It highlights scientific and practical issues related to chemical safety and publishes legislative and regulatory documents concerning chemicals (http://www.rpohbv.ru/magazin/). It should be noted that it is different from the "Toxicological reviews" (ISSN 1176-2551) published since 2003 by Adis International (New Zealand).

In January 2009 we performed a search in PubMed using a combination of the keywords (toxic*) AND ("russian"[Language]) and obtained 16374 hits. The bibliometrical analysis in PubMed for some of the above described journals using the keyword "toxic*" showed that those Russian journals (Table 3) accounted in total for 37% of the hits for the combination "toxic* AND Russian" (6075/16374) and that the "Doklady. . ." titles seem to be the least representative for toxicological studies (Table 3).

6.3. Scientific reviews of Soviet literature on toxicity and hazard of chemicals

The International Register of Potentially Toxic Chemicals (IRPTC or UNEP Chemicals) has collaborated with the Government of the USSR to publish in English and in Russian a series of Scientific Reviews of Soviet Literature on Toxicity and Hazards of Chemicals (http://www-cger.nies.go.jp/cger-e/db/info-e/InfoDBWeb/db/irptc.htm). The current availability of these papers

Table 3
Visibility of the toxicological research published in Russian scientific journals indexed in PubMed database. Indices a and b show the change of the name of the Journal (see also Section 6.2).

Journal	Number of documents in PubMed	Number of documents containing "toxic*"	% of documents found with keyword "toxic*"
1. Hygiene and sanitary (Gigiena i sanitaria)	18622	2980	16.0
2.a. Industrial hygiene and occupational diseases (Gigiena truda i professional'nye zabolevaniia)	7426	1375	18.5
2.b. Occupational medicine and industrial ecology (Meditsina truda i promyshlennaia ekologiia)	2187	207	9.5
3.a. Pharmacology and toxicology (Farmakologiia i toksikologiia)	6350	989	15.6
3.b. Experimental and clinical pharmacology (Eksperimental'naia i klinicheskaia farmakologiia)	2059	329	16.0
4. Doklady Akademii Nauk SSSR	9859	101	1.0
5. Doklady Akademii Nauk	1378	54	3.9
6. Doklady biological sciences	1283	32	2.5
7. Doklady biochemistry and biophysics	815	8	1.0
Total:	49979	6075	

Source: PubMed, 2009. (http://www.ncbi.nlm.nih.gov/pubmed/).

is problematic even though these are constantly cited in various reports and reviews.

6.4. Dissertations

The Russian State Library (RSL) has a unique collection of scientific dissertations. The All-Russian (former All-Union) Collection of Dissertations was founded by the Decree of the All-Union Committee for Higher School. Around 17,000 Candidate of Sciences (*Russian:* кандидат наук, more or less equivalent to Ph.D. degree) and 8000 Doctor of Sciences (*Russian:* доктор наук, the highest post-graduate academic degree in the Soviet Union, Russia and in many post-Soviet states awarded in recognition of a substantial and sustained contribution to scientific knowledge) dissertations in all fields of research are collected every year. At the moment the full access is possible in the reading rooms of the RSL. Digital versions of dissertations have been available since 1996. The search can be performed via the electronic catalogue of the RSL.

7. Impact factor of Russian scientific journals

The impact factor of a journal is a measure of the frequency with which the "average article" in a journal has been cited in a particular year or period (Garfield, 2006). Based mainly on papers published in English, it is calculated each year by Thomson Scientific, previously known as The Institute for Scientific Information® (ISI®). And thus, it was recommended by Garfield (1997) that all Russian journals could provide keywords and abstracts in English.

A Russian Impact Factor of Russian scientific citation index (Impact factor RINTS) has also been established by the Scientific Electronic Library of Moscow (eLIBRARY.RU). The comparison of those two impact factors jointly available for 24 Russian journals shows that these indices are not correlated (data not shown). It should be noted that none of these 24 journals were related to toxicology. However, all journals considered relevant for toxicology (listed in Section 6.2) belonged to top-1000 Russian journals and had a RINTS factor above 0.200 (http://www.physchem.chimfak.rsu.ru/index-eng.html).

8. Comparison of the Russian database "Hazardous Substances" with European and American databases

In order to compare the Russian database "Hazardous Substances" (see also Section 5.4.1.) with the analogous US TOXNET (http://toxnet.nlm.nih.gov/) and US EPA ECOTOX (http://cfpub.epa.gov/ecotox/) databases, three lists of chemicals were used:

- two lists of chemicals from the European Chemical Substances Information System, i.e. the PBT (persistent, bioaccumulative and toxic chemicals) list and the ORATS (Online European Risk Assessment Tracking System) list
- a list consisting of 373 phenolic compounds (Phenols list) was created by the authors of this paper. The list is a result of the search in US EPA database ECOTOX using "phenol" as a keyword.

For all three lists, less than 23.4% of the compounds were registered in the Russian database (Table 4).

Among the Phenols list of 373 chemicals, only 8.3% were recorded in the Russian database whereas 317 chemicals (84.9%) were recorded in the US TOXNET database (data not shown). However, for those 317 chemicals, 207 were associated with toxicological data.

For the Phenols list, the search in TOXNET was also done with the language restriction criterion "limit to: Russian" (years: 1900–2008) using Toxline (Toxicology Literature Online) subdatabase. In this case the hits obtained included articles published in Russian from Russian journals and in addition also from those of former socialist countries (Poland, Czechoslovakia, Hungary, GDR). As a total, 317 chemicals of the Phenols list had 130610 references in Toxline of which only 2372 (1.8 %) were in Russian. However, these Russian references concerned only 112 chemicals (38.7%) of the Phenols list. The comparison of the use of test organisms in toxicological research showed that the pattern of use of test animals is similar in Russian "Hazardous Substances" and TOXNET databases with rodents as the most often used group of animals (Table 5).

Among the 373 chemicals of the Phenols list, 297 had aquatic toxicity data for algae, fish, protozoa, molluscs and crustaceans. In contrast, only 21 of those 373 chemicals had ecotoxicological data in the Russian database. Crustaceans, fish and algae are the most used ecotoxicological test organisms in both databases (Table 6).

Table 4
Availability of toxicity data for different types of chemicals in the Russian database "Hazardous Substances" (Опасные вещества, http://www.rpohbv.ru/arips/online/).

List of chemicals	Total number of chemicals in the list	Number and percentage of chemicals present in Russian database "Hazardous Substances"
PBT list[a]	127	17 (13.8%)
ORATS list[b]	141	33 (23.4%)
Phenols list[c]	373	31 (8.3%)

Search in the Russian database was performed compound-by-compound using CAS numbers.
[a] Persistent, bioaccumulative and toxic chemicals.
[b] Chemicals from Online European Risk Assessment Tracking System.
[c] Phenolic compounds from EPA ECOTOX database.

Table 5
Comparison of Russian on-line database "Hazardous Substances" (Опасные вещества, http://www.rpohbv.ru/arips/online/) and US TOXNET database in terms of usage frequency (%) of test organisms in toxicological research.

Species	Usage frequency (%) in the toxicological research	
	US TOXNET database	Russian database
Dog	4.8	4.8
Cat	4.1	4.0
Guinea pig	8.4	14.4
Mouse	29.7	24.0
Rabbit	10.7	22.4
Rat	27.0	23.2
Hamster	0.4	5.6
Chicken	4.1	0.8

Table 6
Comparison of Russian on-line database "Hazardous Substances" (Опасные вещества, http://www.rpohbv.ru/arips/online/) and US EPA ECOTOX database in terms of usage frequency (%) of test organisms in ecotoxicological research.

Species	Usage frequency (%) in the ecotoxicological research	
	US EPA ECOTOX database	Russian database
Algae	15.3	29.8
Fish	41.5	35.1
Protozoa	8.9	1.8
Mollusks	13.2	1.8
Crustaceans	21.1	31.6

9. Conclusions

The reviewed Russian data sources contain a considerable amount of toxicological information on different pure chemicals, mixtures and preparations, including pesticides and pharmaceuticals. The major limitation of access to this information is knowledge of the Russian language. However, this limitation could be par-

tially overcome by using on-line translation services (translation websites) such as Google™ Translate (http://translate.google.com). Another limitation of the access to Russian toxicological data might be that this information is usually not digital and is available only in libraries. Fortunately, in the libraries of former Soviet republics now part of the EU, such as Estonia, large collection of Russian language literature has been preserved. This makes access to original documents much easier.

In addition to the problems linked to apparently limited access, CAS numbers or IUPAC names of chemicals are not always provided and thus identification of compounds may be difficult for non-chemists. More positively, references to test guidelines are usually given at least in the list of literature references. As a rule, toxicity studies have been or are conducted according to standardized methodologies of the former USSR (Sanotski, 1970) and in the Russian Federation (Ministry of Natural Resources of the Russian Federation, 2001). Currently, many ISO international standards and EPA standard procedures are also used (http://www.bioassay.narod.ru/standards/standards.html).

This review demonstrates that considerable amounts of information from Russian language data sources of potential benefit for the new EU chemical policy REACH (European Parliament, 2006) remains unexplored, mostly due to language barriers. Those hidden data may be used for the development of intelligent testing strategies (ECETOC, 2007) that enable a significant increase in the use of non-testing information for regulatory decision making, thus minimizing the need for animal testing (3R: reduction, replacement, refinement). Indeed, the Annexes VII-X of the REACH directive state: "all available *in vitro* data, *in vivo* data, historical human data, data from valid (Q)SARs and data from structurally related substances (read-across approach) shall be assessed first".

Conflict of interest

There are none.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2009.05.001.

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Russom, C.L., 2002. Mining environmental toxicology information: web resources. Toxicology 173 (1–2), 75–88.

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SJR SCImago Journal & Country Rank

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Country Indicators

Ranking Parameters

Subject Area: Environmental Science
Subject Category: Health, Toxicology and Mutagenesis
Region: Eastern Europe
Year: 1996-2007
Order By: Documents
Display countries with at least: 0 Documents
Refresh

Subject Area: **Environmental Science**.
Subject Category: **Health, Toxicology and Mutagenesis**.
Region: **Eastern Europe**.
Period: **1996-2007**.

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	Country	Publications	Citable Publications	Citations	Self-Citations	Citations per Publication	H index
1	Poland	770	756	5.717	1.576	7,84	31
2	Czech Republic	425	419	4.146	1.310	11,16	30
3	Russian Federation	348	342	2.775	733	8,08	24
4	Hungary	232	223	2.082	562	8,05	21
5	Slovakia	200	198	1.938	412	9,80	19
6	Croatia	184	178	1.335	315	7,15	20
7	Bulgaria	171	167	1.263	227	7,83	18
8	Slovenia	96	94	555	185	8,28	14
9	Ukraine	73	72	513	122	7,06	13
10	Estonia	64	64	329	58	6,34	11
11	Belarus	54	53	474	60	9,64	13
12	Lithuania	54	53	356	51	12,34	10
13	Romania	25	25	92	9	4,06	6
14	Latvia	13	13	155	35	18,52	4
15	Armenia	12	7	52	10	13,00	4
16	Azerbaijan	5	5	29	18	6,83	4
17	Serbia	4	4	0	0	0,00	0
18	Moldova, Republic Of	3	3	7	2	2,50	2
19	Albania	3	3	19	2	6,00	3
20	Bosnia and Herzegovina	2	2	12	0	12,00	1
21	Georgia	1	1	0	0	0,00	0
22	Macedonia, The Former Yugoslav Republic Of	1	0	1	0	1,00	1

Table S1:
Databases/websites cited (updated on April 16, 2009).

Website/database	URL	Major topics covered	Availability in English
INSTITUTIONS, SOCIETIES AND COOPERATION PROGRAMS			
All-Russian Organization for Quality Assurance Committee REACH	http://www.reach.ru	International business and advice to Russian companies under the laws of REACH.	No*
Chemical Weapons Demilitarisation Conferences (CWD)	http://www.dstl.gov.uk/conferences/cwd/	CWD Papers and Presentations since 11 years	Yes
Estonian Society of Toxicology	http://www.kbfi.ee/ets	Activities and members	Yes
EU Cooperation with the NIS in S&T	http://ec.europa.eu/research/nis/en/istc.html	informative	Yes
Russian Academy of Sciences	http://www.ras.ru	Activities and access to the «Publications» Web-portal of Russian Academy of Sciences	Under construction
Russian section of the Cheminformatics and QSAR Society	http://www.ndsu.edu/qsar_soc/aboutsoc/russia.htm	Activities and members	Yes
RESEARCH CENTERS			
Institute of Physiologically Active Compounds (IPAC)	http://www.ipac.ac.ru	Activities of the research center	No*
Institute of Toxicology, St. Petersburg	http://toxicology.ru	Activities of the research center	No*
International Science and Technology Centre (ISTC)	http://www.istc.ru	Activities of the center	Yes
Laboratory of Structure-Function Based Drug Design, Institute of Biomedical Chemistry RAMS	http://www.ibmc.msk.ru/en/departments/drug_design	Software for drug design and QSAR	Yes

Research Center for Applied Microbiology and Biotechnology (SRCAMB)	http://www.obolensk.org	Activities of the research center	Yes
Research Center for Toxicology and Hygienic regulation of Biopreparations (RCT&HRB)	http://www.toxicbio.ru	Activities of the research center	Yes
Research Institute of Hygiene, Toxicology and Occupational Pathology (RIHTOP)	http://www.rihtop.ru	Activities of the research center	Yes
Science and Technology Center in Ukraine (STCU)	http://www.stcu.int	Activities of the center	Yes
TOXICOLOGICAL DATABASES			
ARIPS «Hazardous Substances»	http://www.rpohbv.ru/arips/	On-line version of the database ARIPS	No*
Chemistry and Toxicology	http://chemister.da.ru	Chemical and toxicity data	No*
Research and Applied Toxicology Centre (RTIAC)	http://web.mac.com/sktors/rtiac/index.html	Bank of toxicological data IPCS INTOKS and toxicological journals	No*
LIBRARIES AND ELECTRONIC CATALOGUES			
All-Russian Institute of Scientific and Technical Information (VINITI)	http://www2.viniti.ru	Access to integrated scientific and information (science, technology and economics)	No*
eLIBRARY.RU	http://www.elibrary.ru/defaultx.asp?lng=EN	Scientific electronic database	Yes
Estonian Research Portal ETIS	http://www.etis.ee	Database of Estonian research: researchers, projects and publications	Yes
National Library of Estonia	http://www.nlib.ee	ESTER online catalogue	Yes
National Library of Russia (NLR)	http://www.nlr.ru/eng/opac	Online Catalogues	Yes
Russian State Library (RSL)	http://www.rsl.ru/index.php?f=97	United Electronic Catalogue (UEC) (Alef System)	Yes

SCIENTIFIC JOURNALS

Doklady Akademii Nauk	http://www.maik.ru/cgi-perl/journals.cgi?lang=eng&action=alphabet#	On-line "Doklady Biochemistry and Biophysics", "Doklady Biological Sciences", "Doklady Earth Sciences"	Yes
Experimental and clinical pharmacology	http://ekf.folium.ru/contents.htm	On line contents since 2001	No*
Hydrobiological Journal	http://www.begellhouse.com/journals/38cb2223012b73f2.html	On line papers	Yes
Hygiene and Sanitaria	http://www.medlit.ru/medrus/gigien.htm	On line contents	No*
Inland Water Biology	http://www.springer.com/life+sci/ecology/journal/12212	On line papers available since 2008	Yes
MAIK Nauka/Interperiodica	http://www.maik.ru and http://www.maikonline.com	Scientific Russian journals. Online	Yes
Modern problems of Toxicology	http://www.medved.kiev.ua/MAG/arhiv.HTM	On line papers in Russian available since 1998	Yes
Occupational medicine and industrial ecology	http://www.niimt.ru/publishing/magaz_contents2008.php?lang=en	On line contents since 2008	Yes
Springer's "Russian Library of Science"	http://www.springer.com/life+sci?SGWID=0-10027-12-129141-0	Papers from research institutes and scientific societies in Russia and the surrounding countries	Yes
Toxicological reviews	http://www.rpohbv.ru/magazin	On line with subscription	No*

*On-line translation to English possible with Google Translate

Table S2

Issues of Russian series of handbooks “Hazardous substances in the industry” (“Вредные вещества в промышленности”) available in Estonian libraries.

	Original Russian title	Year of publishing	Title in English (translation by authors of this paper)
	ORGANIC CHEMICALS		
1	Химически вредные вещества в промышленности. Ч. 1, Органические вещества : справочник для химиков, инженеров и врачей / Н.В. Лазарев. Ленинград ; Москва: Государственное научно-техническое издательство химической литературы	1951	Hazardous substances in the industry. Part 1: Organic compounds: a handbook for chemists, engineers and doctors. N.V. Lazarev, Leningrad, Moscow: State Scientific and Technical Publishers of the chemical literature, 1951
2	Вредные вещества в промышленности 1, http://193.40.4.177:80/record=b1190004~S1*est	1963	Hazardous substances in the industry, Part 1, 1963
3	Вредные вещества в промышленности 2, http://193.40.4.177:80/record=b1190005~S1*est	1963	Hazardous substances in the industry, Part 2, 1963
4	Вредные вещества в промышленности. Ч. 1, Органические вещества: справочник для химиков, инженеров и врачей / под редакцией Н.В. Лазарева. Москва : Химия	1965	Hazardous substances in the industry. Part 1: Organic compounds: a handbook for chemists, engineers and doctors. Ed: N.V. Lazarev. Moscow: “Khimiya” Publishing house, 1965
5	Вредные вещества в промышленности : справочник для химиков, инженеров и врачей: [в 3-х томах]. 1, Органические вещества / под общей редакцией Н.В. Лазарева, Э.Н.Левиной. Ленинград : Химия, Ленинградское отделение	1976	Hazardous substances in the industry: a handbook for chemists, engineers and doctors (in 3 volumes). Part 1: Organic compounds.Genral Eds: N.V. Lazarev, E. N. Levina. Leningrad: “Khimiya” Publishing house, Leningrad Department, 1976
6	Вредные вещества в промышленности : справочник : для химиков, инженеров и врачей: [в 3-х томах]. 2, Органические вещества / под общей редакцией Н.В. Лазарева и Э.Н. Левиной, Ленинград : Химия, Ленинградское отделение.	1976	Hazardous substances in the industry: a handbook for chemists, engineers and doctors: (in 3 volumes). Part 2: Organic compounds. General Eds: N.V. Lazarev and E.N. Levina; Leningrad: “Khimiya” Publishing house, Leningrad Department, 1976
7	Вредные вещества в промышленности: органические вещества: новые данные 1974-1984 г.г. : справочник / под общей редакцией Э.Н. Левиной и И.Д. Гадаскиной. Ленинград : Химия	1985	Hazardous substances in the industry: organic compounds: new data on years 1974-1984: a handbook. General Eds. E.N. Levina and I.D. Gadaskina.Leningrad: “Khimiya” Publishing house, Leningrad Department, 1977
8	Вредные химические вещества : справочник, Углеводороды, галогенпроизводные углеводородов / под общей редакцией В.А. Филова. Ленинград : Химия, Ленинградское отделение	1990	Hazardous chemical compounds: a handbook. Hydrocarbons, halogenated derivatives of hydrocarbons. General Ed: V.A. Filov, Leningrad: “Khimiya” Publishing house, Leningrad Department, 1990
9	Вредные химические вещества. Галоген- и кислородсодержащие органические соединения : справочник / А. Л. Бандман, Г. А. Войтенко [и др.] ; под ред. В. А. Филова [и др.] Санкт-Петербург: Химия.	1994	Hazardous chemical substances. Halogen-and oxygen-containing organic compounds: a handbook. A.L. Bandman, G.A. Voitenko et al. Ed. by V.A. Filov et al.St. Petersburg: “Khimiya” Publishing house, 1994

INORGANIC CHEMICALS

10	Химически вредные вещества в промышленности. Ч. 2, Неорганические и металлоорганические соединения : справочник для химиков, инженеров и врачей / Н.В. Лазарев. Ленинград ; Москва: Государственное научно-техническое издательство химической литературы	1951 Hazardous substances in the industry. Part 2: Inorganic and organometallic compounds: handbook for chemists, engineers and doctors. N.V. Lazarev, Leningrad, Moscow: State Scientific and Technical Publishers of the chemical literature, 1951
11	Вредные вещества в промышленности. Ч. 2, Неорганические и элементорганические соединения : справочник для химиков, инженеров и врачей / под редакцией Н.В. Лазарева. Москва : Химия	1965 Hazardous substances in the industry. Part 2: Inorganic and elemental organic compounds: a handbook for chemists, engineers and doctors. Ed: N.V. Lazarev. Moscow: “Khimiya” Publishing house, 1965
12	Вредные вещества в промышленности : справочник для химиков, инженеров и врачей: [в трех томах. Том] II, Неорганические и элементорганические соединения / под общей редакцией Н. В. Лазарева и И. Д. Гадаскиной. Ленинград : Химия. Ленинградское отделение	1971 Hazardous substances in the industry: a handbook for chemists, engineers and doctors: (in three volumes). Part 2: Inorganic and elemental organic compounds. Eds: N.V. Lazarev and I.D. Gadaskina. Leningrad: “Khimiya” Publishing house. Leningrad Department, 1971
13	Вредные вещества в промышленности : справочник : для химиков, инженеров и врачей: [в 3-х томах]. 3, Неорганические и элементоорганические соединения / под общей редакцией Н.В. Лазарева и И.Д. Гадаскиной, Ленинград : Химия, Ленинградское отделение.	1977 Hazardous substances in the industry: a handbook for chemists, engineers and doctors (in 3 volumes).Part 3: Inorganic and elemental-organic compounds. General Eds Lazarev and Gadaskina
14	Вредные химические вещества : справочник, Неорганические соединения элементов I-IV групп / под общей редакцией В.А. Филова, Ленинград : Химия, Ленинградское отделение	1988 Hazardous chemical substances: a handbook, Inorganic compounds of elements of I-IV groups. General Ed: V.A. Filov Leningrad: “Khimiya” Publishing house, Leningrad Department, 1988
15	Вредные химические вещества: справочник, Неорганические соединения элементов V-VIII групп / под общей редакцией В.А. Филова. Ленинград : Химия, Ленинградское отделение	1989 Hazardous chemical substances: a handbook, Inorganic compounds of elements of V-VIII groups / General Ed: V.A. Filov. Leningrad: “Khimiya” Publishing house, Leningrad Department, 1989

ADDITIONAL VOLUME

16	Вредные вещества в промышленности : справочник для химиков, инженеров и врачей : дополнительный том / под общей редакцией Н.В. Лазарева, Ленинград : Химия	1969 Hazardous substances in the industry: a handbook for chemists, engineers and doctors: additional issue. General Ed: N.V. Lazarev, Leningrad: “Khimiya” Publishing house, 1969

PLASTICS

17	Вредные вещества в пластмассах: справочник / В.О. Шефтель, Москва: Химия	1991 Hazardous substances in plastics: a handbook. V.O. Sheftel, Moscow: “Khimiya” Publishing house

RADIOACTIVE SUBSTANCES

18	Вредные химические вещества: справочник, Радиоактивные вещества / под общей редакцией Л.А. Ильина, В.А. Филова. Ленинград : Химия.	1990 Hazardous chemical substances: a handbook. Radioactive substances. General Eds: L.A. Ilyin, V.A. Filov. Leningrad: “Khimiya” Publishing house, 1990.

PAPER II

Sihtmäe, M., Blinova, I., Aruoja, V., Dubourguier, H-C., Legrand, N., Kahru, A. 2010. E-SovTox: An online database of the main publicly-available sources of toxicity data concerning REACH-relevant chemicals published in the Russian language. *Alternatives to Laboratory Animals (ATLA)* 38, 297 - 301.

E-SovTox: An Online Database of the Main Publicly-available Sources of Toxicity Data Concerning REACH-relevant Chemicals Published in the Russian Language

Mariliis Sihtmäe,[1] Irina Blinova,[1] Villem Aruoja,[1] Henri-Charles Dubourguier,[1] Nicolas Legrand[2] and Anne Kahru[1]

[1]Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia; [2]Protopro-websites OÜ, Tartu, Estonia

Summary — A new open-access online database, E-SovTox, is presented. E-SovTox provides toxicological data for substances relevant to the EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) system, from publicly-available Russian language data sources. The database contains information selected mainly from scientific journals published during the Soviet Union era. The main information source for this database — the journal, *Gigiena Truda i Professional'nye Zabolevania* [*Industrial Hygiene and Occupational Diseases*], published between 1957 and 1992 — features acute, but also chronic, toxicity data for numerous industrial chemicals, e.g. for rats, mice, guinea-pigs and rabbits. The main goal of the above-mentioned toxicity studies was to derive the maximum allowable concentration limits for industrial chemicals in the occupational health settings of the former Soviet Union. Thus, articles featured in the database include mostly data on LD50 values, skin and eye irritation, skin sensitisation and cumulative properties. Currently, the E-SovTox database contains toxicity data selected from more than 500 papers covering more than 600 chemicals. The user is provided with the main toxicity information, as well as abstracts of these papers in Russian and in English (given as provided in the original publication). The search engine allows cross-searching of the database by the name or CAS number of the compound, and the author of the paper. The E-SovTox database can be used as a decision-support tool by researchers and regulators for the hazard assessment of chemical substances.

Key words: *ecotoxicity,* in vivo *toxicity, laboratory animals, occupational safety, REACH, Russia, Three Rs.*

Address for correspondence: *Anne Kahru, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia.*
E-mail: *anne.kahru@kbfi.ee*

Introduction

Soviet and Russian toxicologists have studied thousands of chemical substances for their toxic properties, in order to provide scientifically justified values for the maximum allowed concentration (MAC) limits for chemicals in working/occupational environments, ambient air, drinking water, food, surfaces, waters and soil. These data were, and still are, published in peer-review journals and other periodical issues (1–3). Most of these Soviet Union era publications are in Russian, but Estonian toxicologists, for example, have also been publishing in international journals, e.g. on the toxicological properties of various oil-shale chemicals (4–5), as well as on occupational health issues. In a paper entitled *Research Results of Soviet Scientists in Some Problems of Occupational Medicine: Review of the Years 1981–1984*, Kahn stated: "*We have set MAC values for more than 800 chemical substances… the research has become more complicated because the traditional toxicological experiments must now be supplemented by studies of various other biological effects, such as the sensitisation of the organism and mutagenic, teratogenic, carcinogenic and other effects…*" (6).

As an example of the numbers of animals used in some experiments performed by Soviet toxicologists, 180 white rats (180–240g), 130 white mice (18–24g) and 40 rabbits (2–2.5kg), were used to derive a MAC for benzonitrile in the working area air (7). In addition to the LD50 values, papers published on the toxicological properties of industrial chemicals contain data on histopathology, enzymatic and blood-component analysis, and a variety of information on the physiological parameters of exposed test animals. This information can be informative for the industrial toxicologists and/or authorities in charge of the chemical safety dossiers for the EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) system, where new animal tests must only be conducted as a last resort, and all available existing test data on the substances must first be gathered and assessed. Despite this, valuable toxicological data published in the Russian language have not been cited in recent scientific papers (8–17), except when these were the only informa-

tion sources available for a particular compound (18). The poor citation rate of these papers/data is probably due to the language barrier, as well as poor digitalisation of these journals.

The aim of the present work was to create an open-access online database, in which key data on the toxicological properties of REACH-relevant (industrial) chemicals reported in the Russian literature are available and searchable. More-detailed information about these sources can be found in Sihtmäe *et al.* (3).

Characterisation of the Database

— *Name of the Database:* E-SovTox;

— *Web address:* http://kbfi-databases.eu/;

— *Cost:* Free after registration;

— *Year of availability of the demo-version:* 2010;

— *Hardware and software required:* Any computer with Internet capabilities;

— *Programming language:* HTML/PHP/MySQL;

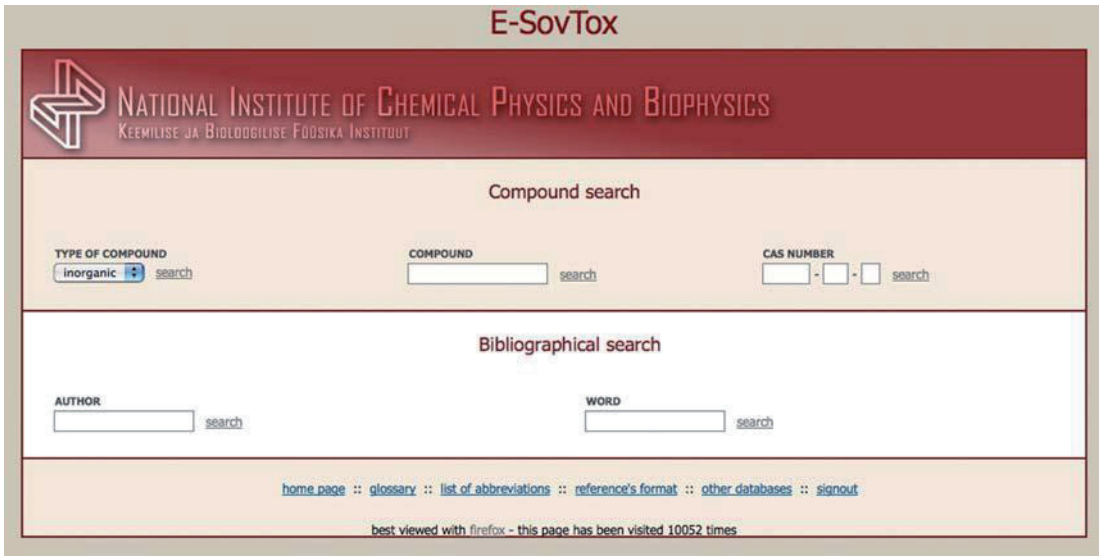
— *Programmed by:* Protopro-websites OÜ (www.dynameeks.com)

Database Description

The database is hosted by the Laboratory of Molecular Genetics (MGL), National Institute of Chemical Physics and Biophysics, Tallinn, Estonia. The MGL is in charge of data collection and editing, database maintenance and dissemination. The scientific leader of the MGL either acts as, or nominates, the administrator who grants user access.

Toxicity data were collected mainly from the Russian scientific journal Гигиена труда и профессиональные заболевания [*Gigiena Truda i Professional'nye Zabolevania*; *Industrial Hygiene and Occupational Diseases*] (ISSN 0016-9919). This journal is referred to in the US PubMed database, but full-text articles are not available on the web. This journal was issued from 1957 to 1992, and included papers on the theoretical and practical problems of occupational health and disease, including toxicological studies on industrial chemicals. Hard copies of the back-issues of the journal, published up to 1992, are available in the National Library in Tallinn, Estonia. In 1993, the name of the journal was changed to Медицина труда и промышленная экология [*Meditsina Truda i Promyshlennaa Ekologia*; *Medicine of Work and Industrial Ecology*] (ISSN 1026-9428), and since 2008, its table of contents has been available online at http://www.niimt.ru/publishing/magazin.php?lang=en. In addition, ecotoxicity data were collected from the Russian scientific journals Гидробиологический журнал [*Gidrobiologicheskii Zhurnal*; *Hydrobiological Journal*] (ISSN 0018-8166), and Биология внутренних вод [*Biologia Vnutrennikh Vod*; *Inland Water Biology*] (ISSN 1995-0829 for the print version, ISSN 1995-0837 for the electronic version).

Figure 1: Screen view of an E-SovTox search-page



For the E-SovTox database (Figure 1), articles were selected from hard copies of the journals, then, following analysis, the printed text was scanned and part of each scanned article was digitalised by optical character recognition. Currently, the database contains data from more than 500 articles, covering over 600 chemicals. The registered users can read the original abstract and full text of the original published work (as well as an English translation of the abstract; Figure 2), and they can validate the data. The search engine allows the user to search either by type of compound (inorganic or organic), by name or CAS number of the compound, or by the author of the Russian article (Figure 1). The search by keyword is performed simultaneously in all the fields of the literature reference page (Figure 2). The query results for a certain chemical will be presented in separate tabs: general information, toxicity to various vertebrate and non-vertebrate organisms, and references. Within the 'toxicity' tabs, each row presents data for a certain organism and the endpoints corresponding to the reference cited (Figure 3). This allows the user to make a direct comparison between different data reported for the same compound. For analysis, data can be copied and transferred to spreadsheet applications (by copy/paste).

Database Access

Access to the online E-SovTox database may be given by the administrator after agreement by the potential user with the 'Terms of Use', and submission of the online application form. For registered users, several levels of access will be established, each of them requiring an application for registration and validation by the administrator. Registered users can propose additional data for inclusion in the database.

Figure 2: Screen view of an E-SovTox literature-reference page

Advantages

The online E-SovTox database is designed for scientists and regulators, to provide them with (eco)toxicological data from literature published in Russian. Advantages of the database are:

— Free access with personal login and password;

— Data are updated according to the comments or references provided by registered users;

— The search engine permits searching with Latin or Cyrillic characters, by category of compound, chemical name, CAS number, author and/or keywords;

— For analysis, data can be copied and transferred to spreadsheet applications (i.e. by copy/paste);

— Abstract and full-text access of the original data sources will be provided when available, permitting validation of the data by the user;

— Updates, potential improvements, and feedback from registered users will be considered by e-mail.

Conclusion

The E-SovTox database can be used as a decision-support tool by researchers and regulators for the hazard assessment of chemical substances.

Acknowledgements

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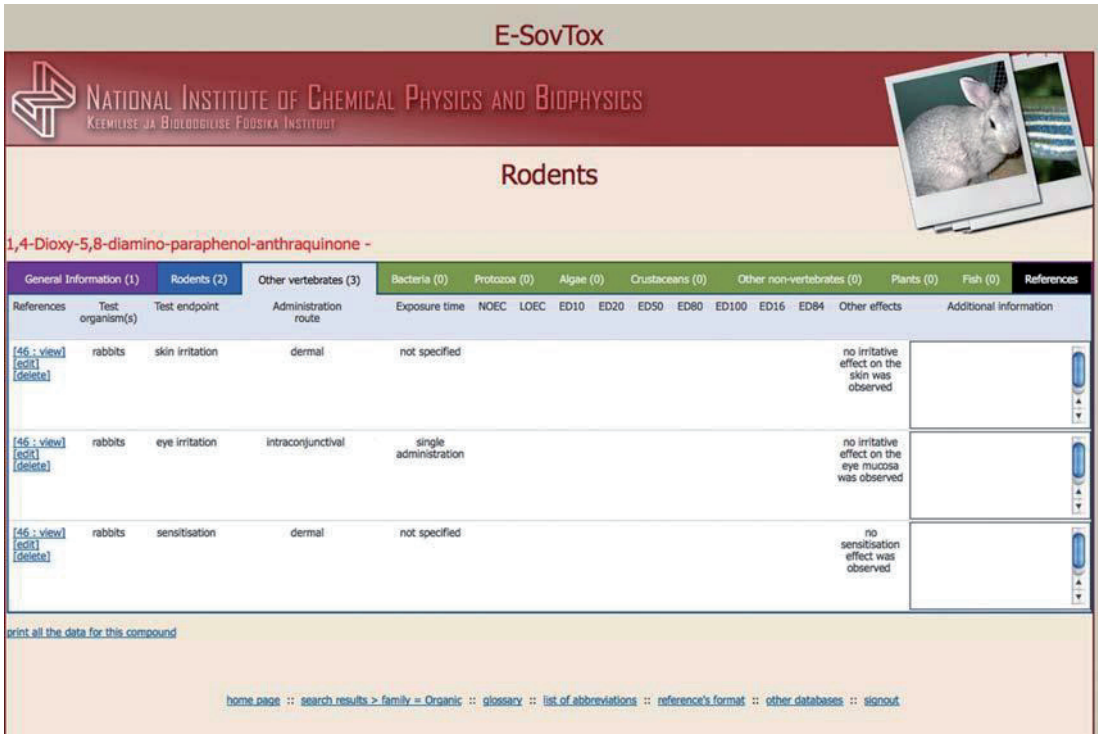
Figure 3: Screen view of an E-SovTox page about toxicity data for a certain chemical (Administrator's view)



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PAPER III

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Toxicity of five anilines to crustaceans, protozoa and bacteria•

MARILIIS SIHTMÄE, MONIKA MORTIMER, ANNE KAHRU and IRINA BLINOVA*

Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn 12618, Estonia

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Abstract: Aromatic amines (anilines and related derivates) are an important class of environmental pollutants that can be released to the aquatic environment as industrial effluents or as breakdown products of pesticides and dyes. The toxicity of aniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline and 3,5-dichloroaniline towards a multitrophic test battery comprised of bacteria *Aliivibrio fischeri* (formerly *Vibrio fischeri*), a ciliated protozoan *Tetrahymena thermophila* and two crustaceans (*Daphnia magna* and *Thamnocephalus platyurus*) were investigated. Under the applied test conditions, the toxicity of the anilines notably varied among the test species. The bacteria and protozoa were much less sensitive towards the anilines than the crustaceans: EC_{50} values 13–403 mg L^{-1} *versus* 0.13–15.2 mg L^{-1}. No general tendency between toxicity and the chemical structure of the anilines (the degree of chloro-substitution and the position of the chloro-substituents) was found in the case of all the tested aquatic species. The replacement of the artificial test medium (ATM) by the river water remarkably decreased the toxicity of anilines to crustaceans but not to protozoa. This research is part of the EU 6th Framework Integrated Project OSIRIS, in which ecotoxicogenomic studies of anilines (*e.g.*, for *Daphnia magna*) will also be performed that may help to clarify the mechanisms of toxicity of different anilines.

Keywords: ecotoxicity; anilines; test battery; river water; ECOSAR.

INTRODUCTION

Aromatic amines (anilines and related derivates) are widely used industrial chemicals and are therefore an important class of environmental pollutants. Aniline is the parent molecule of a vast family of aromatic amines. Since its discovery in 1826, it has become one of the hundred most important building blocks in

* Corresponding author. E-mail: irina.blinova@kbfi.ee
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chemistry. Aniline and its derivatives containing chloro-substituents are used as intermediates in many different fields of applications, such as the production of isocyanates, rubber processing chemicals, dyes and pigments, agricultural chemicals and pharmaceuticals.[1] These compounds can be released into the surface water as industrial effluents or as break-down products of pesticides and dyes.

According to Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation,[2] all substances on the European Market, which are manufactured or imported in a quantity of 1 tonne or more per year will have to be registered by June 1, 2018. The latest evaluation made by Rovida and Hartung[3] in 2009 suggests that around 68,000 to 101,000 chemicals will have to be registered in the EU under the REACH regulation. This is a huge task and responsibility for industry, regulators and scientists to manage the risks that chemicals may pose to health and the environment.

This article focuses on the ecotoxicity of aniline and four of its derivatives: aniline, 2-chloroaniline (2-CA), 3-chloroaniline (3-CA), 4-chloroaniline (4-CA) and 3,5-dichloroaniline (3,5-DCA). According to European Chemical Substances Information System (ESIS) and data on chemical production from 1990–1994, aniline and 2-CA are high production volume (HPV) chemicals (placed on the EU market in volumes exceeding 1000 tonnes per year per producer or importer) and 3-CA, 4-CA and 3,5-DCA are LPV (low production volume) chemicals, *i.e.*, volumes of 10–1000 tonnes per year.[4] Aniline and 4-CA are classified as hazardous substances in Annex I of Directive 67/548/EEC,[5] whereas 2-CA, 3-CA and 3,5-DCA were not evaluated at the EU-level under previous legislation, suggesting the need to collect information on their environmental and health properties and to classify them under REACH-legislation. The (eco)toxicity data available for aniline and its derivates show that 2-CA, 3-CA and 3,5-DCA could also be dangerous to humans and the environment. For example, according to International Agency for Research on Cancer (IARC), 4-CA is classified as possibly carcinogenic to humans. Chen *et al.*[6] showed that 2-CA is also potentially carcinogenic to humans. Aniline and 4-CA are also classified as dangerous for the environment according to European Chemical Substances Information System (ESIS).

The main aim of REACH is not only to provide a high level of protection of human health and the environment, but also to reduce animal testing to a minimum, to promote the use of alternative methods and to combine all sources of data[7] (available existing data, *in silico*, *in vitro* and *in vivo* approaches) for the assessment of the hazardous properties of substances. Thus, expectations towards *in vitro* studies and QSARs (quantitative structure-activity relationship) are very high.

In the field of aquatic toxicology, QSARs have been developed as alternative tools for predicting the toxicity of chemicals, when little or even no empirical data are available. Elaboration of SARs (structure-activity relationships) or some other computational toxicity prediction models is primarily based on experiment-

ally measured toxic effects of chemicals. Therefore, there is a direct relationship between the amount and quality of available information on toxicity of different chemicals towards different test species and adequacy of the models.

The fate and biological effects of chemicals in aquatic ecosystems depend, above all, on the chemical composition of natural water.[8] However, the majority of toxicity data for chemicals available for standard freshwater test organisms has been generated using standard test media, and, as a result, the available information concerning toxicity of chemicals, including anilines, in natural waters is limited. Environmentally irrelevant conditions in standard toxicity tests reduce their predictive power for environmental risk assessment.

The objectives of this study were: 1) to establish the relationship between chemical structure and the toxicity of five anilines (aniline, 2-CA, 3-CA, 4-CA and 3,5-DCA) toward different aquatic test species belonging to different trophic levels and 2) to evaluate the effect of replacement of the artificial test medium by the natural water on the toxicity test results.

EXPERIMENTAL

Chemicals

Aniline, 2-chloroaniline, 3-chloroaniline and 4-chloroaniline were purchased from Sigma-Aldrich and 3,5-dichloroaniline from Acros-Organics. Stock solutions (aniline – 8000 mg L^{-1}, 2-CA – 500 mg L^{-1}, 3-CA – 1100 mg L^{-1}, 4-CA – 550 mg L^{-1} and 2,3-DCA – 200 mg L^{-1}) were prepared in MilliQ water, taking into account their solubility (Table I), and stored in the dark.

TABLE I. Selected characteristics of the five tested anilines

Chemical	CAS No.	Purity %	Measured water solubility mg L^{-1}	Estimated water solubility[a] mg L^{-1}	Measured log K_{ow}[b]	Estimated log K_{ow}[c]
Aniline	62-53-3	≥ 99.5	36000 (25 °C)[b] 34000[d]	20820	0.90	1.08
2-Chloroaniline (2-CA)	95-51-2	≥ 99.5	8160 (25 °C)[b]	2241	1.90	1.72
3-Chloroaniline (3-CA)	108-42-9	99	5400 (20 °C)[b]	2331	1.88	1.72
4-Chloroaniline (4-CA)	106-47-8	98	3900 (25 °C)[b] 2000[e]	2572	1.83	1.72
3,5-Dichloroaniline (3,5-DCA)	626-43-7	98	784 (25 °C)[b] 600 (26 °C)[f]	223	2.90	2.37

[a]EPI Suite™ program WSKOWWIN, v. 1.41; [b]U.S. EPA ECOSAR;[9] [c]EPI Suite™ program KOWWIN™, v. 1.67; [d]Ref. 10; [e]Provider's Material Safety Data Sheet (MSDS) (Sigma-Aldrich); [f]Provider's MSDS (Acros-Organics)

Bioassays

The toxicity of five anilines was studied toward four aquatic organisms: bacteria, protozoa and two crustaceans, using the following bioassays:

The kinetic luminescent bacteria test (modified Flash assay) with *Aliivibrio fischeri* (formerly *Vibrio fischeri*) is based on the inhibition of the light output of naturally bioluminescent bacteria by toxic compounds. The acute test (exposure time 15 min) was performed at room temperature (≈ 20 °C) in 96-well microplates using a modified Flash test protocol described in Mortimer *et al.*[11] Reconstituted *Aliivibrio fischeri* Reagent (Aboatox, Turku, Finland) was used as the test bacteria suspension and all chemicals and their dilutions were tested in 2 % NaCl. Inhibition of bacterial bioluminescence by the tested compounds was calculated as a percentage of the unaffected control (2 % NaCl).

Daphtoxkit F™, Thamnotoxkit F™ and Protoxkit F™ were purchased from MicroBioTests, Inc. (Mariakerke-Gent, Belgium) and tests were performed according to the procedures described in the instruction supplied with the corresponding Toxkits.

The 48-h acute immobilization test with the crustacean *Daphnia magna* (Daphtoxkit F™) adhered to OECD 202 guideline. The tests with neonates less than 24 h old, obtained by the hatching of ephippia, were performed at 20 °C.

The 24-h mortality test with the crustacean *Thamnocephalus platyurus* (Thamnotoxkit FTM) was performed at 25 °C with larvae of shrimp *T. platyurus* (< 24 h old) obtained by the hatching of cysts.

The growth inhibition test (24-h) with the ciliated protozoan *Tetrahymena thermophila* (Protoxkit FTM) is based on the measurement of the population density of protozoa. Briefly, the investigated chemical and *T. thermophila* culture (strain BIII) were added to the food substrate suspension in MilliQ water. While normal proliferating protozoan culture clears the substrate suspension in the test vessels during exposure, inhibition of the growth of protozoa is reflected by the residual turbidity of the food substrate, measured as the optical density (*OD*) of the test samples at 440 nm. The incubation was performed at 30 °C.

The acute inhibition test (24-h) of the viability of *Tetrahymena thermophila* was conducted essentially as described in Mortimer *et al.*[12] Briefly, *T. thermophila* (strain BIII, the growth inhibition test) was grown axenically in nutrient medium. During the exponential growth phase (5×10^5 cells mL^{-1}), the cells were harvested by centrifugation and washed with Osterhout's medium, which was also used as the test medium. The test plates with protozoa were incubated for 24 h at 25 °C without shaking. Cell viability was tested using the fluorescent dye propidium iodide (PI, Fluka) and by measuring the ATP content of the cellular suspensions using the luciferin–luciferase method.

To prevent potential photolytic breakdown of anilines the exposure of protozoan and crustacean tests were conducted in the dark.[13]

The EC_{50} values were determined using Regtox software for Microsoft Excel.[14] The average EC_{50} values and standard deviations (*SD*) were calculated from 3–5 independent experiments, each in several replicates (four for *D. magna*, three for *T. platyurus,* and two for *T. thermophila* and *A. fischeri*).

Test media

The artificial test medium – ATM (test medium used in the standard test procedure) in the crustacean assays had the following composition (mg L^{-1}): for *D. magna* - $CaCl_2{\cdot}2H_2O$, 294; $MgSO_4{\cdot}7H_2O$, 123.25; $NaHCO_3$, 64.75; KCl, 5.75; pH 7.8 ± 0.2 and for *T. platyurus* - $CaSO_4{\cdot}2H_2O$, 60; $MgSO_4{\cdot}7H_2O$, 123; $NaHCO_3$, 96; KCl, 4; pH 7.8±0.2, dissolved in MilliQ water. MilliQ water or Osterhout's medium (NaCl, 104; $MgCl_2$, 8.5; $MgSO_4$, 4; KCl, 2.3; $CaCl_2$, 1 mg L^{-1}; pH 6.6, dissolved in MilliQ water) were used as the standard test medium for *T. thermophila*, and a 2 % solution of NaCl for *A. fischeri*. Thus, the ATM used in the assays did not contain any organic compounds.

Natural waters were sampled from a well (subsurface water) in a small village in northern Estonia and from the River Jägala (Estonia). Chemical analyses of the natural water samples (Table II) were performed using standard analytical methods in an accredited laboratory.

TABLE II. Characterization of the natural waters used as test media

Parameter	Unit	Water from the well	Water from the River Jägala
pH	–	7.5	8
Conductivity	μS	156	282
DOC[a]	mg C L^{-1}	9.6	16.1
BOD_7[b]	mg O_2 L^{-1}	1.4	1.6
Nitrate	mg N L^{-1}	0.27	2.3
Phosphate	mg P L^{-1}	0.195	0.018
Ca^{2+}	mg L^{-1}	33	68
HCO_3^-	mg L^{-1}	96.4	192.2
SO_4^{2-}	mg L^{-1}	4	25
Fe_{tot}	mg L^{-1}	0.21	0.76

Before the biotesting, suspended solids and plankton were separated from the water samples by filtration through a 0.45 μm pore size standard filter (Millipore).

Use of ECOSAR for predicting the aquatic toxicity of anilines

The toxicity of the anilines (EC_{50}) to *D. magna* were calculated using the ECOSAR model – a computerized predictive system used by the United States Environmental Protection Agency (US EPA) to estimate the aquatic toxicity of industrial chemicals. The ECOSAR model uses Structure Activity Relationships (SARs) for the prediction of the aquatic toxicity of untested chemicals based on their structural similarity to chemicals for which aquatic toxicity data are available. The SARs in the ECOSAR model express correlations between the physico–chemical properties and aquatic toxicity of a compound within specific chemical classes. ECOSAR version 1.00a (February 2009), downloadable from the US EPA website,[9] was used in the current study.

RESULTS AND DISCUSSION

The results of the toxicity testing of the five anilines using the above-listed bioassays in ATM (the respective artificial test medium) are presented in Table III. The experimental data on the toxicity of the investigated anilines are comparable with the data published by other authors (Table IV).

It should be mentioned that the 48-h EC_{50} values for *D. magna* available in the literature vary considerably. However, when averaged (Table IV), these data are in agreement with the present results (Table III). Unfortunately, no information on the toxicity of the anilines to *T. platyurus* and *T. thermophila* could be found, but the toxicity of investigated anilines to close protozoan species *T. pyriformis* (Table IV) were comparable to the present data (Table III). In the current study, much higher EC_{50} values were obtained in the acute inhibition test (exposure of protozoa during 24 h with no food added, see Experimental) than in the growth inhibition test with *T. thermophila* (Table III). Exposure of *T. thermophila* to aniline in the acute inhibition test yielded the following EC_{50} values:

2007 mg L^{-1}, measured with propidium iodide, and 2140 mg L^{-1}, according to the measurement of the ATP level. Considering that the EC_{50} value from the acute inhibition test is over 5 times higher than the EC_{50} value of the growth inhibition test (2007 *vs*. 358 mg L^{-1}), it can be assumed that in case of toxicity testing of aniline, the growth inhibition test of *T. thermophila* is more relevant than the acute inhibition test. The difference in the EC_{50} values of the two test formats could be attributed to the mode of action of aniline, which has been classified as a polar narcotic which exerts non-covalent bioreactivity by disturbing the structure and functioning of biomembranes.[19] As a result of the slow narcotic mechanism of action, aniline probably inhibits the normal functioning of the cell, including cell proliferation, but does not kill the cells during that time, rendering the mortality endpoint (propidium iodide assay) less sensitive. However, this supposition has to be verified.

TABLE III. Toxicity of anilines (EC_{50} ,mg L^{-1}, mean±*SD*) towards four aquatic species tested in ATM

Compound	Exposure time			
	24 h	15 min	48 h	24 h
	Protozoa *Tetrahymena thermophila*[a]	Bacteria *Aliivibrio fischeri*	Crustacean *Daphnia magna*	Crustacean *Thamnocephalus platyurus*
Aniline	358±180	403±101	0.13±0.04	2.8±0.6
2-Chloroaniline	252±16	43±19	1.2±0.4	15.2±4.5
3-Chloroaniline	135±9.0	59±14	0.24±0.07	2.0±0.6
4-Chloroaniline	36±3.5	13±0.5	0.19±0.04	4.4±1.1
3,5-Dichloroaniline	29±2.4	36±3.8	0.48±0.24	3.9±0.8

[a]Growth inhibition test (Protoxkit F™)

TABLE IV. EC_{50} values (mg L^{-1}) for the five anilines published by other authors

Chemical	*Tetrahymena pyriformis*	*Aliivibrio fischeri* (*V. fischeri, P. phosphoreum*)[a]	*Daphnia magna*[b]
Aniline	158.1[c]	69 (15 °C)	0.39±0.23
	190[d]	488 (15 °C)	
2-Chloroaniline	188.7[c]	15 (15 °C	0.94±0.68
	200[d]	36.5 (20 °C)	
3-Chloroaniline	76.9[c]	13.4 (15 °C)	0.23±0.13
	100[d]	39.5 (20 °C)	
4-Chloroaniline	113.7[c]	3.77 (15 °C)	0.24±0.13
	10[d]	21 (20 °C)	
3,5-Dichloroaniline	31.6[c]	10.7 (15 °C)	1.16±0.06

[a]Ref.15, exposure time 15 min; testing temperature indicated in the brackets; [b]mean±*STD* from U.S. EPA ECOSAR and Ref.16, exposure time 48 h; [c]Ref.17, exposure time 40 h; [d]Ref.18, exposure time 24 h

The toxicity of investigated anilines varied notably among the test species (Table III). All tested compounds were remarkably more toxic (10–100 times) to

crustaceans than to bacteria and protozoa (both unicellular organisms). *D. magna* was the most sensitive species. Other authors[20,21] also showed that *D. magna* was more sensitive than other aquatic species, *i.e.*, algae and fish, to anilines. Although it was previously demonstrated that *T. platyurus* can be more sensitive than *D. magna*, *e.g.*, to pyrene[22] and insecticides,[23] in case of anilines, *D. magna* was about an order of magnitude more sensitive than *T. platyurus*. It should be emphasized, however, that the acute assays with the two crustacean test species used different exposure times (24-h for *T. platyurus vs.* 48-h for *D. magna*) which could explain the different results obtained. The high sensitivity of *D. magna* to aromatic amines, compared to other crustaceans, was also shown by Ramos *et al.*[24]

Thus, the present study confirms that extrapolation of toxicity data from one species to another (even if the species are taxonomically similar) could lead to incorrect deductions.

Relationship between toxicity and the chemical structure of the anilines

There was no common relationship between the toxicity and chemical structure of the anilines (the degree of chlorosubstitution and the position of chloro-substituents) for all the tested aquatic species (Table III). In case of protozoa, the toxicity of anilines depended on the position of chloro-substituents and increased in accordance with the degree of chlorosubstitution, with aniline (EC_{50} = 358 mg L^{-1}) being about 12-fold less toxic than 3,5-DCA (EC_{50} = 29 mg L^{-1}). Aniline was also approximately 10-fold less toxic than the substituted anilines to the bacteria *A. fischeri* (403 mg L^{-1} *vs*. 13–59 mg L^{-1}; Table III). As mentioned above, both crustaceans and especially *Daphnia magna* were remarkably (up to 3 orders of magnitude) more sensitive towards anilines than protozoa and bacteria. For both crustaceans, it was difficult to recognize a clear relationship between toxicity and the chemical structure of the tested compounds. Interestingly, for both crustaceans, 2-CA was noticeably more toxic than the other four tested anilines. This indicates that, regardless of the different sensitivity of two species, the mechanism of action of anilines is probably the same for both crustaceans.

A comparison of the present results with the predicted toxicity values for *D. magna* obtained with the ECOSAR model (experimentally obtained octanol-water partitioning coefficient, K_{ow}, values were used for the calculations, Table I) shows that the predictive power of the ECOSAR model, at least in case of anilines, is limited. Moreover, the ECOSAR model under predicted the toxicity of four anilines by almost one order of magnitude (Fig. 1).

As a rule, there is a correlation between the toxicity of an organic chemical and its K_{ow} value: the higher the log K_{ow}, the lower the $L(E)C_{50}$ value, *i.e.*, the higher the toxicity. For example, in previous studies on MEIC chemicals, a good correlation was shown between the toxicity of 24 MEIC chemicals to photobacteria and their K_{OW} value; the correlation coefficient of the linear regression

(log–log) was –0.84).[25] Lee *et al.*[26] showed that the toxicity of 16 phenols toward *Selenastrum capricornutum* and *D. magna* was closely related to the log K_{ow} values. In the current study, this trend was observed for 5 tested anilines in the case of protozoa and bacteria. However, the most toxic compound to crustaceans was aniline, which is the least hydrophobic of the five tested compounds (Fig. 2).

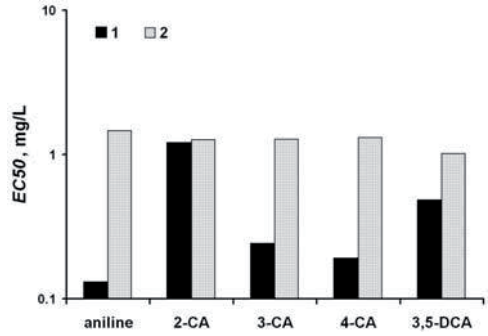

Fig. 1. Toxicity of anilines to crustacean *Daphnia magna:* measured (1) and predicted by ECOSAR (2). Note the logarithmic *y*-scale.

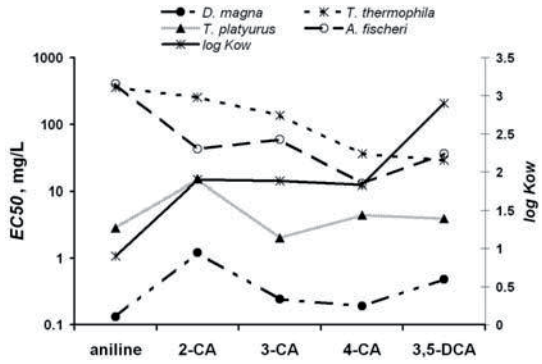

Fig. 2. Toxicity of anilines to the four test species (EC_{50} values obtained in the current study) *vs.* log K_{ow}.

As was shown above, the existing tools for the prediction of the toxicity of aniline (ECOSAR) to aquatic species yields inaccurate toxicity data. There are many reasons why the predictive power of QSAR models is not reliable. Firstly and most importantly, experimentally determined physic–chemical properties should be used to develop QSARs. Secondly, the descriptors should be selected very carefully and the toxicity of chemicals should be predicted by more than one descriptor. Certainly, QSAR models are rapid and cost-effective methods, which can

be used as important alternative screening tools for prioritising and predicting the toxicity of untested chemicals, but it must be born in mind that the calculated values may differ considerably from the experimental ones.

Modulation of the toxicity of anilines in natural water

There are an increasing number of studies showing the modulating effect of the composition of natural water on the toxicity of different chemicals, mostly heavy metals but also metal oxide nanoparticles.[27] The presence of humic compounds in natural water may also modulate the toxicity of organic chemicals. For example, it was shown that dissolved humic materials (DHM) significantly reduced the toxicity of 4-CA to *D. magna*, but the effect of DHM on the toxicity of 4-CA to zebrafish (*Brachydanio rerio*) was not observed.[28] In the present study, the effect of natural water on toxicity of anilines to bacteria, protozoa and crustaceans was evaluated.

It is known that photolysis and microbial degradation are the most important degradative processes affecting anilines in aquatic environments.[29] To prevent breakdown of the chemical structures by photolysis, the exposure of protozoa and crustaceans to the anilines was realised in the dark (see Experimental).[13] In addition, it was previously shown[29,30] that during short incubation periods (up to 3 days in the dark), there was no measurable microbial degradation of aniline and the chloroanilines in natural water. Therefore, it could be presumed that in the short-term tests performed in the current study, the tested compounds remained stable and that the differences between the results obtained with ATM and natural water indicate the impact of water composition on the bioavailability of anilines to different aquatic species.

The mitigation effect of natural water on the toxicity of anilines to four test species is presented in Table V. The tests organisms were exposed to the anilines at concentrations that were close to the EC_{50} values obtained in the respective standard test media (Table II). The results are presented as a ratio of the toxic effect (%) in natural waters and in ATM (Table V). Thus, values lower than one indicate a decrease of toxicity in natural water and values exceeding one, accordingly, indicate an increase in toxicity. For example, when the immobilization of *D. magna* exposed to 2-CA at a concentration 0.2 mg L^{-1} in ATM was 80 % and in natural water only 40 %, the toxicity in natural water decreased 2 times (40/80 = 0.5).

In general, the effect of natural water on the toxicity of anilines was minimal. However, some tendencies were observed: *i*) different to particle-feeding organisms (protozoa and crustaceans), the toxicity of anilines to bacteria was practically the same in natural water and ATM and *ii*) toxicity of anilines for protozoa *T. thermophila* and crustacean *T. platyurus* seemed to be slightly increased when exposed in natural water, and for *D. magna,* natural water slightly decreased the toxic effect of chloroanilines (but not of aniline). These data are in accordance with the data of Lee *et al*.[28] (see above). However, the data on the other crus-

tacean *T. platyurus* did not confirm this tendency (Table V). This discrepancy may be explained by the different sensitivity of the test species to background pollution. It seems that in case of anilines, the mitigation effect of natural water on toxicity to crustaceans depended mainly on the integrated effect of the water composition (including background pollution) and tested chemical, but not on the dissolved organic matter (DOC) content. Thus, the current data on anilines are different from the data of a previous study on the effect of natural waters on the toxicity of CuO nanoparticles to *D. magna* and *T. platyurus*, in which it was shown that natural waters remarkably (up to 100-fold) decreased the toxicity of nano-CuO to both crustaceans and this effect depended mainly on the DOC concentration.[27]

TABLE V. The ratio between the toxicity of anilines in natural water (NW) and artificial test medium (ATM) tested at the same concentrations (effect in NW / effect in ATM)

Compound	*Tetrahymena thermophila*[a]		*Aliivibrio fischeri*		*Daphnia magna*		*Thamnocephalus platyurus*	
	Well	River	Well	River	Well	River	Well	River
Aniline	2.4	2.2	1.1	1	2.1	1.9	1.8	1.5
2-Chloroaniline	1.3	1.5	1.2	1.1	0.6	0.5	0.8	1
3-Chloroaniline	0.95	1.2	0.9	0.85	0.7	0.5	2.2	1.9
4-Chloroaniline	1.2	1.6	0.8	0.9	0.7	0.7	0.8	0.9
3,5-Dichloroaniline	1.8	2.4	1.1	1.1	0.3	0.5	1.1	1.3

[a]Growth inhibition test (Protoxkit F™)

CONCLUSIONS

It may be concluded that the opinion stated 15 years ago: "…at present no prediction about the behaviour of a previously untested chemical can be made, which is based on the physico-chemical or structural properties of the organic chemical."[28] – is still valid, at least in the case of anilines.

QSARs can be used as an initial evaluation of the toxicity of a chemical, however, tests with bioassays must be performed for confirmation.

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ИЗВОД

ТОКСИЧНОСТ ПЕТ АНИЛИНСКИХ ЈЕДИЊЕЊА ПРЕМА ЉУСКАРИМА, ПРОТОЗОАМА И БАКТЕРИЈАМА

MARILIIS SIHTMÄE, MONIKA MORTIMER, ANNE KAHRU и IRINA BLINOVA

Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn 12618, Estonia

Ароматични амини (анилини и деривати) су важна класа загађујућих супстанци које могу бити испуштене у животну средину као индустријски ефлуенти или као производи раз-

градње пестицида и боја. Испитали смо токсичност анилина, 2-хлоранилина, 3-хлоранилина, 4-хлоранилина, и 3,5-дихлоранилина за мултитрофичну тест батерију која се састоји од бактерија *Aliivibrio fischeri* (раније *Vibrio fischeri*), протозоа бичара *Tetrahymena thermophila* и два љускара (*Daphnia magna* и *Thamnocephalus platyurus yypyc*). У примењеним условима токсичност анилина је приметно варирала међу тестираним врстама. Бактерије и протозое су биле много мање осетљиве према анилинима него љускари: вредности EC_{50} су биле 13–403 mg L^{-1} према 0,13–15,2 mg L^{-1}. Није откривен никакав општи тренд између токсичности и хемијске структуре анилина (степен супституције хлора и позиција хлорних супституената) ни у једном случају тестираних водених врста. Замена вештачког тест медијума (ATM) речном водом уочљиво је смањила токсичност анилина за љускаре, али не и за протозое. Ово истраживање је део интегрисаног пројекта OSIRIS у оквиру европског FP6 програма, у коме ће се спровести и екотоксикогеномске студије анилина (нпр. за *D. magna*), које могу помоћи у разјашњавању механизама токсичности различитих анилина.

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PAPER IV

Aruoja, V., **Sihtmäe, M.**, Kahru, A., Dubourguier, H-C. 2011. Toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata* and bacteria *Vibrio fischeri*: comparison with published data and QSARs. *Chemosphere* 84, 1310-1320.

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Toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata* and bacteria *Vibrio fischeri*: Comparison with published data and QSARs

Villem Aruoja [a,c,*,1], Mariliis Sihtmäe [a,b,1], Henri-Charles Dubourguier [a,c,†], Anne Kahru [a]

[a] National Institute of Chemical Physics and Biophysics, Laboratory of Molecular Genetics, Tallinn, Estonia
[b] Tallinn University of Technology, Tallinn, Estonia
[c] Estonian University of Life Sciences, Tartu, Estonia

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ABSTRACT

A congeneric set of 58 substituted anilines and phenols was tested using the 72-h algal growth inhibition assay with *Pseudokirchneriella subcapitata* and 15-min *Vibrio fischeri* luminescence inhibition assay. The set contained molecules substituted with one, two or three groups chosen from -chloro, -methyl or -ethyl. For 48 compounds there was no REACH-compatible algal toxicity data available before. The experimentally obtained EC50 values (mg L^{-1}) for algae ranged from 1.43 (3,4,5-trichloroaniline) to 197 (phenol) and for *V. fischeri* from 0.37 (2,3,5-trichlorophenol) to 491 (aniline). Only five of the tested 58 chemicals showed inhibitory effect to algae at concentrations >100 mg L^{-1}, i.e. could be classified as "not harmful", 32 chemicals as "harmful" (10–100 mg L^{-1}) and 21 as "toxic" (1–10 mg L^{-1}). The occupied para-position tended to increase toxicity whereas most of the ortho-substituted congeners were the least toxic. As a rule, the higher the number of substituents the higher the hydrophobicity and toxicity. However, in case of both assays, the compounds of similar hydrophobicity showed up to 30-fold different toxicities. There were also assay/organism dependent tendencies: phenols were more toxic than anilines in the *V. fischeri* assay but not in the algal test. The comparison of the experimental toxicity data to the data available from the literature as well as to QSAR predictions showed that toxicity of phenols to algae can be modeled based on hydrophobicity, whereas the toxicity of anilines to algae as well as toxicity of both anilines and phenols to *V. fischeri* depended on other characteristics in addition to logK_{ow}.

1. Introduction

REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals (EC, 2006) requires that all substances on the European market, which are manufactured or imported in quantities of 1 tonne or more per year have to be evaluated for hazardous effects to humans and environment by the year 2018. According to a recent evaluation the number of such chemicals is between 68 000 and 101 000 (Costanza and Hartung, 2009), exceeding the earlier estimate of 30 000 chemicals by the European Commision (Pedersen et al., 2003). This translates into expensive and ethically questionable toxicity testing unless alternative methods will be used (Hartung, 2009). For chemicals lacking experimental toxicity data the quantitative structure–activity relationships (QSARs) are expected to fill the gap (for a Review, see Netzeva et al., 2007). QSARs have been occasionally used in the regulatory assessment of chemicals in the EU but under REACH the use of QSARs is expected to increase remarkably. However, the existing QSAR models, including those proposed or developed for regulatory purposes (e.g., US EPA ECOSAR, Danish (Q)SAR Database) still require improvement (Reuschenbach et al., 2008). It has been recognized that their sometimes poor predictive performance is also due to scarce and inconsistent experimental toxicity data on which the models have been built.

According to REACH the basic ecotoxicological information requirements for substances manufactured or imported in quantities of 1–10 tonnes per year include short-term toxicity testing on crustaceans (preferred species *Daphnia,* OECD, 2004) and growth inhibition on aquatic plants (algae preferred, OECD, 2006). In addition, short-term toxicity testing on fish (OECD, 1992) is required in the next tonnage level (>10 tonnes per year). These three organism groups (crustaceans, algae, fish) represent different trophic levels of the aquatic food web, all of which have to be protected. The chemicals are classified according to the response of the most sensitive of these three species. However, REACH-compatible and reliable (eco)toxicity data can be found in few datasets, the biggest of which contains fathead minnow (*Pimephales promelas*) data on

* Corresponding author. Address: Akadeemia tee 23, Tallinn 12618, Estonia. Tel.: +372 6398368; fax: +372 6398382.
E-mail address: villem.aruoja@kbfi.ee (V. Aruoja).
[1] Equal participation.
[†] Deceased.

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about 550 chemicals while the largest *Daphnia* dataset contains about 370 EC50 values. The situation is even worse for algal toxicity as there is no consistent dataset with more than 100 values (Netzeva et al., 2007). In addition, algal test results vary considerably due to the use of many different algal species and methods (see Section 3.2.). A number of studies have found algae more sensitive to chemicals than fish (Weyer et al., 2000; Hutchinson et al., 2003; Kahru and Dubourguier, 2010). This implies that reliable algal toxicity data may help to reduce the number of fish needed for regulatory toxicity testing (Jeram et al., 2005).

Another alternative to higher organisms in toxicity testing is the use of bacterial toxicity assays. The most widely used bacterium for the ecotoxicity analysis is naturally luminescent gram-negative marine bacterium *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*) for which toxicity data are available for more than 1000 chemicals (Kaiser and Palabrica, 1991). There are two ISO standards concerning the luminescence inhibition assay with *V. fischeri*: one for the water samples (ISO, 2007), and the most recent one for sediments, solid and colored samples (ISO, 2010). A number of comparisons of the *V. fischeri* test (e.g., Microtox™) with other toxicity bioassays have been done and significant correlations for many species, including fish, crustaceans and algae have been shown (Kaiser, 1998).

The aim of the current work was to obtain and critically analyze the toxicity data of a congeneric set of anilines and phenols to algae and bacteria in order to support the hazard classification and QSAR development for REACH. For that 58 substituted anilines and phenols were chosen and their toxicity tested with algae *Pseudokirchneriella subcapitata* and bacteria *V. fischeri*. The Algal OECD 201 assay was used due to its regulatory relevance and due to severe shortage of algal toxicity data. Bacteria *V. fischeri* were chosen for the comparison (decomposers *versus* primary producers) and due to the extensive *prior* use of *V. fischeri* data in QSAR modeling (Cronin and Schultz, 1997). In addition the data were compared to the available toxicity data from the literature and databases as well as to QSAR predictions.

Anilines and phenols are compounds of considerable industrial and commercial importance, which makes them important environmental pollutants (Keith and Telliard, 1979; Woo and Lai, 2004). Aniline (aminobenzene) and its derivates are introduced into the environment from many different fields of applications, such as the production of isocyanates, rubber processing chemicals, dyes and pigments, agricultural chemicals and pharmaceuticals (Rapoport, 2007). Phenol (hydroxybenzene) and its derivatives are released from industrial effluents such as those from the coal tar, gasoline, plastic, rubber proofing, disinfectant, pharmaceutical and steel industries and domestic wastewaters, agricultural runoff and chemical spills (Lin and Juang, 2009). However, for many substituted anilines and phenols there are no ecotoxicity data available. Moreover, all the 58 selected anilines and phenols have been pre-registered under REACH referring to European Union production or import quantities of 1 tonne or more per year. According to the European Chemical Substances Information System (ESIS, http://ecb.jrc.ec.europa.eu/esis/) database most of these chemicals have been used in quantities exceeding 10 tonnes per year.

2. Materials and methods

2.1. Chemicals

The 58 anilines and phenols chosen for this study were ⩾95% pure (52 chemicals ⩾98% pure). The chemicals are characterized in Table 1. For *V. fischeri* tests all stock-solutions were prepared in MilliQ-water and for the algae in the algal test medium. No co-solvents were used. If necessary, poorly soluble chemicals were dissolved by shaking the solutions overnight. The solutions were prepared in glass containers, sealed, stored in the dark at room temperature and tested for toxicity within 1–2 weeks. Although algal tests of phenols have been previously also performed in closed conditions (e.g. Chen and Lin, 2006) the volatility of aqueous solutions of our set of chemicals is not a concern in given conditions. The boiling points of phenol and aniline are 182 and 184 °C respectively, i.e. they evaporate at higher temperatures than water, the substituted anilines and phenols are even less volatile.

2.2. 72-h algal growth inhibition assay with P. subcapitata

In general, the OECD 201 algal growth inhibition test protocol (OECD, 2006) was followed. The algae were incubated in vials on a transparent shaking table that allowed simultaneous incubation of up to 136 samples. Algal biomass was measured by optical density at 682 nm directly from the incubation vials using a specially made vial holder for the spectrophotometer (Jenway 6300, Jenway Ltd., Essex, UK). This setting allowed to test 8–9 chemicals in one run in this otherwise laborious assay. In compliance with the OECD 201 guideline exponentially growing algal cultures were exposed to various concentrations of the test chemicals under controlled conditions whereas the concentration of algal cells in the control culture increased at least 16 times during 3 d. The algal biomass measurements were performed at least daily. The *P. subcapitata* stock culture for inoculation was taken from the commercial test system Algal Toxkit F (MicroBioTests Inc., Nazareth, Belgium). The number of the algal cells in the inoculum was determined by counting under microscope in the Neubauer haemocytometer and adjusted to yield 10 000 cells mL^{-1} in the sample after inoculation. The samples were incubated at 24 ± 1 °C for 72 h in 20-mL glass scintillation vials containing 9 mL of algal growth medium described in OECD 201 (2006). The vials were illuminated from below with Philips TL-D 38 W aquarelle fluorescent tubes. The pH of the medium was adjusted to 8.0 and did not change more than 0.5 units by the end of the test. All assays were run twice, all samples in duplicate with eight controls distributed evenly on the transparent table. A dilution series of aniline was included in all experiments as a positive control. In order to reduce the variability between the replicates the vials were single-use. The coefficient of variation of biomass density in replicate control cultures throughout the experiments did not exceed 5%. Each chemical was tested in either 6 or 7 concentrations, depending on previously available toxicity data from literature or preliminary experiments.

2.3. Acute bioluminescence inhibition assay with V. fischeri

The test (exposure time 30-s, 15-min and 30-min) was performed at room temperature (20 °C) in 96-well microplates following the Flash-assay protocol (ISO, 2010). The exact procedure is described in Mortimer et al. (2008) except the inhibition of bacterial bioluminescence was calculated as percentage of the unaffected control (2% NaCl). Reconstituted *V. fischeri* Reagent (Aboatox, Turku, Finland) was used for testing.

Chemicals and their dilutions were tested in 2% NaCl, at pH 6–7. Each chemical was tested in three different days, in 5–7 dilutions each in two replicates. The coefficient of variation of EC50 values obtained in different days did not exceed 20%. The luminescence was recorded with Microplate Luminometer Orion II (Berthold Detection Systems, Pforzheim, Germany), controlled by Simplicity Version 4.2 Software. Samples were not mixed during recording of the luminescence.

2.4. Statistical methods

The toxicity values (EC50) and their confidence intervals were determined from dose–response curves by the REGTOX software

Table 1
Characteristics of the studied chemicals.

No.	Chemical	Abbreviation	CAS no	Provider	Purity (%)	Molecular weight	Hydrophobicity ($\log K_{ow}$)[a]	Solubility (mg L^{-1})[a]
1	Aniline	A	62-53-3	Sigma–Aldrich	⩾99.5	93.1	0.90	36 000
2	2-chloroaniline	2-CA	95-51-2	Sigma–Aldrich	⩾99.5	127.6	1.90	8160
3	3-chloroaniline	3-CA	108-42-9	Sigma–Aldrich	99	127.6	1.88	5400
4	4-chloroaniline	4-CA	106-47-8	Sigma–Aldrich	98	127.6	1.83	3900
5	2,3-dichloroaniline	2,3-DCA	608-27-5	Sigma–Aldrich	99	162.0	2.82	262[b]
6	2,4-dichloroaniline	2,4-DCA	554-00-7	Sigma–Aldrich	99	162.0	2.78	620
7	2,5-dichloroaniline	2,5-DCA	95-82-9	Sigma–Aldrich	99	162.0	2.75	230[b]
8	2,6-dichloroaniline	2,6-DCA	608-31-1	Sigma–Aldrich	98	162.0	2.76	295[b]
9	3,4-dichloroaniline	3,4-DCA	95-76-1	Sigma–Aldrich	98	162.0	2.69	92
10	3,5-dichloroaniline	3,5-DCA	626-43-7	Acros-Organics	98	162.0	2.90	784
11	2,3,4-trichloroaniline	2,3,4-TCA	634-67-3	TCI Europe	>98	196.5	3.33	65[b]
12	2,4,5-trichloroaniline	2,4,5-TCA	636-30-6	Sigma–Aldrich	95	196.5	3.45	52[b]
13	2,4,6-trichloroaniline	2,4,6-TCA	634-93-5	Sigma–Aldrich	⩾98	196.5	3.52	40
14	3,4,5-trichloroaniline	3,4,5-TCA	634-91-3	Sigma–Aldrich	97	196.5	3.32	67[b]
15	2-methylaniline	2-MA	95-53-4	Fluka	⩾99.5	107.2	1.32	16 600
16	3-methylaniline	3-MA	108-44-1	Fluka	⩾99	107.2	1.40	15 000
17	4-methylaniline	4-MA	106-49-0	Fluka	⩾98	107.2	1.39	6500
18	2,3-dimethylaniline	2,3-DMA	87-59-2	Sigma–Aldrich	99	121.2	2.17[b]	5050[b]
19	2,4-dimethylaniline	2,4-DMA	95-68-1	Fluka	>98	121.2	1.68	6070[b]
20	2,5-dimethylaniline	2,5-DMA	95-78-3	Sigma–Aldrich	99	121.2	1.83	5600
21	2,6-dimethylaniline	2,6-DMA	87-62-7	Fluka	>98	121.2	1.84	8240
22	3,4-dimethylaniline	3,4-DMA	95-64-7	Fluka	⩾98	121.2	1.84	3800
23	3,5-dimethylaniline	3,5-DMA	108-69-0	Fluka	⩾97	121.2	2.17[b]	2050[b]
24	2,4,6-trimethylaniline	2,4,6-TMA	88-05-1	Sigma–Aldrich	98	135.2	2.72[b]	617[b]
25	2-ethylaniline	2-EA	578-54-1	Sigma–Aldrich	98	121.2	1.74	5320[b]
26	3-ethylaniline	3-EA	587-02-0	Sigma–Aldrich	98	121.2	2.11[b]	2320[b]
27	4-ethylaniline	4-EA	589-16-2	Fluka	>98	121.2	1.96	2110[b]
28	2,6-diethylaniline	2,6-DEA	579-66-8	Sigma–Aldrich	98	149.2	3.15[b]	670
29	Phenol	P	108-95-2	Merck	⩾95	94.1	1.46	82 800
30	2-chlorophenol	2-CP	95-57-8	Sigma–Aldrich	>99	128.6	2.15	11 300
31	3-chlorophenol	3-CP	108-43-0	Sigma–Aldrich	98	128.6	2.50	26 000
32	4-chlorophenol	4-CP	106-48-9	Sigma–Aldrich	>99	128.6	2.39	24 000
33	2,3-dichlorophenol	2,3-DCP	576-24-9	Sigma–Aldrich	98	163.0	2.84	3600
34	2,4-dichlorophenol	2,4-DCP	120-83-2	Sigma–Aldrich	99	163.0	3.06	4500
35	2,5-dichlorophenol	2,5-DCP	583-78-8	Sigma–Aldrich	99.7	163.0	3.06	2000
36	2,6-dichlorophenol	2,6-DCP	87-65-0	Sigma–Aldrich	99	163.0	2.75	1900
37	3,4-dichlorophenol	3,4-DCP	95-77-2	Sigma–Aldrich	99	163.0	3.33	9260
38	3,5-dichlorophenol	3,5-DCP	591-35-5	Sigma–Aldrich	97	163.0	3.62	5380
39	2,3,4-trichlorophenol	2,3,4-TCP	15950-66-0	Sigma–Aldrich	99	197.4	3.80	98[b]
40	2,3,5-trichlorophenol	2,3,5-TCP	933-78-8	Sigma–Aldrich	99.2	197.4	3.84	90[b]
41	2,3,6-trichlorophenol	2,3,6-TCP	933-75-5	Sigma–Aldrich	99.6	197.4	3.77	450
42	2,4,5-trichlorophenol	2,4,5-TCP	95-95-4	Sigma–Aldrich	99.6	197.4	3.72	1200
43	2,4,6-trichlorophenol	2,4,6-TCP	88-06-2	Sigma–Aldrich	98	197.4	3.69	800
44	2-methylphenol	2-MP	95-48-7	Merck	>99	108.1	1.95	25 900
45	3-methylphenol	3-MP	108-39-4	Merck	>99	108.1	1.96	22 700
46	4-methylphenol	4-MP	106-44-5	Merck	>98	108.1	1.94	21 500
47	2,3-dimethylphenol	2,3-DMP	526-75-0	Fluka	>99	122.2	2.48	4570
48	2,4-dimethylphenol	2,4-DMP	105-67-9	Fluka	>97	122.2	2.30	7870
49	2,5-dimethylphenol	2,5-DMP	95-87-4	Sigma–Aldrich	>99	122.2	2.33	3540
50	2,6-dimethylphenol	2,6-DMP	576-26-1	Sigma–Aldrich	>99	122.2	2.36	6050
51	3,4-dimethylphenol	3,4-DMP	95-65-8	Fluka	⩾98	122.2	2.23	4760
52	3,5-dimethylphenol	3,5-DMP	108-68-9	Sigma–Aldrich	⩾99	122.2	2.35	4880
53	2,3,5-trimethylphenol	2,3,5-TMP	697-82-5	Sigma–Aldrich	99	136.2	3.15[b]	762
54	2,3,6-trimethylphenol	2,3,6-TMP	2416-94-6	Sigma–Aldrich	95	136.2	2.67	1580
55	2,4,6-trimethylphenol	2,4,6-TMP	527-60-6	Sigma–Aldrich	97	136.2	2.73	1200
56	2-ethylphenol	2-EP	90-00-6	Sigma–Aldrich	99	122.2	2.47	5340
57	3-ethylphenol	3-EP	620-17-7	Sigma–Aldrich	98.9	122.2	2.40	11 300[b]
58	4-ethylphenol	4-EP	123-07-9	Sigma–Aldrich	99	122.2	2.58	4900

[a] Data from the SRC PhysProp Database (http://www.srcinc.com/what-we-do/databaseforms.aspx?id=386).
[b] Calculated values.

for Microsoft Excel (Vindimian, 2009) using the Log-normal model. Prism 5 (GraphPad Software Inc. www.graphpad.com) was used for calculations of algal growth rate and statistical significance of correlations. In order to evaluate the fit of QSAR predictions to experimental data, a method suggested by Golbraikh and Tropsha (2002) was used. When observed values are compared to predicted values not only linear correlation but also an exact fit is required and the linear regression should thus have a zero intercept (an intercept other than zero would mean the prediction needs adjustment and is therefore less accurate). The following parameters were calculated: linear correlation coefficient R^2 between observed and predicted values; correlation coefficients (R_0^2) and slopes (K) of linear regressions when intercept was set to zero. In the latter case the predicted *versus* observed and observed *versus* predicted correlation coefficients and slopes are different and designated as R_0^2, K and $R_0'^2$, K' respectively. The prediction is considered acceptable when (Golbraikh et al., 2003):

$$R^2 > 0.6 \quad (1)$$

Table 2
Toxicity (EC50, mg L^{-1}) of 58 substituted anilines and phenols to *Pseudokirchneriella subcapitata* and *Vibrio fischeri*.

No.	Chemical[a]	*P. subcapitata*			*V. fischeri*		
		72-h EC50[b] (mg L^{-1})	95% confidence Interval		15-min EC50[b] (mg L^{-1})	95% confidence Interval	
1	A	54.2	49.5	59.4	491	462	533
2	2-CA	39.1	36.6	43.5	42.8	39.0	47.1
3	3-CA	26.9	26.1	27.5	64.3	62.9	68.9
4	4-CA	3.55	2.31	5.50	15.5	15.2	17.3
5	2,3-DCA	6.75	5.22	7.22	14.2	14.0	14.8
6	2,4-DCA	3.96	3.32	4.27	16.6	16.1	17.5
7	2,5-DCA	16.5	11.7	25.2	16.7	15.4	18.3
8	2,6-DCA	23.2	22.5	26.6	13.0	12.6	13.8
9	3,4-DCA	2.50	1.97	2.99	4.28	4.23	4.71
10	3,5-DCA	4.39	3.71	5.17	35.8	34.3	37.8
11	2,3,4-TCA	3.55	3.16	3.98	10.5	9.25	12.2
12	2,4,5-TCA	3.14	1.88	5.65	7.92	7.14	8.87
13	2,4,6-TCA	4.94	4.74	5.57	>15	–	–
14	3,4,5-TCA	1.43	1.03	1.81	11.7	11.3	12.1
15	2-MA	109	99.6	113	187	179	201
16	3-MA	26.9	21.8	31.9	91.1	88.3	94.4
17	4-MA	42.7	28.8	50.3	41.6	40.3	47.2
18	2,3-DMA	30.8	16.8	35.2	117	104	133
19	2,4-DMA	39.4	34.7	46.3	77.7	72.4	88.0
20	2,5-DMA	70.6	66.7	78.5	66.8	66.1	72.1
21	2,6-DMA	107	105	109	77.8	73.9	84.0
22	3,4-DMA	7.34	5.35	10.1	6.98	6.62	7.66
23	3,5-DMA	27.8	26.1	28.8	71.8	62.9	85.4
24	2,4,6-TMA	20.3	19.3	25.1	89.8	87.7	94.7
25	2-EA	49.2	43.2	52.3	57.4	56.1	59.9
26	3-EA	14.2	10.9	17.9	41.4	40.7	44.0
27	4-EA	8.82	5.28	11.1	1.48	1.35	1.65
28	2,6-DEA	41.5	37.7	44.5	5.53	4.99	6.23
29	P	197	172	209	165	153	185
30	2-CP	51.8	42.8	66.2	69.5	62.8	77.0
31	3-CP	11.5	10.9	13.0	32.3	29.5	34.7
32	4-CP	31.4	29.3	33.5	9.71	9.08	10.6
33	2,3-DCP	10.9	10.1	11.5	13.4	12.5	13.6
34	2,4-DCP	8.13	2.00	15.8	7.14	6.51	7.50
35	2,5-DCP	3.68	2.37	5.21	10.3	9.54	10.7
36	2,6-DCP	16.1	10.7	18.1	16.5	15.3	17.3
37	3,4-DCP	2.19	1.85	2.50	3.60	3.27	3.87
38	3,5-DCP	2.10	1.86	2.82	2.66	2.60	2.70
39	2,3,4-TCP	4.16	3.66	4.69	0.90	0.85	0.91
40	2,3,5-TCP	2.26	1.99	2.67	0.37	0.35	0.38
41	2,3,6-TCP	8.05	7.46	10.2	3.30	3.07	3.41
42	2,4,5-TCP	7.57	5.93	7.99	0.56	0.53	0.57
43	2,4,6-TCP	5.64	4.87	7.02	3.61	3.38	3.72
44	2-MP	127	122	130	38.7	35.6	42.6
45	3-MP	145	141	150	36.1	34.8	39.9
46	4-MP	57.6	45.8	72.6	4.73	4.56	5.06
47	2,3-DMP	48.1	41.7	56.2	11.0	9.84	12.43
48	2,4-DMP	19.3	11.3	25.4	4.91	4.77	5.12
49	2,5-DMP	32.5	28.8	37.1	27.5	26.8	30.1
50	2,6-DMP	41.6	34.0	43.9	54.5	49.2	60.7
51	3,4-DMP	32.0	24.4	42.0	3.12	3.02	3.27
52	3,5-DMP	27.2	26.0	29.3	42.1	41.3	44.3
53	2,3,5-TMP	13.5	13.3	15.0	20.8	19.9	23.0
54	2,3,6-TMP	14.2	13.2	15.8	17.1	16.5	17.4
55	2,4,6-TMP	9.64	8.60	11.1	35.6	35.2	36.6
56	2-EP	31.4	30.5	33.9	39.7	36.1	43.3
57	3-EP	40.3	35.1	44.1	6.97	6.46	7.60
58	4-EP	21.9	19.1	28.3	0.43	0.40	0.48

[a] Abbreviations are explained in Table 1.
[b] The presented toxicity values are based on nominal initial exposure concentrations in a static test.

$$\frac{R^2 - R_0^2}{R^2} < 0.1 \text{ and } 0.85 \leqslant K \leqslant 1.15 \quad (2)$$

or

$$\frac{R^2 - R_0'^2}{R^2} < 0.1 \text{ and } 0.85 \leqslant K' \leqslant 1.15 \quad (3)$$

and

$$|R_0^2 - R_0'^2| \leqslant 0.3 \quad (4)$$

2.5. Previously published toxicity data

In order to compare the experimental data to previously existing toxicity data for the studied anilines and phenols relevant values were obtained from the US EPA ECOTOX (http://cfpub.epa.gov/ecotox/) database and from published papers. US EPA ECOTOX database search for algal toxicity data was performed in December 2010 using the Advanced Database Query and results (LC50, LD50, EC50, ED50, IC50, ID50) were downloaded as a Microsoft Excel

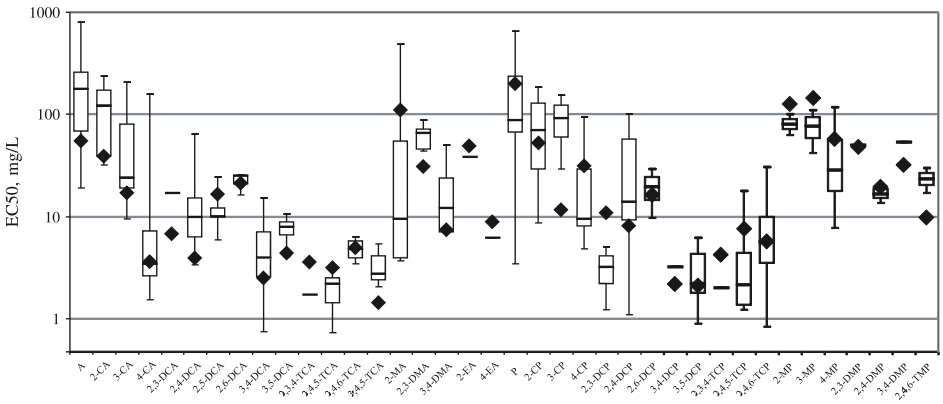

Fig. 1. Variation of *Chlorophyta* toxicity data EC50 (mg L^{-1}) (exposure time 1–4 d, median values) from US EPA ECOTOX database and published papers (see Table S1) on a boxplot. Experimental toxicity data from this study for *Pseudokirchneriella subcapitata* from Table 2 are shown as filled symbols. Abbreviations of the chemical names are explained in Table 1.

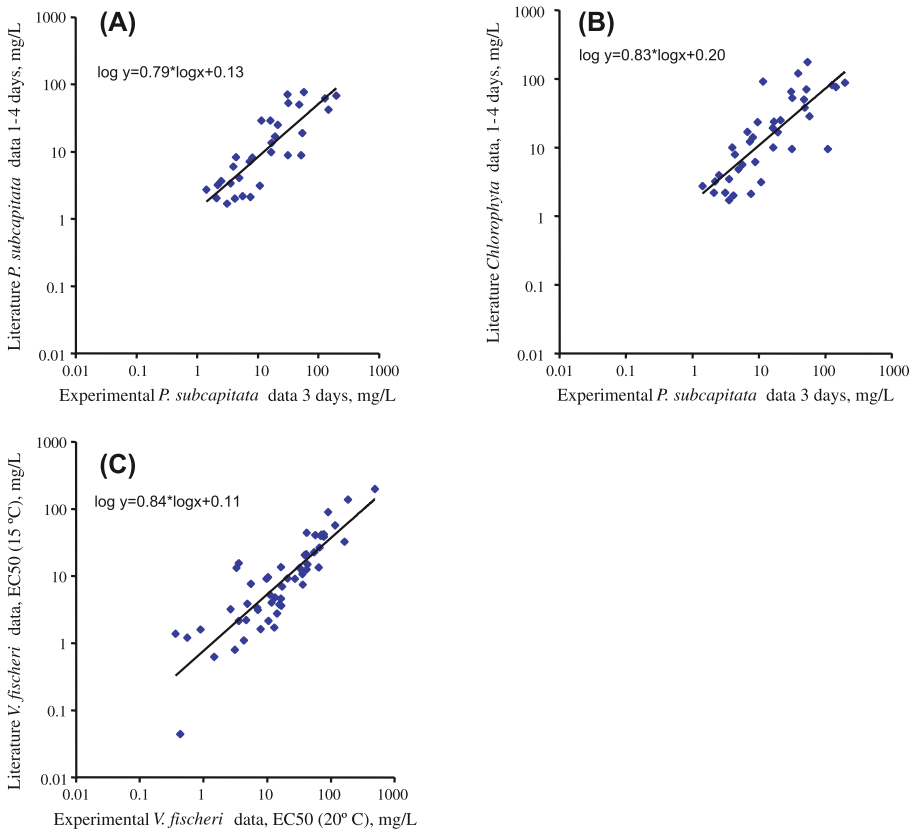

Fig. 2. Literature *versus* experimental data **A** – *Pseudokirchneriella subcapitata* toxicity data (1–4 d, median values) plotted against experimental data, $**R^2 = 0.71$, $p < 0.01$; **B** – *Chlorophyta* toxicity data (1–4 d, median values) plotted against experimental data, $R^2 = 0.64$, $n = 38$, $p < 0.01$, **C** – *Vibrio fischeri* toxicity data (15 min, 15 °C, median values) plotted against experimental data (15 min, 20 °C), $R^2 = 0.74$, $n = 54$, $p < 0.01$. Experimental data were taken from Table 2.

spreadsheet. Literature search of published toxicity data was carried out using Google Scholar, Science Direct and ISI Web of Knowledge. Additional toxicity data on algae and bacteria, which were not present in the US EPA ECOTOX database were collected from the literature (Table S1). The published data on *V. fischeri* luminescence inhibition assay were mainly obtained from the book

"Ecotoxicity of Chemicals to *Photobacterium phosphoreum*" by Kaiser and Devillers (1994).

3. Results and discussion

3.1. Experimental toxicity data

Experimentally determined EC50 values of *P. subcapitata* 72-h growth inhibition, as well as *V. fischeri* 15-min luminescence inhibition for the 28 anilines and 30 phenols are listed in Table 2. The algal EC50 values (mg L^{-1}) ranged from 1.43 (3,4,5-TCA) to 197 (phenol) and bacterial EC50 values from 0.37 (2,3,5-TCP) to 491 (aniline). Thus, the toxicities to algae spanned two orders of magnitude and to bacteria three orders of magnitude. The toxicity of the studied compounds was dependent on the type (chloro-, methyl-, ethyl-), number (mono-, di-, tri-) and position (ortho-, meta-, para-) of the substituents. The chloro-substituted molecules were generally more toxic than alkyl-substituted ones. Among mono-substituted substances the substituent in the para-position tended to increase toxicity whereas most of the ortho-substituted congeners were the least toxic. Similarly, the para-substituent tended to increase the toxicity of di-substituted molecules, especially when combined with the meta-substituent (i.e., 3,4-disubstituted). As a rule, the higher the number of substituents the higher the hydrophobicity and toxicity (Fig. 3). There were also assay-dependent tendencies: phenols were more toxic than anilines in the *V. fischeri* bioluminescence inhibition assay but not in the algal growth inhibition assay.

3.2. Experimental versus published data

Concerning published toxicity data on algae (see Section 2.5, Table S3) there were only 19 values for 10 compounds with strictly the same test conditions available, i.e. for the *P. subcapitata* 72 h growth inhibition test. When other exposure durations between 1 and 4 d were included, 118 data points for 31 substances were found. When all *Chlorophyta* 1–4 d toxicity data were included, altogether 228 data points for 38 substances were obtained (as defined in the ECOTOX database, the *Chlorophyta* included *Pseudokirchneriella*, *Chlamydomonas*, *Chlorella*, *Scenedesmus* and *Chlorococcales*). The variability of these *Chlorophyta* data are illustrated in Fig. 1. The presented toxicity values are based on nominal initial exposure concentrations in a static test. There was wide variation in the toxicity values reported for the same substances/species in different publications, in some cases spanning several orders of magnitude. However, the median values were generally in reasonable agreement with our experimental values (filled symbols in Fig. 1). Our experimental data are compared to median

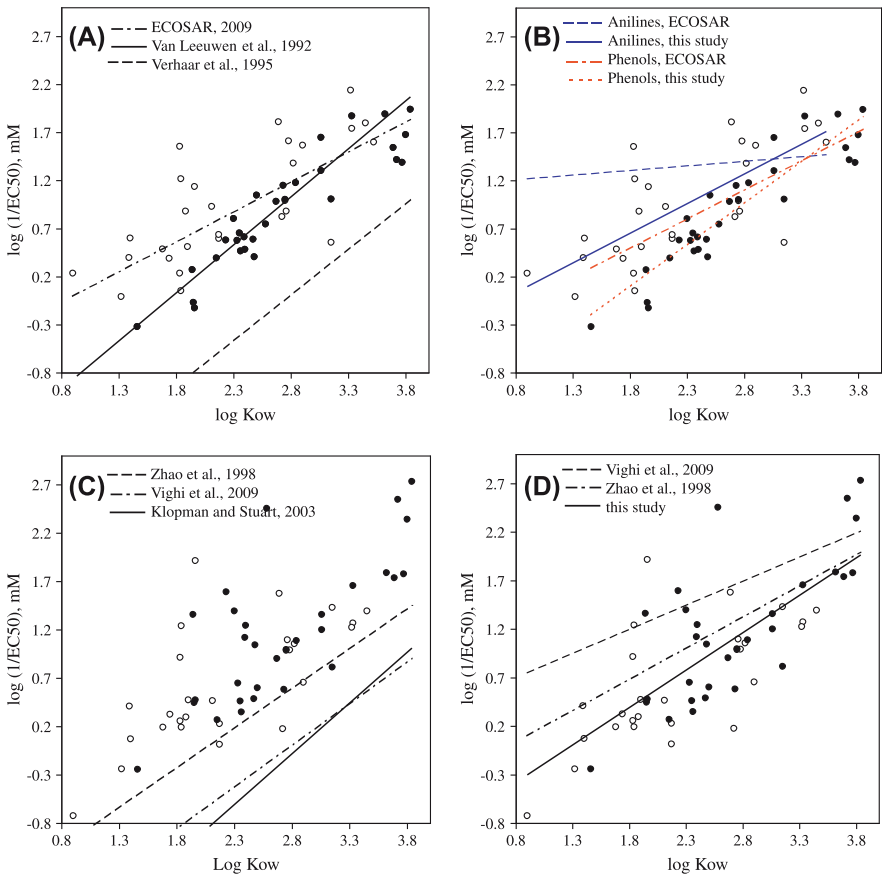

Fig. 3. Comparison of the experimental toxicity data with QSAR predictions. A and B – *Pseudokirchneriella subcapitata* (72-h EC50, mM), C and D – *Vibrio fischeri* (15-min EC50, mM), filled symbols depict phenols and open symbols anilines, equations for the lines are presented in Table 3.

Table 3
logK_{ow} based linear regression equations compared to experimental toxicity data of the studied 58 anilines and phenols. See also Fig. 3.

Organism/test endpoint	Chemical class	Equation	Training set size	R^2 reported for the model	Predicted *versus* observed R^2	R_0^2	K	R_0^2	K'	Figure	Reference
Algae, growth inhibition (EC50, mM)											
Green algae (4d)	Nonpolar narotics	log (1/EC50) = 0.627 * logK_{ow} − 0.569	51	0.60	–	–	–	–	–	Fig. 3A	ECOSAR (2009)
Chlorella vulgaris (5d)	Nonpolar narotics	log (1/EC50) = 0.954 * logK_{ow} − 2.66	34	0.92	–	–	–	–	–	Fig. 3A	Verhaar et al. (1995)
Pseudokirchneriella subcapitata (3–4d)	Nonpolar narotics	log (1/EC50) = 1.00 * logK_{ow} − 1.77	10	0.93	–	–	–	–	–	Fig. 3A	Van Leeuwen et al. (1992)
Pseudokirchneriella subcapitata (3d)	Anilines and phenols	log (1/EC50) = 0.652 * logK_{ow} − 0.712	58	0.60	0.60	0.60	1.00	0.40	0.88		This study
Pseudokirchneriella subcapitata (3d)	Anilines	log (1/EC50) = 0.870 * logK_{ow} − 1.47	28	0.55	0.55	0.51	1.15	0.48	0.75	Fig. 3B	This study
Pseudokirchneriella subcapitata (3d)	Phenols	log 1/EC50 = 0.617 * logK_{ow} − 0.459	30	0.85	0.85	0.75	0.91	0.42	1.02	Fig. 3B	This study
Green algae (4d)	Anilines	log (1/EC50) = 0.095 * logK_{ow} + 1.14	4	0.18	0.55	0.11	0.72	−99.91	1.05	Fig. 3B	ECOSAR (2009)
Green algae (4d)	Phenols	log (1/EC50) = 0.609 * logK_{ow} − 0.599	40	0.67	0.85	0.73	0.92	0.29	1.00	Fig. 3B	ECOSAR (2009)
Algae	Not reported	EC50 Multicase model	476	n.r.	0.17	−0.03	0.65	−0.17	1.08		Danish (Q)SAR Database
Bacteria, luminescence inhibition (EC50, mM)											
Vibrio fischeri (15-min, 20 °C)	Nonpolar narotics	log (1/EC50) = 0.824 * logK_{ow} − 1.71	33	0.85	–	–	–	–	–	Fig. 3C	Zhao et al. (1998)
Vibrio fischeri (5-min, 15 °C)	Nonpolar narotics	log (1/EC50) = 1.05 * logK_{ow} − 3.02	179	0.94	–	–	–	–	–	Fig. 3C	Klopman and Stuart (2003)
Vibrio fischeri (15-min, 15 °C)	Nonpolar narotics	log (1/EC50) = 0.856 * logK_{ow} − 2.42	25	0.84	–	–	–	–	–	Fig. 3C	Vighi et al. (2009)
Vibrio fischeri (15-min, 20 °C)	Anilines and phenols	log (1/EC50) = 0.771 * logK_{ow} − 0.996	57	0.56	0.56	0.56	1.00	0.34	0.84	Fig. 3D	This study
Vibrio fischeri (15-min, 20 °C)	Anilines	log (1/EC50) = 0.619 * logK_{ow} − 0.727	27	0.47	0.47	0.46	0.87	0.26	0.87		This study
Vibrio fischeri (15-min, 20 °C)	Phenols	log (1/EC50) = 0.848 * logK_{ow} − 1.14	30	0.55	0.55	0.55	1.07	0.24	0.82		This study
Vibrio fischeri (15-min, 20 °C)	Polar narcotics	log (1/EC50) = 0.645 * logK_{ow} − 0.48	10	0.84	0.56	0.51	0.87	−0.34	0.94	Fig. 3D	Zhao et al. (1998)
Vibrio fischeri (15-min, 15 °C)	Polar narcotics	log (1/EC50) = 0.497 * logK_{ow} + 0.304	24	0.81	0.56	0.36	0.65	−4.01	1.18	Fig. 3D	Vighi et al. (2009)

Notes:
R^2 – correlation coefficient of linear regression.
R_0^2 – correlation coefficient of predicted *versus* observed linear regression when intercept is set to zero.
K – slope of predicted *versus* observed linear regression when intercept is set to zero.
R_0^2 – correlation coefficient of observed *versus* predicted linear regression when intercept is set to zero.
K' – slope of observed *versus* predicted linear regression when intercept is set to zero.
"–" – not relevant.
n.r. – not reported.

values from the above-described datasets (i.e. for *P. subcapitata* and *Chlorophyta*) in Fig. 2A and B. Expectedly, the data for the same species correlated more closely with our experimental values (log–log $R^2 = 0.71$, $n = 31$) compared to data for *Chlorophyta* (log–log $R^2 = 0.64$, $n = 38$) but both correlations are significant ($p < 0.01$).

In the case of bacteria there were more toxicity data on anilines and phenols available in the literature (Table S3): for the conventional *V. fischeri* bioluminescence inhibition assay (Microtox) there were 111 data points for 54 substances with 15-min exposure time and 15 °C. Again, the median values of the published toxicity data correlated well with our experimental EC50 values on *V. fischeri* performed on 96-well microplates at 20 °C (log–log $R^2 = 0.74$, $n = 54$, $p < 0.01$; Fig. 2C).

3.3. Experimental data compared to QSAR-predicted toxicities

A valid QSAR model should be based on and used for compounds that act through a common or very similar mode of action (Verhaar et al., 1996). The most common method to group chemicals according to the mode of action is the Verhaar scheme that distinguishes four classes based on structural features of the molecules: class 1 – inert chemicals or non-polar narcotics; class 2 – less inert chemicals or polar narcotics; class 3 – reactive chemicals; and class 4 – specifically acting chemicals (Verhaar et al., 1992). The toxicity of the chemicals in classes 1 and 2 is known to be proportional to hydrophobicity (octanol/water partitioning coefficient, K_{ow}). Using toxicity data for a number of species, the class 2 chemicals have been shown to be 5–10 times more toxic than class 1 chemicals with the same K_{ow} (Vaal et al., 1997). This increased toxicity is often called "excess toxicity" as compared to the "baseline toxicity" of class 1 chemicals. All the 58 chosen chemicals belong to Verhaar class 2. The logK_{ow} values of the 58 compounds vary from 0.9 to 3.8 (Table 1), which is useful for logK_{ow} based QSAR modeling as the quality of the model usually increases with increasing logK_{ow} range (Dearden et al., 2009). As a next step in the analysis, experimental data were compared to existing QSAR predictions for algae and bacteria (Table 3, Fig. 3). The US EPA ECOlogical Structure Activity Relationships (ECOSAR, http://www.epa.gov/oppt/newchems/tools/21ecosar.htm) software is a QSAR tool that predicts the toxicity of industrial chemicals to aquatic organisms such as fish, aquatic invertebrates and algae. The classification of chemicals according to the ECOSAR is also included in the OECD QSAR software. In EU, the European Chemicals Bureau and the Danish EPA have jointly produced an internet-accessible version of the Danish (Q)SAR Database which can be used to retrieve predictions of *P. subcapitata* toxicity. In addition, QSAR equations can be found in the EU guidance documents (ECB, 2003; ECHA, 2008) as well as in scientific papers.

3.3.1. Algae: models versus experimental data

In Fig. 3A the experimental algal toxicity data (Table 2) were compared to three baseline QSARs (Van Leeuwen et al., 1992; Verhaar et al., 1995; ECOSAR, 2009). Note that the toxicity values are in the form of log $1/EC_{50}$ (mM). Theoretically all these three baselines describing the toxicity of class 1 chemicals should be similar and the toxicity of our set of chemicals (class 2) should be 5–10 times higher. However, Fig. 3A shows that only the QSAR suggested by Verhaar et al. (1995) and not the other two equations were in agreement with this concept. In comparison to this lowest baseline the toxicity of the anilines and phenols was up to 300-fold higher. Interestingly, the QSAR by Verhaar is not based on *P. subcapitata* but *Chlorella vulgaris* toxicity data (on 34 chemicals; $n = 34$). The other QSAR equations were built on toxicity data of *P. subcapitata*, ($n = 10$; Technical Guidance Document on Risk Assessment: TGD ECB, 2003) or several species including *P. subcapitata*, ($n = 51$; ECOSAR).

Concerning class 2 chemicals the TGD lacks an algal toxicity QSAR for and the Danish (Q)SAR database does not contain detailed information on the models, stating that it uses a Multicase model based on a training set of 476 chemicals (ECB, 2005). Apparently, this model uses other descriptors in addition to logK_{ow}, but still failed to predict our experimental data (predicted *versus* observed $R^2 = 0.166$, see Table 3). Comparison of our experimental toxicity data to the ECOSAR equations is shown in Fig. 3B. Note that ECOSAR provides different equations for anilines and phenols. While neither of the QSARs was acceptable according to strict validation criteria (see Section 2.4; Golbraikh et al., 2003) the observed toxicity of phenols was much closer to the prediction (Fig 3B, Table 3). The ECOSAR model for phenols is built on 40 chemicals whereas the aniline equation is derived from just 4 data points and does not correlate with hydrophobicity. Likewise, our own log 1/EC50 *versus* logK_{ow} regression line for phenols showed better fit than the one for anilines or the whole set of chemicals (Fig. 3B, Table 3). This is a consequence of much wider variation in the aniline data, with up to 30-fold difference in EC50 values for anilines with the same hydrophobicity. Similar results were obtained by analyzing the toxicity values for *P. subcapitata* by Chen et al. (2007). 17 chemicals that overlapped between the two studies, 12 anilines and 5 phenols, were compared. Analogously to our data, there was poor correlation between toxicity and logK_{ow} of anilines ($R^2 = 0.26$, $p > 0.1$) but a good correlation between the toxicity of the phenols and logK_{ow} ($R^2 = 0.85$, $p < 0.03$, data not shown).

3.3.2. Bacteria: models versus experimental data

Although toxicity of chemicals to bacteria is not taken into account in ecotoxicity assessment for regulatory purposes, a number of QSARs for the toxicity of different chemical groups and mixtures to *V. fischeri* can be found in the literature (Lessigiarska et al., 2005) (Table S2). The *V. fischeri* experimental data (Table 2) were compared to baseline equations as well as equations for polar narcotic chemicals (Table 3, Fig. 3C and D). The experimental values (log 1/EC50) were higher than all three baselines (Zhao et al., 1998; Klopman and Stuart, 2003; Vighi et al., 2009) and thus in accordance with the concept of excess toxicity of class 2 chemicals. Still, the toxicity of the tested anilines and phenols to *V. fischeri* was not well explained by hydrophobicity as evidenced by the distribution of values on Fig. 3C. Differently from algae, when the data for anilines and phenols were studied separately the correlations did not improve (Table 3). In addition, the comparison of our data with different class 2 QSARs showed that the best fit was observed for the equation based on toxicity data obtained at 20 °C (Zhao et al., 1998). Also, our work on the toxicities of aniline and phenol to *V. fischeri* at different temperatures has shown 2-fold decrease in toxicity at 20 °C compared to 15 °C in all incubation time-points (5, 15 and 30 min; unpublished data). This should be taken into account when comparing *V. fischeri* toxicity data.

3.4. Classification based on environmental hazard

Classification and labeling involves an evaluation of the intrinsic hazard of a chemical and communication of that hazard *via* the label. This evaluation must be made as set out in the new Classification, Labelling and Packaging Regulation (CLP; EC, 2008) for any substance or mixture/preparation manufactured or imported for the EU. Currently there are more than 7000 hazardous substances listed in the Annex VI to the CLP Regulation (previously Annex I to Directive 67/548/EEC; EC, 1967), however, the number of hazardous chemicals used in EU market is much bigger. By January 3rd 2011, European Chemicals Agency, ECHA (http://echa.europa.eu/) had received 3114 835 notifications of 24 529 substances for the Classification and Labeling Inventory. Comparing the classification and labeling of the selected 58 anilines and phenols it

Table 4
Classification of the studied chemicals.

No.	Chemical[a]	Production volume according to ESIS[b]	Classified under Annex I of directive 67/548/EEC[c]	Classification according to Annex VI of Directive 67/548/EEC[d]			
				Algae[e]	Bacteria[f]	ECOSAR, green algae[g]	Danish (Q)SAR database (multicase)[h]
1	A	HPV	+ (N)	Harmful	Not harmful	Toxic	Harmful
2	2-CA	HPV	–	Harmful	Harmful	Toxic	Harmful
3	3-CA	LPV	–	Harmful	Harmful	Toxic	Harmful
4	4-CA	LPV	+ (N)	Toxic	Harmful	Toxic	Harmful
5	2,3-DCA	#	–	Toxic	Harmful	Toxic	Harmful
6	2,4-DCA	LPV	–	Toxic	Harmful	Toxic	Toxic
7	2,5-DCA	LPV	–	Harmful	Harmful	Toxic	Toxic
8	2,6-DCA	LPV	–	Harmful	Harmful	Toxic	Harmful
9	3,4-DCA	HPV	+ (N)	Toxic	Toxic	Toxic	Toxic
10	3,5-DCA	LPV	–	Toxic	Harmful	Toxic	Harmful
11	2,3,4-TCA	#	–	Toxic	Harmful	Toxic	Toxic
12	2,4,5-TCA	LPV	–	Toxic	Toxic	Toxic	Very toxic
13	2,4,6-TCA	#	–	Toxic		Toxic	Very toxic
14	3,4,5-TCA	#	–	Toxic	Harmful	Toxic	Toxic
15	2-MA	HPV	+ (N)	Not harmful	Not harmful	Toxic	Harmful
16	3-MA	HPV	+ (N)	Harmful	Harmful	Toxic	Harmful
17	4-MA	HPV	+ (N)	Harmful	Harmful	Toxic	Harmful
18	2,3-DMA	LPV	–	Harmful	Not harmful	Toxic	Harmful
19	2,4-DMA	HPV	–	Harmful	Harmful	Toxic	Toxic
20	2,5-DMA	LPV	–	Harmful	Harmful	Toxic	Toxic
21	2,6-DMA	HPV	+ (N)	Not harmful	Harmful	Toxic	Toxic
22	3,4-DMA	LPV	–	Toxic	Toxic	Toxic	Toxic
23	3,5-DMA	LPV	–	Harmful	Harmful	Toxic	Harmful
24	2,4,6-TMA	#	–	Harmful	Harmful	Toxic	Toxic
25	2-EA	LPV	–	Harmful	Harmful	Toxic	Harmful
26	3-EA	#	–	Harmful	Harmful	Toxic	Harmful
27	4-EA	#	–	Toxic	Toxic	Toxic	Harmful
28	2,6-DEA	LPV	+	Harmful	Toxic	Toxic	Toxic
29	P	HPV	+	Not harmful	Not harmful	Harmful	Harmful
30	2-CP	HPV	+ (N)	Harmful	Harmful	Harmful	Harmful
31	3-CP	#	+ (N)	Harmful	Harmful	Harmful	Very toxic
32	4-CP	HPV	+ (N)	Harmful	Toxic	Harmful	Harmful
33	2,3-DCP	LPV	–	Harmful	Harmful	Harmful	Harmful
34	2,4-DCP	HPV	+ (N)	Toxic	Toxic	Toxic	Toxic
35	2,5-DCP	LPV	–	Toxic	Harmful	Toxic	Toxic
36	2,6-DCP	LPV	–	Harmful	Harmful	Harmful	Toxic
37	3,4-DCP	#	–	Toxic	Toxic	Toxic	Toxic
38	3,5-DCP	#	–	Toxic	Toxic	Toxic	Harmful
39	2,3,4-TCP	#	–	Toxic	Very toxic	Toxic	Toxic
40	2,3,5-TCP	#	–	Toxic	Very toxic	Toxic	Toxic
41	2,3,6-TCP	#	–	Toxic	Toxic	Toxic	Very toxic
42	2,4,5-TCP	#	+ (N)	Toxic	Very toxic	Toxic	Toxic
43	2,4,6-TCP	HPV	+ (N)	Toxic	Toxic	Toxic	Toxic
44	2-MP	HPV	+	Not harmful	Harmful	Harmful	Harmful
45	3-MP	HPV	+	Not harmful	Harmful	Harmful	Toxic
46	4-MP	HPV	+	Harmful	Toxic	Harmful	Harmful
47	2,3-DMP	#	+ (N)	Harmful	Harmful	Harmful	Harmful
48	2,4-DMP	LPV	+ (N)	Harmful	Toxic	Harmful	Toxic
49	2,5-DMP	LPV	+ (N)	Harmful	Harmful	Harmful	Toxic
50	2,6-DMP	HPV	+ (N)	Harmful	Harmful	Harmful	Toxic
51	3,4-DMP	#	+ (N)	Harmful	Toxic	Harmful	Toxic
52	3,5-DMP	HPV	+	Harmful	Harmful	Harmful	Harmful
53	2,3,5-TMP	#	–	Harmful	Harmful	Toxic	Harmful
54	2,3,6-TMP	HPV	–	Harmful	Harmful	Harmful	Very toxic
55	2,4,6-TMP	#	–	Toxic	Harmful	Harmful	Toxic
56	2-EP	#	–	Harmful	Harmful	Harmful	Harmful
57	3-EP	#	–	Harmful	Toxic	Harmful	Very toxic
58	4-EP	LPV	–	Harmful	Very toxic	Harmful	Harmful

Notes:
– This substance has not been reported as an HPVC or LPVC.
+ (N) – substance is included in Annex I of directive 67/548/EEC and is classified as dangerous for the environment.
+ – substance is included in Annex I of directive 67/548/EEC, but is not classified as dangerous for the environment.
– substance is not included in Annex I of directive 67/548/EEC and does not have harmonised classification in EU.

[a] Abbreviations are explained in Table 1.

[b] ESIS – European Chemical Substances Information System (http://ecb.jrc.ec.europa.eu/esis/); HPVC – High Production Volume Chemical, production or import volume in EU exceeds 1000 tonnes per year per producer or importer; LPVC – Low Production Volume Chemical, production or import volumes in EU is between 10 tonnes and 1000 tonnes per year per producer or importer.

[c] EC, 1967.

[d] Chemicals are categorized as: very toxic – L(E)C50 $\leqslant$ 1 mg L^{-1}, toxic – 1 mg L^{-1} < L(E)C50 $\leqslant$ 10 mg L^{-1}, harmful - 10 mg L^{-1} < L(E)C50 $\leqslant$ 100 mg L^{-1}. In addition, L(E)C50 > 100 mg L^{-1} were designated as "not classified".

[e] Experimentally determined toxicity to *Pseudokirchneriella subcapitata*, 72-h EC50.

[f] Experimentally determined toxicity to *Vibrio fischeri*, 15-min EC50.

[g] Predicted toxicity, calculated using ECOSAR QSAR models of anilines and phenols for green algae, 96-h EC50.

[h] http://130.226.165.14/User_Manual_Danish_Database.pdf (ECB, 2005).

appeared that 34 of them had not been evaluated on the EU-level under previous legislation and 18 were classified as dangerous to the environment (symbol of danger "N"; Table 4). The list contains 19 high and 19 low production volume chemicals (HPVC and LPVC) for which there was no harmonized classification. This means that the information on environmental and health properties has to be obtained. However, available aquatic toxicity data on algae, daphnids and fish were far from complete, especially data obtained with standard test protocols. Notably, algae were the least represented group, with EC50 values available for only 10 substances, for daphnia and fish respectively 36 and 40 chemicals were covered. In the case of *V. fischeri* almost a complete set of toxicity data for the selected chemicals was available (112 EC50 values for 55 chemicals) (Table S3).

As QSARs are proposed for the hazard classification of chemicals, the 58 studied chemicals were classified using the ECOSAR and the Danish (Q)SAR database. The result of this analysis is presented in Table 4. The ECOSAR classified all anilines as toxic in disagreement with classification based on our experimental data on algae. In case of phenols, ECOSAR classified all 30 phenols as harmful or toxic, in 25 cases matching the classification based on our algal data. The results according to the Danish (Q)SAR database were equally inaccurate for both anilines and phenols, predicting the hazard class in roughly half of the cases (Table 4).

As mentioned, the toxicity of chemicals to bacteria is not taken into account in ecotoxicity assessment for regulatory purposes. However, comparison of the toxicity data for bacteria and algae (Fig. S1) shows that the EC50 values for the majority of the tested compounds for both organisms were between 1 and 100 mg L^{-1}. According to EU classification criteria described in Annex VI of Directive 67/548/EEC (EC, 1991), these chemicals could be considered "harmful" (10–100 mg L^{-1}) or "toxic" (1–10 mg L^{-1}; Table 4). Four compounds would be classified as "very toxic" (<1 mg L^{-1}) only based on bacterial data. The classification would overlap for 59% of substances (for 34 out of the 58 tested substances). This suggests that *V. fischeri* toxicity data may be useful for environmental toxicity screening. The outlook of replacing some of the time-consuming and expensive toxicity testing with the rapid bacterial bioluminescence assay is worth further consideration. In addition, the need to include bacterial data in the ecotoxicological risk assessment has been highlighted by Vighi et al. (2009), who have showed similarities between the QSAR models for *V. fischeri* with those for fish, algae and *Daphnia*.

4. Conclusions and outlook

Often the limiting factor in the development of QSARs is the availability of high quality toxicity data for congeneric chemicals, preferably measured in a single laboratory and using standardized test protocols. Probably the best homogenous toxicity dataset, extensively used for QSARs modeling, contains *Tetrahymena pyriformis* growth inhibition data for 2400 industrial organic compounds (Dimitrov et al., 2003). Indeed, bibliometric analysis (Table S2) shows that currently most of the QSARs have been constructed using toxicity data on fish and protozoa *Tetrahymena,* followed by *Daphnia* and the bacterium *V. fischeri*. Remarkably less QSARs have been developed on algal data. Thus the number of available QSARs is in correlation with the amount of experimental data available and not with the regulatory need.

In this paper a set of homogenous experimental toxicity data was generated for 58 substituted anilines and phenols using algae *P. subcapitata* and bacteria *V. fischeri*. For the 15 HPVC and 17 LPVC in our experimental set, the toxicity data obtained using the OECD 201 algal growth inhibition test were published for the first time. Only five of the tested 58 chemicals showed inhibitory effect to algae at concentrations >100 mg L^{-1}, i.e. could be classified as "not harmful", 32 chemicals as "harmful" (10–100 mg L^{-1}) and 21 as "toxic" (1–10 mg L^{-1}). Comparison of the experimental toxicity data with the predictions made using the existing QSAR models suggests that the toxicity of phenols to algae may be modeled with a simple hydrophobicity-based equation. Aniline toxicity to algae as well as toxicity of both anilines and phenols to *V. fischeri* depended on other characteristics in addition to logK_{ow}.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.05.023.

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Toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata* and bacteria *Vibrio fischeri*: Comparison with published data and QSARs

Villem Aruoja [a,c,*], Mariliis Sihtmäe [a,b], Henri-Charles Dubourguier [a,c], Anne Kahru [a]

[a] National Institute of Chemical Physics and Biophysics, Laboratory of Molecular Genetics, Tallinn, Estonia
[b] Tallinn University of Technology, Tallinn, Estonia
[c] Estonian University of Life Sciences, Tartu, Estonia

*Corresponding author. Address: Akadeemia tee 23, Tallinn 12618, Estonia. Tel.: +372 6398368; fax: +372 6398382.
E-mail address: villem.aruoja@kbfi.ee (V. Aruoja).

Additional information presented as Supporting Information:

Content:

Table S1. References of toxicity data on of 58 substituted anilines and phenols.

Table S2. Bibliometry of peer-reviewed papers published on QSARs according to Thomson Reuters ISI Web of Science for years 1980–2010.

Table S3. Toxicity data (EC50s) availability from US EPA ECOTOX database and from published papers (see Table S1).

Fig. S1. Comparison of the toxicity of substituted anilines (filled symbols) and phenols (open symbols) to *Pseudokirchneriella subcapitata* (72-h EC50, mg/L) with *Vibrio fischeri* (15-min EC50, mg/L).

Table S1. References of toxicity data on of 58 substituted anilines and phenols.

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7.	Lee, Y.G., Hwang, S.H., Kim, S.D, 2006. Predicting the toxicity of substituted phenols to aquatic species and its changes in the stream and effluent waters. Arch. Environ. Contam. Toxicol. 50, 213–219.
8.	Yin, D., Hu, S., Jin, H. and L. Yu, 2003. Deriving freshwater quality criteria for 2,4,6-trichlorophenol for protection of aquatic life in China. Chemosphere. 52, 67-73.
9.	Yin, D., Jin, H., Yu, L., Hu, S., 2003. Deriving freshwater quality criteria for 2,4-dichlorophenol for protection of aquatic life in China. Environ Pollut. 122, 217-222.
10.	Kaiser, K.L.E., Devillers, J., 1994. Ecotoxicity of Chemicals to *Photobacterium Phosphoreum* (Handbooks of Ecotoxicological Data). Gordon and Breach Sciences PublishersS.A., Amsterdam.
11.	Osano, O., Admiraal, W., Klamer, H.J.C., Pastor, D., Bleeker, E.A.J., 2001. Comparative toxic and genotoxic effects of chloroacetanilides, formamidines and their degradation products on *Vibrio fischeri* and *Chironomus riparius*. Environ. Pollut. 119, 195–202.
12.	Rozkov, A., Vassiljeva, I., Kurvet, M., Kahru, A., Preis, S., Kharchenko, A., Krichevskaya, M., Liiv, M., Kaard, A., Vilu, R., 1999. Laboratory study of bioremediation of rocket fuel-polluted groundwater. Water Research. 33(5), 1303-1313.
13.	Kahru, A., Põllumaa, L., Reiman, R., Rätsep, A., Liiders, M., Maloveryan, A., 2000. The toxicity and biodegradability of eight main phenolic compounds characteristic to the oil-shale industry wastewaters: a test battery approach. Environ. Toxicol. 5,431-442.

Table S2. Bibliometry of peer-reviewed papers published on QSARs according to Thomson Reuters ISI Web of Science for years 1980–2010. Search was performed on January 5, 2011.

Keyword(s) used for the search[a]	Results found[b]	Sum of the time cited[c]	Top cited articles (number of citations)
QSAR	9358	122744	Huddleston, J.G., Willauer, H.D., Swatloski, R.P., 1998. Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction. Chem. Comm. 16, 1765-1766. **(1017)**
QSAR and fish	266	5273	Verhaar, H.J.M., van Leeuwen, C.J., Hermens, J.L.M., 1992. Classifying environmental pollutants. 1: Structure–activity relationships for prediction of aquatic toxicity. Chemosphere. 25(4), 471–491 **(303)**
QSAR and Tetrahymena	244	3499	Cronin, M.T.D., Schultz, T.W., 2003. Pitfalls in QSAR. Journal of Molecular Structure: THEOCHEM. 622(1-2), 39-51. (**118)**
QSAR and Daphnia	124	1810	Van Leeuwen, C.J., Van Der Zandt, P.T.J., Aldenberg, T., Verhaar, H.J.M., Hermens, J.L.M., 1992. Application of QSARs, extrapolation and equilibrium partitioning in aquatic effects assessment. I. Narcotic industrial pollutants. Environ Toxicol Chem. 11(2), 267-282. **(111)**
QSAR and QSAR AND Topic=(Vibrio OR Photobacterium OR Microtox)	116	1573	Cronin, M.T.D., Schultz, T.W., 2003. Pitfalls in QSAR. Journal of Molecular Structure: THEOCHEM. 622(1-2), 39-51. **(118)**
QSAR and alga*	88	1035	Van Leeuwen, C.J., Van Der Zandt, P.T.J., Aldenberg, T., Verhaar, H.J.M., Hermens, J.L.M., 1992. Application of QSARs, extrapolation and equilibrium partitioning in aquatic effects assessment. I. Narcotic industrial pollutants. Environ Toxicol Chem. 11(2), 267-282. **(111**)
QSAR and (Selenastrum OR Pseudokirchneriella)	19	188	Eng, G., Brinckman, F.E., Olson, G.J., Tierney, E.J., Bellama, J.M., 1991. Total surface areas of group IVA organometallic compounds: predictors of toxicity to algae and bacteria. Appl. Organomet. Chem. 5(1), 33-37. **(35)**
QSAR AND Topic=(alga* AND Vibrio OR Photobacterium OR Microtox)	79	990	Hansch, C., Hoekman, D., Leo, A., Weininger, D., Selassie, C.D., 2002. Chem-bioinformatics: comparative QSAR at the interface between chemistry and biology. Chem. Rev. 102(3), 783-812. **(100)**
QSAR AND aniline*	111	1731	Hansch, C., Hoekman, D., Gao, H., 1996. Comparative QSAR: toward a deeper understanding of chemicobiological interactions. Chem. Rev. 96(3), 1045-1075. **(133)**
QSAR AND phenol*	424	6252	Lien, E.J., Ren, S.J., Bui, H.Y.H., Wang,. R., 1999. Quantitative structure-activity relationship analysis of phenolic antioxidants. Free Radic. Biol. Med. 26(3-4):285-294. **(212)**
QSAR AND aniline* AND phenol*	46	816	Cronin, M.T., Aptula, A.O., Duffy, J.C., Netzeva, T.I., Rowe, P.H., Valkova, I.V., Schultz, T.W., 2002. Comparative assessment of methods to develop QSARs for the prediction of the toxicity of phenols to Tetrahymena pyriformis. CHEMOSPHERE49(10), 1201-1221. **(70)**
QSAR AND aniline* AND phenol* AND alga*	11	74	Lu, G.H., Yuan, X., Zhao, Y.H., 2001. QSAR study on the toxicity of substituted benzenes to the algae (Scenedesmus obliquus). CHEMOSPHERE 44(3), 437-440. **(23)**
QSAR AND (aniline* AND phenol*) AND Vibrio OR Photobacterium OR Microtox) AND alga*	5	8	Chen, C.Y., Ko, C.W., Lee, P.I., 2007. Toxicity of substituted anilines to Pseudokirchneriella subcapitata and quantitative structure-activity relationship analysis for polar narcotics. Environ. Toxicol. Chem. 26(6), 1158-1164.

[a] Keyword(s) used for the search. The search for "QSAR" was restricted to Article or Proceedings Paper or Review, to yield <10000 papers that allows to perform the further analysis for citation statistics. Keyword "toxic" was used for refining of the previous search.

[b] Number of the papers retrieved by the respective keyword(s)

[c] Total number of citations for retrieved papers ([b]).

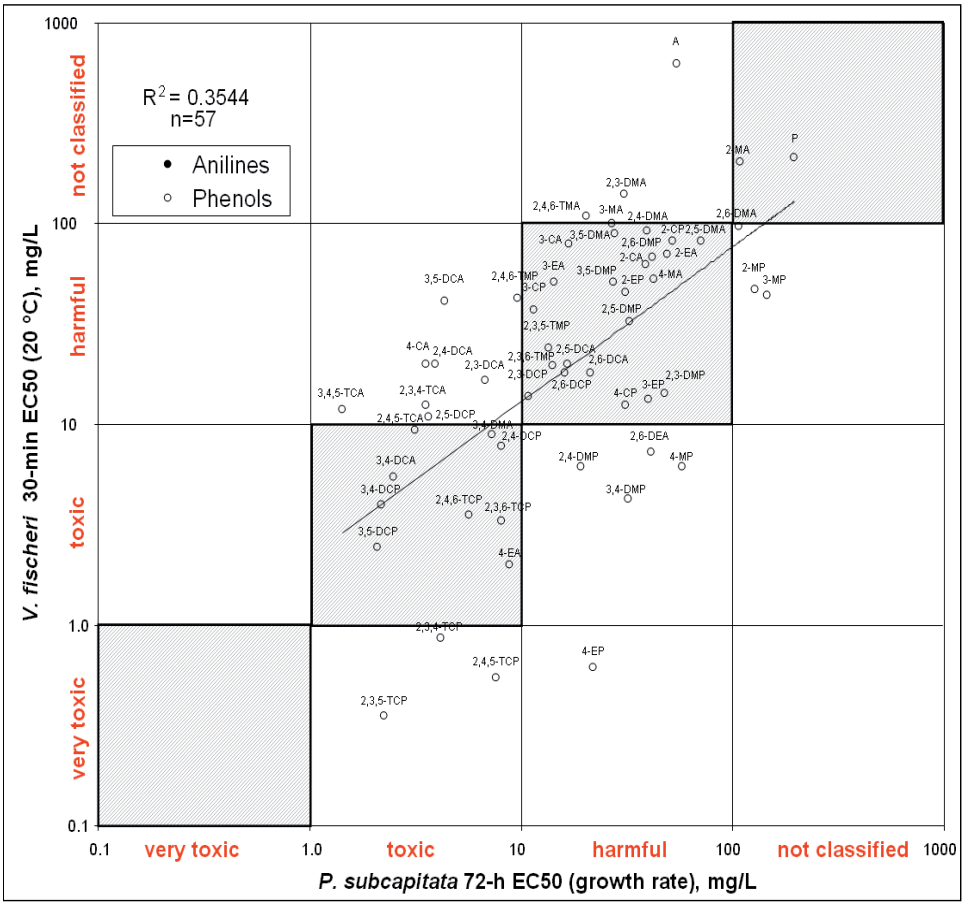
Table S3. Toxicity data (EC50s) availability from US EPA ECOTOX database and from published papers (see Table S1).

No	Chemical	Abbreviation	*V. fischeri*, 15-min (15oC)	*P. subcapitata*, 3 days	*P. subcapitata*, 1-4 days	*Chlorophyta*, 1-4 days	Fish, 96-h	*Daphnids*, 48-h
1	aniline	A	4	n.f	1	9	29	15
2	2-chloroaniline	2-CA	1	n.f	n.f	8	6	6
3	3-chloroaniline	3-CA	1	n.f	3	8	1	3
4	4-chloroaniline	4-CA	1	n.f	3	11	11	3
5	2,3-dichloroaniline	2,3-DCA	1	n.f	n.f	1	1	n.f
6	2,4-dichloroaniline	2,4-DCA	1	n.f	4	9	12	6
7	2,5-dichloroaniline	2,5-DCA	1	n.f	3	5	1	2
8	2,6-dichloroaniline	2,6-DCA	1	n.f	3	3	n.f	1
9	3,4-dichloroaniline	3,4-DCA	3	2	11	24	21	34
10	3,5-dichloroaniline	3,5-DCA	1	n.f	3	4	n.f	2
11	2,3,4-trichloroaniline	2,3,4-TCA	1	n.f	n.f	1	2	2
12	2,4,5-trichloroaniline	2,4,5-TCA	1	2	5	7	1	3
13	2,4,6-trichloroaniline	2,4,6-TCA	1	n.f	3	4	n.f	1
14	3,4,5-trichloroaniline	3,4,5-TCA	1	n.f	3	3	n.f	n.f
15	2-methylaniline	2-MA	2	n.f	n.f	5	n.f	2
16	3-methylaniline	3-MA	2	n.f	n.f	n.f	1	2
17	4-methylaniline	4-MA	2	n.f	n.f	n.f	3	2
18	2,3-dimethylaniline	2,3-DMA	3	n.f	3	5	n.f	n.f
19	2,4-dimethylaniline	2,4-DMA	5	n.f	n.f	n.f	n.f	1
20	2,5-dimethylaniline	2,5-DMA	3	n.f	n.f	n.f	n.f	n.f
21	2,6-dimethylaniline	2,6-DMA	3	n.f	n.f	n.f	n.f	n.f
22	3,4-dimethylaniline	3,4-DMA	3	n.f	3	5	n.f	n.f
23	3,5-dimethylaniline	3,5-DMA	3	n.f	n.f	n.f	n.f	n.f
24	2,4,6-trimethylaniline	2,4,6-TMA	n.f	n.f	n.f	n.f	n.f	n.f
25	2-ethylaniline	2-EA	2	n.f	n.f	1	n.f	3
26	3-ethylaniline	3-EA	2	n.f	n.f	n.f	n.f	1
27	4-ethylaniline	4-EA	3	n.f	n.f	1	2	3
28	2,6-diethylaniline	2,6-DEA	2	n.f	n.f	n.f	n.f	n.f
29	Phenol	P	11	1	22	31	129	49
30	2-chlorophenol	2-CP	1	n.f	3	7	15	8
31	3-chlorophenol	3-CP	1	n.f	1	2	2	n.f
32	4-chlorophenol	4-CP	1	2	9	17	14	10
33	2,3-dichlorophenol	2,3-DCP	1	n.f	2	2	2	1
34	2,4-dichlorophenol	2,4-DCP	2	n.f	4	9	20	8
35	2,5-dichlorophenol	2,5-DCP	1	n.f	n.f	n.f	2	n.f
36	2,6-dichlorophenol	2,6-DCP	1	n.f	1	2	3	2
37	3,4-dichlorophenol	3,4-DCP	2	n.f	1	1	2	n.f
38	3,5-dichlorophenol	3,5-DCP	4	7	10	15	2	n.f
39	2,3,4-trichlorophenol	2,3,4-TCP	1	n.f	1	1	2	n.f
40	2,3,5-trichlorophenol	2,3,5-TCP	1	n.f	n.f	n.f	1	n.f
41	2,3,6-trichlorophenol	2,3,6-TCP	1	n.f	n.f	n.f	3	n.f
42	2,4,5-trichlorophenol	2,4,5-TCP	2	1	6	6	16	4
43	2,4,6-trichlorophenol	2,4,6-TCP	2	n.f	2	5	25	8
44	2-methylphenol	2-MP	2	n.f	1	4	16	15
45	3-methylphenol	3-MP	1	n.f	1	2	6	2
46	4-methylphenol	4-MP	4	1	2	4	11	9
47	2,3-dimethylphenol	2,3-DMP	4	1	1	1	1	1
48	2,4-dimethylphenol	2,4-DMP	4	1	2	2	15	4
49	2,5-dimethylphenol	2,5-DMP	1	n.f	n.f	n.f	1	n.f
50	2,6-dimethylphenol	2,6-DMP	1	n.f	n.f	n.f	1	3
51	3,4-dimethylphenol	3,4-DMP	4	1	1	1	2	1
52	3,5-dimethylphenol	3,5-DMP	1	n.f	n.f	n.f	1	n.f
53	2,3,5-trimethylphenol	2,3,5-TMP	1	n.f	n.f	n.f	n.f	n.f
54	2,3,6-trimethylphenol	2,3,6-TMP	1	n.f	n.f	n.f	1	n.f
55	2,4,6-trimethylphenol	2,4,6-TMP	1	n.f	n.f	2	1	1
56	2-ethylphenol	2-EP	n.f	n.f	n.f	n.f	n.f	n.f
57	3-ethylphenol	3-EP	n.f	n.f	n.f	n.f	n.f	n.f
58	4-ethylphenol	4-EP	1	n.f	n.f	n.f	2	1

Notes:
n.f - not found

Fig. S1. Comparison of the toxicity of substituted anilines (filled symbols) and phenols (open symbols) to *Pseudokirchneriella subcapitata* (72-h EC50, mg/L) with *Vibrio fischeri* (15-min EC50, mg/L).

Data are plotted from Table 2. Log-log R^2=0.35, p<0.01. The toxicological classes were assigned according to the EU criteria as described in Annex VI of Directive 67/548/EEC (EC, 1991). Chemicals are categorized as: very toxic - L(E)C50 >=1 mg/l; toxic - L(E)C50 1-10 mg/L; harmful - L(E)C50 <=100 mg/L). In addition, L(E)C50 >100 mg/L were designated as "not classified".



APPENDIX I

List of available (Q)SARs for the purposes of REACH from the JRC QSAR Model Database (as of September 2011).

No	Title
1.	QSAR for mutagenicity (*Salmonella typhimurium* TA98 strain)*
2.	QSAR for skin sensitisation *via* Schiff base formation
3.	QSAR for the Global Half-Life Index (GHLI) of Persistent Organic Pollutants (POPs)
4.	QSAR for honey bee acute contact toxicity (ester derivatives) *
5.	QSAR for honey bee acute contact toxicity (amine derivatives) *
6.	QSAR for acute toxicity to *Pimephales promelas* (Fathead minnow)
7.	Catalogic model for biodegradation (MITI OECD 301C)
8.	QSAR for eye irritation (Draize test) *
9.	QSAR for acute toxicity to fish (*Danio rerio*) *
10.	QSAR for bioconcentration factor in fish
11.	QSAR for acute aquatic toxicity to *Pimephales promelas* (Fathead minnow)
12.	QSAR for honey bee acute contact toxicity (amide derivatives) *
13.	QSAR model for acute toxicity to rainbow trout*
14.	QSAR for female rat carcinogenicity (TD_{50}) of nitro compounds*
15.	TOPKAT NTP Rodent Carcinogenicity Model (Female Mouse)
16.	QSAR for haloacetic acid mutagenicity
17.	QSAR for Ames test of alpha, beta-unsaturated carbonyl compounds
18.	QSAR for mammalian cell mutagenicity of alpha, beta-unsaturated carbonyl compounds
19.	QSAR for narcosis to fathead minnow, including non-polar and polar narcosis
20.	QSAR for honey bee acute contact toxicity (ether derivatives not containing amide groups) *
21.	QSAR for Relative Binding Affinity to Estrogen Receptor*
22.	Catalogic basesurface narcotic model for aquatic toxicity to *Daphnia Magna*
23.	QSAR for bioconcentration (flow-through fish test) of polychlorinated biphenyls*
24.	QSAR for acute toxicity to Fathead minnow*
25.	QSAR for bioconcentration (flow through fish test) of pesticides*
26.	QSAR for acute oral toxicity (*in vitro*) *
27.	QSAR for human serum albumin binding*

No	Title
28.	QSAR for soil adsorption coefficient Koc*
29.	QSAR for blood-brain barrier (BBB) partitioning*
30.	QSAR for the bioconcentration factor of non-ionic organic compounds*
31.	QSAR for acute toxicity to algae*
32.	QSAR for algae toxicity of benzene derivatives*
33.	QSAR model for acute oral toxicity of benzene derivatives - Acute Toxic Class Method*
34.	Molcode QSAR for abiotic degradation in air (OH tropospheric degradation of volatile organic compounds) *
35.	Molcode QSAR for abiotic degradation in air (NO3 radical reaction of volatile organic compounds) *
36.	Molcode QSAR for abiotic degradation in air (O3 radical reaction of volatile organic compounds) *
37.	Nonlinear QSAR: aritficial neural network for mouse carcinogenicity*
38.	Nonlinear QSAR: artificial neural network for biodegradation: activated sludge respiration inhibition test*
39.	Nonlinear QSAR: artificial neural network for classification of skin sensitisation potential*
40.	TOPS-MODE QSAR for mammalian cell mutagenicity of alpha,beta-unsaturated carbonyl compounds
41.	TerraQSAR - LOGP
42.	Nonlinear QSAR: artificial neural network for classification of repeated dose toxicity*
43.	QSAR model for persistence: abiotic degradation in water*
44.	QSAR for persistence: abiotic degradation in air*
45.	Nonlinear QSAR: artificial neural network for acute toxicity to *Daphnia magna**
46.	QSAR for rat chronic LOAEL*
47.	Nonlinear QSAR: artificial neural network for in vitro chromosome aberrations in mammalian cells*
48.	Toxtree QSAR 6: mutagenicity aromatic amines in *Salmonella typhimurium* TA100, with S9 metabolic activation
49.	Toxtree QSAR 8: carcinogenicity of aromatic amines
50.	Toxtree QSAR 13: mutagenicity of alpha,beta unsaturated aliphatic aldehydes in *Salmonella typhimurium* TA100
51.	Toxtree: Benigni-Bossa rulebase for genotoxic and non-genotoxic carcinogenicity
52.	Toxtree: rulebase for mutagenicity (*in vivo* micronucleus assay)
53.	Nonlinear QSAR: artificial neural network for acute oral toxicity (rat cell line)*

No	Title
54.	QSAR for acute toxicity to *Daphnia magna* (LC_{50})*
55.	QSAR for octanol-water partition coefficient (logP) for pesticides*
56.	TOPS-MODE QSAR for Ames test of alpha, beta-unsaturated carbonyl compounds
57.	QSAR for toxicity to activated sludge*
58.	Nonlinear QSAR: artificial neural network for *in vitro* chromosomal aberration*
59.	Derek for Windows - Mutagenicity
60.	Derek for Windows - Chromosome damage
61.	Derek for Windows - Carcinogenicity
62.	Derek for Windows - Skin sensitisation
63.	Nonlinear QSAR: artificial neural network for long-term toxicity to *Daphnia magna**
64.	Derek for Windows - Chromosome damage
65.	Derek for Windows - Carcinogenicity
66.	Derek for Windows - Skin sensitisation
67.	Derek for Windows - Mutagenicity
68.	Non polar narcosis QSAR for *Tetrahymena pyriformis* acute toxicity
69.	Polar narcosis QSAR for Fathead minnow acute toxicity
70.	Polar narcosis QSAR for *Tetrahymena pyriformis* acute toxicity
71.	Non polar narcosis QSAR for Fathead minnow acute toxicity

Note: * - Developed by the Estonian company, MolCode AS (www.molcode.com).

APPENDIX II

Databases currently participating in eChemPortal (as of September, 2011).

ACToR	U.S. EPA Aggregated Computational Toxicology Resource
AGRITOX	AGRITOX - Base de données sur les substances actives phytopharmaceutiques
CCR	Canadian Categorization Results
CCR DATA	Canadian Categorization Results
CESAR	Canada's Existing Substances Assessment Repository
CHRIP	Information on Biodegradation and Bioconcentration of the Existing Chemical Substances in the Chemical Risk information platform (CHRIP)
ECHA CHEM	European Chemicals Agency's Dissemination portal with information on chemical substances registered under REACH
EnviChem	Data Bank of Environmental Properties of Chemicals
ESIS	European Chemical Substances Information System (ESIS)
GHS-J	The Result of the GHS Classification by the Japanese Government
HPVIS	High Production Volume Information System (HPVIS)
HSDB	Hazardous Substance Data Bank
HSNO CCID	New Zealand Hazardous Substances and New Organisms Chemical Classification Information Database
INCHEM	Chemical Safety Information from Intergovernmental Organizations - INCHEM
J-CHECK	Japan CHEmicals Collaborative Knowledge database
JECDB	Japan Existing Chemical Data Base
NICNAS PEC	Australian National Industrial Chemicals Notification and Assessment Scheme (NICNAS) Priority Existing Chemical Assessment Reports
OECD HPV	Organisation for Economic Cooperation and Development (OECD) Existing Chemicals Database
OECD SIDS IUCLID	OECD Existing Chemicals Screening Information Data Sets (SIDS) Database
SIDS UNEP	OECD Initial Assessment Reports for HPV Chemicals including Screening Information Data Sets (SIDS) as maintained by United Nations Environment Programme (UNEP) Chemicals
UK CCRMP Outputs	UK Coordinated Chemicals Risk Management Programme Publications
US EPA IRIS	United States Environmental Protection Agency Integrated Risk Information System
US EPA SRS	United States Environmental Protection Agency Substance Registry Services

APPENDIX III

Copy of the OSIRIS flyer.

OSIRIS Partners

OSIRIS has 31 Partners from 14 European countries.

24 Research institutes / universities
5 Small and medium-sized enterprises
2 Manufacturers of chemicals and chemical products

Final OSIRIS Meeting

The Final OSIRIS Stakeholder Meeting will be held on 29 September 2011 at the Helmholtz Centre for Environmental Research – UFZ in Leipzig, Germany.

Details on the agenda and registration will be announced on the OSIRIS website www.osiris-reach.eu.

For information and pre-registration contact osiris@ufz.de.

Contact OSIRIS

OSIRIS Co-ordinator
Prof. Dr. Gerrit Schüürmann

OSIRIS Project manager
Dr. Andrea Richarz

Department of Ecological Chemistry
Helmholtz Centre for Environmental Research – UFZ
Permoserstraße 15 I 04318 Leipzig I Germany

Contact OSIRIS office: osiris@ufz.de

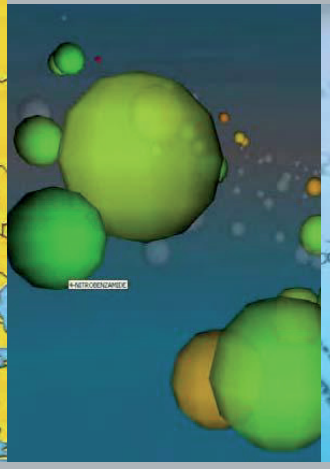
Information on OSIRIS: www.osiris-reach.eu

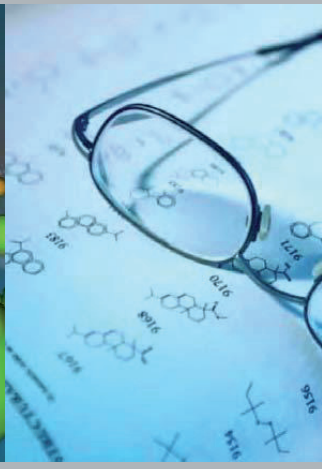
EU Integrated Research Project
SIXTH FRAMEWORK PROGRAMME

Contract no. GOCE-CT-2007-037017

OSIRIS

Optimised Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information





Optimised Strategies for the Risk Assessment of Chemicals

According to REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), the European legislation on chemicals and their safe use, all industrial chemicals produced or imported in quantities above 1 t/y have to be evaluated regarding their toxicological and ecotoxicological effects. Considering the currently used testing schemes, this procedure is expected to result in a significant increase in animal tests. However, REACH also aims at reducing animal testing where possible.

OSIRIS is developing Integrated Testing Strategies (ITS) considering both non-test and test information and thus combining different approaches for the hazard and risk evaluation of chemicals. ITS shift risk assessment from a "box-ticking" approach with extensive animal testing to a more efficient, context-specific and substance-tailored approach. The underlying principle is to take advantage of existing information, to group information about similar substances, and to integrate exposure considerations in the decision making.

The complementary alternative approaches considered include:

- Chemical and biological read-across
- Qualitative/quantitative structure-activity relationships (QSAR)
- *In vitro* testing
- *In vivo* information (existing)
- Chemoassays
- -omics
- Thresholds of toxicological concern (TTC)
- Exposure analysis and exposure-based waiving.

The different information is weighted and the respective uncertainties taken into account in a Weight of Evidence approach.

OSIRIS Webtool

The methods and ITS developed are implemented in the web-based OSIRIS Tool, which will be made freely available for end-users from industry, regulatory authorities and academia.

The following endpoints are included in the Webtool:

- Skin sensitisation
- Mutagenicity & carcinogenicity
- Repeated dose toxicity
- Aquatic toxicity
- Bioconcentration factor (BCF).

Two uncertainty reasoning schemes are implemented in the ITS: Bayesian Networks and Dempster-Shafer theory of evidence. A Chemical Space Navigation Tool has been integrated as a visual aid for pre-screening of substances.

The Webtool also includes interfaces to locally installed QSAR software for generating *in silico* predictions including information about respective applicability domains (e.g. from ChemProp, TIMES etc).

The OSIRIS Webtool indicates what tests (if any) should be performed in order to satisfy REACH data requirements.

OSIRIS Methods and Models

OSIRIS developments include:

Screening methods:

- Cut-off criteria for substance-specific waiving of BCF studies
- Screening method for persistent transformation products
- PBT (persistent, bioaccumulative, toxic) index model
- CMR (carcinogenic, mutagenic, reprotoxic) screening tool

Waiving opportunities:

- Exposure-based waiving (environment, workers, consumers)
- TTC approach for inhalation and dermal exposure
- TTC values for drinking water

Models for toxicity prediction:

- Experimental and computational determination of toxicity-related electrophilicity of chemicals (chemoassays, bioassays, quantum chemistry)
- Prediction of physico-chemical properties and toxicity from structure (ChemProp OSIRIS edition)
- Category formation and read-across approaches
- Prediction of internal exposure
- Prediction of aquatic hazard distribution (NOEC95)

Models for environmental exposure assessment:

- Bioaccumulation of polar and non-polar compounds
- Fate of neutral and ionisable chemicals
- Fate of parent chemicals and their transformation products
- QSAR predictions of fate-related properties
- Probabilistic exposure assessment

Data collation:

- OSIRIS-wide database system, implemented in ChemProp
- E-SovTox: online database on Russian (eco)toxicity data
- Toxicogenomics data

Data and model analysis:

- Data quality assessment strategies
- Determination of the applicability domain

Optimisation possibilities of *in vitro* tests:

- Passive dosing to control exposure concentrations

Optimisation possibilities of *in vivo* tests:

- Guidance on the optimisation of *in vivo* tests
- Acute-chronic ratios to prioritise chemicals for chronic aquatic toxicity testing
- Data quality-related optimisation of testing protocols, *in vitro* and *in silico* approaches for ecotoxicity testing

Decision models:

- Cost-effectiveness analysis model
- Value-of-Information (VOI) model for sequential testing

ITS acceptance:

- Survey on ITS implementation and acceptance

CURRICULUM VITAE

1. Personal data

Name	Mariliis Sihtmäe
Date and place of birth	17.05.1980, Tartu, Estonia

2. Contact information

Address	Akadeemia tee 23, 12618, Tallinn, Estonia
Phone	+327 6398362
E-mail	mariliis.sihtmae@kbfi.ee

3. Education

2005 – 2011	Tallinn University of Technology, PhD student of Chemical and Materials Technology
2002 – 2004	Tallinn University of Technology, Master of Science in Engineering
1998 – 2002	Tallinn University of Technology, Bachelor of Science in Engineering

4. Language competence/skills (fluent; average, basic skills)

Estonian	fluent
English	average
Russian, German	basic

5. Special Courses

Aug. 21-Sept. 1, 2011	USA, Chemical Abstracts Service (CAS), SciFinder® Academic Exchange Program (SAEP 2011)
Jan. 4-30, 2010	Slovenia, University of Ljubljana, Biotechnical Faculty, fatty acid methyl ester analysis by gas chromatography
Aug. 25-29, 2008	Kuopio, Finland, RA-COURSES, Regulatory Toxicology
Jun. 2-5, 2008	Tallinn, Estonia, Estonia Public Service Academy, WMD Commodity Identification Training
Feb. 11-12, 2008	Saku, Estonia, Estonian University of Life Sciences, Methodology of Research
March 1-4, 2006	Toila, Estonia, Doctoral Winter School in “New Production Technologies and Processes at Tallinn University of Technology”
March 20-24, 2005	Elche, Spain, European Chemicals Bureau, Course on IUCLID and EUSES: Tools for Risk Assessment of Chemical Substances

Sept. 2003 – Jan. 2004	Uppsala, Sweden (Sweden-Estonia-Latvia-Lithuania Co-project BTox in Uppsala University), Human Toxicology, Ecotoxicology and Risk Assessment

6. Professional Employment

2007 – ...	National Institute of Chemical Physics and Biophysics, Researcher
2006 – 2007	National Institute of Chemical Physics and Biophysics, Technician
2003 – 2009	Chemicals Notification Centre, Senior Specialist
2002 – 2003	Chemicals Notification Centre, Specialist

7. Defended theses

2004	Master's thesis: „The waste flows of Saku County and possible alternatives for converting waste into energy", Tallinn University of Technology. Supervisors: Vahur Oja, Department of Chemical Engineering and Ülo Kask, Department of Thermal Engineering
2002	Bachelor's thesis: „Integrated waste management opportunities in Kaiu LT", Tallinn University of Technology. Supervisors: Lui Pikkov, Department of Chemical Engineering and Ülo Kask, Department of Thermal Engineering

8. Main areas of scientific work/Current research topics

- Biosciences and environment: research into substances hazardous to the environment
- Natural sciences and engineering: chemistry and chemical technology
- Regulatory toxicology

9. Publications

1) Aruoja, V., Sihtmäe, M., Kahru, A., Dubourguier, H-C. 2011. Toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata* and bacteria *Vibrio fischeri*: comparison with published data and QSARs. Chemosphere 84, 1310-1320.

2) Kurvet, I., Ivask, A., Bondarenko, O., Sihtmäe, M., Kahru, A. 2011. LuxCDABE - transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: comparison with naturally luminous *Vibrio fischeri*. Sensors 11, 7865-7878.

3) Sihtmäe, M., Blinova, I., Aruoja, V., Dubourguier, H-C., Legrand, N., Kahru, A. 2010. E-SovTox: An online database of the main publicly-available sources of toxicity data concerning REACH-relevant chemicals published in the Russian language. *ATLA* 38, 297 - 301.

4) Sihtmäe, M., Mortimer, M., Kahru, A., Blinova, I. 2010. Toxicity of five anilines to crustaceans, protozoa and bacteria. Journal of the Serbian Chemical Society 75, 1291 - 1302.
5) Aruoja, V., Sihtmäe, M., Kahru, A, Dubourguier, H-C. 2010. Toxicity of 66 substituted anilines and phenols to bacteria *Aliivibrio fischeri* and algae *Pseudokirchneriella subcapitata*: Effect of chemical structure. Toxicology Letters 196, 124 - 125.
6) Sihtmäe, M., Dubourguier, H.C., Kahru, A. 2009. Toxicological information on chemicals published in the Russian language: Contribution to REACH and 3Rs. Toxicology 262, 27 - 37.

ELULOOKIRJELDUS

1. Isikuandmed

Ees- ja perekonnanimi	Mariliis Sihtmäe
Sünniaeg ja -koht	17.05.1980, Tartu, Eesti

2. Kontaktandmed

Aadress	Akadeemia tee 23, 12618, Tallinn
Telefon	+327 6398362
E-posti aadress	mariliis.sihtmae@kbfi.ee

3. Hariduskäik

2005 – 2011	Tallinna Tehnikaülikool, keemia- ja materjalitehnoloogia doktorant
2002 – 2004	Tallinna Tehnikaülikool, tehnikateaduste magister keemia- ja keskkonnakaitse tehnoloogia erialal
1998 – 2002	Tallinna Tehnikaülikool, tehnikateaduste bakalaureus keemia- ja keskkonnakaitse tehnoloogia erialal

4. Keelteoskus (alg-, kesk-, või kõrgtase)

eesti	kõrgtase
inglise	kesktase
vene, saksa	algtase

5. Täiendusõpe

21.aug-1.sept. 2011	USA, *Chemical Abstracts Service* (CAS), *SciFinder® Academic Exchange Program* (SAEP 2011)
4.-30.jaan. 2010	Sloveenia Ljubljana Ülikooli biotehnoloogia õppetool, rasvhapete metüülestrite gaaskromatograafiline analüüs
25.-29.aug. 2008	Kuopio, Soome, RA-COURSES, Regulatoorne toksikoloogia
2.-5.juuni 2008	Tallinn, Eesti, Sisekaitseakadeemia, Strateegiliste kaupade tundmise koolitus
11.-12.veeb. 2008	Saku, Eesti, Eesti Maaülikool, Teadustöö metodoloogia
1.-4.märts 2006	Toila, Eesti, Uute tootmistehnoloogiate ja -protsesside doktorikooli talvekool
20.-24.märts 2005	Elche, Hispaania, Euroopa Kemikaalide Büroo, Programmide IUCLID ja EUSES kursus
sept. 2003-jaan. 2004	Uppsala, Rootsi (Rootsi-Eesti-Läti-Leedu haridusprojekt BTox Uppsala Ülikoolis), Toksikoloogia, ökotoksikoloogia ja kemikaalide riski hindamine

6. Teenistuskäik

2007 – ...	Keemilise ja Bioloogilise Füüsika Instituut, teadur
2006 – 2007	Keemilise ja Bioloogilise Füüsika Instituut; tehnik
2003 – 2009	Kemikaalide Teabekeskus, peaspetsialist
2002 – 2003	Kemikaalide Teabekeskus, spetsialist

7. Kaitstud lõputööd

2004	Magistritöö: „Saku valla jäätmevoogude väljaselgitamine ja nende energiaks muundamise võimalused“, Tallinna Tehnikaülikool. Juhendajad: Vahur Oja Keemiatehnika instituudist ja Ülo Kask Soojustehnika instituudist
2002	Bakalaureusetöö: „Integreeritud jäätmekäitluse võimalusest OÜ-s Kaiu LT“, Tallinna Tehnikaülikool. Juhendajad: Lui Pikkov Keemiatehnika instituudist ja Ülo Kask Soojustehnika instituudist

8. Teadustöö põhisuunad

- Bio- ja keskkonnateadused: Keskkonnaohtlikke aineid käsitlevad uuringud
- Loodusteadused ja tehnika: Keemia ja keemiatehnika
- Regulatoorne toksikoloogia

DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *CHEMISTRY AND CHEMICAL ENGINEERING*

1. **Endel Piiroja**. Oxidation and Destruction of Polyethylene. 1993.

2. **Meili Rei**. Lihatehnoloogia teaduslikud alused. Fundamentals of Food Technology. 1995.

3. **Meeme Põldme**. Phase Transformations in Hydrothermal Sintering Processing of Phosphate Rock. 1995.

4. **Kaia Tõnsuaadu**. Thermophosphates from Kovdor and Siilinjärvi Apatites. 1995.

5. **Anu Hamburg**. The Influence of Food Processing and Storage on the N-Nitrosamines Formation and Content in Some Estonian Foodstuffs. 1995.

6. **Ruth Kuldvee**. Computerized Sampling in Ion Chromatography and in Capillary Electrophoresis. 1999.

7. **Külliki Varvas**. Enzymatic Oxidation of Arachidonic Acid in the Coral *Gersemia fruticosa*. 1999.

8. **Marina Kudrjašova**. Application of Factor Analysis to Thermochromatography and Promotion Studies. 2000.

9. **Viia Lepane**. Characterization of Aquatic Humic Substances by Size Exclusion Chromatography and Capillary Electrophoresis. 2001.

10. **Andres Trikkel**. Estonian Calcareous Rocks and Oil Shale Ash as Sorbents for SO_2. 2001.

11. **Marina Kritševskaja**. Photocatalytic Oxidation of Organic Pollutants in Aqueous and Gaseous Phases. 2003.

12. **Inna Kamenev**. Aerobic Bio-Oxidation with Ozonation in Recalcitrant Wastewater Treatment. 2003.

13. **Janek Reinik**. Methods for Purification of Xylidine-Polluted Water. 2003.

14. **Andres Krumme**. Crystallisation Behaviour of High Density Polyethylene Blends with Bimodal Molar Mass Distribution. 2003.

15. **Anna Goi**. Advanced Oxidation Processes for Water Purification and Soil Remediation. 2005.

16. **Pille Meier**. Influence of Aqueous Solutions of Organic Substances on Structure and Properties of Pinewood (*Pinus sylvestris*). 2007.

17. **Kristjan Kruusement**. Water Conversion of Oil Shales and Biomass. 2007.

18. **Niina Kulik**. The Application of Fenton-Based Processes for Wastewater and Soil Treatment. 2008.

19. **Raul Järviste**. The Study of the Changes of Diesel Fuel Properties a its Long Term Storage. 2008.

20. **Mai Uibu**. Abatement of CO_2 Emissions in Estonian Oil Shale-Based Power Production. 2008.

21. **Valeri Gorkunov**. Calcium-Aluminothermal Production of Niobium and Utilization of Wastes. 2008.

22. **Elina Portjanskaja**. Photocatalytic Oxidation of Natural Polymers in Aqueous Solutions. 2009.

23. **Karin Reinhold**. Workplace Assessment: Determination of Hazards Profile using a Flexible Risk Assessment Method. 2009.

24. **Natalja Savest**. Solvent Swelling of Estonian Oil Shales: Low Temperature Thermochemical Conversion Caused Changes in Swelling. 2010.

25. **Triin Märtson**. Methodology and Equipment for Optical Studies of Fast Crystallizing Polymers. 2010.

26. **Deniss Klauson**. Aqueous Photocatalytic Oxidation of Non-Biodegradable Pollutants. 2010.

27. **Oliver Järvik**. Intensification of Activated Sludge Process – the Impact of Ozone and Activated Carbon. 2011.

28. **Triinu Poltimäe**. Thermal Analysis of Crystallization Behaviour of Polyethylene Copolymers and Their Blends. 2011.