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Ecotoxicological Evaluation of Shale Fuel Oils, Metal-Based Nanoparticles and Glyphosate Formulations

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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 doctoral or equivalent academic degree.

Liina Kanarbik

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Põlevkiviküttеõlide, metalliliste nanoosakeste ja glüfosaadipõhiste herbitsiidide ökotoksikoloogilised uuringud

LIINA KANARBIK

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III Blinova, I., **Kanarbik, L.**, Irha, N., Kahru, A. (2015). Ecotoxicity of nanosized magnetite to crustacean *Daphnia magna* and duckweed *Lemna minor*. Hydrobiologia. doi:10.1007/s10750-015-2540-6

IV Blinova, I., Niskanen, J., Kajankari, P., **Kanarbik, L.**, Käkinen, A., Tenhu, H., Penttinen, O., Kahru, A. (2013). Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Environmental Science and Pollution Research. 20, 3456−3463.

V Sihtmäe, M., Blinova, I., Künnis-Beres, K., **Kanarbik, L.**, Heinlaan, M., Kahru, A. (2013). Ecotoxicological effects of different glyphosate formulations. Applied Soil Ecology. 72, 215−224.

Copies of these publications are included in APPENDIX A.

Other publications related to the research:

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Künnis-Beres, K., Sihtmäe, M., **Kanarbik, L**., Heinlaan, M., Blinova, I. (2013). Glüfosaadipõhiste herbitsiidide üledoseerimise või juhureostuse võimalik keskkonnamõju. Keskkonnatehnika. 3, 40−42.

Kanarbik, L., Sihtmäe, M., Künnis-Beres, K., Blinova, I. (2013). Eesti põlevkivikütteõlide võimalik keskkonnamõju. Keskkonnatehnika. 4, 28−29.

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I The author was responsible in design and implementation of the experimental part, data analysis and performed the testing with crustacean *Daphnia magna*, terrestrial plants *Sinapis alba* and *Hordeum vulgare* and participated in manuscript preparation.

II The author participated in study planning, WAF preparation and performed the testing with crustaceans *Daphnia magna* and *Thamnocephalus platyurus*, duckweeds *Lemna minor* and writing of the article in cooperation with other authors.

III The author participated in planning of the experiments and data analysis, performed the testing with crustacean *Daphnia magna* and duckweeds *Lemna minor* and writing of the article in cooperation with other authors.

IV The author was responsible of natural water sampling and data analysis, performed the testing with crustaceans *Daphnia magna* and *Thamnocephalus platyurus* and writing of the article in cooperation with other authors.

V The author performed the testing with crustacean *Daphnia magna*, terrestrial plants *Raphanus sativus* and *Hordeum vulgare* and participated in the analysis of respective data and writing of the manuscript in cooperation with other authors.

INTRODUCTION

The chemical industry is vital to modern society and has rapidly grown in the recent decades. To avoid negative impacts on human health and the environment, chemicals have to be tested for their potential hazard. In European Union (EU), REACH Directive (Registration, Evaluation, Authorisation and Restriction of Chemicals) was adopted requiring that all chemical substances manufactured or imported into the EU in a quantity of more than 1 tonne per year should be provided with the information on physical, health and environmental hazards they may pose (EC, 2006). Although simple and rapid standardized ecotoxicity tests are preferred and recommended, the additional test formats should also be applied to get ecologically relevant data needed for adequate environmental risk assessment and deriving environmental quality standards. The non-standardized tests may contribute important additional information for chemical risk assessment. Simple modifications in test design, such as conducting tests under natural climate conditions or using natural water instead of artificial test water yield environmentally more relevant results with no significant increases in costs.

Although there are methods used for ecotoxicity testing of different groups of chemical products (e.g. industrial chemicals, pesticides, cosmetics) regulated by respective legislatory instruments in details, there are still many gaps to be filled in. For example synthetic nanoparticles (NPs), a relatively new group of chemicals, have different physicochemical properties and different (eco)toxicological profile compared to their microsized analogues (Kahru and Dubourguier, 2010). Therefore, case-by-case risk assessment should be adopted when talking about the environmental risk assessment of synthetic NPs (EC, 2009a).

Some methodological problems also exist in testing petroleum products belonging to UVCB substances (Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials). Oil products are very complex mixtures and difficult to evaluate toxicologically as the results tend to depend on the test procedure applied (Singer et al., 2000).

The toxicity of the Plant Protection Products (PPPs), such as glyphosate-based herbicides, has been extensively studied. The most widely used approach for ecotoxicological evaluation of PPPs is based on the investigation of the active compound's toxicity (e.g. glyphosate) to different test species. However, some recent publications have shown that the additives/surfactants used for the preparation of various PPP formulations may increase the toxicity of the final products to non-target organisms (Edginton et al., 2004; Tsui and Chu, 2003). Moreover, although manufacturers advertise glyphosate-based herbicides as "low toxic and environmental friendly" (Monsanto, 2012), this statement is not in the agreement with the data from the respective material safety data sheets (MSDSs).

In this Thesis, ecotoxicologically insufficiently characterized chemicals were studied using standardized and modified test formats to obtain and contribute to environmentally relevant information on their potential hazard. Various terrestrial and aquatic test organisms from different trophic levels were used to evaluate the toxicity of the following chemicals: Estonian shale fuel oils, metal-based nanoparticles (nanosilver and nanosized magnetite) and different glyphosate formulations.

ABBREVIATIONS

AE	acid equivalents
AFW	artificial freshwater
AMPA	aminomethylphosphonic acid
ASTM	American Society for Testing and Materials
CLP	Classification, Labelling and Packaging
DOC	dissolved organic carbon
EC	European Commission
ECHA	European Chemicals Agency
$E(L)C_{50}$	the median effective concentration of the toxicant that induces a designated effect (or death) in 50 % of the test organisms after a specified exposure time
EQS	environmental quality standard
EU	European Union
IPA	isopropylammonium
ISO	International Organization for Standardization
MSDS	material safety data sheet
NOEC	no observed effect concentration
NP	nanoparticle
OECD	Organisation for Economic Cooperation and Development
OWR	oil-water-ratio
PAHs	polyaromatic hydrocarbons
PNEC	predicted no-effect concentration
POEA	polyethoxylated tallow amine
PPPs	Plant Protection Products
PVP	polyvinylpyrrolidone
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
SFO	shale fuel oil
TPHs	total petroleum hydrocarbons
US EPA	United States Environmental Protection Agency
UVCB Substance	Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials
WAF	water accommodated fraction

1. REVIEW OF THE LITERATURE

1.1. Ecotoxicological hazard evaluation of chemicals

In addition to the great number of chemical compounds already manufactured and used, the chemical industry constantly produces more and more new ones. Many chemicals pose minor or no adverse effects, however, others may be harmful to human health and/or environment. Ecotoxicological evaluation is the cornerstone in the environmental risk assessment of industrial chemicals. Ecotoxicology is still a young discipline of the environmental sciences which studies the toxic effects of substances on species in ecosystems and requires the application of knowledge of three main disciplines: toxicology, ecology and chemistry (Blaise, 2013; van Leeuwen and Vermeire, 2007).

Ecotoxicological effects are changes in the state of the aquatic and/or terrestrial species resulting from exposure to a chemical. These changes may become apparent at different levels of biological organization from sub-cellular to the ecosystem (van Leeuwen and Vermeire, 2007). The aim of ecotoxicological evaluation is to define the concentrations of chemicals in the environmental compartments which are safe to biota. However, the chemical safety assessment is a very complex task due to many abiotic and biotic factors affecting the behaviour of the chemicals in the environmental matrices and their bioavailability.

Ecotoxicological hazard assessment of chemicals is based on the data of the chemicals' properties and behaviour in the natural environment and their effects on living organisms, and is regulated with different international and national legislations. Many countries, e.g. North America, Europe, Australia, New Zealand, have well-developed regulatory frameworks for the management of chemicals in the environment. In the EU, a regulation REACH related to the safe use of chemicals has been implemented (EC, 2006). This directive describes the procedure of determining the hazard (physical, health and environmental hazards) of substances and mixtures produced, imported or used. According to REACH, all substances manufactured or imported in quantities of more than one tonne per year must have basic ecotoxicological information by the year 2018. Information of substances and mixtures in the EU is gathered in a chemical's MSDS that is an integral part of the REACH Regulation (EC, 2006).

REACH requires large amounts of different ecotoxicological information (e.g. aquatic toxicity) on about 100 000 different industrial chemicals. Standard data sets for aquatic toxicity on substances transported 1-10 tons/year includes growth inhibition on aquatic plants (algae preferred, OECD 201, 2011) and short-term toxicity testing on invertebrates such as crustaceans (preferred species *Daphnia*, OECD 202, 2004). On the next tonnage level (10-100 tons/year) short-term toxicity testing on fish as vertebrates (OECD 203, 1992) is required in addition. For tonnages of more than 100 tons/year, long-term toxicity testing on invertebrates (preferred species *Daphnia*) and fish are additionally needed

(Tarazona et al., 2014). However, it should be mentioned that REACH as well as other legislatory acts (e.g. Plant Protection Products Directive: EC, 2009b) regulating the safety assessment of chemicals require data of only a limited number of standardized ecotoxicological tests which are scientifically valid and internationally harmonized (e.g. test guidelines of the Organisation for Economic Cooperation and Development - OECD). The data set required depends on the aims of the risk assessment and regulation involved. Besides, relevant available information from different sources, e.g. published literature, should also be used as supplementary information. The reliability and relevance of data from non-standardized test methods must be evaluated on a case-by-case basis (EC, 2003). However, data from standardized tests has priority in the assessment of the potential environmental hazard of chemicals.

1.1.1. Advantages and limitations of the standardized toxicity tests

Standardized tests are performed following the guidelines provided by official national or international organisations like OECD, US EPA (United States Environmental Protection Agency), ASTM (American Society for Testing and Materials) and ISO (International Organization for Standardization) (Ågerstrand et al., 2011). Standardized tests have specific experimental setup and methods for analysing and reporting the data (Ågerstrand et al., 2011). The major advantages of standardized ecotoxicity tests are following: they are rapid, easy to conduct, not too expensive, the results are comparable across substances and the data is accepted by different regulations (Eriksson et al., 2010; van Leeuwen and Vermeire, 2007). There is a large amount of information from standardized tests available in scientific databases and publications making the comparison of the test results between different laboratories easier. Moreover, it is quite easy to repeat the testing due to detailed description of the experimental, analytical and documentation procedures.

The biggest disadvantage of standardized ecotoxicity test methods is that the ecological relevance of these tests and thus results obtained may be questionable (Eriksson et al., 2010; Merrington et al., 2014). As standardized tests are conducted under controlled laboratory conditions, a lot of factors that may affect the toxicity test results are excluded (e.g. natural climate conditions, interactions with naturally occurring compounds or other biological species). Laboratory toxicity tests use single species, but, in fact, different species interact with each other and pollutants may affect these interactions in biological communities (van Leeuwen and Vermeire, 2007). Also, laboratory toxicity tests usually focus only on a particular life stage of a test species (e.g. *Daphnia magna* acute testing), rather than the full life cycle (Leung et al., 2014). In addition, standardized toxicity tests often study only a single chemical at a time, whereas most aquatic contaminants occur in mixtures that may result in additive or synergistic toxic effects (Leung et al., 2014). Moreover, indirect effects of toxicants, for example, *via* food availability, etc., are often ignored in standardized laboratory tests

(Campell et al., 2003). Due to all the aforementioned limitations it is hard to extrapolate the test results from standardized methods to real ecosystems.

Environmentally more relevant information on chemicals is needed for developing the environmental quality standards (EQSs). In practice, many existing environmental standards are based on the limited data set (van Leeuwen and Vermeire, 2007). The predicted no-effect concentrations (PNEC) may be calculated by applying an appropriate safety factors (generally ranging from 10 to 10 000) to the effect values (e.g. LC_{50} or NOEC) for the most sensitive species from available toxicity data. However, EQSs, derived exclusively from laboratory ecotoxicity data, might under- or overestimate the real risks for ecosystems' concern and such standards should be assessed under field conditions. To get more environmentally relevant information, the number of tested species should be increased and extrapolating the acute data to set chronic limit values should be avoided when deriving EQSs (Merrington and van Sprang, 2013). In addition, available scientific information on chemical's toxicity and bioavailability must also be critically evaluated.

1.1.2. Increasing the environmental relevance of ecotoxicity testing

Whereas standardized tests are mostly performed by commercial laboratories, non-standardized methods are typically applied by scientists investigating the fate and toxicity of chemicals to different test species under different test conditions. Several approaches could be applied to increase the environmental relevance of ecotoxicity data: i) modification of the existing standardized laboratory tests; ii) using microcosms; iii) using mesocosms or field studies.

The first approach is the most cost-effective. Only small changes in the test design allow receiving environmentally more relevant data. For example, most of the ecotoxicity tests with aquatic organisms like crustaceans have been conducted using artificial freshwater (AFW) containing a limited number of chemical compounds compared to the natural water (e.g. surface water). The bioavailability and toxic effects of chemicals depend on the water composition (Witters, 1998). For example, for synthetic nanoparticles (NPs) it is known that the amount of natural organic matter in the test water affects their fate and toxicity (Blinova et al., 2010; Giasuddin et al., 2007; Hyung et al., 2007; Klaine et al., 2008; Lead and Wilkinson, 2006; Wigginton et al., 2007). Thus, the practical value for realistic chemical risk assessment of ecotoxicity tests conducted with AFW is questionable (Handy et al., 2008; Velzeboer et al., 2008). Using unpolluted natural surface water instead of AFW in the tests with crustaceans gives valuable information for ecological risk assessment (Blaise and Ferard, 2005). Also, the prolongation of acute test, namely, registration of mortality and reproduction rate of crustaceans placed into clean water after short-term exposure in acute tests, may give us information on the recovery potential of tested species. Such recovery tests can also be applied in the hazard assessment of contaminated water, soil or sediment. In terrestrial plant toxicity

testing, artificial soil is usually used because it is well-defined, has uniform texture and can easily be used in the laboratory where the physicochemical properties remain the same among tested series (Stephenson et al., 2000). Artificial soil does not contain plant seeds and soil invertebrates but, at the same time, has low content of natural soil bacteria which are very important for biodegradation. Properties of artificial soil are different from natural soil, therefore the extrapolation possibilities are under discussion and natural soil is preferred for more environmentally relevant test results (Hofman et al., 2009).

The multi-species microcosm and mesocosm studies could help to validate the PNECs obtained from standardized laboratory tests (Merrington et al., 2014). The environmental relevance of data from experimental microcosm and mesocosm studies is much higher compared to the results from standardized laboratory tests. The indoor/outdoor microcosm experiments and mesocosm studies may significantly reduce the uncertainty of ecotoxicity data. Such multi-species tests help to predict the fate and bioavailability of contaminants in the ecosystems as well as effect the interactions between species (van Leeuwen and Vermeire, 2007). The mesocosm studies under natural climate conditions are irreplaceable in case of investigating any site-specific contamination. Using mesocosm systems to study the effects of chemicals on aquatic organisms can be important as the first-step screening tool before investigating the effects in the field (Rand, 1995), but they are more expensive and time-consuming than standardized ecotoxicity tests. The full scale field tests are the most costly and complex and their repeatability is very low, therefore their application for EQSs elaboration is very limited

All in all, ecotoxicological testing should be conducted in environmental conditions representing the situation in the natural environment (Laskowski et al., 2010). To enable the use of non-standardized tests in risk assessments they should be included in the legislations in a systematic way and reported in a comprehensive way like when using the standardized test methods (Ågerstrand et al., 2011).

1.2. Selected (model) chemicals for environmental hazard evaluation

1.2.1. Shale fuel oils

Estonia is a country with the largest industrially-used oil shale basin in the world (Kahru and Põllumaa, 2006). Oil shale, a sedimentary rock containing high amount of organic matter, is burnt in local power plants for electricity generation but also used for the production of fuel oil (semi-coking). Currently more than 500 000 tons of different shale fuel oils are produced in Estonia (Estonian Statistics, 2016). These oils are traditionally compared to heavy fuel oils and can be used for the same purposes as those gained from crude oil. The main consumers of shale oil are heat producers, power plants, producers and sellers of

bunker fuels and international traders. Domestic production of oil from oil shale is expanding rapidly and will reach up to one million tons per year in the near future which makes shale oil one of the most produced chemicals in Estonia (Estonian Statistics, 2016). However, experimental data on the potential hazard of shale oil to soil/aquatic ecosystems is limited making the prediction of consequences in case of accidental contamination very difficult.

As a rule, data on hydrocarbons' concentrations is used as an indicator of contamination level or efficiency of remediation actions in oil polluted soil/water ecosystems (Alexander, 2000; Mao et al., 2009; Sammarco et al., 2013; Sung et al., 2013). There are sets of limit values for different oil compounds in various national/international regulations. For example, in Estonia, total petroleum hydrocarbons (TPH; C10–C40), sum of polyaromatic hydrocarbons (PAHs) and some individual hydrocarbons in the soil and groundwater are regulated (RT, 2010a; RT, 2010b). The toxicity of petroleum products is caused by many compounds presented in various amounts in the mixure, but the reporting of all the individual chemicals is not practical (Perkins et al., 2003). Most often, the total concentration of hydrocarbons (C10–C40) and concentration of individual most toxic hydrocarbons (e.g. PAHs) are monitored and compared to existing EQSs. Such approach may lead to over- or underestimation of the real environmental risks as it is difficult to predict the overall toxic effect of the mixure on the basis of a limited number of measured hydrocarbons. Besides, only a part of the hydrocarbons' content measured by chemical analysis is bioavailable to organisms. For example, hydrocarbons strongly sorbed to soil particles are not bioavailable to soil biota and therefore do not pose real risks to these organisms (Bogan and Sullivan, 2003; Haritash and Kaushik, 2009). On the other hand, a reduction in total hydrocarbons' concentration does not always indicate a decrease in toxicity due to the formation of new metabolites during degradation which may be more toxic than initial compounds (Al-Mutairi et al., 2008; Larsson et al., 2013). It should be mentioned that soil/water contamination by different fuel oils with similar content of TPHs may pose different environmental hazard due to the variety in abundance of individual hydrocarbons (e.g. some fuel oils have more toxic or more lighter than heavier fractions of hydrocarbons) in fuel oils' composition. For example, it was shown that the toxicity of water accommodated fractions (WAFs) of crude oil cannot be explained on the basis of the PAHs or TPHs (C10-C40) concentrations (Bellas et al., 2013; Long and Holdway, 2002).

Mineral oils such as shale fuel oils (SFOs) are very complex mixtures and their chemical composition varies depending on the oil shale origin and refining process used. According to REACH and CLP (Classification, Labelling and Packaging), they belong to the category of UVCB-substances (Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials). The petroleum-based fuels are hard to evaluate toxicologially due to their complex and variable composition (Singer et al., 2000). Environmental hazard evaluation of fuel oils (e.g. SFOs) on the basis of

the toxicity of individual components is impossible as i) the complete chemical composition of the specific brand of fuel oil is usually unknown and ii) the bioavailability of the individual compounds may vary depending on the test conditions/environmental factors (Faksness et al., 2008; Gomez-Eyles et al., 2010; Kenaga, 1987; Larsson et al., 2013). Thus, realistic information on the ecotoxicity and behaviour of SFO in soil/water ecosystems may only be received from the experiments with specific type of fuel oil and the data obtained cannot be extrapolated to other oil brands (Rodrigues et al., 2010).

In spite of the different chemical composition of two types of shale fuel oils ("VKG D" and "VKG sweet"), selected for the current investigation, the information on the toxicity to aquatic species in respective MSDSs issued has been gained with tests with an average shale oil sample. However, the use of this data may lead to over- or underestimation when it comes to the risk assessment of specific type of shale oil. In addition, the ecological information of SFOs on European Chemicals Agency (ECHA) datasheet is also limited (ECHA, 2016). Under the section of environmental fate and pathways there is no information on the biodegradation of shale oil in water, sediment or soil, and bioaccumulation in sediment. In addition, there is no ecotoxicological data on the sediment and terrestrial toxicity or long-term toxicity to fish. Only basic ecotoxicologial information required by REACH (e.g. short-term toxicity to fish, short and long-term toxicity to aquatic invertebrates - *Daphnia*, toxicity to aquatic plants - algae) and in addition toxicity to microorganisms (activated sludge) is presented. Lack of information makes the risk assessment of SFOs very difficult. An accidental spill of fuel oils may lead to long-term environmental problems and data on inherent biodegradability (i.e. potential to be biodegraded) of SFOs is one of the most important parameter needed for real risk assessment (Battersby, 2000; Willing, 2001).

The evaluation of the potential negative effects of poorly water-soluble oil products to aquatic ecosystem is based on the toxicological evaluation of oils' water accommodated fractions (WAFs). There are several methods in use for WAF preparation, e.g. WAF of undispersed oil or chemically enhanced WAF of the dispersed oil in the water column (Perkins et al., 2003). A standard protocol proposed by Chemical Response to Oil Spills–Ecological Effects Research Forum (CROSERF) (Aurand et al., 2005) is the most often applied. However, there are still some methodological questions concerning the toxicological evaluation of WAFs to be clarified. Currently, two main approaches for preparing WAF series for toxicity testing are commonly used: variable loading and variable dilution. Variable loading uses different oil-water-ratios (OWRs) to prepare WAF series, whereas in variable dilution the test series are prepared by dilutions of the initial WAF (Barron and Ka'aihue, 2003). In the latter approach the most common OWRs for preparing initial WAF are 1:10 and 1:40, but other OWRs, for example 1:10 000, are also used in the toxicological testing (Anderson, 1985; Barron et al., 1999; Barron and Ka'aihue, 2003). There are still unsolved issues concerning the preparation methods of oil WAF sets for

ecotoxicity testing as both procedures have their advantages and disadvantages (Barron and Ka'aihue, 2003). The reporting of the exposure doses (as loading rate or concentrations of total petroleum hydrocarbon (C10–C36) or total hydrocarbon content (C6–C36)) in water is also crucial for interpretation of the toxicity tests (Perkins et al., 2003). There are different ways to report the toxicity of WAFs: i) as loading rate (mg oil/L), ii) as percentage of initial WAF dilution (v/v %), iii) as concentration of chemical compounds, e.g. TPH (μg/L) or total concentration of 16 PAHs (μg/L), etc. Due to the variability in test designs it is very hard to compare the test results gained from different laboratories (Singer et al., 2000).

1.2.2. Metal-based nanoparticles

According to the definition of European Commission (EC, 2011) nanoparticle (NP) is a particle with one or more external dimensions in the size range of 1-100 nm. NPs can be classified, based on their origin, to natural (volcanic ash, ocean spray, forest fire) or anthropogenic, and the latter can be further divided to unintentional (combustion particulates, diesel exhaust) and intentional (synthetic NPs). Intentionally produced synthetic or engineered NPs are engineered pure materials with controlled properties and can be classified into organic (carbon-based) and inorganic (metal-based) NPs (Srivastava and Kowshik, 2015). Metal-based NPs, in particular, have received increasing interest due to their widespread medical, consumer, industrial and military applications and it is expected that their production and use continues to increase. As of March 20, 2015 listed in Consumer Products Inventory (The Project on Emerging Nanotechnologies, 2015) there were more than 1800 consumer products containing NPs whereas silver, titanium and carbon were the most abundant synthetic nanomaterials used (438, 107, 90 products; respectively) (Juganson et al., 2015). Simultaneously with the rapid growth of nanomaterials' application in different economic sectors (Gottschalk et al., 2013) the hazard of the environmental contamination will also increase.

Although there is no clear reference to nanosize materials under EU chemicals legislation REACH or CLP, in these legislative acts NPs are covered by the definition of "substance" and therefore risk assessment should be performed as part of the chemical safety assessment process using relevant information. To find out the hazards related to NPs, existing test guidelines may need modifications (EC, 2008).

It is already an established fact that metal-based NPs may pose unknown threats to living organisms due to their novel physicochemical properties. With decreasing size, NPs' surface area increases and thus, increases also the reactivity. Some materials (e.g. Ag, Au, and Cu) may induce toxicity in nanosize even if they are relatively inert in their microsized form (Schrand et al., 2010). In addition to particle size, the reactivity and bioactivity of NPs is determined by surface coating and surface functional groups (Lynch et al., 2013). For example,

NPs based on the same element but exhibiting different surface coating may have different ecotoxicological profiles or safety (Rana and Kalaichelvan, 2013). Surface coating may give NPs new bioactive properties and, for example, only NPs with appropriate coatings can be used for biomedical applications (Steitz, 2006).

For different organisms the toxicity mechanisms of NPs vary to a great extent. Bacteria (e.g. *V. fischeri*) as particle non-ingesting organisms are subjected to NPs *via* contact exposure but crustacean (e.g. *D. magna*) as a particle-ingesting organism is exposed *via* both, ”external” and the ”internal exposure” of the chemical. So, daphnids as filter feeders are more vulnerable to the potential toxic effects of the synthetic NPs and have been shown the most sensitive aquatic organisms when exposed to ZnO and CuO NPs (Kahru and Dubourguier, 2010).

Exposure environment is an important element affecting the toxicity test results of NPs. Most of the standardized *in vitro* toxicity assays are performed in artificial test media which composition may affect the bioavailability of metal-based NPs. The stability (solubility) of NPs as well as their dispersion efficacy depends on the interactions of NPs with organic and inorganic components of the test environment. For instance, organic matter may affect the fate of the NPs in test media in two ways: i) NPs may adsorb organic compounds and as a result settle in the test media (Ma et al., 2015); ii) the components of organic-rich test media are able to disperse NPs and prevent their sedimentation (Bondarenko et al., 2013). The interferences between media components and NPs have to be taken into consideration in the test planning and interpretation of test results (Handy et al., 2012).

This Thesis focuses on the evaluation of the environmental hazard of silver and magnetite (iron oxide) NPs.

Silver has been known for its medical properties for over 2000 years and used in various antimicrobial applications since the nineteenth century. Nowadays silver NPs are widely used in more than 400 consumer products such as broad-spectrum antimicrobials in cosmetics, clothing, detergents, electronics, water purification systems, dietary supplements and medical equipment (Juganson et al., 2015). Despite the various benefits of silver NPs, their release into the environment may lead to negative effects to non-target organisms which must be evaluated. According to Piccinno et al. (2012) up to 550 tons of nanosilver are produced annually and potentially may reach the environment via industrial and household wastes (Tashi et al., 2016).

There are several publications that suggest the potential hazard of silver NPs to non-target organisms. However, as discussed above, due to different physicochemical properties (e.g. different surface coatings), silver NPs may exhibit differences in toxicity as well as in their environmental fate. A great deal of papers have argued about the exact mechanism of action of silver NPs.

Whereas some papers suggest that the toxic effects of those particles are caused by nanoparticulates of silver (Chae et al., 2009), in a series of other papers it has been demonstrated that the toxicity of silver in the environment is driven by free silver ions that are released from nanoparticles to the aqueous phase (Bilberg et al., 2012; Ivask et al., 2014; Wijnhoven et al., 2009). Before certain conclusions can be drawn, specific types of synthetic silver NPs must be evaluated individually to understand whether their potential environmental hazards are comparable to water-soluble silver formulations or not (Prabhu and Poulose, 2012). In this study, we chose polyvinylpyrrolidone (PVP) - coated NPs which were selected due to their frequent use in various medical applications (Le Garrec et al., 2004). In addition, protein-coated silver NPs (collargol) that were used in this study have well-known antibacterial properties and have therefore been broadly used in the daily medicine for decades.

Magnetite (iron oxide, Fe_3O_4) is a widespread natural compound that is nowadays increasingly used for technical and biomedical applications (Gustafsson et al., 2010; Karlsson et al., 2015; Marius et al., 2014). Iron oxides (e.g. magnetite) have been proposed to remove organic (Rusevova et al., 2012) and inorganic (Giraldo and Moreno-Piraján, 2013) pollutants from water and soil. Magnetite has proved effective and low-cost adsorbent in drinking and wastewater treatment systems (Horst et al., 2015; Mohan and Pittman, 2007; Shannon et al., 2008; Zelmanov and Semiat, 2008). It has been shown that nanosized magnetite is more efficient in removing environmental contaminants than microsize magnetite (Ngomsik et al., 2005; Vikesland et al., 2007; Yean et al., 2005)

According to Piccinno et al. (2012) up to 5500 tons of nanosized iron oxides are produced annually. Magnetite is considered relatively safe, however, the consequences of large-scale application of both, nano- or microsized magnetite (e.g. for environmental remediation) should be evaluated. Currently, information concerning the potential hazards of synthetic magnetite NPs to aquatic organisms is still limited (Juganson et al., 2015; Li et al., 2016).

1.2.3. Glyphosate formulations

Glyphosate is a chemical herbicide, widely applied in farming, forestry, parks, public spaces and gardens and its use is rising rapidly (Benbrook, 2016). In 2011, about 650 000 tons of glyphosate was used worldwide (CCM International, 2012). The increase of crops (maize, cotton, soybean, sugar beet), genetically modified against glyphosate, may expand the use of this herbicide (UK GM Science Review panel, 2003). In Estonia, more than 40 different glyphosate-based formulations are registered (Register of Plant Protection Products, 2016) making glyphosate the most used active ingredient in pesticide since 2002 and this trend is showing a clear increase (Estonian Agricultural Board, 2014).

Glyphosate's (chemical name N-phosphonomethylglycine) main effect is to block an enzyme that plants need to make amino acids and proteins (Hoagland and Duke, 1982). When the enzyme is blocked, plants die within a few days. The toxicity of glyphosate to organisms listed in the respective legislation act is well investigated. Glyphosate is said to be plant specific and therefore considered not toxic to animals (Giesy et al., 2000) and other non-target (e.g. aquatic organisms) organisms (WHO, 1996). However, recent investigations have shown that it is not as safe as the producers claim (Gomes et al., 2016; Guerrero Schimpf et al., 2016; Monte et al., 2016; Moreno et al., 2014). In available herbicide formulations, glyphosate is usually combined with additives (e.g. surfactants) which help to increase glyphosate's ability to penetrate the plant cells but at the same time may be toxic to non-target organisms (Edginton et al., 2004; Tsui and Chu, 2003). Moreover, the precise chemical composition of a glyphosate-based herbicide is not clearly stated by the manufactures, making the comparison of the toxicity of different glyphosate formulations, containing different surfactants/additives, to target and non-target organisms complicated.

In Europe, the legislation regulating the marketing and use of pesticides on the EU market is Plant Protection Products Regulation 1107/2009 (EC, 2009b). According to the data in the MSDS of glyphosate, required by REACH on aquatic organisms (96 h LC_{50} fish, 48 h EC_{50} crustacean, 72 h EC_{50} alga), it is classified as hazardous to the aquatic environment (toxic to aquatic life with long lasting effects). Still, glyphosate and its main degradation product aminomethylphosphonic acid (AMPA) are not currently included in the list of priority substances under the Water Framework Directive (EC, 2013) and in the EU there are no EQS values set for these substances. Moreover, the information on the persistence and degradability, bioaccumulative potential and mobility of glyphosate in soil varies depending, for example, on the temperature or type of soil used. Therefore, the toxicity of a specific glyphosate formulation to (non)target organisms in different climate conditions should be studied for relevant site-specific risk assessment.

1.3. Aims of the study

All industrial chemicals need to be tested for their potential hazards to human health and the environment. Still, there is a lack of information concerning the ecotoxicity of chemicals already circulating on the market or entering the market. Available data on the ecotoxicity of chemicals is usually received by using standardized tests which however do not reflect real environmental conditions. Application of non-standardized test procedure enables to obtain complementary ecologically relevant information that is crucial for realistic environmental risk assessment.

The aim of the present research was to obtain new scientific knowledge on the potential environmental hazards of selected organic chemicals such as shale fuel oils and different glyphosate formulations and a new emerging class of inorganic chemicals - metal-based nanoparticles. To obtain ecologically relevant toxicological information on these chemicals to different aquatic and terrestrial organisms, the tests were performed in non-standardized formats and the results were compared with those from standard tests.

Each study has addressed its individual aims:

- modification of the standardized test formats of ecotoxicity assays in order to obtain ecologically more relevant information (papers I-V);
- comparative evaluation of the potential environmental hazards of two shale fuel oils to aquatic and terrestrial ecosystems (papers I, II);
- toxicity assessment of metal-based nanoparticles to aquatic species (papers III, IV);
- assessment of the ecotoxicological effect of different glyphosate formulations to non-target organisms in laboratory and outdoor experiments (paper V).

2. MATERIALS AND METHODS

2.1. Tested chemicals

The following chemicals were studied in this Thesis.

Shale fuel oils (papers I, II)
Two shale fuel oils ("VKG D" and "VKG sweet") were obtained from company VKG Oil AS. "VKG D" is a mixture of 70 % heavy shale oil fraction and 30 % middle shale oil fraction and has a density of 1008.5 kg/m^3 at 15 °C (ASTM D 4052) and sulphur content 0.59 % wt. (ASTM D 4294). "VKG sweet" is a mixture of 70 % middle shale oil fraction and 30 % heavy shale oil fraction and has a density of 992.1 kg/m^3 at 15 °C and sulphur content 0.63 % wt. According to the MSDSs of "VKG D" and "VKG sweet", the average shale oil sample has water solubility of 0.22 g/L.

Magnetite (iron oxide, Fe_3O_4) (paper III)
Uncoated nano- and microsized magnetites (iron oxide, Fe_3O_4) were purchased from Sigma-Aldrich (purity ≥ 98 %) and had the following advertised primary particle sizes: nano Fe_3O_4 (< 50 nm) and micro Fe_3O_4 (< 5 μm).

Silver nanoparticles (paper IV)
Polyvinylpyrrolidone (PVP)-stabilized silver NPs were synthesized by H. Tenhu in the Laboratory of Polymer Chemistry, University of Helsinki. Protein-coated silver NPs (collargol) were purchased from an Estonian drugstore and silver nitrate ($AgNO_3$) from Fluka.

Glyphosate formulations (paper V)
Isopropylammonium (IPA) salt of glyphosate 40 % (w/v) in aqueous solution (29.6 % acid equivalents, AE, by weight) was purchased from Sigma-Aldrich. Herbicide formulations, Roundup Quick™ as a spray (0.72 % AE, by weight) consisting of IPA salt of glyphosate 1 %, water 94 %, other not specified additives 5 %, and granulated Roundup Max™ (68 % AE, by weight) which consists of 75 % of ammonium salt of glyphosate, 21 % of surfactant polyethoxylated tallow amine - POEA, 0.5 % of sodium sulphite, 3.5 % other not specified additives, were produced by Monsanto Europe S.A.

Detailed information on the purchased chemicals can be seen in respective articles.

2.2. Characterization of the used ecotoxicity tests

2.2.1. Ecotoxicity tests with crustacean *Daphnia magna* – the model organism for testing chemicals

Water flea *Daphnia magna* (Figure 1) is a small crustacean that lives in freshwater bodies such as ponds, lakes and streams. They are important food source for fish and other aquatic organisms. Water fleas are widely used in the toxicity assessment because they are sensitive to most types of pollutants. Compared to other daphnids, *D. magna* is the largest and the easiest to handle in the laboratory tests (Canton and Adema, 1978). For testing, the daphnia neonates can be obtained by the hatching of ephippia (MicroBioTests Inc, Belgium) or from the healthy laboratory culture. Daphnids are easily cultured in the laboratory, inexpensive to maintain and they mature in just a few days, therefore *D. magna* can be used both, in acute (OECD 202, 2004) and chronic (OECD 211, 1998) toxicity assays.

Figure 1. Crustacean Daphnia magna

Test media (papers I-V)
In the acute tests, artificial OECD 202 freshwater (AFW) is usually used for *D. magna* testing. In papers II, III, IV different 0.45 μm filtered natural waters were used in both, acute and chronic tests in addition to AFW.

48 h immobilization test (papers I-V)
D. magna acute immobilization test is standardized (OECD 202, 2004). In our studies, less than 24-h-old *D. magna* neonates were obtained by the hatching of ephippia (MicroBioTests Inc, Belgium) and fed for 2 h with unicellular alga *Spirulina* sp. After feeding, 20 animals divided into four groups of five animals each (a volume of 10 ml of sample per test well), were used at each test concentration and for the controls and incubated at 20 °C for 48 h in the dark. The toxicity endpoint was the immobilization meaning the daphnids were not able to swim within 15 seconds even if they were able to move their antennae.

21-day reproduction test (paper IV)

D. magna reproduction test was performed according to OECD 211 (1998). Briefly, neonates less than 24-h-old were exposed for 21 days at 21±1 °C with 16 h light/8 h dark photoperiod to different concentrations of tested chemicals. *D. magna* was fed daily with alga *Pseudokirchneriella subcapitata.* Parent animals were transferred to renewed medium every 3 days. Endpoints were mortality of the parent animals during the test and the number of juveniles produced per alive parent animal at the end of the test.

Recovery test (papers II, III)

The aim of the recovery test was to evaluate the long-term effects of short-term exposure to sublethal concentrations of chemicals on crustacean *D. magna* population. Briefly, 24-h-old *D. magna* neonates were exposed to sublethal concentrations of tested chemicals for 48 h, after which actively swimming daphnids were collected from each concentration and further incubated during two-three weeks in clean filtered (0.45 μm pore-size filter) lake water. The medium was renewed once a week and daphnids were fed daily with alga *P. subcapitata*. Dead organisms were removed from the test vessel and the mortality of parent animals and the number of offspring produced was recorded daily. At the end of the test (after 18 days), alive organisms and the total number of living offspring produced per alive parent animal were calculated.

Early life-stage test (paper III)

The aim of the early life-stage test was to investigate the effect of chemicals on the hatching of *D. magna* ephippia. For that, 90 *D. magna* ephippia (MicroBioTests Inc, Belgium) were hatched in different uncontaminated lake waters and in different concentrations of tested chemicals. The ephippia were exposed for 80 h at 20 °C under continuous illumination after which the hatched alive neonates were counted and used in further tests (acute and recovery).

2.2.2. Mortality test with crustacean *Thamnocephalus platyurus* (papers I, II, IV)

Thamnocephalus platyurus (Figure 2) mortality test was performed according to Standard Operational Procedures of Thamnotoxkit F^{TM} (1995). The cysts of *T. platyurus* were purchased from MicroBioTests Inc, Belgium. After hatching of the cysts at 25 °C for 20-22 hours under continuous illumination, less than 24-h-old larvae were used in the test. 30 larvae per every concentration of the tested chemical (3x10 organisms in 1 ml of sample) were incubated for 24 h at 25 °C in the dark. The mortality was used as the toxicity endpoint.

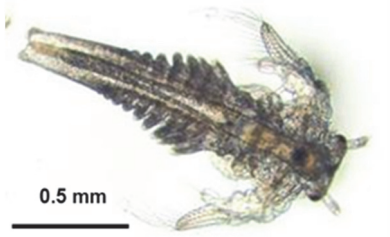

Figure 2. Crustacean Thamnocephalus platyurus

2.2.3. Growth inhibition test with duckweed *Lemna minor* (papers II, III)

Higher water plant duckweed *Lemna minor* growth inhibition test was performed according to OECD 221 (2006). *L. minor* was exposed for 7 days at 25 °C in the continuous illumination (7000 lux) and the number of fronds and dry biomass were used as the toxicity endpoints. The test was performed in standard *L. minor* growth medium (OECD 221, 2006) and in different filtered natural waters.

2.2.4. Tests with terrestrial plants (papers I, V)

Toxicity of spiked soils to terrestrial plants was evaluated using two different test formats. In Phytotoxkit^{TM} plate test (2004), soil and seeds were separated with a filter paper, covered with a lid and incubated at 25 °C in darkness in a vertical position. In traditional OECD 208 bioassay (2006), seeds were placed directly into the soil, not covered and incubated inside or outside under natural light–dark cycle. Toxicity to terrestrial plants mustard *Sinapis alba,* barley *Hordeum vulgare* and red radish *Raphanus sativus* was evaluated using the inhibition of seed germination and root/shoot growth as toxicity endpoints. For more details, see papers I and V.

3. RESULTS AND DISCUSSION

3.1. Environmental hazard evaluation of shale fuel oils (papers I-II)

Behaviour in the soil and toxicity to aquatic species of two widely produced Estonian shale fuel oils "VKG D" and "VKG sweet" with different chemical composition was studied using a combined chemical and biological approach. Different test formats were applied to increase the environmental relevance of the experimental results. According to our knowledge, this is the first study about the behaviour of shale fuel oils in different soil and water matrices giving valuable information on the mobility and degradation of SFOs in the soil needed for the hazard evaluation.

3.1.1. The behaviour of shale fuel oils in two different soil matrices (paper I)

Along with data on the potential hazard of pollutant to the biota, information on the behaviour in the environment, e.g. rates of decontamination as a result of natural procecces and rate of migration within the soil compartment, is needed for forecasting the potential negative environmental impact (Battersby, 2000; Smink and Klaine, 2013; Willing, 2001).

3.1.1.1. Design of the study

To increase the environmental relevance of experimental results, mobility and degradation of SFOs "VKG D" and "VKG sweet" in the soil was studied in microcosms under Estonian environmental conditions, as weather strongly affects the fate of contaminants in the soils (National Research Council, 2003). Two types of soil matrices: organic-rich (C_{org} – 3.5 %) natural soil and sand were used in the study. Soil-filled test containers, open from the bottom, were placed on the sandy ground for one year (Figure 3).

In the migration experiment, SFOs with a pollution load of 1 ml/cm^2 (imitating moderate accidental pollution) were poured directly on the 20 cm thick substrate layer. The hydrocarbons' concentrations and the number of heterotrophic bacteria were assessed in the mixed 3 cm bottom layer, located 10-13 cm under the oil polluted surface (further designated as "bottom layer").

In the degradation experiment, soils were homogeneously mixed with SFOs to obtain concentrations of 10 and 50 g oil/kg soil. Before taking sub-samples for analyses, the oil-spiked layers were mixed to minimise the oils' downward migration impact on the test results. The dynamics of SFOs' hydrocarbons and number of soil heterotrophic bacteria were measured and the toxicity to terrestrial plants *Hordeum vulgare* and *Sinapis alba* was tested.

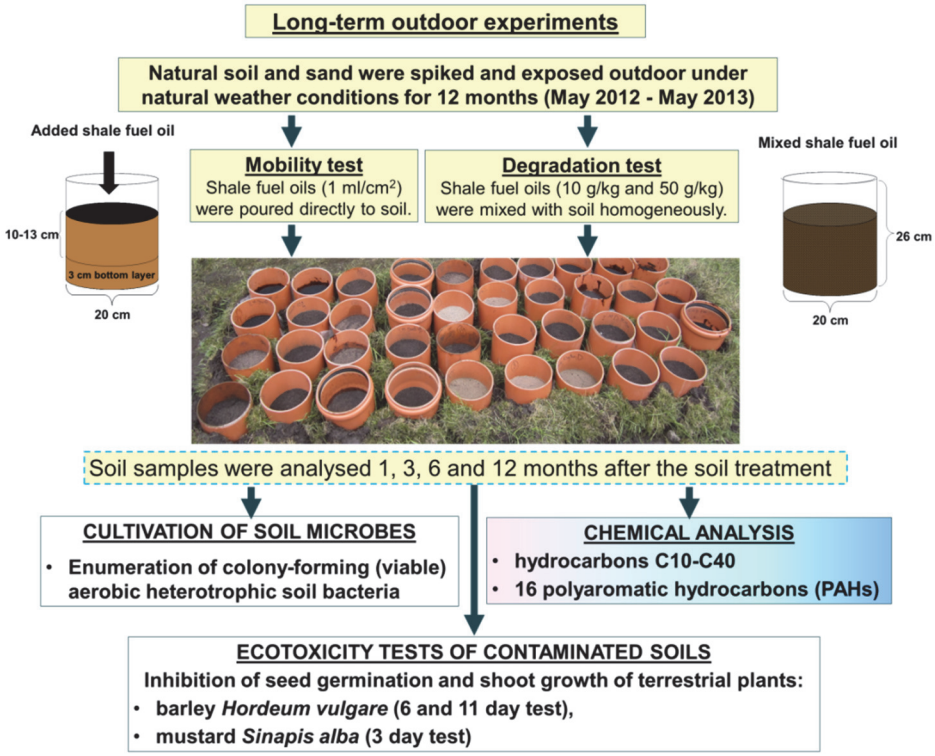

Figure 3. Scheme of the outdoor experiments with shale fuel oils (Figure S2 in paper I).

To evaluate the potential risk of contaminated soils to aquatic ecosystems, the toxicity of aqueous leachates (1:10, i.e. 100 g of dry soil per 1 L of AFW) (Barron and Ka'aihue, 2003) of soil samples to aquatic species *Daphnia magna, Thamnocephalus platyurus* and *Vibrio fischeri* was studied. The content of hydrocarbons (TPHs C10-C40: > C10–C21 and > C21–C40; sum and individual concentrations of 16 PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene) were measured in oil-spiked soil samples and in the respective aqueous eluates.

For more details on the design of the study and the used shale fuel oils, see papers I and II.

3.1.1.2. The migration of shale fuel oils in the soils

In case of an accidental oil spill, it is important to know the migration rate of the fuels to the deeper layers of the soil column. The experiment revealed that the

downward migration of shale fuel oils depended on both, soil matrix and type of SFO.

In natural soil, the mobility of both SFOs was very low. The content of hydrocarbons C10–C40 in the bottom layers of test container increased only 2-fold compared to the control container with non-spiked soil after 3 months of exposure and did not differ from control at 6th and 12th months. On the basis of chemical analysis it could be concluded that one year after spiking the bottom layers (3 cm bottom layer located 10-13 cm under oil-polluted surface) were not contaminated by the SFOs hydrocarbons. However, it should be mentioned that the number of heterotrophic bacteria was higher than in the control soil indicating that small amount of hydrocarbons still continued to migrate from the upper soil layer (Table 1). Moreover, although the concentrations of hydrocarbons in all soil leachates were < 0.03 mg/L, the toxicity of leachates of the bottom soil layer in containers spiked with "VKG sweet" to *D. magna* increased in time from non-toxic at month 3 to toxic at month 12. A decrease in TPH (C10-C40) content in the leachates does not always mean a decrease in toxicity (Loehr and Webster, 1997). The increasing toxicity could be explained by the downward migration of lighter hydrocarbons (< C10) and other mobile toxic compounds formed as a result of hydrocarbons' degradation. Thus, unlike from chemical analysis the biological methods indicate the existence of hydrocarbons' contamination. These results once more confirm that chemical analysis alone is not enough for the realistic assessment of soil contamination level.

In sand, the downward migration of both SFOs was up to 100-fold higher than in natural soils (Table 1) and "VKG sweet" with lower viscosity was much more mobile than "VKG D" with higher viscosity. This is mostly due to the fact that natural soils with higher organic matter content and smaller size of mineral particles sorb hydrophobic organic compounds (bound-phase) more effectively than sand (Means et al., 1980; Nam et al., 1998; Van Gestel et al., 2003).

The toxicity tests with leachates (1:10) from the bottom layers of sands using aquatic species also showed the higher mobility of SFO "VKG sweet". Leachates of the oil-spiked sands were very toxic to crustacean *D. magna* and bacteria *V. fischeri* already after 3 months of spiking.

The mobility test revealed that soils with low organic matter content are not a barrier against diffuse pollution of SFO hydrocarbons and, therefore, in case of an accident on the sand surface, pollution must be removed as soon as possible.

Table 1. Average content of hydrocarbons (C10-C40) and the total number of heterotrophic bacteria in the soils spiked with shale fuel oils (Table 2 in paper I).

Time of sampling	**Month 3 (August)**		**Month 6 (November)**		**Month 12 (May)**	
Sample	**C10-C40,** mg/kg dw	**Bacteria,** 10^6 CFU/g dw	**C10-C40,** mg/kg dw	**Bacteria,** 10^6 CFU/g dw	**C10-C40,** mg/kg dw	**Bacteria,** 10^6 CFU/g dw
Control soil	40	7.00±0.75	25	4.33±0.32	21	8.62±0.96
Soil + "SW"	92.5	8.96±0.70	29	10.74±1.15	< 20	12.87±0.24
Soil + "D"	61	3.97±0.16	31	8.52±0.71	< 20	19.77±0.71
Control sand	< 20	1.47±0.15	20	1.79±0.09	20	1.81±0.05
Sand + "SW"	8200	2.80±0.37	13400	2.37±0.15	n. a.	n. a.
Sand + "D"	4280	1.31±0.08	2290	1.23±0.06	n. a.	n. a.

CFU – colony forming units; dw – dry weight; n. a. – not analysed

3.1.1.3. The degradation of shale fuel oils in the soils

The rate of natural decontamination (the basic parameter for predicting long-term consequences of petroleum contamination) is mainly a result of the following processes which act simultaneously: volatilization, adsorption, migration in the soil matrix, chemical degradation and biodegradation (MacNaughton et al., 1999). Biological degradation is the main process in the soils where degrading bacteria are abundant (Taketani et al., 2010). The rate of hydrocarbon biodegradation depends on many factors like its chemical composition, environmental temperature, bioavailability of the compound, concentration of nutrients and oxygen in the soil (van Leeuwen and Vermeire, 2007).

The long-term exposure under environmental conditions of soils spiked with SFOs showed that degradation efficiency of both SFOs in soils was very low.

TPH C10–C40 concentration decreased only by 20-25 % on the average, both, in natural soil and sand during the first six months after spiking (May-October) and the TPH C10-C40 decrease was neglible during the next 6 months (November–May). However, different processes affect the decontamination in natural soil and sand. As shown in the mobility experiment, the decrease of hydrocarbons' content in the sand samples may be mainly explained by the downward migration. The fact that the total number of heterotrophic bacteria in the spiked sands only slightly differed from the control sand (Table 1) also supports this assumption. On the contrary, in natural soils, biological degradation is a primary process of decontamination. Indeed, the abundance of heterotrophic bacteria in the contaminated natural soils considerably exceeded the number of bacteria in the control soil (Table 1).

The drop in the decontamination efficiency in both soil matrices during the second half of the exposure may be explained by two main reasons: effect of temperature and aging of the contaminants. It is known that the biodegradation rate generally decreases when the temperature falls (Das and Chandran, 2011). In time, petroleum contamination sorbes to soil particles more strongly and is less bioavailable to the bacteria (Kelsey et al., 1997; Tang et al., 1998); as a result the biodegradation slows down. Differences among the soil types also affect the aging-induced changes in biological accessibility (Alexander, 2000) and studies with few soils have suggested that aging is more significant in soils with higher organic matter content (Hatzinger et al., 1995). As at low temperature the water-solubility of hydrocarbons decreases (Delille et al., 2004) and the viscosity of the oil increases (Atlas, 1975), we could assume that low temperature during the second half of the experiment also affected the decrease of hydrocarbons' mobility in the soil matrix.

Our results showed that under natural climate conditions the removal of shale oil contamination from the soil matrix due to evaporation/degradation is much slower than expected from laboratory experiments performed in stable environmental conditions (Goi et al., 2006). Thus, extrapolation of the test

results from the laboratory tests to natural conditions may lead to significant over- or underestimation of real risks associated with shale fuel oil pollution.

PAHs are among the most toxic compounds in the SFOs, posing a risk of causing adverse effects to human health and the environment. The behaviour pattern of the sum of 16 PAHs listed as the "priority pollutants" by the US EPA (Larsson et al., 2013) in the soils was similar to TPH C10–C40, i.e. the most intensive decrease of hydrocarbons' concentration was during the first six months. In the natural soil with low contamination (10 g/kg), the sum of 16 PAHs decreased nearly tenfold during one year of exposure and the residual concentration did not exceed 5 mg/kg (the Estonian EQS target value for soils) for either SFOs. However, the analysis of the dynamics of individual PAHs' concentrations revealed that the reduction of the sum of 16 PAHs both, in natural soil and sand was only due to two low-molecular-weight compounds: naphthalene and acenaphthylene, which are the most abundant in the SFOs (> 60 % of sum mass of 16 PAHs in "VKG D" and nearly 40 % in "VKG sweet"). At the same time the concentrations of the high-molecular-weight PAHs, like benzo(a)pyrene, that are considered the most carcinogenic and toxic among petroleum compounds (Haritash and Kaushik, 2009), decreased only slightly (Figure 4).

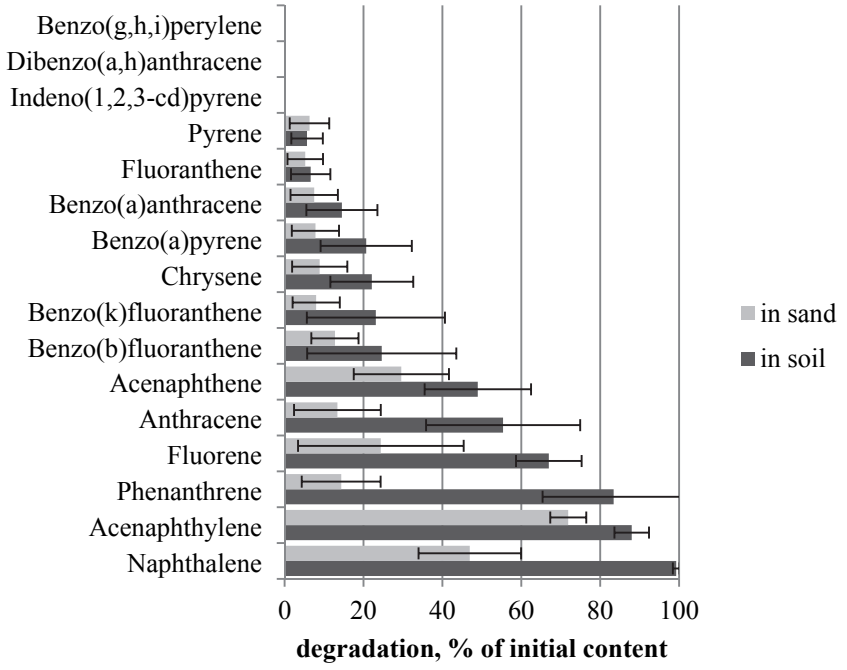

Figure 4. Decrease of the content of individual PAHs in the SFO-spiked natural soil and sand during one year after treatment (Figure 1 in paper I).

The low-molecular-weight PAHs are much easier to biodegrade than highly hydrophobic high-molecular-weight PAHs (Liu et al., 2011; Yi and Crowley, 2007). Our experiments showed that although the decrease of PAHs' content was more intensive in the natural soil than in the sand due to biodegradation, the slowly degradable high-molecular-weight PAHs may pose long-term risks to soil biota in all soil matrices.

The results of toxicological testing of aqueous soil leachates (1:10) to crustacean and marine bacterium confirm the conclusion that "VKG sweet" is more hazardous to the environment than "VKG D". The aqueous leachates of spiked sands were toxic to aquatic organisms *D. magna* and *V. fischeri* during the whole experiment, indicating that sands polluted with SFOs may be a long-term contamination source for groundwater or surface water.

Phytotoxicity testing, a reliable method for the evaluation of soil health (Van Gestel et al., 2003), was performed after 12 months of exposure. The results with terrestrial plants *H. vulgare* and *S. alba* from both test formats (Phytotoxkit™ plate test and OECD 208 bioassay) showed a negative effect on the plant growth. No correlation between the plant growth inhibition and the hydrocarbons' concentrations in the soil matrix was revealed. Evidently, the modification of the soil structure as a result of contamination is the main factor effecting the reduction of the plant development in the contaminated soils. Our experiments once more demonstrated that although the aging of petroleum hydrocarbons reduces the bioavailability to biota (Alexander, 2000) it can also degrade the soil quality by the alteration of water retention, aeration or nutrient supplies.

3.1.2. Water accommodated fractions of shale fuel oils (paper II)

In the evaluation of the potential hazard of oil products to aquatic biota the method used for water accommodated fraction (WAF) preparation is of great importance. The investigation of the toxicity of two SFOs "VKG sweet" and "VKG D" to aquatic organisms belonging to different trophic levels was performed using different WAF preparation approaches: variable loading and variable dilution. For more details of the design of the study see paper II.

3.1.2.1. Design of the study

Three different oil-water-ratios (OWRs): 1:40 (25 g SFO + 1 L of medium), 1:1000 (1 g SFO + 1 L of medium) and 1:10 000 (100 mg SFO + 1 L of medium) were used for WAF preparation (Figure 5). From these parental WAFs the dilution series for toxicity testing were made as described by Barron and Ka'aihue (2003). AFW containing only mineral salts without organic compounds (OECD 202, 2004) and filtered (0.45 μm pore size standard filter) natural water with organic matter was used. Four aquatic test species were used in this study: *Daphnia magna, Thamnocephalus platyurus, Vibrio fischeri,*

Lemna minor. The following chemical analyses from WAFs were performed: TPHs C10-C40 (C10-C21 and > C21-C40), sum and individual concentrations of 16 PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene. The concentrations of hydrocarbons (TPHs and PAHs) were measured in undiluted parental WAF solutions (1:40, 1:1000 and 1:10 000); the concentrations of hydrocarbons in the dilution series were calculated.

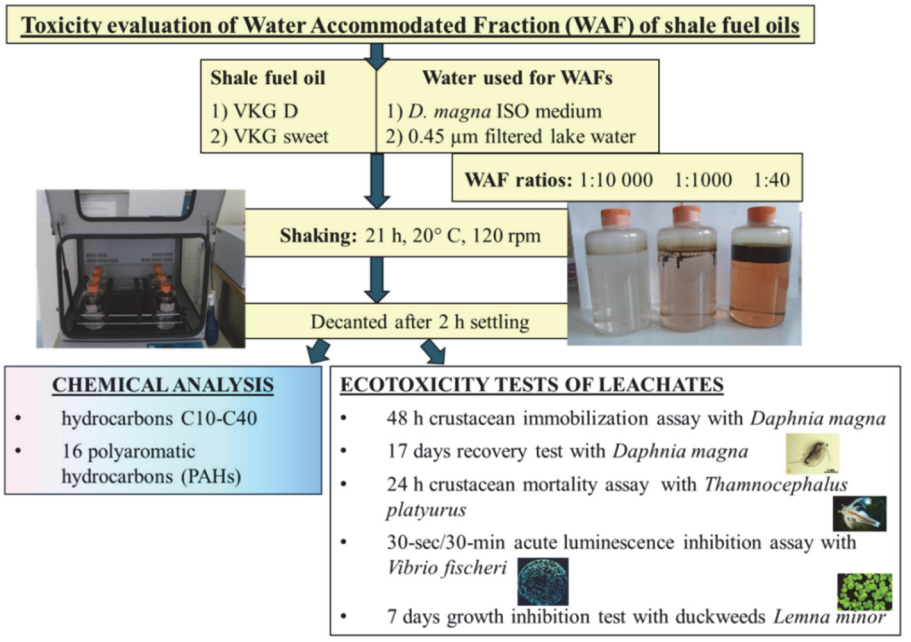

Figure 5. Sample preparation and scheme of experiments with shale fuel oil water accommodated fractions (Figure S1 in paper II).

3.1.2.2. Chemical composition of water accommodated fractions

Information on the chemical composition of WAF solution is a crucial point in the interpretation of the toxicity test results. Although OWR significantly affected the hydrocarbons' concentration in the WAF solutions, no correlations between the applied OWR and the measured chemical parameters (TPHs C10-C40 and sum of 16 PAHs) were found (Table 2). The extraction efficiency of the water soluble hydrocarbons from SFOs was the highest in the WAF solution with OWR 1:10 000.

Table 2. Concentrations of hydrocarbons (TPHs C10-C40 and sum of 16 PAHs) in the water accommodated fractions of two shale fuel oils extracted using different oil-water-ratios and different media (Table 1 in paper II).

Type of WAF	OWR	TPHs (C10-C40)		Sum of 16 PAHs	
		µg/L	% of applied[a]	µg/L	% of applied[a]
"VKG sweet" in AFW	1:10 000	80±7	0.40	13.5±0.7	3.55
	1:1000	260±64	0.13	43.0±22.6	1.13
	1:40	590±14	0.01	56.0±25.0	0.06
"VKG sweet" in lake water	1:10 000	330±179	1.65	19.0±2.8	5.00
	1:1000	930±554	0.47	58.0±42.3	1.53
	1:40	810±390	0.02	57.0±21.0	0.06
"VKG D" in AFW	1:10 000	30±0	0.14	13.0±8.6	3.51
	1:1000	310±71	0.15	66.5±47.4	1.80
	1:40	980±14	0.02	170.0±84.9	0.18
"VKG D" in lake water	1:10 000	70±45	0.33	10.3±2.5	2.77
	1:1000	840±280	0.38	71.5±40.3	1.93
	1:40	910±160	0.02	107.0±18.4	0.12

[a]*percentage of TPHs or PAHs leached into the WAF solutions from the applied (100 %) SFOs for WAF preparation.*
TPHs – total petroleum hydrocarbons; PAHs – polyaromatic hydrocarbons; SFO – shale fuel oil; WAF – water accommodated fraction; AFW – artificial freshwater; OWR – oil-water-ratio

3.1.2.3. Toxicity of water accommodated fractions of shale fuel oils to aquatic species

Aquatic crustaceans are very sensitive to oil pollution and *Daphnia magna* is among the recommended species for toxicity assessment (Bejarano et al., 2006). The testing of SFOs with aquatic organisms from different trophic levels revealed that *D. magna* was the most sensitive among the four tested species. A very good correlation between EC_{50} values was obtained for *D. magna* and other species, namely with *T. platyurus* ($r^2 = 0.93$), *V. fischeri* ($r^2 = 0.87$*), L. minor* ($r^2 = 0.96$). Therefore, the detailed toxicity investigation of SFOs was further continued on *D. magna*.

Acute toxicity test showed that both SFOs were toxic to *D. magna*, whereas "VKG sweet" was more toxic than "VKG D" (Table 3). This may be explained by the different chemical composition of different SFO WAFs. Analysis of the concentration of the individual PAHs revealed that the abundance of more toxic high-molecular-weight PAHs was significantly higher in WAF solutions prepared with "VKG sweet" than with "VKG D" (Figure 6). However, no correlation between the toxic effect and chemical parameters as TPHs and sum of PAHs (Tables 2 and 3) was found. For example, in case of WAF 1:40 prepared in the lake water, the TPHs (µg/L; Table 2) were nearly the same for both SFOs but "VKG sweet" showed higher toxicity to *D. magna* than "VKG D" (TPH, µg/L; Table 3).

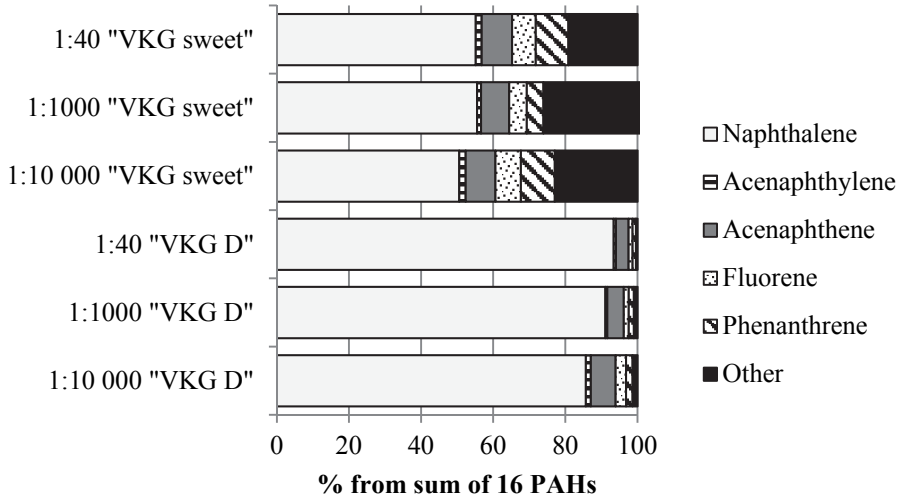

Figure 6. Content of the individual polyaromatic hydrocarbons (% of sum of 16 PAHs) in the water accommodated fractions of shale fuel oils prepared in the lake water (Figure 1 in paper II).
PAHs – polyaromatic hydrocarbons.

Table 3. Toxicity of two shale fuel oil water accommodated fractions (WAFs) to Daphnia magna (Table 2 in paper II).

		48 h EC_{50} for *Daphnia magna*			
Type of WAF	**OWR**	**v/v % of parental WAF**	**Oil load[a],** mg/L	**TPH[a],** µg/L	**Sum of 16 PAHs**, µg/L
"VKG sweet" in AFW	1:10 000	**13.2±3.1**	13.2	10.6	1.8
	1:1000	**3.4±1.0**	34.0	8.8	1.5
	1:40	**0.7±0.5**	175.0	4.1	0.4
"VKG sweet" in lake water	1:10 000	**13.7±10.3**	13.7	45.2	2.6
	1:1000	**4.0±3.0**	40.0	37.2	2.3
	1:40	**1.1±0.9**	275.0	8.9	0.6
"VKG D" in AFW	1:10 000	**37.9±7.7**	37.9	11.4	4.9
	1:1000	**8.8±6.4**	87.7	27.2	5.8
	1:40	**0.3±0.0**	75.0	2.9	0.5
"VKG D" in lake water	1:10 000	**51.1±8.8**	51.1	35.8	5.2
	1:1000	**10.0±4.8**	100.0	84.0	7.2
	1:40	**3.2±1.9**	791.7	28.8	3.4

[a] *calculated from mean EC_{50} values based on the parameters of parental WAFs (OWR, total petroleum hydrocarbons- TPHs and polyaromatic hydrocarbons - PAHs concentrations) and percentage of parental WAF dilution (v/v %; in bold).*

As a rule, after oil spill the concentration of hydrocarbons in water decreases in time due to natural decontamination processes (Short, 2003), therefore the long-term risks for the crustacean population associated with short-term exposure of SFOs hydrocarbons should be evaluated. The recovery test with *D. magna* showed that short-term exposure to high but sublethal concentrations of soluble SFO fractions may increase the reproductive success of the survived organisms. This very interesting finding means that the increased reproduction potential of the survived daphnids after oil spill might promote the recovery of the population after the decrease of hydrocarbons concentration in the water column.

Although different methods for preparing WAF series for toxicity testing are currently used, variable loading (i.e. each test concentration is prepared with individual OWR) is considered more appropriate than variable dilution (Aurand et al., 2001; Barron et al., 1999). However, this method is very time-consuming and application of variable dilution seems to be more practical and cost-effective in case of toxicity evaluation of a multitude of samples (e.g. different SFOs). The 48 h EC_{50} value obtained in the current study from the toxicity test with 1:10 000 WAF "VKG sweet" for *D. magna* (Table 3) expressed as loading rate (13.2 mg/L) was comparable with the 48 h EC_{50} value (9.71 mg/L) of SFO obtained by the application of variable loading approach (ECHA, 2016). Besides, hydrocarbons concentrations in 1:10 000 WAFs are close to the concentrations observed in the water columns after spills (Boehm et al., 2007).

So, on the basis of the results obtained in the current study, the preparation of the test series with variable WAF dilutions with OWR 1:10 000 in natural water may be recommended as environmentally the most relevant and cost-effective approach for toxicity testing of shale fuel oils.

3.2. Evaluation of the potential hazard of metal-based nanoparticles to aquatic organisms (papers III-IV)

As physicochemical properties of nanoparticles (NPs) differ from their microsized analogues (Nanoparticle Technology Handbook, 2007), the data on the environmental hazard presented in the material safety data sheets (MSDSs) for the microsized substances cannot be used for the risk assessment of NPs. Therefore, professional judgement is needed when performing hazard evaluations with NPs.

The behaviour of the metal-based NPs in the environment and their potential ecological hazards depend on NP physicochemical characteristics (size, coating, shape, etc.) (Gatoo et al., 2014). Therefore, for exact ecotoxicological hazard assessment, each NP should be tested separately which however is impractical due to the large demand of experimental resources. Currently, both, scientists and regulators are putting significant resources to read-across, i.e., prediction of the toxicological profile of one nanomaterial from the profile of a similar nanomaterial, and modelling, that would allow the prediction of the hazard of

one nanomaterial using already existing information from other similar nanomaterials. Thus, the finding of common features in biological effects within a group of similar compounds (e.g. silver-based NPs) is of great interest and would significantly help to reduce the number of tests needed for ecotoxicological evaluation of specific type of NP.

In this study we tried to fill some data gaps related to the toxicity of NPs to aquatic organisms.

3.2.1. Toxicity of magnetite (iron oxide, Fe_3O_4) nanoparticles (paper III)

Magnetite (iron oxide, Fe_3O_4) NPs have been proposed for effective removal of environmental contaminants. It has been shown that the efficiency of pollutants' deactivation by magnetite increase with decreasing particle size (and accompanying increase in specific surface area) (Habuda-Stanic´ and Nujic´, 2015; Vikesland et al., 2007). The increased application of iron-based nanomaterials in environmental remediation (Karn et al., 2009) is also expected to rise the risk of environmental contamination by iron oxide NPs, e.g. during their large-scale use or transportation. Our study will provide information needed for assessing the risks related to the application of magnetite nanoparticles for *in situ* remediation and/or in case of accidental pollution.

Toxicity of nano- and microsized magnetite to aquatic organisms belonging to different trophic levels (crustacean *Daphnia magna*, duckweed *Lemna minor*) was investigated. To increase the environmental relevance of the experiments, all tests were performed using AFW and two natural waters with different content of dissolved organic carbon (DOC). In addition to standard acute toxicity tests (OECD 202, 2004), the effect of used chemicals on daphnids' different life stages and their recovery potential was investigated.

Nano- and microsized magnetite used in this study induced very low toxicity (EC_{50} > 100 mg/L) to *D. magna* and *L. minor* in the standard acute assays in all tested media (AFW and natural waters from lakes Ülemiste and Raku). However, in the early life-stage test the water with higher DOC concentration (Lake Ülemiste) had a bigger negative effect on the hatching efficiency, indicating that the bioavailability of magnetite may depend on the content of DOC in the water (no difference between nano- or microsized magnetite was observed). *D. magna* recovery test was the most sensitive test format, revealing that the reproductive potential of crustaceans may significantly be affected even by short-term exposure to magnetite. Interestingly, although during the recovery test the mortality of parent animals was higher in the groups pre-exposed to nano- or microsized magnetites (for two days, 10-100 mg/L), the total number of offspring in the pre-exposed groups was higher or comparable to the control. This information has practical value for risk assessment as the greater mortality of pre-exposed daphnids may be compensated by the higher number of offspring produced by the survived females. Our investigation revealed that contamination

of aquatic ecosystems by magnetite NPs may disrupt stability of the crustacean populations and before magnetite NPs can be widely allowed for *in situ* remediation of polluted aquatic ecosystems more studies are needed to prove their environmental safety. No significant differences between the biological effects of nano- and microsized magnetite to the tested species were revealed.

3.2.2. Toxicity of silver nanoparticles (paper IV)

The mechanism by which silver NPs exhibit their toxic effects is not yet clear. According to the published data, particle dissolution and release of silver ions, organism dependent cellular uptake and induction of oxidative stress are the key properties driving the toxicity of these NPs (Ivask et al., 2014). However, although the dissolution of silver NPs is considered the most important factor for their toxicity, it was also demonstrated that exposure of *D. magna* to silver NPs resulted in remarkably distinct gene expression profiles compared to silver ions (Poynton et al., 2012), and that suggested nanoparticle-specific toxicity. Also, other papers have presented that dissolution of silver NPs and release of silver ions do not explain all the toxic effects (Ivask et al., 2014). It was shown that daphnids accumulated silver more intensively when they were exposed to silver NPs than when they were exposed to $AgNO_3$ and bioaccumulation efficiency depended on the NP characteristics (e.g. surface coating, size) (Zhao and Wang, 2011). In this study we proposed that comparison of toxic effects of different types of silver NPs to the same test species under similar exposure conditions may help to understand the reasons behind differences in ecotoxicological profiles of silver particles with different physicochemical properties. The adverse effects of PVP- stabilized silver NPs and collargol to two aquatic crustaceans *Daphnia magna* (acute and reproduction tests) and *Thamnocephalus platyurus* (acute test) were investigated in the waters with different chemical composition. Soluble silver salt $AgNO_3$ was used to evaluate the role of silver ions, which may be released from silver NPs, in the toxic effects of NPs.

The test results showed that both tested silver NPs as well as silver ions were very toxic to crustaceans in all used test media and the sensitivity of *D. magna* and *T. platyurus* to the same silver compound was comparable. The toxicity of $AgNO_3$ to the test organisms was noticeably higher than that of silver NPs which has also been shown by other authors (Allen et al., 2010; Griffitt et al., 2008; Zhao and Wang, 2011). Toxicity of silver salts and silver NPs in natural water decreased compared to the standard AFW, which is also in the agreement with the previous data from the literature (Erickson et al., 1998; Gao et al., 2009). However, the variation of silver NPs and $AgNO_3$ toxicity in both natural waters was different (Table 4). It could be suggested that the differences between used silver NPs were caused by the differences in their surface coatings (Bone et al., 2012). Our experiment revealed a good correlation (R^2=0.88) between the toxicity and concentration of DOC in water only for $AgNO_3$.

Table 4. Acute toxicity, E(L)C_{50}, of the silver compounds to crustaceans in six different test media, µg compound/L (µg Ag/L).

Test compound	Test medium	*Daphnia magna* 48 h EC_{50}		*Thamnocephalus platyurus* 24 h LC_{50}	
		Mean	**STD**	**Mean**	**STD**
Collargol	AFW	49.4 (36.6)	19.7 (14.6)	256 (189)	51.8 (38.3)
	River 1	59.4 (44.0)	9.4 (6.9)	178 (132)	41.0 (30.4)
	River 2	40.2 (29.8)	16.1 (11.9)	147 (109)	5.9 (8.0)
	Lake 1	74.9 (55.5)	18.6 (13.8)	n.d.	n.d.
	Lake 2	50.8 (37.6)	1.8 (1.3)	250 (185)	13.8 (10.2)
	Lake 3	65.7 (48.7)	7.0 (5.2)	n.d.	n.d.
PVP-Ag4	AFW	54.0 (15.7) *	1.4 (0.4)	68.8 (20.0) *	1.4 (0.4)
	River 1	191 (55.5)	80.5 (23.4)	191 (55.5)	13.4 (3.9)
	River 2	98.7 (28.7)	31.3 (9.1)	n.d.	n.d.
	Lake 1	176 (51.1)	15.1 (4.4)	252 (73.3)	62.3 (18.1)
	Lake 2	236.3 (68.7) *	97.7 (28.4)	605 (176.0)*	113 (33.1)
	Lake 3	162 (47.2)	59.8 (17.4)	n.d.	n.d.
$AgNO_3$	AFW	2.2 (1.4) *	0.5 (0.3)	5.7 (3.6) *	0.6 (0.4)
	River 1	12.4 (7.9)	4.6 (2.9)	10.7 (6.8)	1.7 (1.1)
	River 2	15.9 (10.1)	2.4 (1.5)	n.d.	n.d.
	Lake 1	8.3 (5.3)	0.5 (0.3)	11.6 (7.4)	0.5 (0.3)
	Lake 2	12.9 (8.2)	4.1 (2.6)	24.3 (15.5)	7.2 (4.6)
	Lake 3	6.8 (4.3)	0.2 (0.1)	n.d.	n.d.

* *statistically significant differences ($p < 0.05$) from other test media*
n.d. - not determined

Comparison of the data from acute (48 hours) and reproduction (21 days) tests with *D. magna* showed that the toxicity of both silver NPs (collargol and PVP-coated NPs) to *D. magna* was lower in reproduction test. For example, in acute test, EC_{50} value for PVP-Ag4 was 28.7 µg Ag/L (river 2; Table 4), but in the reproduction assay all the adult daphnids were alive at 58 µg Ag/L even after 21 days of exposure. The main reason could be the addition of algae as food during the reproduction assay; the latter was also shown by Allen et al. (2010). In addition, our results indicated that adult mortality during 21 days of silver NP exposure was more sensitive toxicity test endpoint than the reproduction (the number of offspring per adult). This finding once more points out that the

extrapolation of the results from acute tests to real ecosystem may lead to over- or underestimation of risks related to the water pollution by silver NPs.

In summary, the bioavailability of PVP-coated NPs and collargol was test media dependent and their toxicity to crustaceans was always lower than the toxicity of $AgNO_3$ (Table 4). Thus, it could be concluded that there is no reason to consider silver NPs more dangerous to the aquatic ecosystems than silver ions and the evaluation of risks associated with the contamination of different types of silver NPs could be based on total silver content in the environment.

3.3. Ecotoxicological effects of different glyphosate formulations (paper V)

Toxicity of glyphosate to target organisms is widely studied but not many experiments have been conducted with glyphosate formulations in northern temperate climate zone. Repeated applications of glyphosate may result in its accumulation in soil due to the low degradation rate in cold climate (Laitinen et al., 2009). Glyphosate-based herbicides include various additives/surfactants in different concentrations and some glyphosate formulations may be more toxic to the biota than glyphosate itself (Thompson, 2014). Therefore, the toxicity of every glyphosate formulation to target and non-target organisms should be investigated separately. In this Thesis, the potential ecotoxicity of two glyphosate-based herbicides Roundup Max™ and Roundup Quick™ as well as IPA salt of glyphosate was evaluated in short-term laboratory and long-term outdoor experiments. This study gives valuable information on the effects of the tested chemicals on soil health in Estonian temperate climate conditions.

3.3.1. Design of the study

Short-term toxicity of glyphosate as an active substance (IPA salt of glyphosate) and two commercial glyphosate formulations Roundup Max™ (containing surfactant polyethoxylated tallow amine, POEA) and Roundup Quick™ (without surfactant POEA) to aquatic crustacean *Daphnia magna*, marine bacterium *Vibrio fischeri*, soil bacterium *Pseudomonas putida*, intestinal bacterium *Escherichia coli* and three soil bacteria, isolated in our laboratory from the local soil, was studied.

In addition, outdoor experiments were performed. The bottom of the test containers was first filled with sand and then about 11 cm of natural soil was added. Soils were spiked with Roundup Quick™ and Roundup Max™ in different doses: recommended for herbicidal use (245 mg/m^2) and 100-fold; 300-fold and 1000-fold higher than recommended. Containers were exposed under natural climate conditions for four months (April-September 2012) during which the number of heterotrophic bacteria was assessed. Residual contamination in the spiked soils was evaluated with two crop species,

commonly used in Estonia: horticultural crop, red radish (*Raphanus sativus*) and agricultural crop, field-grown barley (*Hordeum vulgare*) 24, 47, 68, 82 and 110 days after the spiking. According to the producer's information, seeding of crops should take place not less than 30 days after applying glyphosate formulations (Monsanto, 2012).

To evaluate the potential risk of contaminated soils to aquatic ecosystem, the toxicity of aqueous leachates (1:10) of the soils, collected 10 days after the spiking, to crustacean *D. magna* and bacterium *V. fischeri* was investigated (Figure 7).

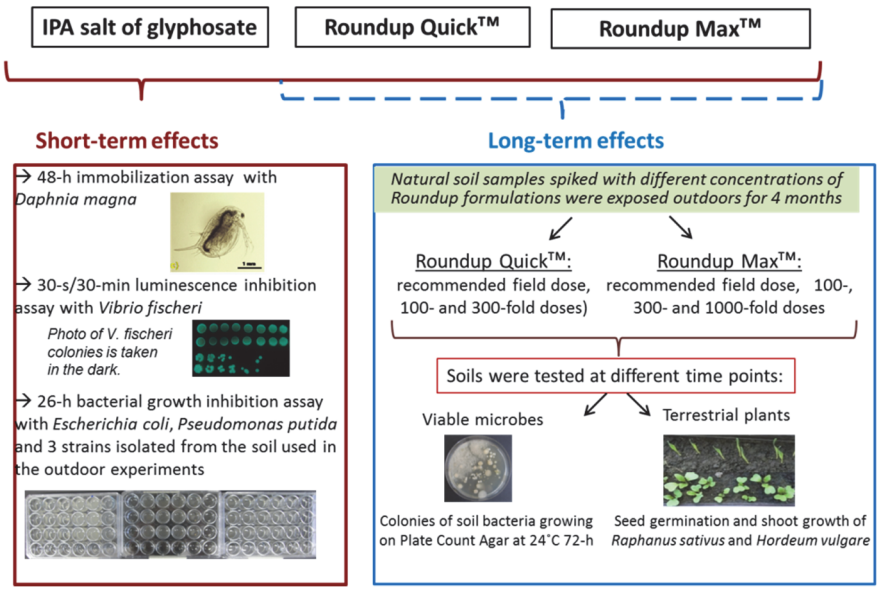

Figure 7. Scheme of experiments with glyphosate formulations

3.3.2. Toxicity of glyphosate formulations to different organisms

Laboratory studies

Tested chemicals showed different toxicity to (non)target organisms. The tested soil microbial strains were less sensitive to glyphosate formulations than aquatic species. IPA salt of glyphosate was about 10-fold more toxic to *D. magna* than Roundup formulations whereas there was no significant difference ($p > 0.05$) between Roundup Max™ and Roundup Quick™. Roundup Quick™ was slighly more toxic to *V. fischeri* than Roundup Max™ and IPA salt of glyphosate. Different toxicity of the tested chemicals to biota may be explained by the different composition of Roundup Max™ and Roundup Quick™.

Field studies

According to Monsanto, there are no glyphosate residues left in the soil after 30 days of application of the glyphosate-based formulations, following

application instructions provided (Monsanto, 2012). Indeed, our experiments revealed that the recommended doses of Roundup Max™ and Roundup Quick™ did not show any toxicity to the target organisms, plants (*R. sativus* and *H. vulgare*), already after 24 days of contamination. Application of glyphosate formulations at higher doses, on the other hand, resulted in toxicity to the two tested plant species, with red radish being less sensitive than barley. In addition, the test results with terrestrial plants and soil microbes showed that it may take longer than the duration of the vegetative period in Estonia for all the glyphosate formulation in the soil to be degraded in case of the highest applied dose of glyphosate (1000-fold of recommended application rate). Surprisingly, the leachates (1:10) of the soils with the highest dose of herbicide were not toxic to non-target aquatic species, *D. magna* and *V. fischeri* after 10 days of spiking.

The experiment revealed that in case of an accidental pollution by glyphosate formulations (e.g. more than 100-fold of recommended field rate), the time needed for self-remediation in typical Estonian climate conditions may exceed the duration of the vegetative period, and applying glyphosate formulations on fields should be planned according to the regulations. No direct relationship between the chemical composition of the tested formulations and toxicity to different (non)target organisms was observed. Roundup Quick™ (without POEA) showed higher toxicity to non-target aquatic bacteria *V. fischeri* but was less toxic to different soil bacteria and terrestrial plants than Roundup Max™. As tested plant species showed different sensitivity, it could be recommended that species from different families should be used to assess the toxicity of soils contaminated with glyphosate formulations.

4. CONCLUSIONS

This Thesis shows the complexity of ecotoxicological testing and interpretation of the test results for different types of pollutants (shale fuel oils, metal-based nanoparticles, glyphosate formulations). It also highlights the importance of the use of non-standardized test methods to perform adequate environmental risk assessment which is essential for deriving environmental quality standards. The main outcomes of the Thesis are following:

- The long-term experiment assessing ecotoxicity of two shale fuel oils (SFOs) under Estonian climate conditions revealed different behaviour of SFOs ("VKG D" and "VKG sweet") in soil matrices. The degradation of both investigated SFOs in the soils was very slow; whereas the mobility of "VKG sweet" with lower viscosity was higher than that of "VKG D". This suggests potential for higher ecotoxicity and environmental hazard of "VKG sweet" than "VKG D".
- No good correlation between ecotoxicological test results and chemical composition of tested SFO samples was revealed. It was shown that such chemical endpoints as total petroleum hydrocarbons C10-C40 or concentrations of polycyclic aromatic hydrocarbons in contaminated soil/water samples do not give an indication on potential toxicity to biota. Based on our results we would suggest using the water accommodated fraction with oil to water ratio 1:10 000 in natural water in the ecotoxicity testing as environmentally the most relevant and cost-effective approach for evaluating the potential hazard of SFOs to aquatic species.
- Analysis of aquatic toxicity of silver nanoparticles (NPs) to crustaceans showed that physicochemical properties of the NPs dictate their toxicity. Moreover, the chemical composition of the exposure medium may substantially mitigate silver NPs toxicity to aquatic crustaceans likely due to their influence on silver bioavailability. Our findings suggested that silver NPs will probably not pose higher hazard to crustaceans than silver ions.
- The test results confirmed low acute toxicity of both, nano- and microsized magnetite (iron oxide, Fe_3O_4) particles to aquatic cructacean *D. magna* and duckweed *L. minor*. However, two days exposure of *D. magna* ephippia or neonates to magnetite, both, nano- and microsized, at concentration of 10 mg/L was significantly affecting the long-term survival and reproductive potential of daphnids.
- Toxicological characterization of glyphosate-based herbicides (Roundup Max™ and Roundup Quick™) showed no direct relationship between ecotoxicity to plants, bacteria and aquatic crustaceans, and chemical composition of respective formulations. We also found that in Estonian climate conditions, the vegetative period may not be long enough for the

biodegradation of glyphosate formulations in soil after an accidental pollution. Therefore, applying glyphosate-based herbicides should be carefully planned to avoid overdosing.

All in all, (i) in addition to the standardized tests other test formats should be used to increase the environmental relevance of the toxicity testing; (ii) even small modifications in the test design (e.g. *D. magna* recovery test; using natural water/soil instead of the artificial one; conducting tests in natural climate conditions) give valuable additional information for environmentally relevant risk assessment.

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ABSTRACT

Ecotoxicological Evaluation of Shale Fuel Oils, Metal-Based Nanoparticles and Glyphosate Formulations

Increasingly more new chemicals are used in all sectors of our fast growing and developing society. As most of the chemicals may be released into the environment through various human activities, their potential hazard to biota and behaviour in the environment needs to be evaluated. The standardized ecotoxicity testing, regulated by legislation, provides basic information on the toxicity of chemicals to aquatic and terrestrial species. However, in addition to standardized ecotoxicological assays, non-standardized formats of ecotoxicological tests that allow various modifications in test parameters should be applied to obtain ecologically more relevant data. The latter is crucial for comprehensive environmental risk assessment and for developing new environmental quality standards.

The aim of this Thesis was to obtain new scientific knowledge on the potential environmental hazards of selected organic chemicals such as shale fuel oils and different glyphosate formulations, and a new emerging class of inorganic chemicals - metal-based nanoparticles. Along with the standardized tests, modified laboratory test formats, e.g. *D. magna* recovery test and the use of natural water/soil as a test medium, were applied. In addition to laboratory tests, outdoor experiments were performed to better understand the behaviour of the chemicals in environment under Estonian climate conditions.

The potential hazard of two types of shale fuel oils (SFOs), produced in Estonia, to aquatic and terrestrial ecosystem was evaluated using toxicological and chemical methods. According to our knowledge, this is the first study of the fate of SFOs in natural soil, performed in Estonian climate conditions. Our experiments showed that the fate of the two SFOs in the environment as well as the toxicity to the tested organisms was different: the lighter SFO was more mobile in soil matrices and more toxic to aquatic and terrestrial species. The comparison of the main chemical parameters (such as the content of C10-C40 hydrocarbons or the sum of 16 polyaromatic hydrocarbons) and ecotoxicity test results of SFO-polluted water and soil samples did not show good correlation, indicating that chemical analysis alone is not sufficiently informative for hazard evaluation of environmental pollution by petroleum hydrocarbons. In addition, we showed that in Estonian climate conditions, the degradation of SFOs in the soils was very slow, and mobility as well as bioavailability to soil organisms depended on the soil type.

The production and use of metal-based nanoparticles (NPs) has increased and the possibility of their release into the environment is expected to rise accordingly. In this Thesis new data on the toxicity of widely used nanosized magnetite (iron oxide, Fe_3O_4) and nanosilver to aquatic organisms is presented. Our data indicated that the toxicity of silver NPs depended on the surface

coating of the particles. We also observed that the chemical composition of the test media (artificial freshwater, natural surface water) significantly affected the bioavailability of NPs to aquatic organisms. Although silver NPs were highly toxic to tested aquatic organisms, their toxicity remained still below the toxicity of soluble silver, which suggests that there is probably no need to consider silver NPs more dangerous to aquatic organisms than soluble silver salts.

Both, nano- and microsized magnetite (iron oxide, Fe_3O_4) induced very low toxicity to aquatic organisms in acute assays. However, it was demonstrated that even short-term exposure to magnetite may affect the daphnids' reproductive potential and, as a result, may lead to changes in the population structure. Thus, more investigations are needed before using magnetite NPs for *in situ* remediation of polluted waterbodies.

Though the toxicity of glyphosate-based herbicides has been widely studied, recent studies have shown that different formulations of this herbicide may not be as safe as assumed, mostly due to the additives (e.g. surfactants). In this study, the toxicity of two different glyphosate formulations to (non)target organisms was evaluated in laboratory and under Estonian climate conditions. We showed that the toxicity of glyphosate to the aquatic (crustaceans and bacteria) as well as terrestrial (bacteria and plants) test organisms depended on the specific composition of the glyphosate formulation. Our experiment revealed that in case of pollution by glyphosate-based herbicides the process of soil self-remediation in Estonian climate conditions is slow. Thus, the application of these herbicides should be carefully planned to prevent overdosing and accumulation in the soil.

In summary, this Thesis showed that to produce relevant data for realistic environmental risk assessment of chemicals, modified test formats in addition to the standardized ones should be applied. In particular, the relatively cost-efficient recovery test with crustacean *D. magna* was found informative for evaluating the recovery potential of crustaceans' population after accidental pollution. The use of different environmental matrices (natural waters and soils) instead of standard test media proved useful to estimate the changes in bioavailability and toxicity of tested chemicals. The use of outdoor experiments in Estonian climate conditions provided essential information about the degradation potential of shale fuel oils and glyphosate-based herbicides. All these data can be used as valuable inputs for evaluating the risks related to the release of these chemicals (shale fuel oils, metal-based nanoparticles and glyphosate formulations) into environment.

KOKKUVÕTE

Põlevkivikütteõlide, metalliliste nanoosakeste ja glüfosaadipõhiste herbitsiidide ökotoksikoloogilised uuringud

Meie arenevas ja kasvavas ühiskonnas võetakse kasutusele aina rohkem uusi kemikaale. Kuna enamik kemikaale võivad erinevate inimtegevuste käigus sattuda ka keskkonda, tuleks nende potentsiaalset ohtlikkust elusloodusele hoolikalt hinnata. Erinevate õigusaktidega reguleeritud standardiseeritud ökotoksilisuse testid annavad kemikaalide (öko)toksilisuse kohta üldinformatsiooni, kuid põhjalikumaks keskkonnaohtlikkuse hindamiseks ja uute keskkonnakvaliteedi standardite väljatöötamiseks tuleks teatud juhtudel täiendavalt kasutada ka modifitseeritud testmeetodeid.

Käesoleva doktoritöö eesmärgiks oli uurida valitud orgaaniliste kemikaalide – põlevkiviküttеõlide ja glüfosaadipõhiste herbitsiidide, ja anorgaaniliste kemikaalide – metalliliste nanoosakeste potentsiaalset keskkonnaohtlikkust kasutades võrdlevalt standardiseeritud ja modifitseeritud (nt. *D. magna* kemikaalistressist taastumise test, loodusvee ja erineva koostisega muldade kasutamine) testmeetodeid. Mõistmaks paremini kemikaalide käitumist reaalsetes keskkonnaoludes viidi katseid läbi nii labori- kui ka välitingimustes Eesti kliimas.

Töös uuriti kahe erineva Eestis toodetud põlevkiviküttеõli potentsiaalset keskkonnaohtlikkust vee- ning mullaorganismidele kasutades nii bioloogilisi kui ka keemilisi meetodeid. Meile teadaolevalt ei ole nende põlevkiviküttеõlide käitumist pinnases Eesti ilmastikutingimustes varem uuritud. Tulemused näitasid, et kahe uuritud põlevkiviküttеõli käitumine pinnases ja toksilisus testorganismidele oli erinev: kergem põlevkiviküttеõli oli pinnases liikuvam ning kõikidele uuritud vee- ning mullaorganismidele mürgisem. Põlevkiviküttеõlidega saastatud vee- ja mullaproovide keemilise koostise (nt. süsivesinike C10-C40 ja 16 polüaromaatse süsivesiniku sisaldus) ja ökotoksikoloogiliste katsete tulemuste vahel tugevat seost ei olnud, mistõttu ei saa põlevkiviküttеõlide keskkonnaohtlikkust hinnata ainult nende keemilise analüüsi põhjal. Põlevkiviküttеõlide lagunemine pinnases oli Eesti ilmastikutingimustes aeglane ning nii õlide liikuvus kui ka biosaadavus mullaorganismidele sõltus mulla tüübist.

Metalliliste nanoosakeste tootmise ja kasutamise kasv suurendavad nende keskkonda sattumise võimalust. Nanoosakeste toksilisus ja käitumine keskkonnas sõltuvad suurel määral osakeste füüsikalis-keemilistest omadustest (nt. suurus, pinnakate). Käesolevas töös uuriti magnetiidi (raudoksiid, Fe_3O_4) ja hõbeda nanoosakeste toksilisust veeorganismidele. Tulemused näitasid, et testvee keemiline koostis mõjutas oluliselt nanoosakeste biosaadavust testorganismidele. Hõbeda nanoosakeste ökotoksilisus sõltus ka nende osakeste pinnakattest. Kuigi hõbeda nanoosakesed olid uuritud veeorganismidele väga

toksilised, ei saa neid tõenäoliselt saadud tulemuste põhjal pidada ohtlikumaks lahustuvatest hõbeda sooladest.

Nii nano- kui ka mikrosuuruses magnetiit (raudoksiid, Fe_3O_4) oli lühiajalistes katsetes veeorganismidele vähetoksiline. Samas näitasid tulemused, et isegi lühiajaline ekspositsioon magnetiidile võib mõjutada vesikirp *D. magna* paljunemisvõimet ning viia tema arvukuse vähenemiseni. Seetõttu oleks enne nanosuuruses magnetiidi laialdast kasutuselevõttu saastunud veekogude puhastamisel *in situ* tehnoloogiaga vaja läbi viia täiendavaid uuringuid.

Kuigi glüfosaadipõhiste herbitsiidide toksilisust on laialdaselt uuritud, näitavad mitmed hiljutised uuringud, et antud taimekaitsevahendid võivad erinevate lisandite/pindaktiivsete ainete tõttu olla mitmetele organismidele siiski ohtlikud. Käesolevas töös uuriti kahe erineva koostisega glüfosaadipõhise herbitsiidi toksilisust (mitte)sihtmärk organismidele nii labori– kui ka välitingimustes Eesti kliimas. Tulemused näitasid, et uuritud herbitsiidide toksilisus vee (vähilised ja bakterid) – ja mullaorganismidele (bakterid ja taimed) sõltus nende koostisest. Juhureostuse puhul oleks pinnase isepuhastumine meie parasvöötme ilmastikutingimustes aeglane ning nii üledoseerimise kui ka jääkreostuse vältimiseks tuleks glüfosaadipõhiste herbitsiidide kasutamist hoolikalt planeerida.

Töö tulemused näitavad, et kemikaalide keskkonnaohtlikkuse hindamisel tuleks lisaks standardiseeritud testformaatidele kasutada ka täiendavaid testmeetodeid. Näiteks on vesikirp *D. magna* kemikaalistressist taastumise test väga informatiivne ja kuluefektiivne võimalus hinnata populatsiooni taastumise potentsiaali. Erinevate keskkonnamaatriksite (nt. loodusveed ning erineva koostisega mullad) kasutamine standardiseeritud testmaatriksite asemel võimaldab hinnata kemikaalide biosaadavust ja toksilisust reaalsetes keskkonnatingimustes. Välikatsete läbiviimine võimaldab hinnata kemikaalide toksilisust ning degradatsiooni Eestile unikaalsetes kliimatingimustes. Kogu eelpool loetletud teave kemikaalide (põlevkivikütteõlid, metallilised nanoosakesed, glüfosaadipõhised herbitsiidid) toksilisuse ja käitumise kohta on oluline nende keskkonnaohtlikkuse hindamisel.

APPENDIX A. PUBLICATIONS

PAPER I

Kanarbik, L., Blinova, I., Sihtmäe, M., Künnis-Beres, K., Kahru, A. (2014). Environmental effects of soil contamination by shale fuel oils. Environmental Science and Pollution Research. 21, 11320–11330.

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RESEARCH ARTICLE

Environmental effects of soil contamination by shale fuel oils

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Abstract Estonia is currently one of the leading producers of shale oils in the world. Increased production, transportation and use of shale oils entail risks of environmental contamination. This paper studies the behaviour of two shale fuel oils (SFOs)—'VKG D' and 'VKG sweet'—in different soil matrices under natural climatic conditions. Dynamics of SFOs' hydrocarbons (C10–C40), 16 PAHs, and a number of soil heterotrophic bacteria in oil-spiked soils was investigated during the long-term (1 year) outdoor experiment. In parallel, toxicity of aqueous leachates of oil-spiked soils to aquatic organisms (crustaceans *Daphnia magna* and *Thamnocephalus platyurus* and marine bacteria *Vibrio fischeri*) and terrestrial plants (*Sinapis alba* and *Hordeum vulgare*) was evaluated. Our data showed that in temperate climate conditions, the degradation of SFOs in the oil-contaminated soils was very slow: after 1 year of treatment, the decrease of total hydrocarbons' content in the soil did not exceed 25 %. In spite of the comparable chemical composition of the two studied SFOs, the VKG sweet posed higher hazard to the environment than the heavier fraction (VKG D) due to its higher mobility in the soil as well as higher toxicity to aquatic and terrestrial species. Our study demonstrated that the correlation between chemical parameters (such as total hydrocarbons or total PAHs) widely used for the evaluation of the soil pollution levels and corresponding toxicity to aquatic and terrestrial organisms was weak.

Keywords Shale fuel oil pollution · Natural attenuation · Biodegradation · PAHs · Ecotoxicity · Risk assessment · Crustaceans · Bacteria

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Introduction

In Estonia, there is the largest industrially used oil shale basin in the world. Oil shale is a sedimentary rock containing up to 50 % of organic matter. In 2012, over 85 % of the mined oil shale was used to produce electricity and for heat generation; the rest was used to produce shale oil, retort gas and chemicals (Khitarishvili 2013).

Shale oil, also known as kerogen oil or oil shale, is unconventional oil produced from oil shale by pyrolysis, hydrogenation or thermal dissolution. These processes convert the organic matter (kerogen) from the rock into synthetic oil and gas. The resulting oil can be used as a fuel in, e.g., boiler houses, industrial furnaces, marine engines and boilers. In Estonia, currently, different fractions of shale fuel oil (SFO) are produced and annual production of SFOs is about 0.5 million tons (Estonian Statistics 2014) that will be increased up to 1 million tons in the near future. Growing production, transportation and use of SFOs entail risks of environmental contamination. According to the European Chemicals Agency, (ECHA 2014) shale oils are high-production volume industrial chemicals (produced in Europe 100,000–1,000,000 tonnes per annum) having a proxy for high exposure for environment. Thus, SFOs must be evaluated for their potential hazardous effects to the environment.

According to the information from the respective chemical material safety data sheets, SFOs are classified as hazardous to the environment as they may cause long-term adverse effects to the aquatic organisms. However, the knowledge on their behaviour and toxicity in the soil and water ecosystems is still very limited. The physicochemical properties of raw shale oil vary with the shale's composition and the extraction technology. Raw shale oils typically contain 0.5–1 % oxygen, 1.5–2 % nitrogen and 0.15–1 % sulphur and have different density, viscosity and content of phenols (Lee 1991). As a rule, the detailed composition of the specific brand of fuel oil is not available, which impedes the prediction of the toxicity of SFOs and evaluation of real risks to living organisms on the basis of concentrations of individual hazardous components. Moreover, toxicity of the mixture depends on the bioavailability of individual compounds in soil and water matrices. The complexity of SFOs containing hundreds of toxic compounds and their large variability in terms of composition complicates the assessment of human and environmental health risks associated with accidental oil spills.

For the evaluation of the realistic environmental risks, the information on the behaviour of SFOs in the soil and water compartments (migration in the soil, degradation rate, etc.) and hazard for living organisms is needed. In addition, in case of accidental oil spills, relevant information on the mobility and toxicity of SFOs is needed for the remediation actions. Currently, the relevant information on the behaviour of SFOs assessed in conditions mimicking the natural environment is largely absent. This study aims to fill these data gaps.

The main goal of our study was to generate new scientific knowledge on the behaviour and ecotoxicity of SFOs in the soils by combining chemical and ecotoxicological approaches.

To increase the relevance of the new data for the risk assessment, the experiments were performed (i) using two commercial SFOs, both produced in high quantities in terms of REACH; and (ii) outdoors, i.e. under natural Estonian (boreal) climate conditions.

Materials and methods

Shale fuel oils (SFOs)

Two different commercial distillate fractions of refined shale oil ('VKG D' and 'VKG sweet') produced by Estonian industry VKG Oil AS were investigated. VKG D (further designated as 'D') is a shale oil fractions mixture (70 % heavy+30 % middle) with a density of 1,008.5 kg/m^3 at 15 °C (ASTM D 4052) and sulphur content of 0.59 % wt. (ASTM D 4294). VKG sweet (further designated as 'SW') is a shale oil fractions mixture (70 % middle+30 % heavy) with a density of 992.1 kg/m^3 at 15 °C and sulphur content of 0.63 % wt. According to product material safety data sheet, water solubility of average shale oil sample is 0.22 g/l.

Experimental design

Natural soil and sand samples used in this study were collected from the Lake Ülemiste catchment area which may be regarded as unpolluted as this lake is the main drinking water source for Tallinn—the capital of Estonia—and its watershed has been under protection during the past decades. Natural top soil samples (20 cm) were collected from clean agricultural land non-treated with pesticides. Sand samples represent the subsoil horizon widely spread around Lake Ülemiste; this sand is also often used in road building. Soil and sand samples were dried at room temperature and were sieved through a 2-mm mesh. The main chemical and physical characteristics of the samples are given in Table 1.

Samples of natural soil and sand were spiked with SFOs (D and SW) and exposed during 1 year in polyvinyl chloride (PVC) plastic containers (20 cm in diameter and 26 cm in height) outdoors under natural Estonian weather conditions (Supplementary material, Fig. S1). Test containers had no bottom and were placed on the sandy ground to ensure good drainage and to model natural water regime. The water passing through the soil/sand samples in the test containers was not monitored. The experiment was performed in two different settings to evaluate (i) the migration and (ii) the degradation of SFOs in the natural soil and sand (Supplementary material, Fig. S2). For each treatment, the experiments were performed in two replicates.

In the migration experiment, the test containers were filled with 20-cm thick substrate layer. The initial (at the beginning of the experiment) density for natural soil and sand were 1.2 and 1.3 g/cm^3, respectively. At the start of the experiment, SFOs were evenly poured directly on the soil/sand surface with a pollution load of 1 ml/cm^2.

Table 1 Main parameters of natural soil and sand used in the experiments

	Natural soil	Sand
pH [KCl]	7.0	8.5
C_{org} [%]	3.5	<0.3
P, mg/kg	128	13
K, mg/kg	196	40
Ca, mg/kg	4,773	386
Mg, mg/kg	114	32
Specific surface area [cm^2/g]	16,627	1,200
Particle size distribution		
0.5–0.063 mm [%]	7.0	91.4
0.063–0.002 mm [%]	77.1	7.8
<0.002 mm [%]	15.9	0.8

In the degradation experiment, natural soil and sand were mixed with SFOs to obtain concentrations of 10 and 50 g/kg fresh weight (soil moisture content ~10 %). In order to obtain homogenous mixing, the procedure described by Brinch et al. (2002) was used. Briefly, SFO was first mixed with acetone and then added to part of the soil (~20 %), mixed and after evaporation of the acetone, the oil-spiked soil was mixed with the remaining part (80 %) of the soil. Soils were sampled for the ecotoxicity testing and chemical analyses from unused test containers after 1, 3, 6 and 12 months of spiking. Sampling time was chosen on the basis of preliminary assay performed in laboratory conditions (data not shown).

Chemical analysis

From the oil-spiked and control soils and their aqueous leachates the following parameters were determined: hydrocarbons > C10–C21 and > C21–C40; sum and individual concentrations of 16 PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene. Hydrocarbons C10–C40 in soils (ISO 16703:2004) and in the aqueous leachates (ISO 9377–2:2000) were measured using a gas chromatograph with a flame ionisation detector (CD-FID). The content of 16 PAHs in soil and water samples was measured using a gas chromatograph with a mass spectrometric detector (GC-MS) according to ISO 18287:2006.

Number of the soil heterotrophic bacteria

The number of soil heterotrophic bacteria in oil-spiked soils was detected by the plate count method using spread plate technique and Difco Plate Count Agar (PCA). Briefly, 30 ml of sterilised Milli-Q water was mixed with 1 g of soil/sand sample and shaken for 30 min on an orbital shaker (160 rpm, room temperature). The soil/sand slurry was then used for the preparation of the serial decimal dilutions. At least two replicate plates were inoculated from each dilution at room temperature (22–24 °C) in the dark. The colony forming units (CFU) were counted after 3 days and the counting was repeated after 5 days of incubation. The results are presented as CFU of bacteria per gramme of dry soil.

Phytotoxicity assays

Residual toxicity of contaminated soils in the degradation experiment was evaluated towards two common agricultural plants (mustard *Sinapis alba* and barley *Hordeum vulgare*) using the inhibition of seed germination and root/shoot growth as toxicity endpoints. Two different test formats were used: (i) a traditional terrestrial plant test format as described in OECD 208 (2006) and (ii) a modified test format (Phytotoxkit™ 2004).

In the Phytotoxkit™ test format (plate test), the contaminated soil samples (90 cm^3) were placed on transparent test plates (21×15.5×0.8 cm), covered with a black filter paper and ten plant seeds were placed on the filter paper (Supplementary material, Fig. S3). The test plates were closed with a transparent lid and incubated in a vertical position at 25 °C in darkness for 3 days (*S. alba*) or for 6 days (*H. vulgare*). Different test durations were used as different time is needed for germination of these species. At the end of the incubation, the seed germination, the length of roots, and shoots was measured. The test was made in three replicates.

In traditional OECD 208 test, 10–15 seeds of *H. vulgare* were put in contact with tested soil samples (300 g) and were exposed, not covered (Supplementary material, Fig. S3). The test containers were incubated at room temperature (20–21 °C) at natural light–dark cycle for 11 days. At the end of the incubation, the seed germination and the length of the shoots was recorded. The test was carried out at least in two replicates.

Toxicity to aquatic species

The acute assays with crustaceans *Daphnia magna* and *Thamnocephalus platyurus* and bacteria *Vibrio fischeri* were used for toxicity evaluation of the aqueous leachates of oil-spiked soils. The leachates of the soils were prepared in mineral medium used for the crustacean *D. magna* acute immobilization assay (OECD 202 2004) in ratio 1:10 (100 g of dry soil per 1 L of medium), shaken at 145 rpm at 21 °C for 24 h in the dark, suspensions were let to settle during 3 h, supernatant was decanted and filtered (Whatman filter paper, grade 1, pore size 11 μm; Supplementary material, Fig. S4).

The immobilization test with *D. magna* was performed according to modified standard test procedure (OECD 202 2004). Namely, less than 24-h-old *D. magna* neonates, obtained by the hatching of ephippia (MicroBioTests Inc, Belgium) were exposed to different concentrations of soil aqueous leachates at 20 °C for 48 h in the dark. In the mortality test with *Thamnocephalus platyurus,* the <24-h-old larvae obtained from cysts were incubated in the dark at 25 °C for 24 h (Thamnotoxkit F™ 1995). The tests with *D. magna* were performed in four and with *Thamnocephalus platyurus* in three replicates.

Acute luminescence inhibition assay with bacteria *V. fischeri* (exposure time 30 s and 30 min) was performed at 20 °C using automated tube-luminometer 1251 connected to a computer operated by Multiuse software (both BioOrbit, Finland) following the Flash-assay protocol (ISO 21338:2010). Analyses were made in polypropylene assay cuvettes containing 0.4 ml of the reaction mixture. The exact

procedure is described in Põllumaa et al. (2000) and Mortimer et al. (2008) except that the inhibition of bacterial bioluminescence was calculated as percentage of the unaffected (negative) control (2 % NaCl). Reconstituted *V. fischeri* reagent (Aboatox, Turku, Finland) was used for testing. NaCl was added to aqueous soil leachates (1:10) to adjust the salinity to 2 % NaCl in the test. Each test was performed in 5–7 replicate dilutions. Controls, both negative (2 % NaCl) and positive (3,5-dichlorophenol), were included in each measurement series. Samples were continuously mixed during the recording of luminescence.

All aquatic assays were carried out at least in three replicates. The EC50 values are expressed as percentage of the leachate in the test medium (*v/v*).

Results and discussion

Shale fuel oils (SFOs), similarly to other petroleum-based fuels, are complex mixtures of various compounds in different ratios: hydrocarbons (paraffins, naphtenes, olefins, aromatic compounds), neutral and acidic oxygen compounds (phenols, etc.), as well as sulphur- and nitrogen-containing compounds, etc. (Veldre and Jänes 1979). Therefore, in case of contamination by SFOs, the measurement of the concentration of each individual compound is practically not applicable due to very high costs of available analytical methods. The most common approach to monitor the petroleum contamination level in the environment is the determination of total petroleum hydrocarbons and concentration of PAHs. Estonian environmental legislation regulates the total content of petroleum hydrocarbons (C10–C40) and PAHs in the soil (RT 2010a) as well as in the groundwater (RT 2010b). In the current study, the fate of SFOs in spiked soils was followed using total concentration of C10–C40 hydrocarbons and individual and sum of 16 PAHs listed as the 'priority pollutants' by the US Environmental Protection Agency (Larsson et al. 2013) and European Commission (EC 2006).

The toxicity of bioavailable fraction of SFO in spiked soils was evaluated by measurements on ecological receptors relevant to (i) soil ecosystem (analysis of the effects on soil microbial numbers and germination efficiency of seeds of higher plants) and (ii) aquatic ecosystem organisms (bacteria *V. fischeri* and crustaceans *D. magna* and *T. platyurus*). As the analysis of experimental data revealed a very good correlation (r^2=0.92) between the toxicity of leachates from contaminated soils to crustaceans *D. magna* and *T. platyurus*, only data for *D. magna* are presented.

The fate of the contaminants in soils is strongly influenced by the physicochemical properties of pollutants and the site-specific variables such as soil matrix and weather conditions (temperature, precipitations, etc.) (National Research Council 2003). The latter is a very important factor which limits the application of the laboratory-scale results for the prediction of contaminant behaviour in the soils under real environmental conditions (Harms and Bosma 1997; Kirso et al. 2007). In particular, the viscosity (and accordingly migration rate) of SFOs may change depending on the soil temperature. Another important determinant factor, which is very difficult to simulate in the laboratory, is water regime in the soils. Therefore, to increase the practical value of the results, the long-term experiment (from May 2012 to May 2013) was performed outdoors to mimic natural weather conditions.

The migration of shale fuel oils (SFOs) in the soils

In case of an accidental oil spill, one of the essential parameters needed for the risk assessment is the migration rate of the fuels to the deeper layers of the soil column. The vertical migration of the SFOs SW and D in natural soil and sand was evaluated using chemical and biological methods. The SFO pollution load 1 ml/cm^2 used in the experiments corresponds to the contamination level in case of moderate accident. The dynamics of hydrocarbons' concentrations and number of heterotrophic bacteria were assessed in the 3-cm bottom slice located 10 cm (10–13 cm) under the treated surface (Supplementary material, Fig. S2). As SFOs percolated through soil/sand column non-uniformly, the evaluation of the hydrocarbons' mobility was based on the average concentration in the 3 cm bottom layer which was separated from the soil column, mixed and analysed.

The experiment revealed that the downward migration of both tested SFOs in the natural soils was very slow. Indeed, 3 months after the treatment, the total content of hydrocarbons (C10–C40) in the bottom layers only slightly increased (Table 2) and then decreased to the background (control) level during the next 6 months. The concentration of PAHs (sum of 16 PAHs) in the bottom layers was below the detection limit of the analytical methods applied (<0.2 mg/kg for individual PAHs or <3 mg/kg for the sum of 16 PAHs). Thus, it could be concluded that the most intensive migration of the mobile components of the SFOs in the natural soil occurred during the first 3 months (June–August) and afterwards, the migration of both tested SFOs was negligible. However, though chemical analysis did not show hydrocarbons' contamination in the bottom layers of soils after 6 months of exposure, the higher number of heterotrophic bacteria compared to the control soil (Table 2) indicates that bacteria were active in the biodegradation process. Hence, some organic contaminants still migrated to the bottom layers, but the bacteria were capable to biodegrade that.

As the migration of SFOs in the soil column may pose a threat to the surface and groundwater, the potential risk of contaminated soils to aquatic ecosystems was also evaluated. The toxicity testing of the soil leachates (1:10) from the bottom layers of soils were performed. According to our

Table 2 Average content of hydrocarbons and the total number of heterotrophic bacteria at different sampling times of the soils spiked with different shale fuel oils (SW and D) in the soil layer at a depth of 10–13 cm

Time of sampling	Month 3 (August)		Month 6 (November)		Month 12 (May)	
Sample	C10–C40, mg/kg dw	Bacteria, 10^6 CFU/g dw	C10-C40, mg/kg dw	Bacteria, 10^6 CFU/g dw	C10–C40, mg/kg dw	Bacteria, 10^6 CFU/g dw
Control soil	40	7.00±0.75	25	4.33±0.32	21	8.62±0.96
Soil + SW	92.5	8.96±0.70	29	10.74±1.15	<20	12.87±0.24
Soil + D	61	3.97±0.16	31	8.52±0.71	<20	19.77±0.71
Control sand	<20	1.47±0.15	20	1.79±0.09	20	1.81±0.05
Sand + SW	8,200	2.80±0.37	13,400	2.37±0.15	NA	NA
Sand + D	4,280	1.31±0.08	2,290	1.23±0.06	NA	NA

CFU colony forming units, *dw* dry weight, *NA* not analysed

results, the leachates from the bottom layers of soils spiked with D were not toxic to aquatic organisms during the whole duration of the experiment, whereas in case of soils spiked with SW, the toxicity of leachates to *D. magna* increased from non-toxic at month 3 and month 6 to toxic (LC50=25 %, *v/v*) at month 12 (data not shown). The concentrations of hydrocarbons in tested leachates were very low (below analytical detection limit, <0.03 mg/L) and cannot explain the observed toxic effect. Therefore, it could be assumed that SFO SW contained also other mobile toxic compounds which migration rate was slower than that of C10–C40 hydrocarbons and these contaminants reached the bottom layers only after 6 months of exposure. These results once more confirm that chemical analysis alone is not enough for the realistic assessment of soil contamination level.

Interestingly, after 3 months of treatment, live earthworms were found in non-treated (control) test containers as well as in the bottom layers of SFO-treated soils indicating that the concentration of total hydrocarbons up to 92.5 mg/kg (Table 2) is not toxic to earthworms.

It is known that the higher organic matter content and the smaller soil particles size support the higher sorption of hydrophobic organic compounds (bound-phase) to the soil matrix (Means et al. 1980; Van Gestel et al. 2003; Nam et al. 1998). Therefore, in the sand, both SFOs were remarkably more mobile than in the humus-rich natural soil (Table 2). The content of hydrocarbons in the bottom layers of sands exceeded that in the soil about 90-fold for SW and about 70-fold for D already at month 3 after the spiking. The higher mobility of SFO SW resulted also in higher toxicity of the respective aqueous leachates (1:10) from the bottom layers of sands to tested aquatic species. The toxicity testing showed that leachates from the bottom layers of the sand were very toxic to crustacean *D. magna* and bacteria *V. fischeri* already after 3 months of spiking. The toxicity of the leachates of the sand samples spiked with SW and D to *D. magna* (EC50, %, *v/v*) was 4.4 and 13.7 %, respectively, and to *V. fischeri* (30 min, EC50, %, *v/v*) 1.2 and 3.6 %, respectively. At month 6 after the spiking, the toxicity of the sand's leachates to aquatic species slightly decreased in case of D, whereas it remained unchanged (no statistically significant differences) in case of SW that is in the agreement with chemical analysis (Table 2). Thus, experimental results showed that in oil-spiked sand analogously to oil-spiked natural soil the highest rate of migration of pollutants occurred during the first 3 months. The share of lighter hydrocarbons' fractions of SFOs (>C10–C21) in total (C10–C40) hydrocarbons' content in the bottom layers of sands gradually increased in time indicating that heavy fraction (>C21–C40) is more prone to adhere to the sand matrix. Both chemical and biological methods showed that SFO SW with lower viscosity was more mobile in sands than heavier fraction D.

As the number of heterotrophic bacteria in the bottom layers of SFO-spiked sands did not significantly differ from the bacterial number in the control sand (Table 2), the degradation of SFOs in the bottom layers of sands by bacteria was probably negligible.

The experiment showed that in case of an accidental spill of fuel oil in the area covered by sand or soil with low content of organic matter the contaminated upper surface layer must be promptly removed in order to prevent the spreading of the pollution and contamination of the surface or groundwater.

The degradation of shale fuel oils (SFOs) in the soils

Dynamics of C10–C40 hydrocarbons' content in SFO-contaminated soils

Unlike the air or water compartments, the photodegradation of SFOs in the soils can be considered negligible since only a very thin surface layer of soil is affected by the sun and thus, the removal of SFOs from the contaminated soils is mainly a result of three processes: biodegradation, migration in the soil matrix and volatilization (MacNaughton et al. 1999). Volatilization usually concerns the volatile low-molecular weight compounds (C <10) and therefore the effect of volatilization

on the decrease of C10–C40 hydrocarbons' concentration in tested soils may be considered negligible. Moreover, the crust formed on the soil surfaces in the test containers already by month 3 also reduced the air exchange between the soil and atmosphere and, as a result, possible volatilization. Therefore, the microbial degradation and migration were probably the main processes regulating the fate of hydrocarbons in tested soil matrices. To minimise the impact of the oils' migration on the test results, the 20-cm thick layer in the test containers was thoroughly mixed before the sampling. Pollutants' biodegradation rates were evaluated separately for two contamination levels: 10 and 50 g of SFO kg^{-1} of soil.

The results of the outdoor experiment showed very low degradation rates for both tested SFOs. The content of total hydrocarbons (C10–C40) in both tested matrices (natural soil and sand) decreased only by 20–25 % on average after 6 months of spiking whereas the decline of hydrocarbons' concentrations in soils contaminated with SW was slightly lower (but the difference was not statistically significant) than in case of spiking with D. During the next 6 months (November–May) of the experiment, the decrease of the content of hydrocarbons in spiked soils continued but no statistically significant changes were observed for both tested SFOs.

It could be assumed that in case of oil-spiked natural soils, the main factor affecting the concentration change of hydrocarbons was the biodegradation as the migration of SFOs in soils was very slow (see above). The number of heterotrophic bacteria in the spiked natural soils considerably exceeded the bacterial number in the control soil indicating that SFOs can be growth substrates for autochthonous soil bacteria. A good correlation (r^2=0.7) between hydrocarbons' concentrations in tested natural soils and number of heterotrophic bacteria at month 3, 6 and 12 illustrate the biodegradation potential of SFOs in natural soils.

The biodegradation rate depends on the availability of the biodegradable pollutants to microorganisms in the soil matrix. The organic contaminants exist in the soils in bound-phase and released state (liquid phase and dissolved phase). The degradation of contaminants in the bound-phase is slow or absent even under optimal conditions (Eriksson et al. 2000; Weissenfels et al. 1992; Bogan and Sullivan 2003; Haritash and Kaushik 2009). It has been demonstrated that the older the contamination, the more strongly it is sorbed to soil particles. Moreover, during ageing, the movement of molecules into the soil micropores can result in their inaccessibility even to the smallest soil organisms (Smith et al. 1997; National Research Council 2003). Thus, the bioavailability to microorganisms and consequently pollution biodegradation rate tend to decrease in time. Among physical factors, temperature plays an important role in biodegradation of hydrocarbons by directly affecting the properties of the pollutants as well as the physiology and biodiversity of microbes (Das and Chandran 2011). At low temperatures, the viscosity of the oil increases delaying the onset of biodegradation (Atlas 1975). Also, temperature affects the solubility of hydrocarbons (Delille et al. 2004). Although the biodegradation of hydrocarbons can occur over a wide range of temperatures, the rate of biodegradation generally decreases with the decreasing temperature (Das and Chandran 2011). Therefore, low temperature along with contamination ageing is a realistic explanation for the decrease of biodegradation rates after month 6 (since October). During months 1–6 (May–October) the temperature was noticeably higher than during the second half of the experiment (November–May; Supplementary material, Fig. S1).

In the sand samples, the dynamics of hydrocarbons in tested 20-cm layers mainly depended on the migration of SFOs since the degradation of SFOs in the sand was negligible. Indeed, in the SFO D-spiked sand, the number of heterotrophic bacteria was 1.5–3-fold lower and in case of SW-spiked soil, only 1.5–2-fold higher than in the control matrices during the entire experiment.

The results obtained from the current study significantly differ from the data presented by Goi et al. (2006) for the shale oil of the same origin. Namely, in laboratory conditions (30 days, 20 °C, in the dark) the biodegradation of SFO in the soil and sand led to removal of 42 and 50 % of the pollution, respectively. The different results obtained by Goi et al. (2006) and in the current study can be explained by the following: (i) although the producer of the SFO studied by Goi et al. (2006) is similar, the chemical composition of 'our' SFOs is different (in the current study heavier fraction was used); (ii) more intensive volatilization of low-molecular weight compounds due to the smaller test volumes used in laboratory tests, (iii) different test conditions and (iv) different analytical methods used. As a rule, the actual biodegradation of organic pollutants in the natural ecosystems is slower than expected from laboratory experiments (Harms and Bosma 1997). It is very difficult to compare the results from laboratory tests performed in stable environmental conditions (temperature, moisture, etc.) and outdoor experiment exposed at changing weather conditions as microbial biodegradation activity heavily depends on all these factors (Atlas 1975; Van Gestel et al. 2003). For example, half life of diesel oil in laboratory experiments at room temperature was 133 days and at elevated temperature 38 days (Van Gestel et al. 2003). In the current study, the impact of the crust formed on the soil/sand surface on water and air flow within test containers and as a result on bacterial activity should be regarded as normal behaviour of the oils in the soil matrix in case of oil pollution.

Dynamics of PAHs in the polluted natural soil and sand

Analogously to other hydrocarbons, the degradation of PAHs in the soil matrix depends on the contaminant type, soil properties and microbial activity (Eriksson et al. 2000). The analysis of the experimental results revealed a good

correlation between the removal of PAHs from the same solid matrix treated either with D or SW (r^2=0.9 in natural soil and r^2=0.7 in sand) after 1 year of exposure (month 12), therefore, in Fig. 1, all microbial degradation results from experiments with both SFOs are pooled. High variability of data may be explained by (i) the pooling of results from different experiments (low and high contamination, different SFOs) and (ii) non-uniform spatial distribution of pollutants in the soil/sand column.

It is known that the low-molecular-weight PAHs composed of two or three aromatic rings are easily biodegraded by microbes but highly hydrophobic high-molecular-weight PAHs with more than four aromatic rings such as benzo[a]pyrene are resistant to microbial degradation (Kastner and Mahro 1996; Juhasz and Naidu 2000; Yi and Crowley 2007; Liu et al. 2011). Our experiment also proved higher degradation rate of low-molecular-weight PAHs (Fig. 1). In the soil samples, 90 % of naphthalene and acenaphthylene was degraded already during the first 3 months (June–August; data not shown).

The chemical analysis of the oil-spiked *natural soils* revealed decline in a total concentration of 16 PAHs at month 12. In case of low treatment with both SFOs, (10 g/kg), the concentration of 16 PAHs in the soils decreased nearly tenfold by month 12 and the residual concentration of PAHs did not exceed 5 mg/kg, the Estonian Environmental Quality Standard target value for soils (RT 2010a). In soils treated with D, the total concentrations of 16 PAHs decreased more rapidly than in soils treated with SW which can be explained by the different composition of tested SFOs. Indeed, in both SFOs, the total content of 16 PAHs was comparable: 1.76 % (D) and 1.9 % (SW) of total hydrocarbons' (C10–C40) content, whereas the abundance of individual compounds was different (Table 3). In particular, SW contained noticeably less naphthalene (the most easily biodegradable compound from these 16 PAHs) than D and more phenanthrene, pyrene and anthracene (very toxic to aquatic species). As naphthalene forms 56 % of the sum concentration of 16 PAHs in case of D and only <1 % remained in the soil after 1 year of exposure, a larger decrease of 16 PAHs in soils treated with D compared to SW was expected.

In the *oil-spiked sands*, the content of PAHs decreased less than twice by month 12 and the residual concentration exceeded target value threefold (D) and ninefold (SW).

As shown in the current study and by other authors (Chung and Alexander 2002; Revitt et al. 2014), the mobility of PAHs in natural soils is negligible and microbial degradation is the dominant mechanism of the breakdown of bioavailable PAHs in the soil (Wang et al. 2014). On the contrary, in the sand, the bacterial growth is limited due to the lack of essential nutrients (nitrogen, phosphorus, potassium) needed for the vital functions of bacteria (Harms and Bosma 1997; Eriksson et al. 2000; Juhasz and Naidu 2000). As biodegradation of the hydrocarbons in the sand was negligible, the observed decrease of PAHs may be explained by leaching from the investigated soil layer and/or evaporation. Though low relationship between solubility and decrease of PAHs concentrations in the sand was revealed, the significant decrease of naphthalene and acenaphthylene content (most soluble of 16 PAHs) may be explained mainly by leaching.

Toxicity of the leachates from oil-spiked natural soils and sands

The leachates (1:10) from oil-spiked natural soil and sand samples collected at different time points (month 3, 6 and 12

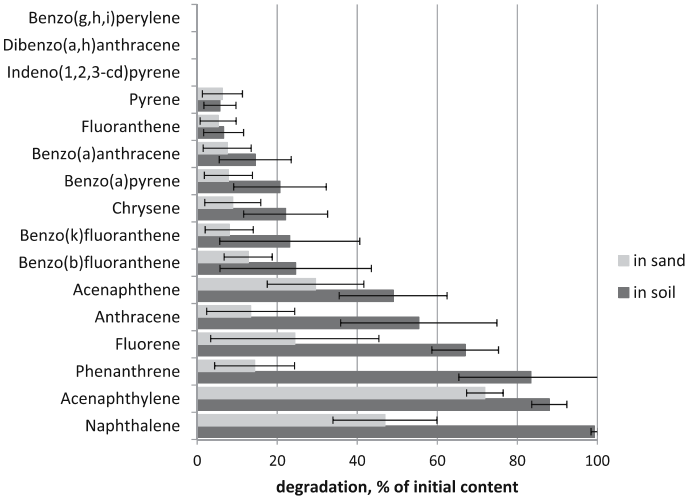

Fig. 1 Decrease of the content of individual PAHs in the shale fuel oil (SFO)-spiked natural soil and sand during 1 year of treatment

Table 3 Content of individual PAHs (% of sum of 16 PAHs) in the shale fuel oils

PAH	Number of aromatic rings	Shale fuel oil D (%)	Shale fuel oil SW(%)
Naphthalene	2	57.5	18.6
Acenaphthene	3	9.3	12.1
Phenanthrene	3	6.0	20.8
Pyrene	4	5.8	9.7
Anthracene	3	4.1	12.1
Benzo(a)anthracene	4	3.8	4.2
Acenaphthylene	3	2.7	3.4
Fluorene	3	2.4	10.8
Benzo(a)pyrene	5	2.0	1.2
Fluoranthene	4	1.9	3.7
Chrysene	4	1.8	2.0
Benzo(b)fluoranthene	5	0.84	0.60
Benzo(g,h,i)perylene	6	0.73	0.20
Benzo(k)fluoranthene	5	0.67	0.39
Indeno(1,2,3-cd)pyrene	6	0.32	0.15
Dibenzo(a,h)anthracene	5	0.14	0.06
Sum of 16 compounds		*100*	*100*

after spiking) were analysed. Although the concentration of the hydrocarbons C10–C40 in all leachates tended to decrease in time, these changes were not statistically significant. In case of lower treatment (10 g/kg), leachates from natural soils treated with both SFOs were not toxic to aquatic organisms *D. magna* and *V. fischeri*. In case of higher treatment (50 mg/kg), the toxicity of soil leachates to aquatic species decreased in time (Table 4). One year after treatment, only the leachate of SW-spiked natural soil was toxic. However, all leachates from contaminated sand samples were toxic to both tested organisms due to (i) lower hydrocarbons sorption capacity of the sand matrix and (ii) negligible degradation in the sand compared to the natural soil. All leachates of SW were more toxic to aquatic species than leachates of D. The latter can be explained by the different chemical composition of these two SFOs. In addition to different PAHs composition, the phenolic compounds may also determinate different toxicity of leachates (Eesti Energia Technology Industries 2014). It has been shown that acute toxicity of shale oils to humans and mammals is generally determined by the toxicity of oil shale phenols (Veldre and Jänes 1979). However, as phenols biodegrade in the soils comparatively rapidly (Baker and Mayfield 1980; Shiu et al. 1994; Kahru et al. 2000; Kahru et al. 2002) it could be assumed that their ecotoxic effect in the long-term perspective is not remarkable. Unfortunately, the concentration of phenols in tested SFOs was not analysed.

Phytotoxicity of shale fuel oil (SFO)-contaminated soils

The determination of the soil phytotoxicity is rapid and reliable method for the evaluation of the bioavailability of contaminants and soil health (Van Gestel et al. 2003). In the

Table 4 Toxicity of natural soil and sand leachates to crustacean *Daphnia magna* and bacteria *Vibrio fischeri* after 3, 6 and 12 months of spiking with shale fuel oils

Aquatic organism		*D. magna,* EC50, %, *v/v*		*V. fischeri,* EC50, %, *v/v*		*D. magna,* EC50, %, *v/v*		*V. fischeri,* EC50, %, *v/v*	
Spiked with	Time since spiking, months	Shale fuel oil D				Shale fuel oil SW			
		10 g/kg	50 g/kg	10 g/kg	50 g/kg	10 g/kg	50 g/kg	10 g/kg	50 g/kg
Natural soil	3	Not toxic	61.7±10.0	Not toxic	23.4±1.5	Not toxic	29.5±8.0	Not toxic	10.4±1.6
	6	Not toxic	89.3±8.0	Not toxic	Not toxic	Not toxic	42.4±7.0	Not toxic	12.9±1.5
	12	Not toxic	Not toxic	Not toxic	Not toxic	Not toxic	47.9±3.0	Not toxic	18.1±1.1
Sand	3	40.4±13.5	9.5±1.5	11.9±1.3	2.7±1.5	17.7±3.2	5.7±2.0	3.6±1.6	1.9±1.5
	6	22.9±5.0	12.1±0.5	12.9±1.2	9.6±1.0	15.7±3.0	5.3±0.3	10.5±0.8	3.2±1.0
	12	47.8±6.2	19.7±2.0	20.0±1.6	7.6±1.2	19.9±1.0	8.7±1.5	12.0±0.9	2.5±1.3

current study, two test formats (see "Materials and Methods"; Supplementary Material, Fig. S3) were used to evaluate the toxicity of treated soils to mustard *S. alba* and barley *H. vulgare*. The plate test format (filter paper separates soil and seeds) shows bioavailability of the released fraction of SFOs, whereas classical bioassay (seeds are sown directly into the soil) evaluate the effect of all fractions of pollutants presented in the soil (including the soil-bound bioavailable fraction).

In the plate assay, toxic effect of soils treated with both SFOs on inhibition of seed germination and growth of shoots and roots decreased nearly twofold by month 12. By the end of the experiment, soils spiked with 10 g oil/kg were not toxic to tested plants, but soils spiked with 50 g oil/kg were still very toxic (inhibition of seed germination was nearly 50 % and shoot growth inhibition ~60 %). These results are in agreement with toxicity of soil leachates to aquatic species and confirm a conclusion that the toxicity of water soluble oil fraction in soils decreases in time.

In case of direct contact of seeds with soil, phytotoxicity of spiked soils increased up to 30 % with time even in case of lower treatment (10 g oil/kg), probably due to the ageing of the contamination. Usually, the effect of ageing of oil contamination is discussed in the context of the reduction of bioavailability of toxic compounds in time. Although ageing reduces the exposure and thus toxicity and risks for biota, it does not eliminate the exposure and risk (Alexander 2000). In case of slowly biodegradable mineral oils (Battersby 2000), the change of soil mechanical and physicochemical properties as a result of hydrocarbons' adsorption on the soil matrix may also significantly deteriorate the condition of plant growth. Indeed, soils treated with SFOs became more and more 'oily' to the touch in time. Different hydrocarbons were strongly bound to soil particles, making nutrients less available to plants. The effect of secondary low soluble metabolites originated from not completely degraded hydrocarbons (Van Gestel et al. 2003) which could be more toxic to plants than initial compounds also cannot be excluded; these results emphasise the importance of bioassays in the risk assessment of contaminated soils. Furthermore, to obtain relevant data for risk assessment the appropriate test format must be used. Results from this study highlighted that in case of contamination by SFOs, the classical test procedure of phytotoxicity assays is more relevant for risk assessment than the plate assay.

It is important that although the residual concentrations of PAHs did not exceed Estonian Environmental Quality Standard target value for soils (5 mg/kg), tested soils were still toxic to plants. The correlation between chemical parameters (hydrocarbons or PAHs) and toxicity of spiked soils to plants was weak.

It was previously shown that phytoremediation could be considered as an alternative management option for remediation of oil shale industry solid waste (Truu et al. 2007). As the results of the current study revealed that inhibition of the seed germination and plant growth did not exceed 60 % even in case of higher treatment (50 g oil/kg), phytoremediation can be applicable also in case of SFO-contaminated soils. Interestingly, in spite of the fact that low-contaminated soils (10 g oil/kg) showed toxicity to tested higher plants after 1 year of exposure, the surface of the treated soils in the containers with both SFOs was completely covered with mosses *Marchantia polymorpha* and *Tortula ruralis* by that time (Supplementary material, Fig. S5). Thus, these moss species are much more tolerant to contamination with SFOs than higher plants used in the current tests.

Conclusions

The long-term outdoor experiments performed under natural climatic conditions yield more relevant data on behaviour of shale fuel oils (SFOs) in soils than short-term laboratory tests.

Although both investigated SFOs (D and SW) had comparable composition in terms of integrated chemical parameters such as hydrocarbons C10–C40 and sum of 16 PAHs, their hazard to the environment was different. SFO SW with lower density poses higher hazard to the environment than the heavier fraction D due to higher mobility in different soil matrices. Also, SFO SW was more toxic to tested aquatic and terrestrial species than SFO D.

The study demonstrated that it is impossible to predict the toxicity of contaminated soils to living organisms only on the basis of integrated chemical parameters (hydrocarbons C10–C40 or sum of 16 PAHs) widely used for evaluation of the soil pollution levels, since these parameters do not have strong correlation with the results from ecotoxicity tests.

We also showed that although autochthonous soil microbial community can utilise the hydrocarbons of SFOs, the degradation of SFOs in the oil-spiked soils was slow. In tested weather conditions (northern part of the temperate climate zone), the decrease of total hydrocarbons' content in the contaminated soils did not exceed 25 % by month 12.

The study revealed that in case of an accidental oil pollution, the territory covered with at least 10-cm layer of natural soil with relatively high content of organic matter (Corg >2–3 %) is much more protective than sandy surface as the risk of surface or groundwater contamination in the former case can be considered low even during several months after accident. However, even in case of moderate (1 ml/cm^2) accidental spill of SFOs in the area covered with sand or poor soil, SFO must be promptly removed to prevent the diffusion of the pollution to the surface or groundwater.

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PAPER II

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Toxicity of Water Accommodated Fractions of Estonian Shale Fuel Oils to Aquatic Organisms

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Abstract Estonia is the worldwide leading producer of the fuel oils from the oil shale. We evaluated the ecotoxicity of water accommodated fraction (WAF) of two Estonian shale fuel oils ("VKG D" and "VKG sweet") to aquatic species belonging to different trophic levels (marine bacteria, freshwater crustaceans and aquatic plants). Artificial fresh water and natural lake water were used to prepare WAFs. "VKG sweet" (lower density) proved more toxic to aquatic species than "VKG D" (higher density). Our data indicate that though shale oils were very toxic to crustaceans, the short-term exposure of *Daphnia magna* to sub-lethal concentrations of shale fuel oils WAFs may increase the reproductive potential of survived organisms. The weak correlation between measured chemical parameters (C10–C40 hydrocarbons and sum of 16 PAHs) and WAF's toxicity to studied species indicates that such integrated chemical parameters are not very informative for prediction of shale fuel oils ecotoxicity.

Abbreviations

AFW	Artificial fresh water
EC50	The median effective concentration of the toxicant that induced a designated effect in 50 % of the test species after a specified exposure time
LR	Loading rate
OWR	Oil-to-water ratio
PAH	Polyaromatic hydrocarbons
SFO	Shale fuel oil
THC	Total hydrocarbons
TPH	Total petroleum hydrocarbons (C10–C40)
WAF	Water accommodated fraction

Oil shale is a sedimentary rock rich in organic matter that is widely distributed around the world. One of the world's largest industrially used oil shale basin is situated in Estonia. Approximately 90 % of the oil shale excavated in Estonia is used for the electricity production in the local thermal power stations and the rest is used for the shale fuel oil (SFO) production (Kahru and Põllumaa 2006). SFOs are used as a supplement to ship fuel in heating boilers and industrial furnaces. Estonia is the world's leading producer of synthetic crude oil (SFO) derived from oil shale by retorting (Francu et al. 2007). Currently, the annual production of SFOs in Estonia exceeds 500,000 tons and tends to increase (Estonian Statistics 2014; ECHA 2015). It is evident that growing production, transportation and use of SFOs as a ship fuel may pose environmental hazard. However, the aspects concerning environmental effects of the SFOs are insufficiently studied. Besides, the inherent variability of different fractions/types of SFOs may result in different toxicological and environmental behaviour depending on the chemical composition of these oils. The information on environmental fate and effects of SFOs is

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crucial for the elaboration of efficient methods to minimize the negative consequences in case of water contamination.

There are two main objectives in the current investigation: (1) to provide new knowledge on the potential environmental hazard of two different SFO fractions to aquatic ecosystem; (2) to address the problems associated with the interpretation of data obtained from laboratory toxicity tests. The latter concerns difficulties in extrapolating the laboratory toxicity data to real water ecosystem and comparing the results between different laboratories.

Materials and Methods

Preparation of Water Accommodated Fractions (WAFs) from Shale Fuel Oils (SFOs) for the Toxicity Testing

Two SFOs, VKG sweet (SW) and VKG D (D), were obtained from the Estonian oil shale enterprise VKG Oil AS. SFO "SW" contains 30 % heavy and 70 % middle fraction of shale oil, whereas "D" is a mixture of 70 % heavy and 30 % middle fraction of shale oil with density of 992.1 and 1008.5 kg/m^3 at 15 °C (ASTM D4052-11 2011) respectively. The sulphur content in both SFOs is ~0.6 wt.% (ASTM D4294-10 2010).

SFOs' WAF solutions were prepared according to the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) method in a closed, low-energy mixing system (Singer et al. 2000; Aurand and Coelho 2005). Briefly, SFO (SW or D) was carefully added to 1-L dilution medium and stirred at 120 rpm (to avoid vortex) at 21 ± 1 °C for 21 h (Fig. S1). The sample was allowed to "rest" for 2 h to confirm the phase separation and carefully decanted from the bottom of the bottle and used for testing within 24 h. WAFs were prepared using three different oil–water ratios (OWR): 1:40 (25 g oil per 1 L of medium), 1:1000 (1 g oil per 1 L of medium), and 1:10,000 (100 mg oil per 1 L of medium) and used as the parental WAFs to prepare the further dilution series for toxicity testing as described by Barron and Ka'aihue (2003).

Two media were used for WAF preparation: (1) artificial fresh water (AFW) for *Daphnia magna* toxicity testing—a solution of mineral salts with no added organics (OECD 2004) and (2) filtered (0.45 μm pore-size filter) water from unpolluted lake (used as water supply source for Tallinn) containing also natural dissolved organic matter (DOC—10.5 mgC/L). No dispersants were used.

Chemical Analysis

The chemical composition of WAFs was described by (1) total petroleum hydrocarbons (TPHs C10–C40: C10–C21 and >C21–C40) and (2) sum and individual concentrations of 16 polyaromatic hydrocarbons (PAHs): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene, and benzo(g,h,i)perylene.

Chemical analysis was performed in a certified laboratory. TPHs (ISO 2000) were measured using a gas chromatograph with a flame ionization detector (GC-FID). The concentration of PAHs was measured using a gas chromatograph with a mass spectrometric detector (ISO 2006).

The concentrations of TPHs and PAHs were measured in undiluted parental WAF solutions (1:40, 1:1000 and 1:10,000); the concentrations of hydrocarbons in the dilution series were calculated.

Toxicity to Aquatic Species

The test battery included four aquatic species belonging to different trophic levels: freshwater crustaceans *D. magna* and *Thamnocephalus platyurus*, marine bacterium *Vibrio fischeri*, and freshwater duckweed *Lemna minor*. The dilution series for bioassays were prepared from each parental WAF (for example, SFO D in lake water with OWR—1:40).

In the acute immobilization test (48 h) with *D. magna* (OECD 2004), <24-h-old neonates obtained by the hatching of ephippia were exposed to different concentrations of WAFs at 20 °C for 48 h in the dark. The tests were performed in 4 replicates.

In the recovery test (18 days) with *D. magna,* <24-h-old neonates (as in the acute test) were exposed to three sublethal concentrations of tested WAFs. After 48 h, 10 actively swimming daphnids were collected from each concentration and further incubated in clean filtered (0.45-μm pore-size filter) lake water. Each neonate was kept separately in 40-mL flask. The medium was renewed once per week, and daphnids were daily fed with the algae *Pseudokirchneriella subcapitata*. The mortality of parent animals and the number of offspring produced was recorded daily. Dead organisms were removed from the test vessel. At the end of the test (after 18 days), the number of organisms alive and the total number of living offspring produced per parent animal alive were calculated.

Acute test (48 h) with *T. platyurus* was performed as described in Standard Operational Procedures of Thamnotoxkit F^{TM} (1995). Briefly, less than 24-h-old larvae obtained by the hatching of cysts were incubated for 24 h at 25 °C in the dark and the mortality was used as the toxicity endpoint. The acute tests with *T. platyurus* were performed in triplicate. *Daphnia magna* ephippia and *T. platyurus* cysts were purchased from MicroBioTests Inc. (Mariakerke-Gent, Belgium).

Acute bioluminescence inhibition assay (30 min) with bacterium *V. fischeri* was performed at room temperature (20 °C) using an automated tube-luminometer 1251 (ThermoLabsystems, Finland) connected to a computer operated by Multiuse software (BioOrbit, Finland) following the modified Flash-assay protocol (Mortimer et al. 2008; ISO 2010). Reconstituted *V. fischeri* reagent (Aboatox, Turku, Finland) was used for testing. All test samples were tested in 2 % NaCl. Inhibition of bacterial bioluminescence by the tested sample was calculated as a percentage of the unaffected control (2 % NaCl).

The growth inhibition test (7 days) with duckweeds *L. minor* was performed as described in OECD 221 (2006) in the filtered lake water. Number of fronds and dry biomass of the plant were used as the test endpoints.

In all bioassays, the toxicity values (EC50) were determined from dose–response curves by the REGTOX software for Microsoft Excel (Vindimian 2009) using the log-normal model. The WAF preparation and its toxicity evaluation were performed at least 3 times. A Student's *t* test was used to assess whether average values of parameters were statistically different from each other. The differences were considered significant, when $p < 0.05$.

Results and Discussion

Chemical Composition of Water Accommodated Fraction (WAF) of Two Shale Fuel Oils

The chemical characterisation of the water accommodated fraction (WAF) plays an important role in the interpretation of the ecotoxicity results. SFOs are very complex mixtures containing a large number of potentially toxic compounds, which cannot be individually measured in the WAF solutions for practical reasons (cost, detection limits of analytical methods etc.). In Table 1, the concentrations of the total petroleum hydrocarbons (TPHs, C10–C40) and the sum of 16 PAHs (the chemical parameters commonly used for the characterisation of oil WAFs) in the investigated WAF stock solutions are presented.

There was a large variation in chemical composition between the WAFs replicates, indicating that the leaching efficiency depends on various factors which may slightly vary in the laboratory, e.g. temperature. High variability of the results also shows that in real environmental conditions leaching of contaminants from the same pollution load of SFO into the water column may significantly vary.

Effect of Oil–Water Ratio on WAF Composition

The chemical analysis showed that, analogously to crude oils (Shiu et al. 1990; Hokstad et al. 1999; Page et al. 2000), the loading rates significantly affected the concentration as well as the composition of individual hydrocarbons in the WAF solutions of SFOs (Table 1). However, unlike Page et al. (2000) who observed a direct correlation between oil loading and TPH concentrations in the petroleum WAFs, no such correlation was revealed in our study. The absence of the strong relationship between loading rate and TPH concentration may be explained by the fact that the oil–water equilibrium in WAFs with the highest OWR was obtained by solution saturation due to the excess of applied oil, whereas in the lowest OWR by maximal leaching of most soluble oil components (Hokstad et al. 1999). Indeed, the percentage of hydrocarbons leached from oil increased sharply with the

Table 1 Concentrations of total petroleum hydrocarbons (TPHs) and the sum of 16 polycyclic aromatic hydrocarbons (PAHs) in the water accommodated fractions (WAFs) of two shale fuel oils (SFOs) extracted using different oil–water ratios (OWR) and media

Type of SFO and extraction medium	Oil-to-water ratio (OWR)	TPHs (C10-C40)		Sum of 16 PAHs	
		μg/L	% of applied[a]	μg/L	% of applied[a]
"SW" in AFW	1:10,000	80 ± 7	0.40	13.5 ± 0.7	3.55
	1:1000	260 ± 64	0.13	43.0 ± 22.6	1.13
	1:40	590 ± 14	0.01	56.0 ± 25.0	0.06
"SW" in lake water	1:10,000	330 ± 179	1.65	19.0 ± 2.8	5.00
	1:1000	930 ± 554	0.47	58.0 ± 42.3	1.53
	1:40	810 ± 390	0.02	57.0 ± 21.0	0.06
"D" in AFW	1:10,000	30 ± 0	0.14	13.0 ± 8.6	3.51
	1:1000	310 ± 71	0.15	66.5 ± 47.4	1.80
	1:40	980 ± 14	0.02	170.0 ± 84.9	0.18
"D" in lake water	1:10,000	70 ± 45	0.33	10.3 ± 2.5	2.77
	1:1000	840 ± 280	0.38	71.5 ± 40.3	1.93
	1:40	910 ± 160	0.02	107.0 ± 18.4	0.12

[a] Percentage of TPH or PAHs leached into the WAF solutions from the applied (100 %) SFOs for WAF preparation

decrease of applied oil load (column *"% of load"* in Table 1). Thus, the extraction efficiency of the water soluble components from the oil phase to aqueous phase was the highest in the OWR 1:10,000. The same trend (namely, extraction percentage of trace elements from the ashes to water was much higher for 1:10,000 solid–liquid ratio than for 1:10) was observed for fly ashes generated during the oil shale combustion (Blinova et al. 2012).

Effect of Dilution Medium on WAF Composition

AFW not containing any dissolved organic matter was used to prepare WAFs as recommended by the European standard EN 14,735:2005 (CEN 2005). The water from the local lake also was used as a dilution medium to increase the ecological relevance of the experiment (Perkins et al. 2003) and to reveal the effect of the water composition on the WAF's toxicity.

As shown in Table 1, the amount of leached hydrocarbons (calculated as % of load) from applied SFO (SW and D) also depended on the chemical composition of the water used for the WAF preparation. The concentration of TPH was higher in the lake water than in the AFW. There were no statistically significant differences between TPH concentrations in 1:40 and 1:1000 WAF solutions prepared in lake water ($p > 0.3$) for both SFOs. However, then AFW was used as dilution media the TPH concentrations in WAF solutions 1:1000 made were more than twofold smaller than in 1:40 ($p < 0.05$). This fact indicates different solubility of the components of SFOs in the dilution media, namely, the equilibrium in oil–water system in AFW has been reached at lower concentrations than in the lake water.

The abundance of different hydrocarbons' fractions in WAFs also depended on the test medium. In all investigated SFOs' WAFs the largest share of TPH was presented by the lighter hydrocarbon fractions (C10–C21). However, the average share of this fraction was less than 1 % of TPHs (C10–C40) in the WAFs prepared in AFW, but increased up to 15.7 % in WAFs made in lake water.

Composition of Individual PAHs in WAF

It has been shown (Lu et al. 2013) that the dissolved organic matter in natural waters may affect the solubility of individual PAHs; therefore, only the data on WAFs prepared in the lake water are presented in Fig. 1. The abundance ratios of individual PAHs significantly differed depending on the type of SFO as well as on OWR (Fig. 1; Table 1). In WAFs made with SFO D, the naphthalene formed the largest portion of total concentration of 16 PAHs and the other 15 PAHs were presented in very small amounts. Differently, in WAFs prepared with SW, naphthalene represented only about 50 % of the extracted 16 PAHs. Therefore, in spite of very similar total concentrations of sum of 16 PAHs (Table 1), e.g. in the WAFs 1:10,000 (10.3 and 13.5 μg/L for D and SW, respectively), the different toxicity of WAFs prepared with SFOs SW and D could be expected due to different concentration of individual PAHs. Thus, the analysis of the composition of PAHs in WAF solutions once more confirmed that such integrated parameter as the sum of 16 PAHs is probably not very informative for toxicity prediction.

Toxicity of Shale Fuel Oil (SFO) Water Accommodation Fractions (WAFs) to Aquatic Species

Sensitivity of Different Aquatic Species to SFOs

Four test species representing different trophic levels were used for the evaluation of the potential toxicity of SFOs to

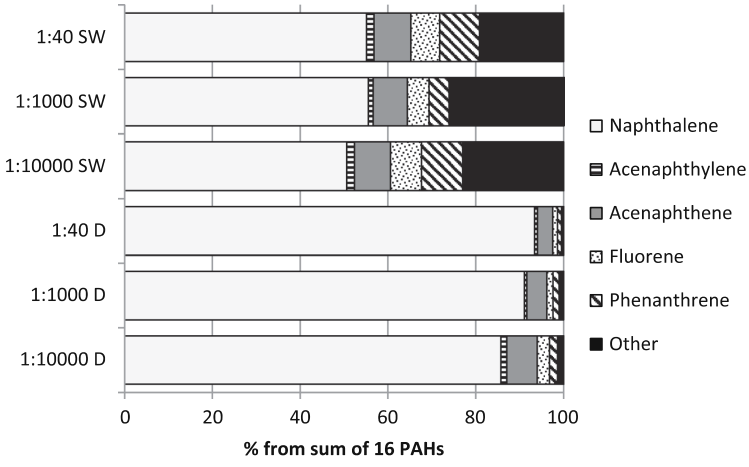

Fig. 1 Concentration of the individual polyaromatic hydrocarbons (% of sum of 16 PAHs) in the water accommodated fractions (WAFs) of shale fuel oils prepared in the lake water

aquatic organisms. Marine bacterium *V. fischeri* and freshwater crustacean *D. magna* are among the most commonly used species for toxicity assessment of different contaminants in aquatic biota. Because crustaceans are very sensitive to oil contamination (Bejarano et al. 2006), an additional crustacean species *T. platyurus* was used in the test battery to compare the sensitivity of the species belonging to the same class *Branchiopoda* but to different orders (*Cladocera* and *Anostraca*). The widespread duckweed *L. minor* was chosen for the phytotoxicity evaluation because data on the toxicity of different crude and refined oils to freshwater plants is very limited (Lewis and Pryor 2013).

The tested crustaceans and bacterium *V. fischeri* showed similar sensitivity to the SFOs. The differences in EC50 values for *D. magna*, *T. platyurus*, and *V. fischeri* did not exceed a factor of 2, whereas EC50 was up to tenfold higher (i.e., SFOs were remarkably less toxic) for *L. minor* than for crustaceans. As an example, EC50 values (presented as WAF v/v %) for WAF 1:40 "D" in lake water were 2.5 (*D. magna*), 3.4 (*T. platyurus*), 4.8 (*V. fischeri*), and 14.2 (*L. minor*). It should be mentioned that the exposure to undiluted WAF 1:10,000 "D" even caused the growth stimulation of duckweed. The stimulatory effect of soluble fraction of SFOs is in agreement with the results of other researches also showed that low concentrations of petroleum WAFs could even promote the growth of phytoplankton in the seawater (Ohwada et al. 2003; Nayar et al. 2005; Jiang et al. 2010).

The toxicity of SFOs' WAF to the test species used in the current study was as follows: *D. magna* ≥ *T. platyurus* > *V. fischeri* > *L. minor* (i.e., crustaceans were the most sensitive and duckweeds the least sensitive species). Such "sensitivity order" is probably valid only for SFOs. In case of other types of contamination, the comparative sensitivity of test species may essentially change. For example, it was shown that *T. platyurus* and *L. minor* were slightly more sensitive to pyrene than *D. magna* (Blinova 2004), but *D. magna* was much more sensitive to phenolic compounds (Kahru et al. 2000) and anilines (Sihtmäe et al. 2010) than algae, protozoa and bacteria.

In the current study, a very good correlation between the toxicity of SFO's WAFs to *D. magna* and to other tested species [*T. platyurus* ($r^2 = 0.93$), *V. fischeri* ($r^2 = 0.87$), *L. minor* ($r^2 = 0.96$)] was revealed. Therefore, the toxicity data only for the most sensitive species (*D. magna)* from the applied test battery are presented in Table 2. The high variability of WAF's toxicity data presented, in particular, may be explained by the high variability of chemical composition between WAF replicates (Table 1).

Influence of Natural Water on the Toxic Effects of SFOs

The chemical composition of natural water may affect bioavailability of hydrocarbons to aquatic biota (Akkanen et al. 2001; Sadani et al. 2011). Our test results showed that despite the higher TPHs and PAHs concentrations in the WAF solutions prepared in the lake water (Table 1), the toxicity of these WAFs was comparable (or slightly lower) than WAFs prepared in AFW (Table 2). The statistically significant differences in toxicity between WAFs prepared in AFW and lake water were recorded only for OWR 1:10,000. So, it may be concluded that the presence of natural dissolved organic matter may decrease the

Table 2 Toxicity of two shale fuel oil water accommodated fractions (WAFs) to *Daphnia magna*

Type of WAF	Oil-to-water ratio (OWR)	48-h EC50 for *Daphnia magna*			
		v/v % of parental WAF	Oil load[a] (mg/L)	TPH[a] (μg/L)	Sum of 16 PAHs[a] (μg/L)
"SW" in AFW	1:10,000	**13.2 ± 3.1**	13.2	10.6	1.8
	1:1000	**3.4 ± 1.0**	34.0	8.8	1.5
	1:40	**0.7 ± 0.5**	175.0	4.1	0.4
"SW" in lake water	1:10,000	**13.7 ± 10.3**	13.7	45.2	2.6
	1:1000	**4.0 ± 3.0**	40.0	37.2	2.3
	1:40	**1.1 ± 0.9**	275.0	8.9	0.6
"D" in AFW	1:10,000	**37.9 ± 7.7**	37.9	11.4	4.9
	1:1000	**8.8 ± 6.4**	87.7	27.2	5.8
	1:40	**0.3 ± 0.0**	75.0	2.9	0.5
"D" in lake water	1:10,000	**51.1 ± 8.8**	51.1	35.8	5.2
	1:1000	**10.0 ± 4.8**	100.0	84.0	7.2
	1:40	**3.2 ± 1.9**	791.7	28.8	3.4

[a] Calculated from mean EC50 values based on the parameters of parental WAFs (loading rate. Total petroleum hydrocarbons (*TPH*) and polyaromatic hydrocarbons (*PAHs*) concentrations and percentage of parental WAF dilution (v/v %; bold)

bioavailability of some toxic substances leached from SFOs into water column to aquatic species.

Recovery of WAF-Exposed Daphnia magna: Survival and Reproduction

In real environmental conditions (e.g. after oil spills), the exposure concentration rapidly declines in time due to the dilution, volatilization, and natural degradation of the pollutants as well as measures applied for the pollution elimination (Short 2003). Therefore, it is unlikely that organisms in the polluted water ecosystem would be continuously exposed to the same WAF concentration for a long period of time, as it is required in the standardized reproduction toxicity test (OECD 1998). The purpose of the recovery test was to evaluate the effect of short-term exposure of *D. magna* neonates to sublethal WAF concentrations on survival and reproduction potential. For that, actively swimming 24-h-old neonates were exposed to sublethal concentrations of WAFs during 2 days ("Materials and Methods" section). After the transfer into the clean water, the mortality of daphnids was observed mainly during the first week (before first brood). However, strong correlation between the pre-exposure concentration and mortality after transferring the neonates into the clean water was not revealed.

Interestingly, the reproductive performance of daphnids exposed to subtoxic concentrations of toxicants was even higher than in the control. Moreover, a good correlation ($r^2 = 0.89$) between the mortality during the recovery test in groups pre-exposed to different WAF concentrations and reproduction potential of survived organisms was revealed (Fig. S2). Namely, the higher the mortality in the group, the higher number of offspring per alive parental female was registered. The same trend was observed for neonates hatched from *D. magna* ephippia in oil-spiked lake water (10 % WAF 1:10,000 "D"). The number of neonates hatched in spike water was in an average 2.5-fold lower than in the control, but the number of offspring per alive organisms was threefold higher than in the control.

Increased reproductive success of another Daphnia species, *Daphnia pulex,* exposed to low concentrations of water soluble fraction of fuel oils was observed also by Geiger and Buikema (1982). Thus, the higher reproduction potential of survived organisms after short-term exposure to subtoxic concentration of fuel oils may to a certain extent compensate the mortality during oil spills and support the recovery of the population after the spill. The investigation of the recovery potential is an ecologically significant as it shows the potential for the restoration of the zooplankton populations after oil spills (Rico-Martínez et al. 2013).

Interpretation of the Results of WAFs' Toxicity Testing

SFOs are traditionally compared to heavy fuel oils. Toxicity of investigated SFOs' WAFs (expressed as THC or PAHs concentrations) to *D. magna* was much higher than previously reported for biodiesel and petro-diesel oils (Hollebone et al. 2008) as well as for different types of crude oils (Hokstad et al. 1999; Jiang et al. 2012; Bellas et al. 2013). However, is such a comparison appropriate to address the toxicity issues?

The evaluation of the hazard of partially soluble compounds or mixtures to aquatic organisms, as a rule, is based on the toxicity investigation of WAFs of these materials. For crude oils or petroleum products, term WAF is applied to "a laboratory prepared solution derived from low energy (no vortex) mixing of test material which is essentially free of particulates of bulk (>1 micron diameter) material" (Aurand and Coelho 2005). The chemical composition and, accordingly, toxicity of oil' WAFs depend on many factors such as oil loading rates, mixing protocols, dilution medium etc. (Barron et al. 1999; Hokstad et al. 1999; Perkins et al. 2003). Thus, the methods used for WAFs preparation play a crucial role in the assessment of potential hazard of oil contamination.

For ecotoxicological investigation of oils and oil products, two approaches for preparing WAF series for toxicity testing are commonly used: (1) variable loading (different OWR are applied to prepare WAF series) and (2) variable dilution, i.e., test series are prepared from one parental WAF solution (e.g. 1:10) by dilution from the same test medium what was used for preparation of parental WAF solution (Singer et al. 2000; Barron and Ka'aihue 2003; Aurand and Coelho 2005). Moreover, in case of variable dilution approach different OWR have been applied to the preparation of the initial (parental) WAF, and the OWRs 1:10 or 1:40 are used most often (Barron et al. 1999; Long and Holdway 2002; Bejarano et al. 2006; Hollebone et al. 2008; Hansen et al. 2011; Hemmer et al. 2011; Bellas et al. 2013). There is still no consensus concerning the preparation procedure of the oil WAF sets for bioassays as both approaches have their advantages and limitations (Barron and Ka'aihue 2003). Due to the methodological variability in tests' designs, it is very difficult to compare the test results obtained in different laboratories (Singer et al. 2000). In the current study, the combination of two approaches was used for the preparation of WAF solutions for toxicity testing (Fig. S1).

Since the highest leaching efficiency of hydrocarbons from the SFO was observed in OWR 1:10,000 (Table 1), this leaching protocol could be recommended as the most appropriate for proactive forecasting of the environmental adverse effects of oil spills. Moreover, the concentrations measured in the WAF solutions 1:10,000 were more

realistic than in the WAFs with OWR 1:40 or 1:1000. For example, according to Boehm et al. (2007), the concentrations of total PAHs in the upper water column under an oil slick did not exceed 41.6 ppb.

In addition to the variability of WAF preparation procedures (see above), the problem that makes comparing the toxicity tests results obtained in different laboratories difficult, is the reporting of exposure concentrations. For oil WAFs, the toxicity is usually presented as: (1) the concentration of pollutant(s) in WAF, i.e., total petroleum hydrocarbons, total hydrocarbons, PAHs, or a certain individual pollutant, (2) the loading rate (LR) (i.e., the amount of oil added to obtain 1 L of WAF), or (3) the percentage of parental WAF dilution (Barron et al. 1999; Long and Holdway 2002; Aurand and Coelho 2005; Hansen et al. 2011; Hemmer et al. 2011; Jiang et al. 2012; Bellas et al. 2013). The various approaches used to report the test results can lead to significantly different conclusions (Perkins et al. 2003, 2005). Latter also may be illustrated by our results on SFOs: in the current study (Table 2), the toxicity of SFO's WAFs (EC50) is expressed alternatively as (1) the percentage of parental WAF dilution (v/v %), (2) the concentration of TPH (μg/L), (3) the total concentration of 16 PAHs (μg/L), and (4) the loading rate (mg oil/L). Three last values were calculated based on the toxicity expressed as the percentage of parental WAF dilution (Table 2, column 3) and parameters of parental WAF (Table 1).

According to the information from the respective chemical Material Safety Data Sheets (ECHA 2015), SFOs were classified as hazardous to the aquatic environment, but no differences between SFOs "D" and "SW" were noted. However, the current study shows that when the LC50 values are expressed as WAF dilution (v/v %) or the sum of 16 PAHs (Table 2), the "SW" WAF solutions were more toxic than that of "D" in both test media (AFW and lake water). These conclusions are in agreement with the data of Kanarbik et al. (2014): SFO "SW" was more toxic to soil ecosystem than "D." However, when the toxicity is reported as the concentration of TPH (Table 2), the difference between two SFOs is not so evident.

In the current study, the relationship between toxicity and concentrations of hydrocarbons (TPH or PAHs) in parental WAFs was not revealed. For example, the differences between the TPH concentrations measured in WAF solutions with OWR 1:1000 and 1:40 prepared in lake water (correspondingly, 840 and 910 μg/L for "D"; 930 and 810 μg/L for "SW") were not statistically significant for either oils ("D" and "SW"). However, the WAFs 1:40 were nearly threefold more toxic compared to the WAFs 1:1000 (EC50 presented as v/v % of parental WAF) for both SFOs (Table 2). The absence of correlation between the toxicity and chemical parameters used in the current study may be explained by the different solubility of individual toxic hydrocarbons in SFOs' WAFs prepared with different OWRs. Also, the presence of other very toxic soluble hydrocarbons (missed by the used analytical methods), e.g. phenols, may play an important role.

Although most of the published data on toxicity of crude oils and oil products are reported based on integrated parameters such as TPH or total PAHs concentrations, this approach has been criticized by many authors. It was shown that often the toxic effect of oil WAFs cannot be explained on the basis of the PAHs concentration (Barron et al. 1999; Bellas et al. 2013). Long and Holdway (2002) stressed that the integrated parameter "TPH" does not include the lower molecular weight hydrocarbons (<C10) that may be more toxic to aquatic organisms than PAH components of crude oil WAFs. Thus, it is also recommended to report the concentrations of the volatile hydrocarbons (<C10) (Singer et al. 2000). Our experiments showed that the toxicity of studied SFO WAFs (1:10,000) to crustaceans significantly decreased after 21 days of ageing of the test solutions in the dark at room temperature (data not presented). The reduction of toxicity of weathered WAFs may be regarded as indirect evidence of an important role of low molecular weight volatile components (for example, $C < 10$ hydrocarbons or phenolic compounds) in the WAF toxicity.

The test results showed that when the toxicity of WAF was expressed as calculated loading rate (Table 2), the WAFs 1:10,000 proved the most toxic (e.g. EC50 for "SW" WAF in lake water was 275 mg oil/L for OWR 1:40, but only 13.7 mg oil/L for OWR 1:10,000). As the amount of the toxic compounds leached from the applied oil depends on the OWR (as shown in Table 1), the expression of WAF toxicity calculated on oil load is incorrect and misleading. Therefore, reporting the toxicity values as the loading rate is appropriate only when the variable loading approach is used for the preparation of WAF solutions series, i.e., different OWR are applied to make WAF series for toxicity testing.

It is important that 48 h EC50 values for *D. magna* of WAFs 1:10,000 in AFW expressed as loading rate (Table 2) were quite comparable with the data available for SFOs in ECHA's database (ECHA 2015) which were obtained by the application of variable loading approach (EC50 for both heavy and light shale oils—9.71 mg/L). This also confirms that the use of OWR 1:10,000 is the most appropriate for the evaluation of the toxicity of substances poorly soluble in water.

Conclusions

The data obtained within the current study showed that both investigated SFOs were very hazardous to the aquatic organisms, whereas lower density SFO ("SW") was more toxic than higher density SFO ("D").

The weak correlation between concentrations of total petroleum hydrocarbons or sum of 16 PAHs and WAF's toxicity to aquatic species indicated that such integrated chemical parameters are not very informative for toxicity prediction, at least in case of SFOs.

Different results obtained with different protocols used for WAF preparation once more raises the question on interpretation of the laboratory toxicity data for the prediction of ecological effects. The analysis of the chemical and toxicological results of SFO's WAFs testing showed that WAFs 1:10,000 prepared in natural water contained the highest concentration of extracted pollutants. Thus, this OWR could be considered most environmentally relevant and informative for risk assessment models.

We showed that short-term exposure to sublethal concentrations of soluble SFOs fraction may increase the reproductive potential of *D. magna*. The information on the recovery potential of crustaceans' population is very important for the realistic prediction of consequences for aquatic ecosystems in case of oil spills.

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PAPER III

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CLADOCERA

Ecotoxicity of nanosized magnetite to crustacean *Daphnia magna* and duckweed *Lemna minor*

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Abstract Along with the development of nanotechnology, an increase in production and application of nanosized magnetite (Fe_3O_4) is expected. Though magnetite is considered relatively safe, information concerning potential hazards of synthetic magnetite nanoparticles with unique physico-chemical characteristics to aquatic organisms is still limited. In this study, we evaluated the toxicity of nanosized (27.2 ± 9.8 nm) and bulk (144.2 ± 67.7 nm) magnetite particles to different life stages of the aquatic crustacean *Daphnia magna*. In addition, phytotoxicity of the magnetite was evaluated using duckweed *Lemna minor*. The study did not reveal any statistically significant differences between the biological effects of nanosized and bulk magnetite particles. Both forms of magnetite induced very low toxicity (EC50 > 100 ppm) to *D. magna* and *L. minor* in the standard acute assays. However, it was demonstrated that at acutely subtoxic magnetite concentrations (10 and 100 ppm), the number of neonates hatched from *D. magna* ephippia was decreased. Moreover, short-term (48 h) exposure of neonate daphnids to these concentrations may significantly affect the long-term survival and reproductive potential of daphnids. These results indicate that substantial contamination of aquatic ecosystems by magnetite may disrupt the stability of cladoceran populations.

Keywords Fe_3O_4 · Iron oxide nanoparticles · Ecotoxicity · *Daphnia magna* · *Lemna minor* · Pollution

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Introduction

Magnetite (Fe_3O_4) is a naturally widespread compound, also widely used for technological and biomedical applications (Karlsson et al., 2015). Iron oxides (in particular magnetite) have been shown to be efficient in removal of organic (Rusevova et al., 2012) and inorganic (Giraldo & Moreno-Piraján, 2013) pollutants from water and soil. Magnetite has also been used as a low-cost adsorbent in drinking and wastewater treatment systems (Mohan & Pittman, 2007; Shannon et al., 2008; Zelmanov & Semiat, 2008). Nanosized magnetite particles have unique

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physico-chemical characteristics (Blaney, 2007) and due to the development of nanotechnology drastic increase in the production volume of nanosized iron oxides, compared to the bulk (microsize) form, is expected. Magnetite nanoparticles have proven efficient in removal of environmental contaminants whereas the rates of pollutants' deactivation increased with decreasing particle size (and accompanying increase in specific surface area) (Yean et al., 2005; Ngomsik et al., 2005; Vikesland et al., 2007; Habuda-Stanić & Nujić, 2015). The increased application of iron-based nanomaterials in the field of environmental remediation (Karn et al., 2009) increases the risk of environmental contamination by iron oxide nanoparticles (NPs) during use or transportation.

Many investigations have demonstrated that the bioavailability/hazard of nanosized metal oxide particles to organisms may significantly differ from that of their bulk (microsized) analogues (Kahru & Dubourguier, 2010; Kahru & Ivask, 2013). Iron is an essential nutrient for most life forms and nanosized magnetite crystals occur naturally in the earth's crust, soils and natural water. It can therefore be assumed that nanosized magnetite is safe. On the other hand, despite its abundance in nature, the effects of elevated concentrations of nanosized magnetite on aquatic biota should be evaluated (Karn et al., 2009). In the case of iron oxides, most of the studies conducted so far have focused on the potential adverse effects in various biomedical applications. In general, the studies have proven iron oxide nanoparticles to be of low cytotoxicity, although magnetite has been shown to cause oxidative stress and inflammation if applied at high concentrations (Singh et al., 2010). Therefore, it was suggested that iron oxide NPs may cause health effects in biomedical applications (Karlsson et al., 2015).

According to Vuori (1995), the increasing load of iron may change the structure of the lotic ecosystems, but our knowledge of the ecological effects of iron compounds is still poor. Information on the behaviour and ecotoxicity of iron containing NPs in the aquatic ecosystems is also limited (Zhu et al. 2012; Habuda-Stanić & Nujić, 2015). The uncertainty regarding the environmental impact of magnetite NPs impedes their large-scale application for in situ remediation of different environmental compartments.

The main aim of the current study was to evaluate the toxicity of nano- and bulk magnetite particles to the crustacean *Daphnia magna*, a keystone species in many pelagic environments and an important model organism in ecotoxicology. For the comparison, the duckweed *Lemna minor,* representing a different trophic level in the aquatic ecosystem (primary producers), was tested. The duckweed *Lemna minor* was chosen for the evaluation of phytotoxicity of magnetite since (i) macrophytes are one of the main autotrophs in the ecosystems of small lakes and rivers, (ii) growth inhibition test with *Lemna* sp. (OECD221) is widely used in the ecotoxicological investigations. Such experiments are needed for the assessment of the risks related to the application of magnetite for in situ remediation and/or in the case of accidental contamination.

Materials and methods

Chemicals

Nanosized (<50 nm) and bulk (<5 μm) forms of iron (II, III) oxide (magnetite, Fe_3O_4) were purchased from SIGMA-ALDRICH (CAS: 1317-61-9, purity ≥98%). Magnetite particles were not coated. The primary size and morphology of magnetite particles (MPs) was checked by transmission electron microscopy (TEM) in 100 ppm nanoparticle (NP) suspension in deionized water (Fig. 1). The particle size was determined by measurement of 50 particles from two TEM images for each magnetite form. Due to very rapid agglomeration and sedimentation (Online resource 1), it was impossible to measure zeta potential and particle size distribution of both forms of magnetite in the test media as suspensions in these media did not meet the measurement criteria for Zetasizer Nano ZS (Malvern Instruments, UK).

The stock suspensions (1000 ppm) of nano or bulk magnetite were prepared in test media, sonicated (Branson Digital Sonifier) for 4 min at 40 W and immediately used for toxicity testing.

Test media

Natural water from two unpolluted Estonian lakes (Ülemiste and Raku) filtered through 0.45 μm pore size filter (Millipore) and artificial fresh water (AFW) (OECD, 2004) were used as test media in the experiments. Main hydrochemical parameters of the

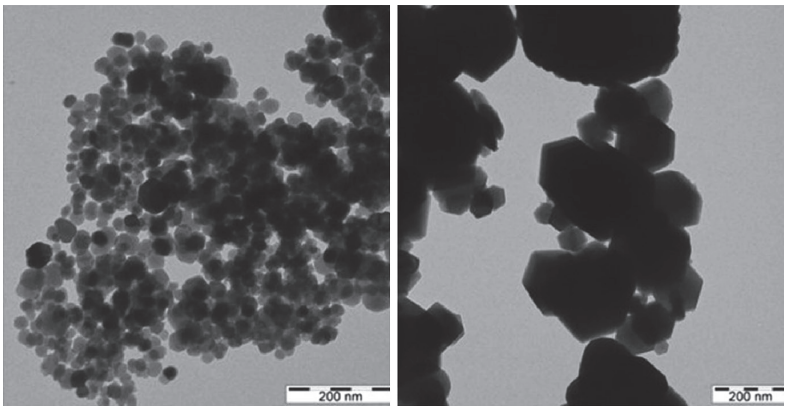

Fig. 1 TEM image of nanosized (*left*) and bulk (*right*) Fe_3O_4 particles

lake water are presented in Table 1. The AFW (pH 7.8 ± 0.2) contains only inorganic compounds: $CaCl_2 \cdot 2H_2O$ (294 ppm), $MgSO_4 \cdot 7H_2O$ (123.25 ppm), $NaHCO_3$ (64.75 ppm) and KCl (5.75 ppm).

Assays with *Daphnia magna*

The *D. magna* ephippia used in the current study were purchased from MicroBio-Tests, Inc. (Mariakerke-Gent, Belgium). All the tests were performed in three different media (AFW and two lake waters; Table 1) in 2–3 independent experiments with 3–4 replicates per sample. The experiment included several steps to evaluate the toxicity of magnetite to different *D. magna* life stages (Fig. 2).

In the *early life-stage test* (Fig. 2), equal number of eggs (90 ephippia per each treatment) of *D. magna* ephippia was hatched in two different lake waters in the absence (control) and in the presence (10 and 100 ppm) of magnetite particles. All ephippia had the same colour and contained two eggs. The ephippia were incubated for 80 h at 20°C under continuous illumination. Upon 80 h the number of hatched alive neonates was counted and used in acute and recovery tests (by this time the age of all neonates was less than 24 h).

Table 1 Characterisation of the lake waters used in the tests

Parameter	Lake Ülemiste	Lake Raku
pH	8.50	8.49
Conductivity, 20°C (μS cm^{-1})	387	251
Ca^{2+} (mg l^{-1})	67	41
DOC (mg C l^{-1})	10.2	6.4
COD_{Mn} (mg O_2 l^{-1})	9.0	4.2
P_{tot} (mg P l^{-1})	0.057	0.036
N_{tot} (mg N l^{-1})	1.06	0.49
Fe_{tot} (μg l^{-1})	74.6	<10

DOC dissolved organic carbon, N_{tot} total nitrogen, P_{tot} total phosphorus, N_{tot} total nitrogen, Fe_{tot} total iron, COD_{Mn} chemical oxygen demand

In *D. magna acute immobilization test* (OECD, 2004), the actively swimming <24 h old neonates, hatched from the ephippia (from the early life-stage test) were fed with algae *Pseudokirchneriella subcapitata* for 2 h and then exposed to different nominal concentrations (0.1; 1.0; 10.0; 100.0; 1000.0 ppm) of magnetite particles at 20 ± 2°C in the dark for 48 h. At the end of the exposure the number of dead or immobilized neonates was counted. The survived neonates were used in the recovery test.

In the *recovery tests*, two groups of *Daphnia* were used: (1) <24 h old neonates from the early life-stage test and (2) <72 h old neonates from the acute immobilization tests. In both cases, 20 actively swimming neonates from each test concentration were collected and transferred into clean lake water (Fig. 2). Every individual was exposed separately in a 40 ml vessel for 15 days and fed daily with algae *Pseudokirchneriella subcapitata* (0.2–0.3 mg C per Daphnia day^{-1} to guarantee that lack of food did not limit the reproduction rate). The produced offspring from individual parental females was counted and removed daily. At 15 days, the mortality and the number of offspring per survived parental females (1st generation) were evaluated. The offspring removed

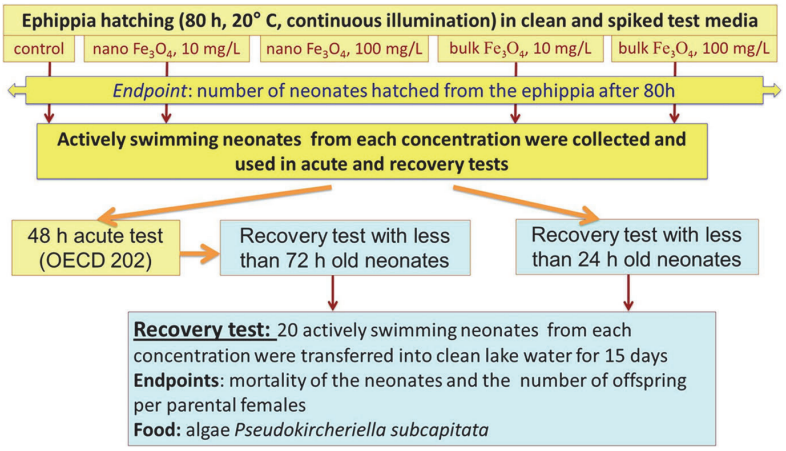

Fig. 2 Scheme of experiments with *Daphnia magna*: all tests were performed in three different test media (artificial fresh water, and two lake waters; Table 1)

from each treatment were collected in 200 ml vessels and fed with algae. The survival of the 1st generation neonates and the number of the 2nd generation offspring were recorded on the 22th day from the beginning of the experiment.

Growth inhibition test with the duckweed *Lemna minor* (OECD, 2006)

The duckweed *Lemna minor* was exposed to two nominal concentrations (10 and 100 mg l^{-1}) of magnetite particles (bulk and nanosized) for 7 days at 25 ± 1°C with continuous illumination (7000 lux). The test was performed in three media: Swedish Standard *Lemna* growth medium (OECD, 2006) and natural waters from Ülemiste and Raku lakes (Table 1). At the end of the test the number of fronds was counted and the biomass dry weight determined. Growth inhibition compared to the control was calculated for each tested concentration and expressed as % inhibition of growth.

Statistical methods

One-way analysis of variance (ANOVA) followed by t-tests were used to determine statistical significance of the differences between toxic effects of the investigated compounds. The differences were considered significant, when $P < 0.05$ (Crane et al., 2000).

Results

Suspensions of magnetite particles

The mean primary size of the magnetite particles measured from the TEM image was 27.2 ± 9.8 nm for the nanosized form and 144.2 ± 67.7 nm for the bulk form. It should be mentioned that the bulk form also included a small amount (~14%) of nanosized particles in the size range of 60–100 nm. In the current study, both tested forms of magnetite (nanosized and bulk) rapidly agglomerated and settled once suspended in the test media (natural water or AFW) (see photodocumentation in Online Resource 1). Most of the suspended magnetite settled on the flask bottom already 30 min after sonication. However, despite the rapid settling, some magnetite particles remained suspended even after 2 weeks. Sedimentation rate was somewhat slower for the nanosized magnetite and in natural lake water compared to AFW, which does not contain organic matter (see Online Resource 1).

Effect of magnetite on the hatching of *Daphnia magna* ephippia (early life-stage test)

A slight inhibitory effect (<20%) on the ephippia hatching was observed already at quite low nominal magnetite concentration of 10 ppm (Table 2). At 100 ppm the number of hatched neonates in Lake Ülemiste water decreased nearly twofold compared to

Table 2 Hatching efficiency (% from the control) of the *Daphnia magna* ephippia in the presence and absence (control) of Fe_3O_4 particles in two different lake waters (Table 1)

Test medium (natural water)	Concentration of Fe_3O_4, mg l^{-1}	Hatching efficiency (%) mean (min–max)	
		Nano Fe_3O_4	Bulk Fe_3O_4
Ülemiste Lake	0 (control)	100	100
(TOC 10 mg C/L)	10	100	81.3 (78.5–83.9)*
	100	56.3 (49.8–60.2)*	60.4 (57.1–65.3)*
Raku Lake (TOC 6.4 mg C/L)	0 (control)	100	100
	10	90.3 (86.2–94.5)	109.7 (97.1–115.3)
	100	87.1 (82.3–96.2)*	112.9 (101.3–118.0)

TOC total organic carbon

* $P < 0.05$ (statistically significant differences from the control)

Table 3 Acute toxicity of nano- and bulk Fe_3O_4 to *Daphnia magna* in artificial freshwater (AFW) and two different lake waters (Table 1)

	Test medium[a]	48 h immobilization of daphnids (%) mean (min–max)				
		Fe_3O_4 nominal concentration (ppm)				
		0.1	1.0	10.0	100.0	1000.0
nano Fe_3O_4	Ülemiste	6.3 (0–12.5)	17.0 (10.0–24.0)	15.0 (10.0–22.5)	1.4 (0–4.3)	0.0
nano Fe_3O_4	Raku	0.0	0.0	3.8 (0–7.5)	13.8 (10.0–17.5)	nd
nano Fe_3O_4	AFW[b]	0.0	3.3 (0–10.0)	8.3 (4.8–10.0)	9.6 (7.1–21.6)	45.6 (40.0–51.3)
bulk Fe_3O_4	Ülemiste	9.3 (5.0–13.6)	0.0	10.8 (0–23.1)	21.3 (0–47.6)	22.5 (10.0–35.0)
bulk Fe_3O_4	Raku	0.0	0.0	20.0	22.5 (10.0–35.5)	nd
bulk Fe_3O_4	AFW	4.9 (4.8–5.0)	7.5 (0–15.0)	7.3 (5.0–9.5)	23.0 (0–49.0)	33.3 (10.0–56.5)

[a] The same test medium was used for pre-exposure and testing

[b] AFW—OECD202 artificial freshwater

nd not determined

the control, however, in the Lake Raku water no statistically significant difference from the control was observed. The effect of magnetite particles on the hatching efficiency was more pronounced in Lake Ülemiste water, characterized by higher DOC concentration (Table 1) indicating that the bioavailability of magnetite particles may depend on the content of dissolved organic matter in the water. Although we observed the effect of water composition on hatching efficiency, there was no clear effect of magnetite particle size (nano *versus* bulk).

Toxicity of magnetite to *Daphnia magna* neonates (acute immobilization test)

Acute toxicity of the magnetite particles to *D. magna* was evaluated applying standard and modified test formats. In the first case, neonates hatched in the untreated test media were used, in the modified test the neonates were hatched in the presence of magnetite particles (Fig. 2). The aim of the modified test was to compare the sensitivity to magnetite particles of the neonates hatched in the clean water and in water spiked with different concentrations of magnetite.

The acute immobilization tests showed that in general the magnetite particles were not acutely toxic to *D. magna*: even at 100 ppm the inhibitory effect ranged from 1.4% (nano magnetite in Ülemiste water) to 23% (bulk magnetite in AFW) (Table 3). Due to the low toxicity of magnetite, we also evaluated the toxic effects at 1000 ppm although according to the test guidelines (OECD, 2004) the highest concentration of test compound in the acute test should not exceed 100 ppm. However, even at 1000 ppm the 50%

inhibitory effect was not observed (Table 3). Also, the sensitivity of neonates to either form of magnetite did not depend on the hatching conditions, i.e. pre-exposure to different magnetite concentrations during the first 24 h of life (ANOVA, $P > 0.2$), or test medium composition (ANOVA, $P > 0.3$).

Recovery of *Daphnia magna* after exposure to magnetite (a recovery test)

The aim of the recovery test was to evaluate the effect of short-term exposure to relatively high but still subtoxic concentrations of magnetite particles on further survival and reproduction potential of *D. magna* upon transfer to clean medium. In the recovery tests, two groups of actively swimming neonates were used (Fig. 2): (1) hatched in the 'early life-stage test' (<24 h old) in the lake water in the absence or presence of magnetite particles and (2) neonates (72 h old) from the acute toxicity tests in the lake water.

The neonates from the first group, i.e. exposed to magnetite (10 and 100 ppm) during the hatching stage, did not show statistically significant differences (ANOVA, $P > 0.2$) from control in their survival and reproduction. However, the 48 h exposure to magnetite particles significantly affected the survival and reproductive potential of the neonates in the second group (i.e. surviving individuals originating from the acute toxicity test). The mortality of daphnids was mainly occurring during the first week after initiation of the recovery test (transfer into clean water) and depended on the concentration of magnetite particles to which daphnids had been exposed in the acute test. The average mortality of the control group was $17.0 \pm 3.1\%$, whereas the mortality of daphnids pre-exposed in acute test to 10 ppm magnetite was $41.3 \pm 3.6\%$ and in the case of daphnids pre-exposed to 100 ppm magnetite it was $68.0 \pm 5.8\%$. It is interesting that the reproductive performance of daphnids in all groups pre-exposed to subtoxic magnetite concentrations (10 or 100 ppm) was 1.5–2.7-fold higher than in the control. As a result, by the end of the recovery test, the total number of offspring in the groups pre-exposed to 10 ppm was even higher (58 ± 7) than in the control (49 ± 5) and in the groups pre-exposed to 100 ppm (45 ± 6) it was comparable to the control in spite of the higher mortality of parent animals. The dependence of survival or fertility of *D. magna* during recovery experiments on test medium (water from Lake Ülemiste or Lake Raku) or size of the magnetite particles (nanosized or bulk) used in the acute tests was not revealed, i.e. differences in the test results were not statistically significant (ANOVA, $P > 0.1$).

The survival and reproductive performance of the 2nd generation did not differ significantly ($P > 0.1$) in all tested groups of daphnids.

Toxicity to duckweeds

Different concentrations of the main nutrients in the standard test medium and waters from lakes Ülemiste and Raku resulted in different *Lemna minor* growth rates: 0.269 ± 0.008, 0.182 ± 0.011 and 0.169 ± 0.015 days^{-1}. However, there was no significant difference (ANOVA, $P = 0.406$) between the duckweed growth in the magnetite-spiked water and the corresponding control in all media. At the end of the test, the differences between the biomass of the duckweed exposed to magnetite particles (10 and 100 ppm) and corresponding controls did not exceed 13.5% regardless of the medium. Also, there was no dose-dependent effect of magnetite on duckweed growth in case of both particle sizes (nano and bulk).

Discussion

To increase the environmental relevance of the experimental results of the current investigation, two natural waters with different DOC concentrations (Table 1) were used as test media for the crustacean assays in addition to the standard *D. magna* test medium (OECD202 artificial freshwater, AFW). The chemical composition of water determines the speciation and/or the rate of particle agglomeration and, as a result, the bioavailability (and toxicity) of metal oxides NPs in the aquatic ecosystems (Klaine et al., 2008; Nowack et al., 2012; Baumann et al., 2014). We have previously shown that the toxicity of CuO to aquatic organisms depended on the test medium composition (Heinlaan et al., 2008), as did the bioavailability of Cu-ions, being the lowest in the test media containing high concentrations of organic compounds (Käkinen et al., 2011). Indeed, the toxicity of nano and bulk forms of CuO to crustaceans differed from five to tenfold depending on water composition

and was lowest in natural water with high concentration of dissolved organics (Blinova et al., 2010). It has been reported that the fate of iron oxides in the aquatic environment also depended on the presence of dissolved organic matter (Vuorinen et al., 1998), in particular, natural organic matter may affect aggregation and disaggregation of iron oxide NPs (Baalousha, 2009). In the current study, agglomeration and sedimentation of magnetite particles was very rapid in all test media. Thus, it could be expected that in the case of contamination of water bodies, the largest part of the suspended magnetite NPs will concentrate in the sediments and may alter the quality of benthic habitats (Vuori, 1995), in particular, affect the dormant stages of aquatic organisms. We assume that sedimentation of the magnetite particles during the acute test still resulted in substantial exposure concentrations, since *D. magna* is a particle-ingesting organism (filter-feeding crustacean), ingesting particles both from the water column as well as from the bottom layer.

Several studies have reported that early life stages of aquatic organisms are more sensitive than adults to pollution by iron compounds. Myllynen et al. (1997) showed that in Finnish rivers the hatchability of lamprey roe and survival of the newly hatched larvae was especially affected by the increase in total iron concentration to 4–6 ppm. Nations et al. (2011) reported that although the exposure of amphibians *Xenopus laevis* to Fe_2O_3 NPs concentrations of up to 1000 ppm did not increase their mortality, the high concentration of iron oxide NPs can negatively affect amphibians during development. Similarly, Zhu et al. (2012) demonstrated that iron oxide nanoparticle (nFe_2O_3) concentrations higher than 10 ppm affected embryonic development of the zebrafish (*Danio rerio*). Chen et al. (2012a) showed that nFe_3O_4 suspension (0.5–87.2 ppm) did not cause larval mortality, but low concentration (<5 ppm) may interrupt antioxidant activities. The *early life-stage test* performed in the current study (Table 2) revealed that high concentrations of magnetite particles decreased the number of neonates hatched from the resting eggs (Table 2), but had no effect on the fertility of the hatched neonates. Thus, accumulation of magnetite particles in the bottom sediments may have a negative effect on the hatching not only of *D. magna* ephippial eggs but probably also on other cladoceran species and genera. However, even if only *Daphnia* spp. were affected, changes in their population structure in turn may influence other cladocerans through competitive interactions. The current study confirmed that investigation of the effect of magnetite NPs on the development of early stages of aquatic organisms has practical value for risk assessment.

It has been reported (Fjällborg et al., 2006) that iron ions are toxic to *D. magna* (EC_{84}-8.8 ppm), however, so far, little data on toxicity of iron oxide NPs to crustaceans are available and these data remarkably vary. In the current study, the acute immobilization test showed very low toxicity of both magnetite forms (nanosized and bulk) to *D. magna* (Table 3). This is in agreement with Baumann et al. (2014) who showed that in 48 h acute tests with *D. magna* the inhibitory effect of synthesized magnetite NPs (different coatings; primary size $\sim$5 nm) at 100 mg Fe l^{-1} was below 50%, and with Hu et al. (2012) who indicated low toxicity of nano-Fe_2O_3 (no lethal effect at concentration 200 ppm) to *Ceriodaphnia dubia*. García et al. (2011) reported high toxicity of nanosized magnetite ($\sim$6 nm) to *D. magna* ($LC_{50} < 1$ ppm), however, as a solvent was used for stabilizing NPs in the liquid medium in their investigation, such data cannot be directly compared to the results of the current study.

In the case of accidental pollution, the aquatic organisms are exposed to relatively high contaminant concentrations during a short time period followed by a rapid decrease in the pollution level due to dilution with clean water. Accordingly, the information on the recovery potential of organisms from acute toxicant stress is useful for realistic risk assessment. The results of the recovery test demonstrated that in spite of low mortality of the neonates during short-term exposure to magnetite particles, the subsequent effect on survival and reproductive potential of the organisms was dose-dependent. Moreover, mortality during the recovery test (in clean water) in the groups exposed to magnetite in the acute test was significantly higher than in the acute test. On the other hand, the higher mortality of pre-exposed daphnids was compensated by higher number of offspring produced by the survived females.

Our experiments revealed that magnetite nanoparticles were not toxic to duckweed at concentrations $\leq$100 ppm in waters with various hydrochemical compositions. Chen et al. (2012b) also showed that low toxic effect of the same 'brand' of magnetite NPs as in the current study on *Chlorella vulgaris* was

observed only at concentrations higher than 100 ppm. Relatively low toxicity of manufactured Fe_3O_4 NPs ($\sim$10 nm) has also been reported for *Chlorella vulgaris* (Barhoumi & Dewez, 2013) and *Lemna gibba* (Barhoumi et al., 2015). All in all, according to the current test results, magnetite nanoparticles in concentrations less than 100 ppm seemingly do not pose a threat to aquatic vegetation.

It is important to note that, according to the data of long-term national hydrochemical monitoring (1990–2010), the total iron concentration in Estonian lakes and rivers varies from <0.01 to 15 ppm and usually remains below 1.5 mg ppm. Thus, the applied nominal concentration of 10 ppm (equal to 7 mg Fe l^{-1}) did not exceed the highest observed level of total iron concentration and the 100 ppm concentration models the situation of high (e.g. accidental) contamination. The effect of background iron concentrations in the test media on the experimental results can be considered negligible as the concentration of total iron (Table 1) was 100-fold lower than the lowest exposure concentration used in the experiment.

Conclusion

The current study did not reveal any differences between the biological effects of nanosized and bulk magnetite particles (Fe_3O_4) to the aquatic species, used in testing. Both forms of magnetite induced very low toxicity (EC50 > 100 ppm) to *D. magna* and *L. minor* in the standard acute assays. However, we demonstrated that a decrease in the number of neonates hatched from *D. magna* ephippia may already occur at the nominal magnetite concentration of 10 ppm. Moreover, the recovery test revealed that even short-term exposure to 10–100 ppm concentrations may significantly affect the long-term survival and reproductive potential of cladocerans.

These results indicate that contamination of aquatic ecosystems by magnetite may disrupt the stability of *D. magna* population, and presumably also of other zooplankton species. Therefore, case by case evaluation of behaviour and fate of magnetite nanoparticles should be performed before any large-scale application of magnetite nanoparticles for in situ remediation of aquatic ecosystems.

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PAPER IV

Blinova, I., Niskanen, J., Kajankari, P., **Kanarbik, L.**, Käkinen, A., Tenhu, H., Penttinen, O., Kahru, A. (2013). Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Environmental Science and Pollution Research. 20, 3456−3463.

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RESEARCH ARTICLE

Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*

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Abstract Although silver nanoparticles (NPs) are increasingly used in various consumer products and produced in industrial scale, information on harmful effects of nanosilver to environmentally relevant organisms is still scarce. This paper studies the adverse effects of silver NPs to two aquatic crustaceans, *Daphnia magna* and *Thamnocephalus platyurus*. For that, silver NPs were synthesized where Ag is covalently attached to poly(vinylpyrrolidone) (PVP). In parallel, the toxicity of collargol (protein-coated nanosilver) and $AgNO_3$ was analyzed. Both types of silver NPs were highly toxic to both crustaceans: the EC50 values in artificial freshwater were 15–17 ppb for *D. magna* and 20–27 ppb for *T. platyurus*. The natural water (five different waters with dissolved organic carbon from 5 to 35 mg C/L were studied) mitigated the toxic effect of studied silver compounds up to 8-fold compared with artificial freshwater. The toxicity of silver NPs in all test media was up to 10-fold lower than that of soluble silver salt, $AgNO_3$. The pattern of the toxic response of both crustacean species to the silver compounds was almost similar in artificial freshwater and in natural waters. The chronic 21-day toxicity of silver NPs to *D. magna* in natural water was at the part-per-billion level, and adult mortality was more sensitive toxicity test endpoint than the reproduction (the number of offspring per adult).

Keywords Ecotoxicology · Crustaceans · Silver nanoparticles · Collargol · Bioavailability · Natural water

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Introduction

Silver nanoparticles are increasingly used in consumer applications. The Woodrow Wilson Database (2011), although not comprehensive, has listed 1,317 nanoparticle (NP)-based consumer products currently on the market, 311 of which contain silver nanoparticles (AgNPs). Colloidal silver has been used for more than 100 years (Nowack et al. 2011). Already since the late nineteenth century, colloidal silver such as collargol (protein-stabilized nanosilver) was used for numerous medical purposes (Fung and Bowen 1996). According to Mueller and Nowack (2008), approximately 500 tonnes of nanosilver is produced per year. Thus, there is a high risk of environmental pollution by nanosilver due to its leaching from consumer products, as well as through industrial waste streams. Even though an increasing number of reports on the toxicity of different AgNPs to aquatic species have been published during the last decade, the information is still scarce (Fabrega et al. 2011; Kahru and Dubourguier 2010; Wijnhoven et al.

2009). Among the papers on silver NPs, the number of articles published on the effect of nanosilver to bacteria and environmentally relevant aquatic organisms such as crustaceans, algae, and fish is still low. Majority of the reports concern the effects on bacteria, mostly *Escherichia coli*, showing the importance of research on the antibacterial applications of nanosilver (Fig. S1 in Supplementary material). Often, AgNPs used in various commercial products are coated with organic compounds to promote their dispersability. The coatings/capping agents may in turn influence the bioavailability and fate of the particles in the environment.

The experimentally measured toxicity of nanosilver varies depending on the test species. For example, a recent review by Kahru and Dubourguier (2010) showed that AgNPs were toxic to algae and crustaceans even at very low concentration (EC50<1 mg/L), but the toxicity to protozoa was relatively low, EC50~40 mg Ag/L. Therefore, any additional information on the toxicity of different formulations of silver NPs to environmentally relevant test species in varying test conditions will be helpful for realistic risk assessment of AgNPs. As the bioavailability of silver NPs owes to numerous factors determined already in the synthesis stage (Tolaymat et al. 2010; Xia et al. 2011), the need to report in detail the preparation methods is evident.

In the current study, the toxicity of two types of silver NPs, i.e., (1) poly(vinylpyrrolidone) (PVP)-stabilized AgNPs and (2) collargol (protein-coated silver NPs), are reported for two aquatic crustaceans, *Daphnia magna* and *Thamnocephalus platyurus*. The toxicity of silver NPs was compared with the toxic effect of a soluble silver salt, $AgNO_3$, to evaluate the contribution of the dissolved silver to the overall toxic effect of silver NPs.

PVP is a nontoxic polymer widely used as an additive in pharmaceutical applications (Haaf et al. 1985), in cosmetics (Goddard and Gruber 1999), and even as a blood plasma expander (Kuo et al. 1997). It is important to note that most often PVP is used as a stabilizing agent for various types of NPs, as silver, without binding it covalently to the particles (Greulich et al. 2011; Ledwith et al. 2007). In the current work, PVP was first synthesized by reversible addition–fragmentation chain transfer radical polymerization (RAFT) (Chiefari et al. 1998), and next, the obtained polymer was used in the preparation of water-dispersible silver NPs. In this way, the polymer was covalently attached to the particles via sulfur linkages. This is a technique that has been formerly used in the preparation of gold nanoparticles (Lowe et al. 2002; Shan et al. 2003). Essentially, the same chemistry to graft silver nanoparticles with rubbery polymeric (meth)acrylates was recently used by Niskanen et al. (2010).

D. magna was chosen as a test species because it is among the most sensitive freshwater test species to silver (Ratte 1999). In addition, for *D. magna*, an OECD test method has been developed both for short-term and long-term endpoints. Both of these endpoints are listed as necessary environmental ones for hazard evaluation of synthetic nanomaterials in *Phase-One of the OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials* (List of manufactured nanomaterials and list of endpoints for phase one of the sponsorship programme for the testing of manufactured nanomaterials: revision 2010). The majority of the information published so far on the toxicity of silver NPs to environmentally relevant aquatic species concerns *D. magna*; this information allows the comparison of results. Another crustacean, *T. platyurus*, was used as an additional test species to compare species sensitivity. To increase the environmental relevance of our studies, the toxicity of silver NPs to crustaceans was evaluated also in natural water.

Materials and methods

Chemicals

For the synthesis of PVP-Ag, the following chemicals were used. The initiator azobisisobutyronitrile (Fluka, >98.0 %) was recrystallized from methanol. The monomer *N*-vinyl-2-pyrrolidone (Polysciences, 99 %) and solvent 1,4-dioxane (Aldrich, >99.5 %) were distilled prior to use. The chain transfer agent cyanopentanoic acid dithiobenzoate was synthesized according to Shan et al. (2003). Diethyl ether (J.T. Baker, anhydrous), silver nitrate (VWR International, Ph. Eur.), sodium borohydride (Sigma-Aldrich, >98.5 %), tetrahydrofuran (THF; LabScan, 99.8 %), silver nitrate (Fluka), and collargol (protein-coated colloidal silver, purchased from an Estonian drugstore) were used for the toxicity testing as received.

Synthesis of PVP-stabilized silver nanoparticles (PVP-Ag)

For the preparation of polyvinyl pyrrolidone (PVP) and for the reduction of the polymer dithiobenzoate end group into a thiol (PVP-SH), *N*-vinyl-2-pyrrolidone (4 mL, 37.5 mmol), 1,4-dioxane (5 mL), cyanopentanoic acid dithiobenzoate (50.48 mg, 0.18 mmol), and azobisisobutyronitrile (5.25 mg, 0.03 mmol) were placed in a Schlenk flask. Oxygen was removed by four freeze–thaw cycles using a Schlenk line. The reaction flask was placed in an oil bath at 60 °C for 20 h. The polymer was purified by precipitation in diethyl ether twice and freeze-dried. Altogether 1.98 g of polymer was obtained. The molar mass (Mn) of the polymer was determined by size exclusion chromatography to be 13,600 g/mol with a polydispersity index of 1.2. For the reduction of the polymer end group, 1.0 g polymer was dissolved in 100 mL THF/water (1:1) mixture. $NaBH_4$ (59.3 mg, 1.6 mmol) was then added to the solution, and the reaction was left overnight in an open vessel. The polymer obtained was purified by dialysis against water and freeze-dried.

Altogether, four batches of PVP-Ag were produced using PVP with a dithiobenzoate end group or PVP with a thiol end group (PVP-SH). Three of them were used for the current study, designated as PVP-Ag1, PVP-Ag3, and PVP-Ag4 (Table 1). Briefly, PVP (234 mg) was dissolved in 40 mL water followed by the addition of $AgNO_3$ (141 mg, 0.83 mmol). A solution of $NaBH_4$ (312 mg, 8.3 mmol) in 10 ml water was slowly added to the polymer solution. The formation of NPs could be observed as the color changed from colorless to brown. After stirring overnight in an open vessel a clear, dark brown solution of nanoparticles was obtained. The particles were purified by dialysis against water and freeze-dried.

Characterization of the Ag nanoparticles used for the toxicity tests

The PVP-AgNPs were characterized by thermogravimetric analysis (TGA) and transmission electron microscopy (TEM). The hydrodynamic diameter and zeta (ζ) potential of collargol and PVP-Ag4 (concentration 10 mg Ag/L) in media used for the toxicity testing, i.e., in distilled water, OECD *Daphnia* medium and natural water, were determined at 25 °C using Zetasizer Nano ZS equipped with a 633-nm He–Ne laser (Malvern Instruments, UK). Before the addition of NPs, natural waters were filtered through PTFE filters (pore size, 0.45 μm). The intensity weighted average hydrodynamic radii were obtained using the software by the manufacturer. ζ potential measurements were performed using Doppler electrophoresis as the basic principle of operation. The instruments for TGA as well as the methods used to determine the molar masses of the polymers are presented in Supplementary material, Annex 2.

Toxicity tests

In the acute immobilization tests with *D. magna* (water flea), the neonates less than 24 h old obtained by the hatching of ephippia were exposed to different concentrations of Ag compounds at 20 °C in the dark for 48 h (OECD 202). Differently from the standard test procedure (OECD 202), neonates used for the toxicity tests were not from a laboratory culture but were hatched from dormant eggs. Both, the ephippia of *D. magna* and cysts of *T. platyurus* were purchased from MicroBio-Tests, Inc. (Mariakerke-Gent, Belgium). In the acute mortality test with the crustacean *T. platyurus* (fairy shrimp), the larvae hatched out from the cysts were exposed at 25 °C in the dark for 24 h. The tests were performed three to four times each in four (*D. magna*) and three (*T. platyurus*) replicates.

In the *D. magna* reproduction test (OECD 211), the neonates less than 24 h old were exposed at 21±1 °C with 16:8 h light/dark photoperiod during 21 days. Endpoints were the mortality of the parents during the test and the total number of juveniles produced per parent alive at the end of the test. *D. magna* was fed daily with the algae *Pseudokirchneriella subcapitata* (0.1–0.2 mg C/*Daphnia*/day). The test medium was renewed every 3 days; the parent animals were transferred to vessels with fresh medium with a glass pipette.

The artificial fresh water (AFW) and natural water from rivers and lakes were used as test media. The moderately hard AFW (US EPA 2005) was used in tests with *T. platyurus* and ISO medium (OECD 202) for *D. magna*. Samples of natural water were collected in Estonia and Finland, filtered through 0.45-μm-pore-size filters and stored at 4 °C.

The stock suspensions of AgNPs (500 mg/L) were prepared in MilliQ water, sonicated for 30 min, and stored in the dark. The silver concentrations in the stock solutions were determined with a Varian SpectrAA 220Z Atomic Absorption Spectrometer.

Table 1 Characterization of AgNPs

AgNP	Coating[a]	Size of the NPs[b], nm	
	%	Mean	St. dev.
PVP-Ag1	73	6.3	2.3
PVP-Ag3	56	6.0	3.0
PVP-Ag4	71	8.4	2.8
Collargol	30	12.5	4.0

[a] Mass fraction, analyzed by thermogravimetry (see also Table S1)

[b] Measured from the TEM images (see Figures S2–5)

Results and discussion

Physical–chemical characterization of AgNPs

Poly(vinyl pyrrolidones) with narrow molar mass distributions and dithiobenzoate end groups were prepared by the RAFT polymerization technique. In the syntheses of AgNPs, the polymers were used as such or after reducing the end groups into thiols, both methods being as effective. Silver ions were reduced into metallic silver in the presence of the end-functionalized polymers, and in this way, stable NPs stabilized with PVP were obtained. Several batches of water-dispersible silver NPs with PVP bound to the particles with sulfur bonds were produced. It is worth noticing that usually PVP has been used in stabilizing metallic NPs by simply adsorbing the polymer to the particle surfaces, and thus, the concept of using covalently bound polymers as stabilizing agents is fairly novel. Some variation in the amount of the grafted polymer in different batches of the silver nanoparticles was observed. PVP-SH was used in the

first batch (PVP-Ag1). This produced smaller particles with a higher amount of polymer than what was obtained in the last two batches with PVP carrying dithiobenzoate end groups (PVP-Ag3 and 4). Both methods yielded similar particles where the polymer is bound to the metal surface with a sulfur bond (Table S1 in the Supplementary material).

For AgNPs, TEM micrographs and light scattering data are presented in the Supplementary material (Fig. S2–5). As shown in Table 1, the core size of synthesized PVP-AgNPs (6.3–8.4 nm) was slightly smaller than the size of protein-coated AgNPs (collargol), whereas the mass fraction of the organic coating in the case of PVP-Ag was noticeably larger (56–73 %) than that in collargol (30 %).

Acute toxicity of AgNPs to crustaceans

The toxicity of three batches of PVP-Ag (Table 1) was first screened with the crustacean *T. platyurus* acute assay as this test needs a smaller sample volume and can be conducted more rapidly than the *D. magna* acute assay. The results revealed that small differences in particle core size, percentage of synthetic polymer on the surface, and thus hydrodynamic size (Tables 1 and S1) did not influence the acute toxic properties of PVP-AgNPs to *T. platyurus* (data not shown); therefore, only one batch of three (PVP-Ag 4) was used for further investigation.

Acute toxicity of PVP-AgNPs, collargol, and silver nitrate was tested with two crustaceans. Five natural water samples with different hydrochemical characteristics were used in addition to AFW to study the bioavailability/toxicity of the silver compounds to aquatic crustaceans in environmentally relevant test media (Table 2). The wide concentration range of inorganic salts (observed as conductivity varying 4-fold) and different amount of dissolved organic carbon (DOC) in natural waters used as test media reflect the hydrochemical diversity of surface waters in Estonia and Finland (Table 2).

The analysis of the hydrodynamic diameter (D_h) and zeta potential of the silver NPs (10 ppm Ag) showed that the D_h of collargol in all tested media was about half of that of PVP-Ag4 (50.7 versus 105.7 nm in AFW). Apparently, the protein coating of collargol behaves differently from electroneutral PVP in stabilization of nanosilver suspensions. Also, the higher absolute zeta potential of collargol (−16… −42 mV) than that of PVP-Ag4 (about −5 mV) shows that the behavior of the NPs in aqueous media is different. However, the chemical composition of different test media did not affect the D_h of AgNPs (collargol), or the effect was not remarkable (PVP-Ag4) (Table 3; Fig. 1).

Table 2 Characterization of the test media

Test medium	Conductivity 20 °C (μS/cm)	DOC[a] mg C/L	pH
River 1	560	15.3	8.3
River 2	421	35	7.45
Lake 1	439	11.8	8.3
Lake 2	143	22	7.6
Lake 3	315	5.3	7.1
$AFW_{Daphnia}$	640	0	7.8–8.0
$AFW_{Thamnocephalus}$	665	0	7.8–8.0

[a] Dissolved organic carbon

Acute toxicity (EC50) of collargol, PVP-AgNPs, and $AgNO_3$ to crustaceans *D. magna* and *T. platyurus* in six different test media (see also Table 2) is presented in Table 4. The E(L)C50 values for $AgNO_3$ in AFW were very low: 1.4 ppb for *D. magna* and 3.6 ppb for *T. platyurus* (Table 4) and comparable with the earlier reported data on toxicity of soluble silver salts to *D. magna* (Erickson et al. 1998).

The results of acute tests revealed that toxicity of both studied AgNPs (collargol and PVP-Ag) to crustaceans was similar when calculated based on the silver content. Here, it is important to note that the share of the organic coating of PVP-AgNPs was up to 73 % whereas in the case of collargol, the share of the coating was 30 %. Silver from both types of NPs (Table 4) was up to 10-fold less bioavailable to crustaceans in all test media than Ag from the soluble silver salt ($AgNO_3$). The lower toxicity of different silver NPs to daphnids compared to a soluble silver salt has also been reported by Allen et al. (2010), Griffitt et al. (2008), and Zhao and Wang (2011).

The acute toxicity (E(L)C50 values) of all studied silver compounds to *D. magna* (water flea) and *T. platyurus* (fairy shrimp) was of the same order of magnitude, but the latter test species was slightly less sensitive to silver. As shown previously by Blinova et al. (2010), the sensitivity of these two aquatic species was similar also to CuO nanoparticles. Both crustacean species showed similar trends for toxicity of investigated silver compounds in different test media (Table 4). The big variation (STD in Table 4) of E(L)C50 values may be explained firstly by the steep slope of the dose–response curve, i.e., a small increase in silver dose caused a large increase in toxicity, and, secondly, by the instability of NP suspensions (dissolution, aggregation, settling) in the test media (Liu and Hurt 2010). The measurements showed that the absolute value of zeta potential of both nanosilver suspensions in MilliQ water was higher (indicating higher electrostatic stability) than in other test media (Table 3). The observed remarkably high toxicity of nanosilver compounds (in the parts-per-billion range) to crustaceans indicates that these organisms are a vulnerable link in the aquatic food chain concerning contamination by nanosilver.

Bioavailability of all the studied silver compounds (Table 4) decreased in natural water by a factor of 3–5 as compared with AFW. These results are in general agreement with the data of

Table 3 Hydrodynamic diameter and zeta potential of collargol and PVP-Ag4 suspensions (10 ppm Ag) in three different test media

Analysis medium	Collargol		PVP-Ag 4	
	Hydrodynamic diameter (D_h), nm	Zeta potential, mV	Hydrodynamic diameter (D_h), nm	Zeta potential, mV
Milli Q water	50.7	−42.7	122.4	−6.97
$AFW_{Daphnia}$	50.7	−16.5	105.7	−4.86
River 2	50.7	−16.1	141.8	−4.93

other authors, showing that in natural water, the toxicity of silver salts (Erickson et al. 1998) and silver NPs (Gao et al. 2009) was remarkably lower than in AFW. The interaction with different organic and/or inorganic components (water hardness, DOC, sulfides, etc.) in natural water may significantly modify the silver speciation and bioavailability/toxicity to living organisms (Bianchini and Wood 2008; Ratte 1999). In the current study, the relationship between the silver toxicity and concentration of dissolved organic matter was revealed only for $AgNO_3$ (R^2=0.88). A higher DOC was accompanied by a lower toxicity (Table 4). This indicates that the behavior of AgNPs in natural water may differ from that of Ag ions, and thus, the fate of silver NPs in an aquatic environment most probably cannot be predicted on the basis of the existing knowledge on behavior of silver ions in natural water. The commercially available nanosilver particles have different coatings and correspondingly, different surface properties. Thus, it may be assumed that their fate in the environment may be different. Bone et al. (2012) have recently shown that the behavior of different AgNPs in the same test media was coating dependent.

Chronic toxicity of silver nanoparticles to *D. magna*

The dose–effect data on the mortality of the daphnids and the reduction of the offspring per surviving adult upon 21 days of exposure to PVP-Ag and collargol are presented in Table 5. A very steep dose–effect response was typical for *D. magna* mortality. The data show that in the current test setting, it was not possible to determine the EC50 values for the reproduction endpoint, because the tested organisms died before a 50 % decrease in reproduction was reached. Thus, in the case of AgNPs, the 21-day mortality endpoint could be considered more relevant and more sensitive than the reduction of offspring per adult (reproduction). We agree with Nebeker et al. (1983) that for silver, "a simpler and less expensive test using just long-term survival might be adequate to predict toxicity." In the case of chronic tests with silver compounds, some additional endpoints such as body length of daphnids may be more informative than mortality.

The comparison of the acute and chronic toxicity data for silver NPs toward *D. magna* in two different natural waters (river 2 and lake 3; Table 2) shows that upon the long-term (21 days) exposure, the toxicity of PVP-AgNPs was reduced compared with the acute tests (Tables 4 and 5). For example, the 48-h EC50 of PVP-Ag4 (river 2) was 28.7 ppb Ag whereas in the chronic assay, all the daphnids were alive even after 21-day exposure to 58-ppb Ag. Analogously, the 48-h EC50 for collargol (river 2) was 51.3, whereas the 21-day exposure to 37-ppb collargol did not cause any mortality. The 48-h EC 50 of collargol (lake 3) was 48.7 whereas the 21-day exposure to 74-ppb collargol (lake 3) caused only 30 % mortality.

One reason for the reduced toxicity in the chronic test could be the addition of algae as food. It should be

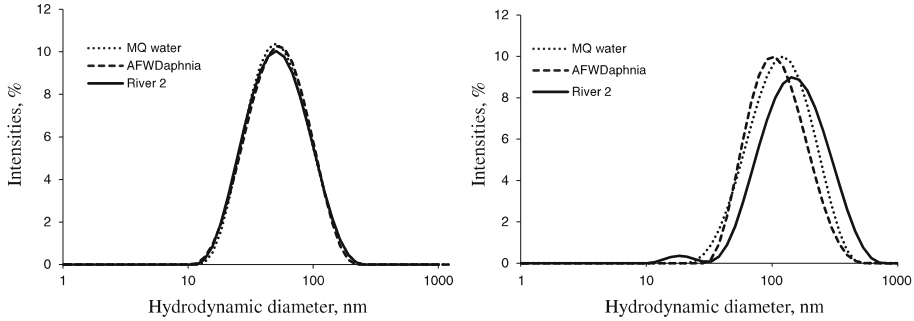

Fig. 1 Hydrodynamic diameter (D_h) of PVP-Ag4 NPs (*right panel*) and collargol (*left panel*) in three different test media. Analysis was performed using dynamic light scattering

Table 4 Acute toxicity, E(L)C50, of the Ag compounds to crustaceans in six different test media, in micrograms Ag/L

Test compound	Test medium	*Daphnia magna*		*Thamnocephalus platyurus*	
		48 h EC50		24 h LC50	
		Mean	STD	Mean	STD
Collargol	AFW	49.4 (36.6)	19.7 (14.6)	256 (189)	51.8 (38.3)
	River 1	59.4 (44.0)	9.4 (6.9)	178 (132)	41.0 (30.4)
	River 2	40.2 (29.8)	16.1 (11.9)	147 (109)	5.9 (8.0)
	Lake 1	74.9 (55.5)	18.6 (13.8)	n.d.	n.d.
	Lake 2	50.8 (37.6)	1.8 (1.3)	250 (185)	13.8 (10.2)
	Lake 3	65.7 (48.7)	7.0 (5.2)	n.d.	n.d.
PVP-Ag4	AFW	54.0 (15.7)*	1.4 (0.4)	68.8 (20.0)*	1.4 (0.4)
	River 1	191 (55.5)	80.5 (23.4)	191 (55.5)	13.4 (3.9)
	River 2	98.7 (28.7)	31.3 (9.1)	n.d.	n.d.
	Lake 1	176 (51.1)	15.1 (4.4)	252 (73.3)	62.3 (18.1)
	Lake 2	236.3 (68.7)*	97.7 (28.4)	605 (176.0)*	113 (33.1)
	Lake 3	162 (47.2)	59.8 (17.4)	n.d.	n.d.
$AgNO_3$	AFW	2.2 (1.4)*	0.5 (0.3)	5.7 (3.6)*	0.6 (0.4)
	River 1	12.4 (7.9)	4.6 (2.9)	10.7 (6.8)	1.7 (1.1)
	River 2	15.9 (10.1)	2.4 (1.5)	n.d.	n.d.
	Lake 1	8.3 (5.3)	0.5 (0.3)	11.6 (7.4)	0.5 (0.3)
	Lake 2	12.9 (8.2)	4.1 (2.6)	24.3 (15.5)	7.2 (4.6)
	Lake 3	6.8 (4.3)	0.2 (0.1)	n.d.	n.d.

AFW artificial freshwater, *n.d.* not determined

*$p<0.05$ (statistically significant differences from other test media)

mentioned that toxicity of silver NPs to *P. subcapitata* (used as food in the current test setting) is nearly 5-fold lower than that to the crustaceans (Griffitt et al. 2008). It has been shown that the addition of algae may decrease the toxicity of silver ions and AgNPs up to ten times (Allen et al. 2010; Erickson et al. 1998; Nebeker et al. 1983). Koukal et al. (2007) observed that *P. subcapitata* exudates markedly decreased the toxicity of metals. Unrine et al. (2012) reported

Table 5 Long-term toxic effect of silver nanoparticles to *D. magna* exposed in two types of natural water for 21 days

Test compound	Test media	(μg Ag/L)	Mortality of the adults, %	Decrease of the offspring per adult, %
PVP-Ag4	River 2	50.0 (14.5)	0	No effect*
PVP-Ag4	River 2	100 (29)	0	No effect*
PVP-Ag4	River 2	200 (58)	0	No effect*
PVP-Ag4	River 2	300 (87)	90	No effect*
PVP-Ag4	Lake 3	5 (1.4)	0	No effect*
PVP-Ag4	Lake 3	10 (2.9)	0	No effect*
PVP-Ag4	Lake 3	25 (7.2)	0	No effect*
PVP-Ag4	Lake 3	50 (14.5)	0	No effect*
PVP-Ag4	Lake 3	85 (25)	0	No effect*
PVP-Ag4	Lake 3	100 (29)	0	23 %**
PVP-Ag4	Lake 3	175 (50)	100	–
Collargol	River 2	50.0 (37)	0	No effect*
Collargol	River 2	100 (74)	20	No effect*
Collargol	River 2	200 (148)	100	No effect*
Collargol	Lake 3	50.0 (37)	0	No effect*
Collargol	Lake 3	100 (74)	30	43 %**
Collargol	Lake 3	200 (148)	100	–

Dose–effect data are presented

*$p>0.05$ (no statistically significant differences from the control)

**$p<0.05$ (statistically significant decrease of the offspring per adult)

that the presence of the aquatic plants *Potamogeton diversifolius* and *Egeria densa* in the microcosms reduced the toxicities of both gum arabic-coated and PVP-coated AgNPs. This was suggested to owe to the changes in the surface chemistry of the particles upon the release of organic substances from the plants.

Thus, the presence of algae in the test media may have contradictory effects. On one hand, the algae may reduce the concentration of toxic silver ions/NPs in the test medium. On the other hand, silver adhered on ingested algae may increase the dietary intake of AgNPs by crustaceans. Zhao and Wang (2010) showed that more than 70 % of AgNP accumulated in the daphnids was through ingestion of silver sorbed to algae. The finding highlights the importance of AgNP transport along the food chain as the AgNPs could not be completely depurated from the daphnids.

It has been shown that the observed toxicity of AgNPs to bacteria (Fabrega et al. 2009), algae (Navarro et al. 2008), and *Daphnia pulex* (Griffitt et al. 2008) was the result of both Ag^{+} ions and particles of nanosilver. To reveal the role of the silver ions in the overall toxic effect of AgNPs, the release of metal ions from NPs must be quantified. However, currently, the appropriate analytical methods to detect and to quantify NPs in the complex matrix (e.g., natural water) are limited (Weinberg et al. 2011). Moreover, ingestion and excretion of NPs and adhesion of aggregates of NPs to the outer surface of test organisms (such as the exoskeleton of crustaceans) during the test may change the speciation of silver. Thus, complex chemical and biological processes that constantly modify Ag speciation during the test as well as different exposure pathways for tested silver to organisms (via food or from the test medium) make the analysis of the contribution of silver ions to the net toxicity of AgNPs very complicated.

The major finding of our research, based on the data on two crustacean species, is that there is apparently no reason to consider silver NPs more dangerous for aquatic ecosystems than silver ions. Therefore, the environmental risks of manufactured AgNPs assumingly do not exceed the risks related to environmental contamination by soluble Ag salts.

However, these conclusions are based on the results from conventional OECD test procedures and may need to be reconsidered in case of using more relevant endpoints for toxicity testing of nanomaterials containing silver.

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Supplementary material

Environmental Science and Pollution Research

Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*

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Annex 1: Scientific publications on silver nanoparticles

The increasing importance of the use of nanosilver in nanotechnologies is proven by the bibliometrical survey made in Thomson Reuters ISI Web of Science in April, 2012 (Fig. S1): although the papers on silver nanoparticles (NPs) started to emerge since the beginning of 1990s, currently already over 18,000 papers on that subject have been published. About 1,000 of these papers concern the effects on bacteria, mostly *Escherichia coli*, showing the importance of research into nanosilver's antibacterial applications. Indeed, silver NPs are increasingly used as antimicrobial additives in detergents, food packaging and textiles such as socks and underwear (Wijnhoven et al. 2009).

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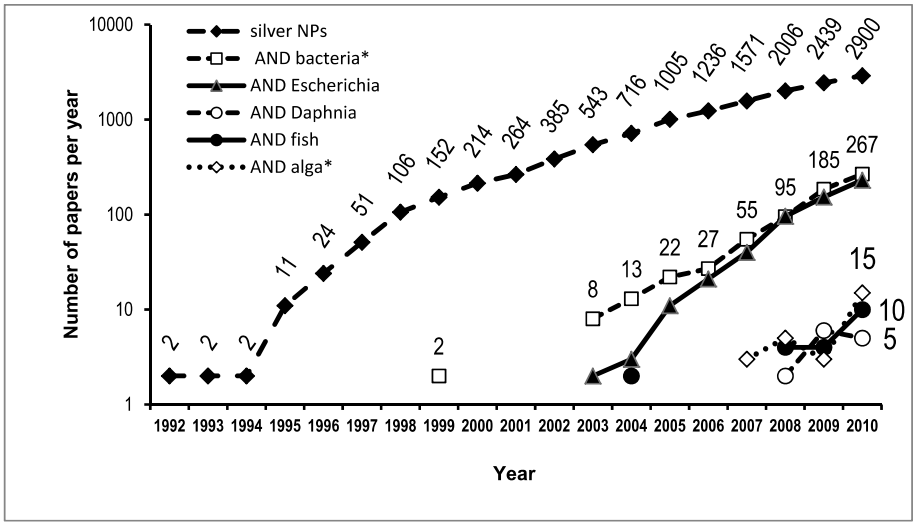

Fig. S1. Number of papers per year in Thomson Reuters ISI Web of Science on silver nanoparticles (uppermost line) and on silver nanoparticles concering certain organisms (lower lines). The search terms are indicated as data lables. To obtain data for different organisms, the search term 'nanoparticles' was refined using the additional search terms (i.e., bacteria – 2nd uppermost line; *Escherichia* – 3rd uppermost line; algae, fish, *Daphnia*- three bottom lines). The search was made at April 11, 2012.

Annex 2: Characterization of the tested polyvinylpyrrolidone (PVP)-coated silver nanoparticles (PVP-Ag)

Table S1. Additional information on PVP-Ag nanoparticles

Ag-NP	PVP[a] mg	PVP-SH[b] mg	$AgNO_3$ mg (mmol)	$NaBH_4$ mg (mmol)	m (Ag-NP) mg	TGA[c] %-polymer	Size[d] nm	st.dev.	d_h[e] nm
PVP-Ag1		234	141 (0.83)	312 (8.25)	270	73	6.3	2.3	104
PVP-Ag3	191	40	141 (0.83)	314 (8.31)	260	56	6.0	3.0	122
PVP-Ag4	232		141 (0.83)	315 (8.32)	310	71	8.4	2.8	112

[a]PVP with dithiobenzoate end group that was reduced simultaneously with the silver nitrate
[b]PVP in which dithiobenzoate was reduced prior to the nanoparticle synthesis
[c] Thermogravimetric analysis (TGA) performed with a MettlerToledo 850. 70 µl Al_2O_3 crucibles were used and the samples were heated from 25 to 700 °C (10 °C/min) under nitrogen atmosphere
[d] calculated from TEM micrographs, obtained with a Hitachi S4800 FE-SEM using a TEM-probe and Inca X-sight software (Oxford instruments) (Fig S3).
[e]Hydrodynamic diameter obtained from 90° angle dynamic light scattering studies

Methods used for the characterization of PVP-Ag nanoparticles

Size exclusion chromatography (SEC) was used to determine the molar masses of the polymers. PMMA standards from PSS Polymer Standards Service GmbH were used for calibration. Eluent was THF with tetrabutyl ammonium bromide (1 mg/ml). To prevent disulfide formation of the thiols ascorbic acid was added to the sample. The apparatus included following instruments: Biotech model 2003 degasser, Waters 515 HPLC pump, Waters 717plus auto sampler, Viscotek 270 dual detector, Waters 2487 dual λ absorbance detector, Waters 2410 refractive index detector and the software OmnisecTM from Viscotek. Styragel HR 1, 2 and 4 columns and a flow rate of 0.8 ml/min was used in the measurements.

Light scattering analysis

Light scattering experiments were conducted using a LS setup composed of a Brookhaven Instruments BI-200SM goniometer, a BIC-TurboCorr digital pseudo-cross-correlator, and a BI-CrossCorr detector, including two BIC-DS1 detectors; pseudo-cross-correlation functions of the scattered light intensity were collected with the self-beating method (Chu, 1991); a Sapphire 488-100 CDRH laser from Coherent GmbH operating at λ_o = 488 nm. Samples were aqueous dispersions containing 0.1 mg/ml silver nanoparticles and 1 mg/ml $NaNO_3$ and were measured at 25°C and 90°.

Detailed aspects of the data analysis of the light scattering (results summarized in Table S1)

In the DLS experiment, $G_2(t)$ can be converted into a correlation function of the scattered electric field, $g_1(t)$, using the Siegert's relationship (Brown, 1993; Schärtl, 2007). For monodisperse particles, having smaller diameter compared to the wavelength of light as well as for hard spheres of any size, the relaxation time of $g_1(t)$, τ, is related to the relaxation rate of $g_1(t)$, Γ, and the translation diffusion coefficient, D, by the relationship

$$g_1(t) = e^{-t/\tau} = e^{-\Gamma t} = e^{-Dq^2 t} \quad \text{and} \quad \Gamma = \tau^{-1} = Dq^2 \qquad (1)$$

The hydrodynamic radii of particles can thus be obtained from the diffusion coefficient, D, via the Stokes-Einstein equation

$$R_h = \frac{kT}{6\pi\eta_o D} \qquad (2)$$

where k, T and η_o are the Boltzmann constant, the absolute temperature and the solvent viscosity.

Mean peak values of the size distributions, obtained at fixed *q* and *c*, were used to estimate the apparent hydrodynamic radii, R_h^{app}. The true hydrodynamic radius, R_h, as well as the true radius of gyration, R_g, were then obtained by extrapolating to q = 0 and c = 0. Decay rates of $g_1(t)$ were calculated from R_h^{app}.

Additional methodological aspects of dynamic light scattering (DLS) can be found elsewhere (Brown, 1993; Schärtl, 2007).

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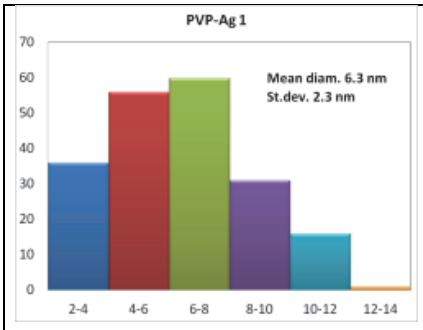
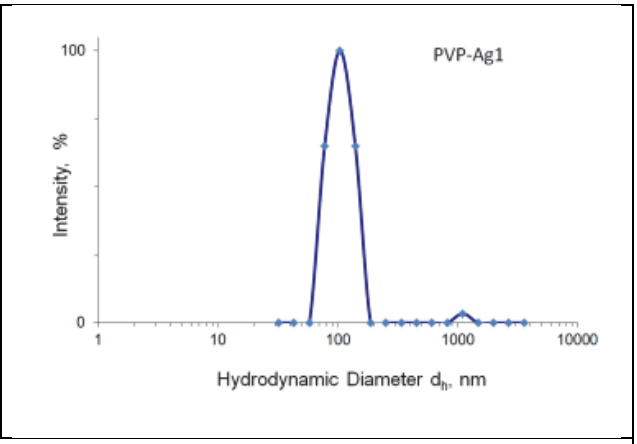




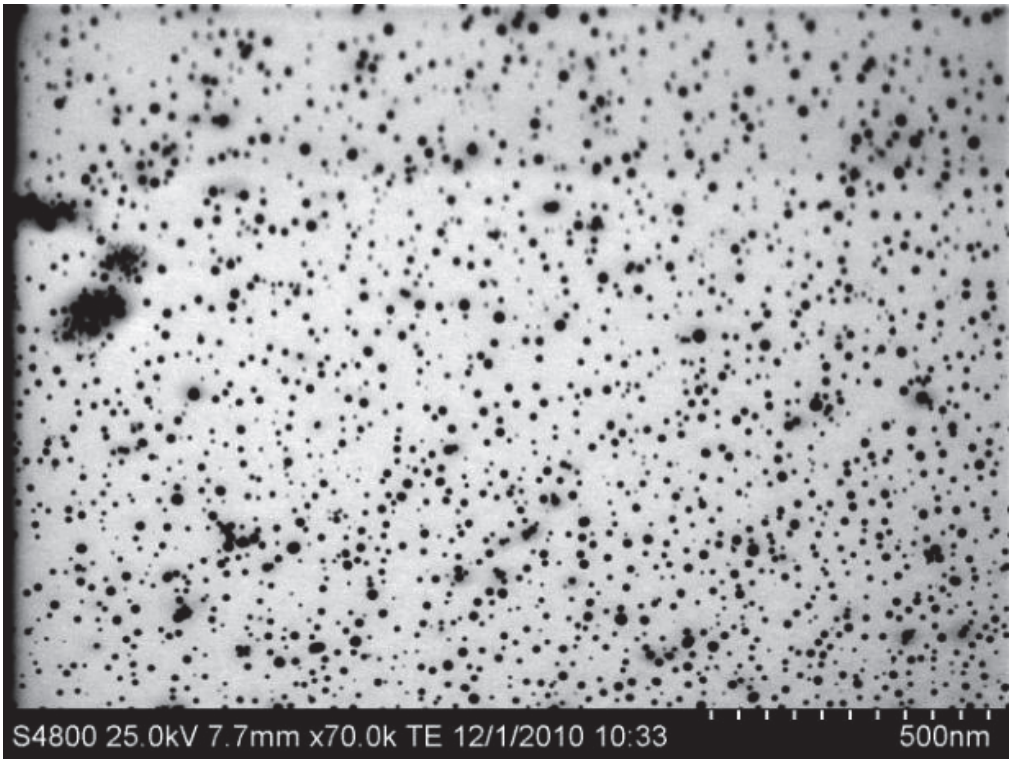
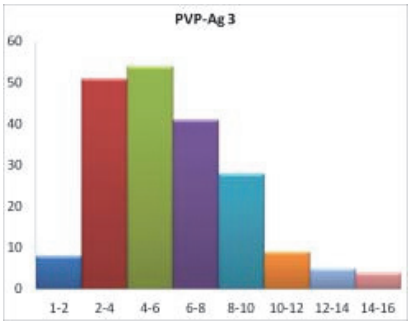
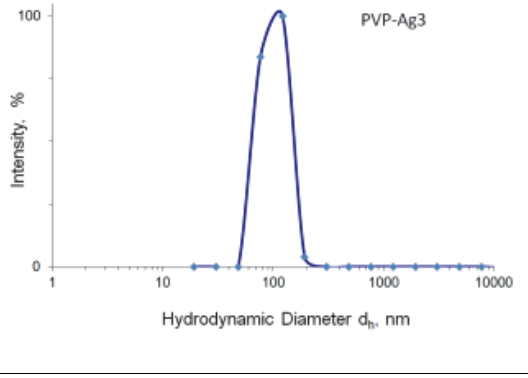


Figure S2: Size distribution (upper left panel), DLS data (upper right panel) and TEM image (lower panel) of PVP-Ag1 nanoparticles. Sizes of the NPs (upper left panel) were measured from TEM images using 200 particles (see also Table 1 & S1).





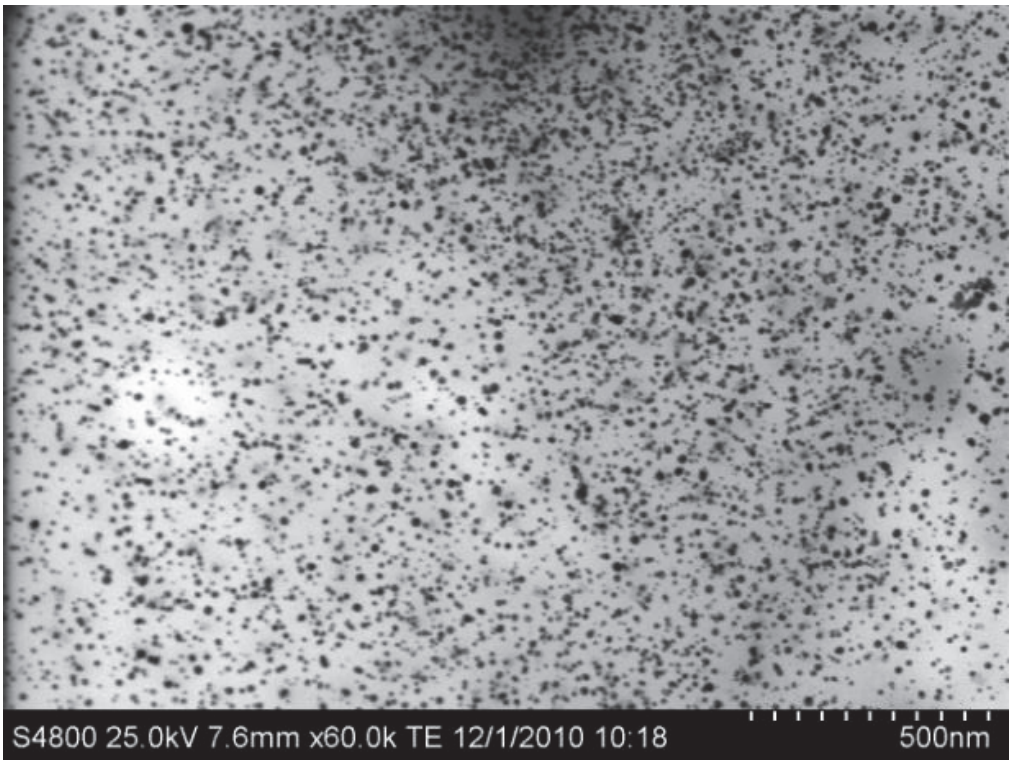
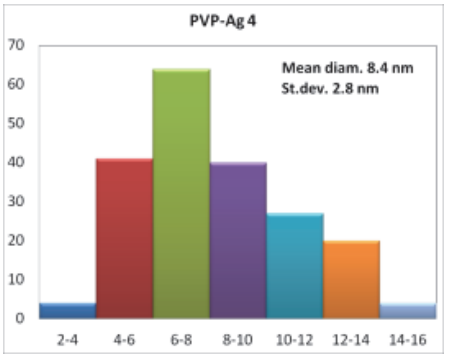
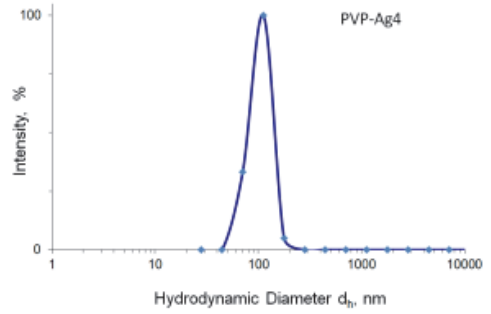


Figure S3: Size distribution (upper left panel), DLS data (upper right panel) and TEM image (lower panel) of PVP-Ag3 nanoparticles. Sizes of the NPs (upper left panel) were measured from TEM images using 200 particles (see also Table 1 & S1).





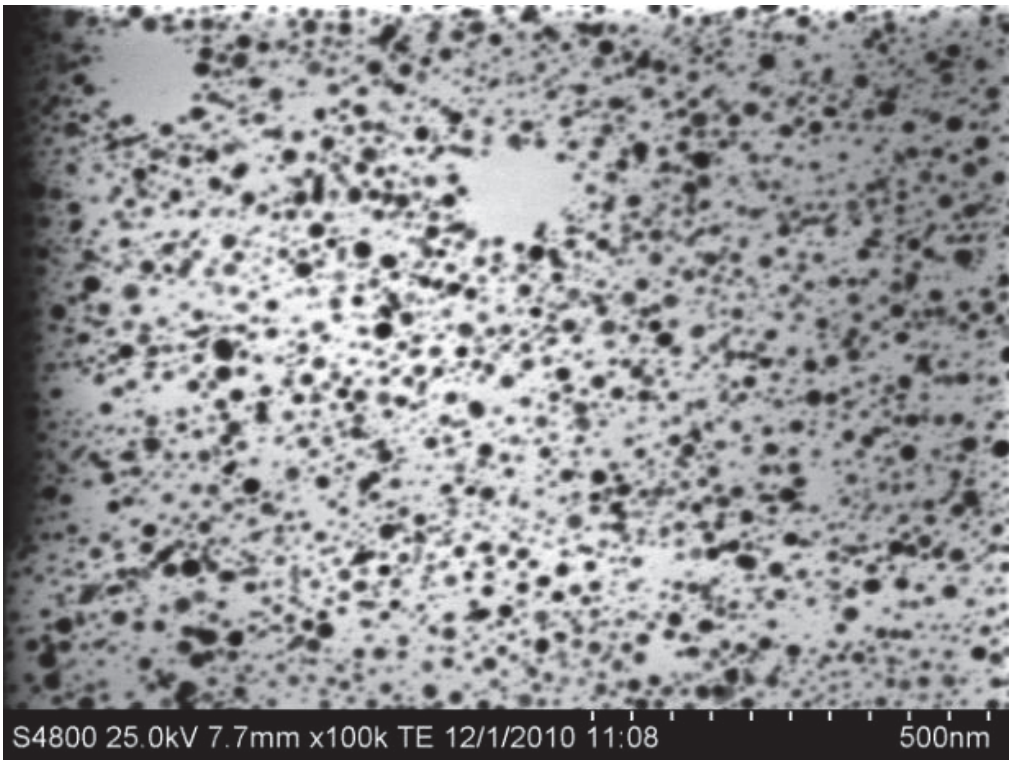
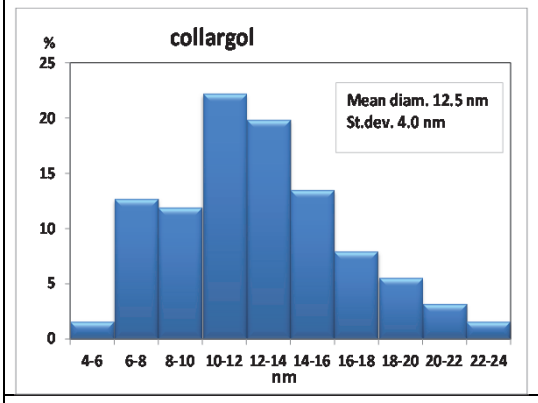
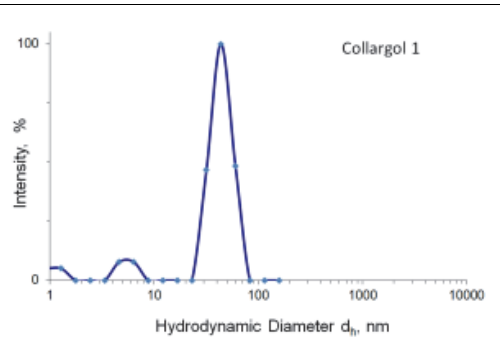


Figure S4: Size distribution (upper left panel), DLS data (upper right panel) and TEM image (lower panel) of PVP-Ag4 nanoparticles. Sizes of the NPs (upper left panel) were measured from TEM images using 200 particles (see also Table 1 & S1).





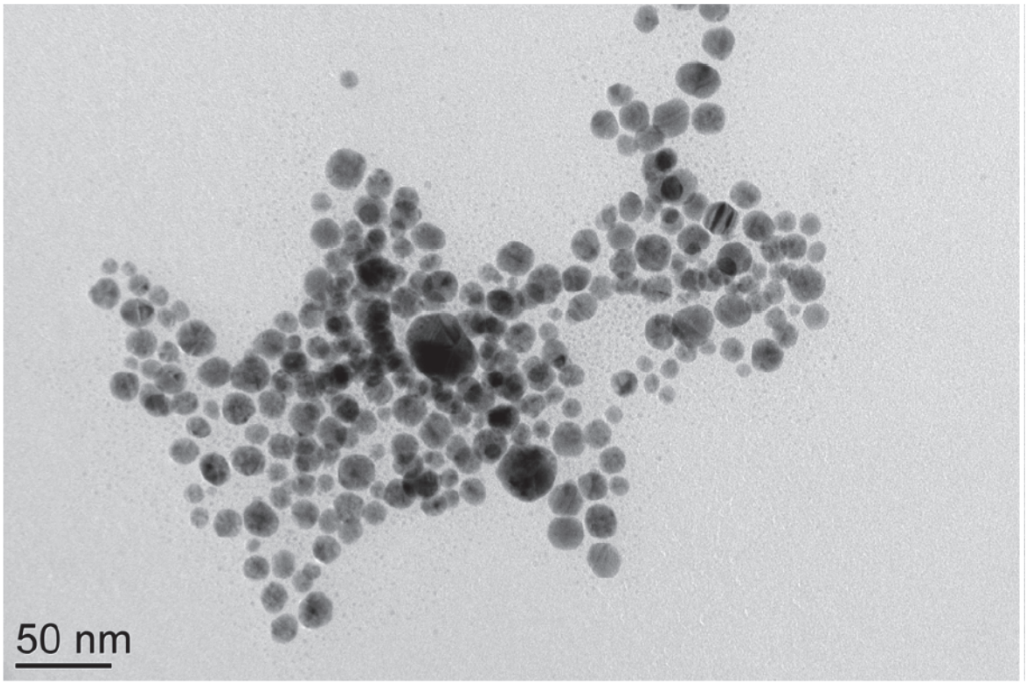


Figure S5: Size distribution (upper left panel), DLS data (upper right panel) and TEM image (lower panel) of collargol. Sizes of the NPs (upper left panel) were measured from TEM images using 200 particles (see also Table 1 & S1).

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ERRATUM

Erratum to: Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*

Irina Blinova · Jukka Niskanen · Paula Kajankari · Liina Kanarbik · Aleksandr Käkinen · Heikki Tenhu · Olli-Pekka Penttinen · Anne Kahru

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Unfortunately, there was an error on the title of Table 4 in the original publication of this article.

Instead of "Acute toxicity, E(L) C50, of the Ag compounds to crustaceans in six different test media, in micrograms Ag/L" it should have been "Table 4 Acute toxicity, E(L) C50, of the Ag compounds to crustaceans in six different test media, in micrograms/L (μg Ag/L)"

The online version of the original article can be found at http://dx.doi.org/10.1007/s11356-012-1290-5.

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PAPER V

Sihtmäe, M., Blinova, I., Künnis-Beres, K., **Kanarbik, L.**, Heinlaan, M., Kahru, A. (2013). Ecotoxicological effects of different glyphosate formulations. Applied Soil Ecology. 72, 215−224.

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Ecotoxicological effects of different glyphosate formulations

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ABSTRACT

Glyphosate is an active substance of the most used herbicides worldwide. Nevertheless, questions on safety of glyphosate-based herbicides are periodically raised and recent studies indicate that glyphosate may not be as safe as assumed, mostly due to the additives/surfactants in its formulations. The aim of this study was to evaluate the effects of isopropylamine salt of glyphosate and two glyphosate-based herbicides, Roundup Max™ (containing surfactant polyethoxylated tallow amine, POEA) and Roundup Quick™ (without POEA), on non-target species. Special focus was on the evaluation of long-term effects of high concentrations (simulating accidental pollution, e.g. transportation spills) of glyphosate formulations on soil health. Laboratory ecotoxicity testing was conducted with (i) two aquatic organisms – crustaceans *Daphnia magna* and marine bacteria *Vibrio fischeri*, (ii) five bacterial strains (*Escherichia coli* MG1655, *Pseudomonas putida* KT2440 and three bacterial isolates from the soil) and (iii) terrestrial plants *Raphanus sativus* and *Hordeum vulgare*. Laboratory toxicity results showed that among the non-target test species, *D. magna* and *V. fischeri* were the most sensitive to glyphosate formulations: acute EC50 values ranged from 4 to 49 mg L^{-1}. Direct relation between the toxicity of the tested formulations and the presence/absence of the surfactant POEA was not evident. Long-term outdoor experiments (April to September 2012) showed that the number of heterotrophic microbes in Roundup-spiked (up to 1000-fold the recommended field rate) soils during two months after the treatment were significantly higher than in the control soils, especially in case of Roundup Quick™. Residual toxicity of the treated soils to terrestrial plants decreased more rapidly in Roundup Quick-spiked soils. It was shown that in temperate climate conditions the recovery of soil health in case of (accidental) pollution by glyphosate formulations is slow and may even exceed the duration of the vegetation period. The mobility of glyphosate in the soils proved very low thus risks to aquatic ecosystems due to application of glyphosate-based herbicides may occur rather in case of direct contamination of surface water.

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1. Introduction

Herbicides account for about 40% of the pesticide volume used worldwide. Glyphosate is one of the most common herbicides used in agriculture, but also in forestry and horticulture (including home use) (EPA, 2011). Moreover, glyphosate is one of the first herbicides against which crops (e.g. soy, maize, cotton) have been genetically modified (James and Krattiger, 1996; UK GM Science Review panel, 2003). Thus, it is expected that the expansion of glyphosate-resistant crops will further increase the use of this pesticide.

Glyphosate is a broad spectrum, post-emergent herbicide that inhibits the growth of plants through interfering with the biosynthetic pathway of the essential aromatic amino acids phenylalanine, tyrosine and tryptophan. Specifically, it is an inhibitor of the enzyme in shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Vencill, 2002; Tomlin, 2006) which exists in plants and in some microorganisms but not in animals (Steinrücken and Amrhein, 1980). Due to its assumingly plant-specific mode of action, it is generally considered of low toxicity to animals (Giesy et al., 2000) and other non-target organisms (WHO, 1996). The manufacturers advertise the "low toxicity and environmental friendliness" of glyphosate-based herbicides (Monsanto, 2012). However, according to the information from the respective chemical safety data sheets, the glyphosate-based herbicides are classified as hazardous to the aquatic environment (toxic to aquatic life with long lasting effects).

Glyphosate (IUPAC name *N*-(phosphonomethyl)-glycine) as an active substance as well as glyphosate-based herbicides have been extensively studied for the properties to produce adverse effects on human health and ecosystems (U.S. EPA, 1993; Giesy et al., 2000; Williams et al., 2000; EC, 2002; Govindarajulu, 2008). Nevertheless, questions on safety of glyphosate-based herbicides are periodically raised and recent independent studies indicate that glyphosate may not be as safe as previously assumed (Paganelli et al., 2010; Guilherme et al., 2012; Koller et al., 2012; Moore et al., 2012). Moreover, only a small fraction of the applied herbicides reaches the

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target species (Pimentel, 1995) and the residual amount of herbicides in soil and water may pose hazard to human, animal and crop health.

In the European Union (EU), the marketing and use of pesticides is regulated by Regulation 1107/2009/EC (EC, 2009). Active substances (e.g. glyphosate) are approved at the EU level, while the plant protection products (e.g. Roundup) containing these substances are authorised at Member State level. Currently, glyphosate and its main degradation product aminomethylphosphonic acid (AMPA) are not included in the existing list of priority substances under the Water Framework Directive (EC, 2000) and there are no environmental quality standard (EQS) values set for these substances at the EU level. Some countries have proposed various EQS values for glyphosate in the surface water, ranging from 10 $\mu g\,L^{-1}$ to 27,000 $\mu g\,L^{-1}$ (Kreuger and Asp, 2005; CCME, 2012; UKTAG, 2012). This orders of magnitude range points to the uncertainties in the existing assessment of the environmental risks of glyphosate contamination.

When discussing the toxicity of pesticides to non-target species, the focus is mostly on the active substance. However, pesticides are formulated products and usually contain additives (e.g. surfactants) which enhance their effectiveness but at the same time may increase the toxicity to non-target biota (Tsui and Chu, 2003; Edginton et al., 2004). Glyphosate-based pesticides are commercialised in many different formulations such as Roundup, Rodeo, Aquamaster etc. Frequently, the information on the surfactants and additives is not clearly stated by the manufactures. As the toxicity of glyphosate-based products may differ from the toxicity of pure glyphosate, the information on toxicity and environmental fate of a specific glyphosate formulation is needed for relevant risk assessment.

In Estonia, glyphosate-based formulations are the most used pesticides since 2002 and this trend shows a clear increase (Estonian Agricultural Board, 2012). Currently, there are about 30 different glyphosate-based formulations registered in Estonia (Register of Plant Protection Products, 2012) and additional information on environmental hazard of different formulations of glyphosate-based herbicides in the local climatic conditions would be useful for relevant risk assessment.

In the current study the potential harmful effects of two glyphosate-based herbicides Roundup Quick^{TM} and Roundup Max^{TM} assigned for home and industrial use, respectively, were evaluated in both, short-term laboratory and long-term outdoor experiments. The study aimed (i) to compare the potential harmful effects of different commercial formulations of glyphosate to non-target species; (ii) to evaluate the influence of high concentrations (e.g. as a result of an accident during transportation) of glyphosate-based herbicides on soil microbes in the northern (part of the) temperate climate zone.

2. Materials and methods

2.1. Test chemicals

Isopropylamine (IPA) salt of glyphosate (CAS no 38641-94-0), 40% (w/v) solution in water (29.6% acid equivalents, AE, by weight), was purchased from Sigma–Aldrich (Schnelldorf, Germany). Herbicide formulations, Roundup Quick^{TM} (spray, 0.72% AE by weight) with the composition of IPA salt of glyphosate (CAS no 38641-94-0) 1%, water 94%, other additives 5% (not specified by the manufacturer) and Roundup Max^{TM} (granulated, 68% AE by weight) consisting of ammonium salt of glyphosate (CAS no 114370-14-8) 75%, surfactant POEA (CAS no 61791-26-2) 21%, sodium sulphite 0.5%, other additives 3.5% (not specified by the manufacturer), were produced by Monsato Europe S.A. (Antwerpen, Belgium). For the experiments (Supplementary material, Fig. S1), stock solutions of IPA salt of glyphosate and Roundup formulations were prepared in MilliQ water, stored in the dark at room temperature and tested for toxicity within one week. pH of the initial glyphosate solutions was not adjusted in order to mimic real use conditions. The initial pH values of the stock solutions of IPA salt of glyphosate (6.8 g AE L^{-1}), Roundup Max^{TM} (6.8 g AE L^{-1}) and Roundup Quick^{TM} (7.2 g AE L^{-1}) were 4.6, 4.2 and 7.0, respectively.

2.2. Laboratory toxicity assays

2.2.1. Acute immobilization assay with crustacean Daphnia magna

Testing with the freshwater crustacean *D. magna* adhered to OECD 202 guideline (OECD, 2004). The neonates of *Daphnia* less than 24 h old, obtained by the hatching of ephippia, were exposed to different concentrations of herbicide solutions or soil extracts at 20 °C for 48 h in the dark. The ISO medium (OECD 202) was used as the test medium.

2.2.2. Acute luminescence inhibition assay with bacterium Vibrio fischeri

The test (exposure time 30-s and 30-min) was performed at 20 °C on automatic tube-luminometer 1251 (ThermoLabsystems, Finland), connected to a computer operated by Multiuse software (BioOrbit, Finland) following the Flash-assay protocol (ISO, 2010). The exact procedure is described in Põllumaa et al. (2000) and Mortimer et al. (2008) except the inhibition of bacterial bioluminescence that was calculated as percentage of the unaffected (negative) control (2% NaCl). Reconstituted *V. fischeri* Reagent (Aboatox, Turku, Finland) was used for testing. IPA salt of glyphosate and Roundup formulations were tested in 2% NaCl. Each test was performed three times, each in 5–7 replicate dilutions. Controls, both negative (2% NaCl) and positive (3,5-dichlorophenol; 3,5-DCP), were included in each measurement series. Samples were continuously automatically mixed during the recording of luminescence. pH values in *V. fischeri* tests were in the range of 5.3–6.0 in tests with IPA salt of glyphosate and Roundup Max^{TM} and 6.5–7.0 with Roundup Quick^{TM}.

2.2.3. Bacterial growth inhibition assay

The effects of IPA salt of glyphosate and Roundup formulations on growth of bacteria during 26 h were studied. Bacterial biomass was evaluated by optical density ($OD_{600\,nm}$) of the bacterial suspensions. Altogether five bacterial strains were used: *Escherichia coli* MG1655, *Pseudomonas putida* KT2440 and three strains isolated by us from the control soil used in the outdoor experiments. One soil isolate was identified as *Bacillus mycoides* (gram-positive endospore forming aerobic bacilli), the other two were named soil bacterium M1 (gram-positive non-sporulating aerobic bacilli) and soil bacterium M2 (gram-negative aerobic coccobacilli), respectively (Supplementary material, Fig. S2). All these three bacterial isolates represented dominant viable strains in the untreated soil samples. Half-strength Luria-Bertani (LB) medium (trypton 5.0 g L^{-1}, yeast extract 2.5 g L^{-1}) was used for the cultivation of bacteria and dilution of chemicals. For the experiments bacterial cultures incubated at 24 °C overnight were adjusted to $OD_{600\,nm}$ 0.08–0.1 and an equal amount of the chemical or half-strength LB growth medium (control) was added. The tested concentrations of Roundup Quick^{TM}, Roundup Max^{TM} and the IPA salt of glyphosate were 3600, 1800, 900, 450 and 225 mg AE L^{-1}. All the stock solutions were UV-sterilised (2 × 6 W Hg lamp) before testing. The tests were performed in sterile 24-well polypropylene microplates (Falcon) at room temperature (24 °C) on microplate shaker (Titramax 1000, Heidolph). Optical density (OD_{600}) was recorded by Multiskan Spectrum microplate spectrophotometer (Thermo Electron

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Corporation, Finland) by interval of one hour during the first ten hours (soil isolates) or thirteen hours (*E. coli, P. putida*) and then after 22, 24 and 26 h. All the tests were performed in at least 3 replicates. Bacterial cultures were grown at 24 °C, optimal for the natural soil isolates and also acceptable for most of the mesophilic bacteria, including *E. coli, P. putida* and *B. mycoides*.

2.2.4. Bacterial viability assay (a 'spot'-test)

The 'spot'-test (described in detail by Kasemets et al., 2013) was used as an additional viability endpoint for the bacterial growth inhibition assays to test the ability of the toxicant-exposed bacteria to form colonies on nutrient agar after 26 h exposure to the tested chemicals. For that, 3 μl of the culture from each microplate well (treated and not-treated) was pipetted ('spotted') onto nutrient agar and incubated at 24 °C for 48–72 h. The growth of bacteria (formation of colonies) was evaluated visually on Difco™ Plate Count Agar (PCA) (formula as gram per litre: pancreatic digest of casein 5.0, yeast extract 2.5, dextrose 1.0, agar 15.0).

2.2.5. Analysis of the bacterial numbers from the Roundup-spiked soils

Colony-forming units (CFU) of heterotrophic bacteria (HB) from different time points of spiked and control soil samples were analysed on Difco™ Plate Count Agar (PCA) by spread plate technique. Briefly, 29 ml of sterile MilliQ water was mixed with 1 g of fresh soil in sterile plastic tube, shaken for 30 min on an orbital shaker and then vortexed for 30 s. The obtained slurry was used for preparing of the serial decimal dilutions in sterilised tap-water. Three replicate plates were inoculated from the three subsequent dilutions per sample. The inoculated plates were incubated at the room temperature (22–24 °C) in the dark and after 3 and 5 days incubation the CFU were counted. The results are presented as bacteria (CFU) per gram of dry soil.

For the microscopic enumeration of total number of bacteria in the soil, 1 g of fresh soil was mixed with 29 ml of sterile MilliQ water in 40 ml sterile screw cap cell culture tube and fixed with 37% formaldehyde (final concentration in sample 0.8–1.2%). The tubes were shaken on orbital shaker for 30 min. Then large soil particles were allowed to settle (5 min) and 0.5 ml of the upper layer containing bacterial suspension was filtered through 0.2 μm pore size black polycarbonate membrane filter (Nuclepore Track-Etch membrane Whatman, diameter 25 mm). For the enumeration of the total number of soil bacteria the acridine orange (AO) stained bacteria (Zimmermann, 1977) were counted using fluorescence microscope Olympus CX41.

2.2.6. Statistical methods

The toxicity values (EC50 – the median effective concentration of the toxicant that induces a designated effect in 50% of the test organisms after a specified exposure time) were determined from dose–response curves by the REGTOX software for Microsoft Excel (Vindimian, 2009) using the Log-normal model. One-way analysis of variance (ANOVA) followed by *t*-tests were used to determine statistical significance of the differences between toxic effects of the investigated compounds. The differences were considered significant, when $p < 0.05$.

2.3. Long-term outdoor experiments

Soil samples used for the long-term outdoor experiments were collected from the upper 5–20 cm layer of uncontaminated arable land not used during the past 10 years. Soil samples were air-dried at room temperature and sieved at 2 mm before use. Main characteristics of the soil: pH 7.0, C_{org} – 3.5%, soil texture – loam.

Tests were performed in containers with the total volume of the soil 6075 cm^3 and surface area of 486 cm^2. The containers were first filled with unpolluted fine sand (2 cm on the bottom) and then field-moist (initial water content about 23%) organic soil (10.5 cm) was added. The moisture content of the soil was determined by drying the soil samples at 105 °C for 24 h. All the results were calculated per gram of dry soil.

Soils were spiked with different doses (recommended for herbicidal use, 245 mg m^{-2}; 100-fold; 300-fold and 1000-fold rates) of two herbicide formulations, Roundup Quick™ and Roundup Max™, and were exposed outdoors during four months (from April to September 2012). 486 ml of the aqueous solutions of Roundup formulations of appropriate concentration was added to each container, while control containers received an equal amount of distilled water.

To evaluate the effects of the different concentrations of herbicides on soil microbes the number of viable heterotrophic bacteria (HB) was analysed after 10, 21, 45 and 108 days of spiking the soils. At the end of the experiment, after 108 days, the fluorescence microscopic counting of total number of the bacteria in treated and untreated (control) soils were performed in addition to viable plate counts.

Residual toxicity of the treated soils to non-target organisms was monitored using terrestrial (higher plants) and aquatic test species during several weeks after the spiking. In the plant assay, the inhibition of seed germination and shoot growth of the red radish *Raphanus sativus* and barley *Hordeum vulgare* in the treated soils were evaluated. The 48-h acute immobilization assay with crustacean *D. magna* and 30-min acute luminescence inhibition assay with bacterium *V. fischeri* were used for determining potential hazardous effects of leaching of herbicides from the treated soils into waterbodies. For that, aqueous extracts of the treated soils were prepared in ISO medium used in *D. magna* assay. The soil was added to ISO medium in 1:10 ratio and shaken at 200 rpm at 21 °C for 24 h. The suspension was clarified by centrifugation and supernatants were used in toxicity testing with crustacean *D. magna* and bacteria *V. fischeri*.

3. Results and discussion

The use of herbicides may pose hazard to non-target species. Commercial glyphosate formulations contain various additives, including surfactants. One of the most well-known additives is the surfactant polyoxyethyleneamine or polyethoxylated tallow amine, both abbreviated as POEA. Several studies have reported that the toxicity of glyphosate-based herbicides to aquatic organisms is largely due to this surfactant in the mixture (Folmar et al., 1979; Servizi et al., 1987; Buhl and Faerber, 1989; Mann and Bidwell, 1999; Tsui and Chu, 2003; Edginton et al., 2004; Moore et al., 2012). Specifically, commercial glyphosate formulations containing POEA were considerably more toxic to amphibians (Mann and Bidwell, 1999; Perkins et al., 2000; Edginton et al., 2004; Howe et al., 2004), to aquatic microalgae, protozoa and crustaceans (Tsui and Chu, 2003) than pure active substance or some glyphosate formulations with other surfactants (the identity and composition of these surfactants are often trademark protected).

In the current study, the short-term toxicity of glyphosate as an active substance (IPA salt of glyphosate) and two commercial glyphosate formulations with POEA (Roundup Max™) or without POEA (Roundup Quick™) to freshwater crustacean *D. magna*, laboratory test bacteria marine bacterium *V. fischeri*, soil bacterium *P. putida*, intestinal bacterium *E. coli* and three soil bacteria isolated by us from the local soil was studied. In addition, outdoor experiments were performed to assess/compare the long-term effects of different Roundup formulations on natural soil microbial numbers at recommended and elevated (simulating accidental pollution, e.g. spill during transportation) field application rates. Biological

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methods were used to evaluate the residual toxicity of treated soils at different time points.

3.1. Laboratory studies

3.1.1. Toxicity to aquatic species

Crustaceans *D. magna* and bacteria *V. fischeri* are aquatic species widely used in ecotoxicology. The toxicity of IPA salt of glyphosate and both Roundup formulations to these species ranged from 4.2 to 48.9 mg AE L^{-1} (Table 1). In case of *D. magna*, IPA salt of glyphosate (48-h EC50 = 4.2 mg AE L^{-1}) was about 10-fold more toxic than the tested Roundup formulations and no statistically significant differences ($p > 0.05$) between toxicities of Roundup Max™ and Roundup Quick™ were found. In case of *V. fischeri* Roundup Quick™ (30-min EC50 = 5.4 mg AE L^{-1}) was slightly more toxic ($p < 0.05$) than IPA salt of glyphosate (EC50 = 7.5 mg AE L^{-1}) and Roundup Max™ (EC50 = 7.6 mg AE L^{-1}) (Table 1). Hence, *V. fischeri* 30-min luminescence inhibition assay was up to 9 fold more sensitive than 48 h *D. magna* assay towards Roundup formulations but slightly less sensitive towards IPA salt of glyphosate (Table 1). However, the ability of *V. fischeri* to yield colonies on agar after the exposure to Roundup Max™ (the 'spot'-test, Fig. S3) showed that *V. fischeri* minimal bactericidal concentration (MBC) values were higher than the respective EC50 values and quite similar for *D. magna* 48-h EC50 values (Table 1). Thus, these tests did not reveal any link between the toxicity and presence/absence of surfactant (POEA) in the studied glyphosate products.

The toxicity results for *D. magna* in the current study (Table 1) are comparable to the data of the Safety Data Sheets (SDS) (Monsato Europe S.A. 2000, 2010). In *D. magna* tests, the effect of pH on the test results can be excluded as there was only a slight difference in the pH of the test solutions (pH at EC50: IPA salt of glyphosate-7.2; Roundup Quick™-7.1 and Roundup Max™-6.7). It can therefore be concluded that both investigated glyphosate formulations were about 10-fold less toxic to crustaceans than IPA salt of glyphosate. This conclusion, however, differs from the results presented by Tsui and Chu (2003), who demonstrated that Roundup was 77 fold more toxic than IPA salt of glyphosate to another aquatic crustacean, *Ceriodaphnia dubia*. This controversy may be explained by different formulations of Roundup and/or different test species used.

As mentioned above, the toxicity of Roundup formulations and IPA salt of glyphosate for *V. fischeri* was approximately in the same range (30-min EC50 values from 5.4 to 7.6 mg AE L^{-1}), but Roundup Quick™ was slightly more toxic ($p < 0.05$) than IPA salt of glyphosate and Roundup Max™. For the visualisation of the changes of the bacterial bioluminescence immediately after the contact with pesticide, kinetic bioluminescence inhibition test *V. fischeri* was conducted. This assay is very rapid and sensitive: the effect of toxic organic compounds on bacterial bioluminescence is noticeable already in few seconds after the exposure (Mortimer et al., 2008; Kurvet et al., 2011). Comparing the pattern of the 30-s kinetics of the glyphosate products it can be concluded that the mechanism of toxic action of Roundup Quick™ to *V. fischeri* is different from that of IPA salt of glyphosate and Roundup Max™ (Fig. 1). Indeed, in case of Roundup Quick™ the most remarkable effect on bacterial luminescence occurred already within the first few seconds and the effect reached plateau after 10 s showing rapid deleterious effects on bacterial cell membrane. However, in case of Roundup Max™ and especially IPA salt of glyphosate the effects were not so rapid and by 30 seconds the effect had still not reached the plateau (Fig. 1). However, the final EC50 values after 30-min of exposure were comparable (Table 1).

The *V. fischeri* luminescence inhibition assay EC50 values of this study are in agreement with the data from Kahru et al. (1996), Chang et al. (1981) and McFeters et al. (1983) but up to 20 fold lower than those reported by Tsui and Chu (2003), Hernando et al. (2007) and Bonnet et al. (2007). Such variations could be explained by different composition of the tested Roundup formulations but also by different test conditions (temperature, exposure time) and/or bacterial preparations used.

Indeed, due to many factors that may influence the test results it is often difficult to compare data originating from different literature sources. According Giesy et al. (2000), data reported on the short-term toxicity of glyphosate and its formulated products for the aquatic organisms vary remarkably: (1) microorganisms (3–7 day EC50 0.64–590 mg AE L^{-1}); (2) macrophytes (7–14 day EC50 1.6–25.5 mg AE L^{-1}); (3) invertebrates (2–10 day EC50 7 – >1000 mg AE L^{-1}); (4) fish (2–4 day LC50 5.8 – >1000 mg AE L^{-1}). The large variation of toxicity values may be mostly explained by the wide variety of the tested glyphosate-based herbicides but also by different test organisms, test conditions (temperature, test media) and test designs. In addition, there is a number of different formulations with analogous brand name (e.g. Roundup), which exhibit varying degrees of toxicity (Nandula, 2010). The toxic effect is a resultant of all the components in the formulation, incl. so-called "other additives". The unknown additives (e.g. up to 5% of weight in case of Roundup Quick™ used in this study) may also modulate the toxicity of the main components (active compound and surfactant) of herbicide formulations to aquatic species. Moreover, it is often not clear whether the toxicity data of glyphosate-based herbicides in literature are presented as the toxicity of the whole formulation or expressed as the acid equivalent, complicating the comparison with the existing information on the toxicity.

Due to the extensive use of glyphosate-based herbicides, glyphosate and its major metabolite AMPA have frequently been detected in water bodies. Both glyphosate and AMPA are water soluble and can persist in aquatic environments for several weeks (Giesy et al., 2000). According to a review (WRc Plc, 2009), glyphosate was detected in about 30% and AMPA in 50% of samples collected across Europe in 1993–2009. The highest observed concentrations were: glyphosate – 50 μg L^{-1}, AMPA – 49 μg L^{-1}. AMPA has usually been detected in higher concentrations and in a larger proportion of samples than glyphosate. High concentrations of glyphosate (up to 328 μg L^{-1}) have also been recently reported in USA (Battaglin et al., 2009). As mentioned in the Introduction, the proposed environmental quality standards for freshwater ecosystems vary largely (10–27,000 μg L^{-1}). According to the Water Framework Directive (EC, 2000), the permitted level of chemicals in surface water may be evaluated using a safety factor 1000 for the lowest acute EC50 value from the applied test set. If we divide the lowest EC50 value (4.2 mg L^{-1}, Table 1) for glyphosate obtained in the current study by 1000, the non-hazardous concentration of glyphosate for aquatic ecosystems may be roughly estimated as 4.2 μg L^{-1}, which is 10 to 100-fold lower than the highest observed concentrations. In Estonia, the screening data on glyphosate and AMPA have recently been made available and in some cases the concentrations of glyphosate and AMPA have remained below the limits of quantification (BaltActHaz, 2011, 2012). However, the highest reported concentrations of glyphosate and AMPA in surface water were 0.29 and 0.93 μg L^{-1}, respectively (Maves, 2010).

3.1.2. Inhibition of the growth of different bacterial strains by studied Roundup formulations

Microbial community of natural soil normally contains both, rapidly growing and slowly growing heterotrophic bacteria. The ratio of these two groups of bacteria depends on several environmental factors of which the most important are temperature and the availability of biodegradable organic substrates. Therefore, freshly isolated soil bacterial strains with different growth characteristics (Table 2) – *B. mycoides*, soil bacterium M1 and

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Table 1
The short-term toxic effect of Roundup Max™, Roundup Quick™ and IPA salt of glyphosate to crustacean *Daphnia magna* and bacteria *Vibrio fischeri*.

	Daphnia magna	*Vibrio fischeri*	
Toxicity	Immobilization of crustaceans 48-h EC50[a]	Inhibition of the luminescence 30-min EC50[a]	Inhibition of the further growth MBC[b]
Roundup Max™ (with POEA)	38.1 ± 6.7	7.6 ± 0.9	34
Roundup Quick™ (without POEA)	48.9 ± 5.5	5.4 ± 1.3*	Not tested
IPA salt of glyphosate	4.2 ± 1.8*	7.5 ± 1.4	Not tested

[a] EC50 – the median effective concentration, mg AE L^{-1}, of the toxicant that induces a designated effect in 50% of the test organisms upon specified exposure time.
[b] MBC – minimum bactericidal concentration, mg AE L^{-1}. The lowest tested concentration that completely inhibited the visible growth of bacteria on the agarized test medium at room temperature in the dark after 30 min of incubation to Roundup Max™ (Supplementary material, Fig. S3).
* Statistically significant ($p < 0.05$) difference from the other tested products in this toxicity test.

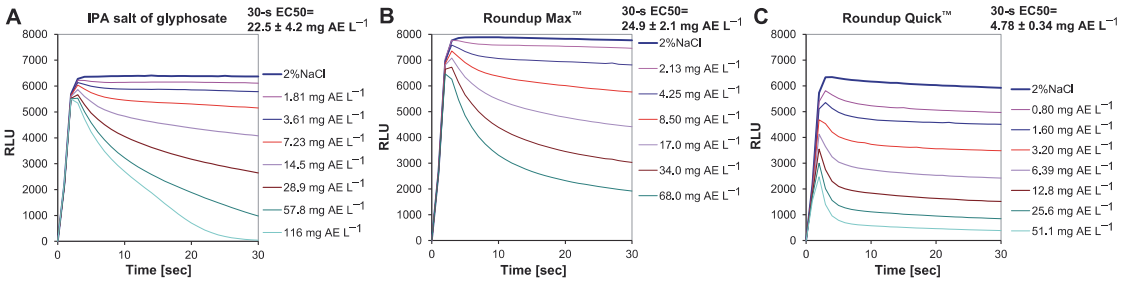

Fig. 1. The kinetic 30-s dose-effect curves of luminescence of *Vibrio fischeri* exposed to the different glyphosate products: A – IPA salt of glyphosate; B – Roundup Max™; C – Roundup Quick™. RLU – relative light units; 2% NaCl – negative control and diluent.

soil bacterium M2 – were used in addition to widely used laboratory test bacteria *E. coli* and *P. putida*, to study the toxic effect of glyphosate-based herbicides to various heterotrophic bacteria.

The results showed that toxicity of the tested glyphosate products to heterotrophic bacteria depended on the origin and growth characteristics of the tested bacterial strains. Despite the identical growth conditions, the growth pattern of the five tested bacterial strains (*E. coli*, *P. putida*, *B. mycoides*, soil bacteria M1 and M2) and their response to the glyphosate products varied largely (Table 2). The growth inhibition assay with the bacteria isolated from the soil demonstrated very different sensitivity of soil bacteria to the Roundup formulations and IPA salt of glyphosate. The results showed that the tested products of glyphosate totally inhibited the growth of indigenous gram-positive soil bacterial strains (*B. mycoides*, soil bacterium M1) already at the lowest test concentration (225 mg AE L^{-1} of glyphosate). However, glyphosate may also support the growth of some bacteria (e.g. *Pseudomonas* spp) (Gimsing et al., 2004). The results of the current study showed that the tested Roundup formulations were not toxic to gram-negative bacteria *P. putida*, *E. coli*, and soil bacterium M2 (Table 2): the EC50 values of tested products based on bacterial growth inhibition were 340–515, 506–706 and 845–1855 mg AE L^{-1}, respectively. Analogously to *V. fischeri* bioluminescence inhibition assay (Table 1), the EC50 values based on growth inhibition on five bacterial strains for three glyphosate products did not depend on the presence/absence of POEA.

In addition, a 'spot'-test was performed at the end of the growth inhibition tests (after 26-h incubation). Treated and non-treated bacterial cultures were examined for their ability to grow (form colonies) on the nutrient agar medium but also to verify the absence of the contamination of the test culture. The results of the 'spot'-test were coherent with the results of the growth inhibition test (Fig. 2 and Table 2).

Table 2
The effect of Roundup Max™, Roundup Quick™ and IPA salt of glyphosate on bacterial growth in laboratory conditions.

	Laboratory test strains				Bacterial strains isolated from the soil					
Test bacteria	*Pseudomonas putida*		*Escherichia coli*		Soil bacterium M2		Soil bacterium M1		*Bacillus mycoides*	
Duration of the lag phase (h)[a]	0.5		1		4.5		10		1	
μ_{max} (h^{-1})[b]	0.58		0.21		0.47		0.21		0.69	
	5h-EC50	MBC	5h-EC50	MBC	8h-EC50	MBC	16h-EC50	MBC	5h-EC50	MBC
Roundup Max™ (with POEA)	340	Not tested	548	Not tested	846	3600	<225	<225	<225	<225
Roundup Quick™ (without POEA)	515	Not tested	706	Not tested	1855	>3600	<225	<225	<225	<225
IPA salt of glyphosate	343	Not tested	506	Not tested	845	3600	<225	<225	<225	<225

[a] Duration of the lag phase – the period of time between the introduction of the control bacteria (i.e., no toxicants added) into the culture medium and the time it begins to increase exponentially.
[b] μ_{max} – maximum specific growth rate of the control bacteria.
EC50 – the concentration of the toxicant, mg AE L^{-1}, that inhibited bacterial growth by 50% after a specified exposure time.
MBC – minimal bactericidal concentration, mg AE L^{-1}. The lowest tested concentration that completely inhibited the visible growth of bacteria on the Difco™ Plate Count Agar (PCA) medium at room temperature in the dark after 26 h of incubation to the three glyphosate products (Roundup Quick™, Roundup Max™ and IPA salt of glyphosate). For more detail, see Section 2 and also Fig. 2.

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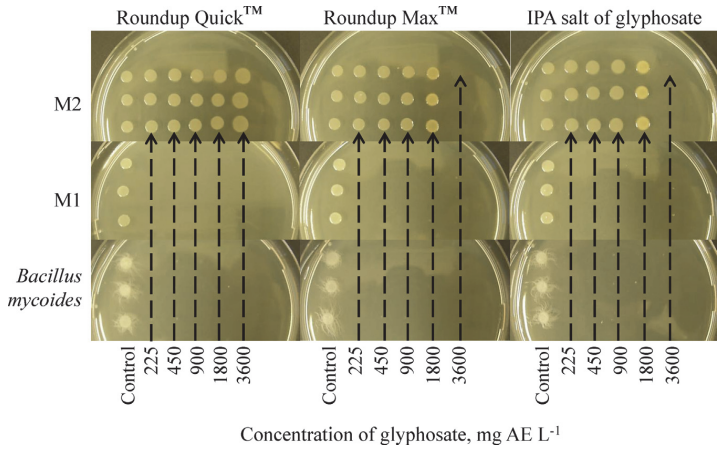

Fig. 2. Ability of bacterial strains isolated from the soil (M1, M2 and *Bacillus mycoides*) to yield colonies on the Difco™ Plate Count Agar (PCA) medium after 26 h of incubation at room temperature in the dark to different glyphosate concentrations of the three glyphosate products (Roundup Quick™, Roundup Max™ and IPA salt of glyphosate). For more details, see Section 2 and also Table 2 for minimal bactericidal concentration (MBC) values.

3.2. Long-term outdoor experiments

The persistence of glyphosate in the soil depends on soil type, climatic conditions and soil microbial activity. According to literature data, the half-life of glyphosate in field soils varies from 2 to 197 days (Giesy et al., 2000). The mean degradation half-life of glyphosate in the field soil has been suggested to be 30 days (Monsato, 2005). However, it should be mentioned that little research has been done on pesticide degradation in northern environmental conditions (temperate climate zone). Cold climate may influence glyphosate degradation in the soil and repeated applications could lead to accumulation in the soil (Dibyendu et al., 1989; Stenrød et al., 2005; Laitinen et al., 2009).

In the current study, the experiments were performed in climatic conditions typical for Estonia characterised by short and cool vegetative season (Supplementary material, Fig. S4). Soil samples were spiked with different doses (recommended, 100-fold, 300-fold and 1000-fold application rates) of Roundup formulations and exposed outdoors for four months. In practice, the application rate of glyphosate varies depending on the target species, the growth stage of the weed, the application method as well as on the specific formulation used (e.g. the recommended application rate for Roundup Max™ is 0.75–4.0 kg ha^{-1} and Roundup Quick™ 2.45 kg ha^{-1}). For soil experiments, the average treatment rate (2.45 kg ha^{-1}) recommended by the producer for weed control was chosen.

3.2.1. The impact of Roundup formulations on soil microbes

The fundamental role of the microorganisms in the biological and biochemical processes in the soil is generally acknowledged (Nielsen and Winding, 2002). In case of glyphosate contamination, the soil microbes are the main biological agents in bioremediation (Ermakova et al., 2010). Indeed, glyphosate degradation occurs quite rapidly in the presence of microorganisms but not in sterile conditions (Friestad and Brønstad, 1985; Torstensson, 1985; Giesy et al., 2000; Busse et al., 2001; Vereecken, 2005). At the recommended application rates and even at moderately increased concentrations (up to 100× field rate) glyphosate has generally been found harmless to soil microorganisms (Olson and Lindwall, 1991; Stratton and Stewart, 1992; Ratcliff et al., 2006; Zabaloy et al., 2012). Moreover, soil microorganisms can use glyphosate as an alternative phosphorus source (Borggaard and Gimsing, 2008).

In the current study, the approach based on the estimation of the number of viable aerobic heterotrophic bacteria (HB) by plate count method and by microscopic counting of total bacterial numbers (TBN) was used for the evaluation of the impact of Roundup formulations on soil microbes. The number of HB in the soils depended on the exposure time since treatment and on the applied Roundup formulation. The mean number of viable HB in non-treated (control) soil during the four month exposure (April 27 to August 21, 2012) was quite stable, ranging from $20{\cdot}10^6$ to $9{\cdot}10^6$ CFU g^{-1} dry soil (Table 3). The results are comparable to the data presented by other authors for unpolluted soils (Grayston et al., 2004; Lawlor et al., 2000; Black et al., 2003). The total heterotrophic bacterial numbers, evaluated by viable cell counts, had a tendency to increase in Roundup-exposed soils even in case of the highest application rates of the tested herbicide (Table 3). In case of the recommended field rate of Roundup formulations the number of HB in soils 10 days after the treatment was only slightly higher (2–4 times) than in the control soil. Remarkable increase in the number of HB (45 to 48-fold) was observed at the 100-fold and 300-fold recommended field rate of Roundup Quick™ at 10 and 21 days after the treatment, respectively. In the soil treated with Roundup Max™ the increase in the number of HB was maximum at the 1000× recommended field application rate after 10 days (4 fold) and at the 300-fold recommended field rate after 45 days (5 fold) when compared to the control soil (Table 3). Significantly higher number of HB (10 days after the exposure) in soil treated with Roundup Quick™ compared to Roundup Max™ indicates different impact of two formulations on soil microbial community (Supplementary material, Fig. S5). Thus, the impact of the glyphosate-based herbicides on the soil bacterial number depended not only on the concentration of glyphosate (the active substance), but even more on the additives, i.e. on the full composition of the herbicide formulations.

It should be mentioned that some microbial species involved in the glyphosate degradation are unable to grow *in vitro* and form visible colonies on the standard nutrient agar (Forlani et al., 1999). As a result, only part of soil bacteria can be enumerated by the colony forming units (CFU). In the current study, about 0.1% of the soil bacteria that were estimated by the direct microscopic counting were capable to grow/form colonies on the Difco™ Plate Count Agar (PCA) medium. Therefore, direct fluorescence microscopic counting of total bacteria number (TBN) was additionally used for the enumeration of the bacteria at the end of the experiment

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Table 3
The effect of Roundup Max™ and Roundup Quick™ on the soil bacteria.

Spiked concentrations of Roundup		Number of viable aerobic heterotrophic bacteria, HB (CFU g^{-1})[a] 10^6 g^{-1} dry soil				Total number of bacteria, TBN (cells g^{-1})[b] 10^6 g^{-1} dry soil
		10 d[c]	21 d[c]	45 d[c]	108 d[c]	108 d[c]
Roundup Max™	As recommended (245 mg m^{-2})	33.8 ± 2.8*	14.0 ± 0.1	27.6 ± 2.1*	8.6 ± 2.1	27.3 ± 2.5
	100× as recommended	39.9 ± 3.7*	26.9 ± 1.3*	48.4 ± 2.8*	8.6 ± 0.4	25.7 ± 1.7*
	300× as recommended	37.9 ± 1.4*	25.6 ± 1.1*	76.4 ± 3.1*	7.6 ± 0.3	24.0 ± 1.1*
	1000× as recommended	77.3 ± 1.5*	21.3 ± 1.4	52.6 ± 0.9*	7.1 ± 0.1	22.7 ± 0.9*
Roundup Quick™	As recommended (245 mg m^{-2})	85.1 ± 5.6*	20.0 ± 1.4	28.7 ± 0.8*	9.3 ± 0.2	22.4 ± 1.4*
	100× as recommended	953.0 ± 56*	256.2 ± 14*	91.0 ± 4.8*	15.2 ± 0.1*	24.3 ± 1.5*
	300× as recommended	360.8 ± 28*	708.6 ± 35*	134.6 ± 11*	24.1 ± 3.2*	22.5 ± 0.8*
Control	–	19.8 ± 2.8	15.8 ± 3.2	14.0 ± 3.7	8.5 ± 2.2	34.8 ± 3.6

[a] The number of viable aerobic heterotrophic bacteria (HB) in the soil was obtained by plate counts using Difco™ Plate Count Agar (PCA) and designated as colony forming units (CFU).
[b] The total number of bacteria in the soil (TBN) was analysed by fluorescence microscopy.
[c] Days passed from the spiking of the soil with indicated Roundup formulations.
* Statistically significant ($p < 0.05$) difference from the control.

(108 days after the spiking, Table 3). The microscopically evaluated total bacterial numbers in treated soils were slightly lower than in the control soil after 108 days of the soil treatment with the Roundup formulations.

3.2.2. *The residual toxicity of treated soils*

As plants are target organisms for the herbicides, the residual contamination of the treated soils was evaluated with two common crop species belonging to different plant orders and commonly used in Estonia: horticultural crop red radish (*Raphanus sativus*) and agricultural crop, field-grown barley (*Hordeum vulgare*). In addition to shoot growth inhibition, visible changes in morphological traits (colour and shape) of the plants were evaluated (data not shown). According to the producer's information, seeding of vegetables should take place 21 days after application of the glyphosate-based formulation (Monsato, 2002), therefore plant tests were performed 24, 47, 68, 82 and 110 days after soil treatment with the Roundup formulations.

Our experiments showed that barley was slightly more sensitive to Roundup formulations than red radish (Figs. 3 and 4). Therefore, the tests with radish were performed only twice (24 and 47 days after the spiking of soil with Roundup formulations) and further effects of soil treatment with Roundup formulations were evaluated with barley.

Results of the plant tests demonstrated that the recommended dose of the Roundup formulations did not affect the plant growth of either test species from already 24 days after the soil treatment. However, residual toxicity of soils treated with 100-fold and 300-fold doses disappeared only after 68 days and the toxic effect of the highest concentration of Roundup Max™ (1000-fold the recommended application rate) to barley after 110 days. In general, toxicity of Roundup Quick-spiked soils to barley decreased more rapidly than toxicity of Roundup Max-spiked soils (Fig. 4). The more rapid 'detoxification' of the Roundup-Quick treated soil compared with Roundup-Max was coherent with the higher bacterial numbers in the Roundup-Quick treated soil (Table 3).

In the view of the aforesaid, it could be concluded that in case of high (up to 1000-fold of recommended dose) contamination, the time needed for the decrease of glyphosate content in the soils to safe level may exceed the duration of vegetative period in climatic (temperate) conditions typical for Estonia. This conclusion is in agreement with the data on the effects of glyphosate on the soil microbes presented above.

In addition, as the two tested plant species (*R. sativus* and *H. vulgare*) showed different sensitivity to Roundup Max™ and Roundup Quick™ (Figs. 3 and 4), it is advisable to use at least two species from different families to monitor the remediation process at the contaminated sites.

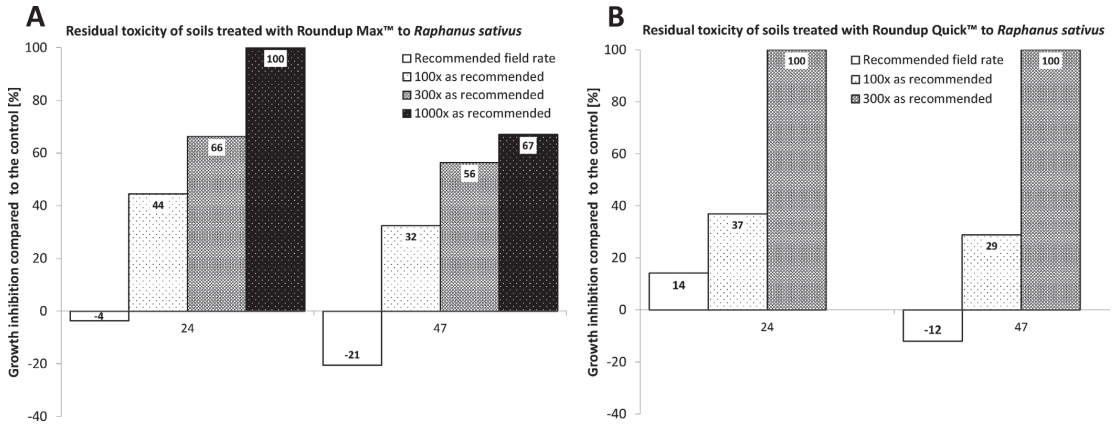

Fig. 3. Residual toxicity of soils treated with Roundup Max™ (A) and Roundup Quick™ (B) to red radish *Raphanus sativus*. Seeds of red radish were sown 24 and 47 days after the application of Roundup formulations.

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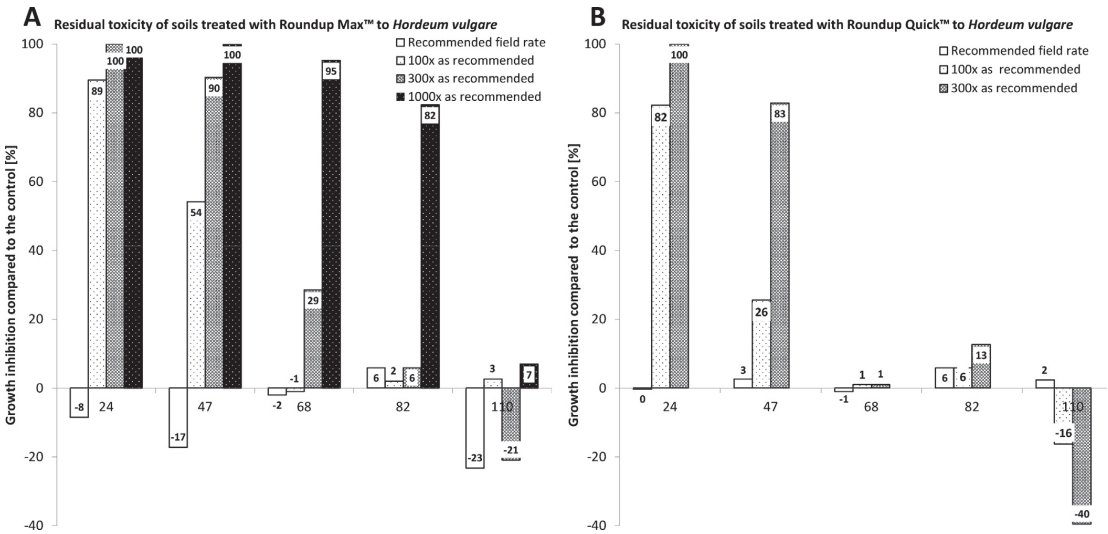

Fig. 4. Residual toxicity of soils treated with Roundup Max™ (A) and Roundup Quick™ (B) to barley *Hordeum vulgare*. Seeds of barley were sown 24, 47, 68, 82 and 110 days after the application of Roundup formulations.

The toxicity assessment of water leachates from soils helps to predict the risk of contamination of ground- and surface waters as well as to provide information on the potential hazard to organisms which may come into contact with soil water. The aqueous extracts (1:10) of the soils sampled 10 days after spiking with even the highest dose (1000-fold the recommended) of herbicide were not toxic (compared to the control, the toxic effect did not exceed 10%) to aquatic species, *D. magna* and *V. fischeri*. This shows that mobility of the glyphosate-based herbicides in soils with comparatively high content of organic matter is very low even in the case of high contamination. This is in agreement with the recent studies on the mobility of glyphosate in soil showing that the loss rate of glyphosate from agricultural fields is lower than for other herbicides (Laitinen et al., 2006; Shipitalo et al., 2008).

4. Conclusions

The results of the current study demonstrated that the toxicity of the glyphosate-based herbicides to non-target aquatic (crustaceans and bacteria) and terrestrial organisms (soil bacteria and plants) vary within a wide range.

Short-term toxicity tests showed that non-target aquatic species were much more sensitive to glyphosate formulations than the tested soil microbial strains. The results from the tests with the three natural bacterial strains, isolated from soil, showed that the indigenous gram-positive soil bacteria are seemingly more sensitive to glyphosate based herbicides than the gram-negative bacteria.

The long-term outside experiments revealed the different effects of the two tested glyphosate formulations (Roundup Quick™ and Roundup Max™) on the soil bacteria that are the main biological detoxifiers of glyphosate in the soil.

Direct relation between the toxicity of the tested formulations and the presence/absence of surfactant POEA was not evident as tested species showed different effects. Roundup Quick™ (without POEA) was more toxic to aquatic bacteria *V. fischeri* but less toxic to soil bacteria strains and terrestrial plants than Roundup Max™ containing POEA. Thus, the difference in toxicity of the two investigated glyphosate products (Roundup Max™ and Roundup Quick™) depended not only on POEA addition but also on other additives used in the specific formulation. Therefore, when reporting results of toxicity testing of glyphosate formulation it is very important to provide the complete name of the tested product and all the possible information on its chemical composition.

In typical Estonian climatic conditions, characterised by short vegetative period and relatively long cold period, the time needed for self-remediation of the soils in case of accidental pollution by glyphosate formulations (e.g. more than 100-fold the recommended field rate) may exceed the duration of the vegetative period. The mobility of the glyphosate in soils proved very low. Thus, application of glyphosate-based herbicides may pose risk to aquatic ecosystem stability mostly in case of direct contamination of surface water.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2013.07.005.

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APPENDIX B. CURRICULUM VITAE

ELULOOKIRJELDUS

1. Isikuandmed

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2. Hariduskäik

2011 – 2017	Tallinna Tehnikaülikool, keemia- ja materjalitehnoloogia doktorant
2008 – 2010	Tallinna Tehnikaülikool, keemia- ja keskkonnakaitse tehnoloogia, tehnikateaduste magistrikraad
2005 – 2008	Tallinna Tehnikaülikool, keemia- ja keskkonnakaitse tehnoloogia, tehnikateaduste bakalaureusekraad
1993 – 2005	Tamsalu Gümnaasium, keskharidus

3. Keelteoskus

eesti	emakeel
inglise	kõrgtase
hispaania, saksa	algtase

4. Täiendusõpe

2009	õpingud ERASMUS programmi raames Carlos III Ülikoolis Madriidis

5. Teenistuskäik

2009 – ...	Keemilise ja Bioloogilise Füüsika Instituut, insener
2007	Keskkonnainspektsioon Harjumaa osakond, praktikant keskkonnainspektorina

6. Kaitstud lõputööd

Tehnikateaduste magistrikraad: Saasteainete keskkonnaohu hindamine: ökotoksikoloogiline uuring. Tallinna Tehnikaülikool, 2010.
Juhendajad: Irina Blinova ja Marina Trapido

7. Teadustegevus

1. Bio- ja keskkonnateadused; 1.9. Keskkonnaohtlikke aineid käsitlevad uuringud; CERCS ERIALA: P305 Keskkonnakeemia

CURRICULUM VITAE

1. Personal data

Name:	Liina Kanarbik
Date and place of birth:	03.02.1986, Rakvere
Citizenship:	Estonian
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2. Education

2011 – 2017	Tallinn University of Technology, PhD student of Chemical and Materials Technology
2008 – 2010	Tallinn University of Technology, Master of Science in Engineering
2005 – 2008	Tallinn University of Technology, Bachelor of Science in Engineering
1993 – 2005	Tamsalu Gymnasium, High school education

3. Language competence/skills

Estonian	fluent
English	fluent
Spanish, German	basic

4. Special courses

2009	ERASMUS framework studies in Carlos III University in Madrid

5. Professional employment

2009 – ...	National Institute of Chemical Physics and Biophysics, engineer
2007	The Environmental Inspectorate, trainee

6. Kaitstud lõputööd

M. Sc. Thesis: Environmental hazard assessment of pollutants: ecotoxicity testing. Tallinn University of Technology, 2010.
Supervisors: Irina Blinova and Marina Trapido

7. Research activity

4. Natural sciences and Engineering; 4.11. Chemistry and Chemical Technology; P305 Environmental chemistry

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.

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