Eesti Maaülikool
Estonian University of Life Sciences

ALGAE *PSEUDOKIRCHNERIELLA SUBCAPITATA* IN ENVIRONMENTAL HAZARD EVALUATION OF CHEMICALS AND SYNTHETIC NANOPARTICLES

VETIKAD *PSEUDOKIRCHNERIELLA SUBCAPITATA* KEMIKAALIDE JA SÜNTEETILISTE NANOOSAKESTE KESKKONNAOHTLIKKUSE HINDAMISEL

VILLEM ARUOJA

A Thesis
for applying for the degree of Doctor of Philosophy in
Environmental Protection

Väitekiri
filosoofiadoktori kraadi taotlemiseks keskkonnakaitse erialal

Tartu 2011

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Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences

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To Henri-Charles

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LIST OF ORIGINAL PUBLICATIONS

I **Aruoja, V.**, Kurvet, I., Dubourguier, H-C., Kahru A., 2004. Toxicity testing of heavy-metal-polluted soils with algae *Selenastrum capricornutum*: a soil suspension assay. Environmental Toxicology, 19(4), 396 – 402.

II **Aruoja, V.**, Dubourguier, H-C., Kasemets, K., Kahru, A., 2009. Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. Science of the Total Environment, 407(4), 1461 – 1468.

III **Aruoja,V.**, Sihtmäe, M., Dubourguier, H-C., Kahru, A., 2011. Toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata* and bacteria *Vibrio fischeri*: Comparison with published data and QSARs. Chemosphere, 84(10), 1310 – 1320.

AUTHOR'S CONTRIBUTION TO PUBLICATIONS

I Villem Aruoja implemented and modified the algal testing procedure and used it to test the soil samples. He participated in data interpretation and preparation of the manuscript.

II Villem Aruoja participated in the study design and modifyed the experimental setup in order to study shading effects. He was responsible for most of the data interpretation and most of the writing of the manuscript.

III Villem Aruoja participated in the study design and performed the algal tests. He was the major interpreter and writer of the part of the manuscript that concerned algae. He is the corresponding author of this paper.

ABBREVIATIONS

AAP test	Algal assay bottle test
AAS	Atomic absorption spectroscopy
APIs	Active pharmaceutical ingredients
DNA	Deoxyribonucleic acid
EC	European Commission
EC50	Median effective concentration refers to the concentration of a toxicant which induces a response halfway between the baseline and maximum
ECHA	European Chemicals Agency (www.echa.europa.eu)
ECOSAR	ECOlogical Structure Activity Relationships (www.epa.gov/oppt/newchems/tools/21ecosar.htm)
ECOTOX	ECOTOXicology database (cfpub.epa.gov/ecotox)
ED50	Median, i.e. half maximal effective dose
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
IC50	Median, i.e. half maximal inhibitory concentration
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ID50	Median, i.e. half maximal inhibitory dose
ISO	International Organization for Standardization
K_{ow}	Octanol-water partitioning coefficient
LC50	Median lethal concentration, i.e. a concentration required to kill half the members of a tested population
LD50	Median lethal dose
LED	Light-emitting diode
LOEC	Lowest observed effect concentration
MDA	Malondialdehyde
MWCNT	Multi-walled carbon nanotube
NOEC	No observed effect concentration
NPs	Nanoparticles
OECD	Organisation for Economic Co-operation and Development
PNEC	Predicted no effect concentration
QSAR	Quantitative Structure Activity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive oxygen species
SWCNT	Single-walled carbon nanotube
U.S. EPA	United States Environmental Protection Agency
UV	Ultraviolet

1. INTRODUCTION

Algae are primary producers in the aquatic ecosystem and, as such, create the base of aquatic food chains. While they may seem insignificant compared to rainforests or other accumulated biomass on land, algae contribute approximately half of the global primary production as well as atmospheric oxygen. Due to their importance, algal growth response to existing and potential new environmental threats has to be clarified. In addition, being easily grown under laboratory conditions and sensitive to a wide array of both inorganic and organic chemicals, they are useful for the study of ecotoxicology in general.

In order to protect human health and the environment through better identification of the properties of chemical substances, a regulation called Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has been passed into law in Europe in 2007. REACH requires that ecotoxicological information, including toxicity to freshwater algae, is provided for all substances manufactured or imported into the European Union in quantities exceeding 1 tonne per year. In some cases, the toxicity data obtained using algae can even be used instead of vertebrate animal testing, thus saving costs and animal lives. Potential new environmental pollutants, such as synthetic nanoparticles, that are being increasingly used in consumer producs, are also required to be assessed for environmental toxicity, however the bioassays, which have been developed for soluble chemicals, have to be critically evaluated for this purpose.

In this thesis, a growth inhibition assay, using fresh-water microalgae *Pseudokirchneriella subcapitata* (also known as *Selenastrum capricornutum*), was developed and employed in the study of soils polluted with heavy metals, metal oxide nanoparticles as well as for the hazard analysis of a set of 58 industrially important anilines and phenols.

2. REVIEW OF THE LITERATURE

2.1. Ecotoxicology: chemicals and the environment

Ecotoxicology is a relatively new science that deals with the effects of toxic chemicals on biological organisms, especially at the population, community and ecosystem level. The term "ecotoxicology" was coined by René Truhaut in 1969 (Truhaut, 1977) and is by definition concerned with toxic effects of chemical and physical agents on living organisms, especially on populations and communities within defined ecosystems; it includes the transfer pathways of those agents and their interactions with the environment (Butler, 1978). As recently reviwed by Kahru and Dubourguier (2010 – Toxicology), ecotoxicological research gained importance due to the pollution brought on by industrial development and the research has been sped up by severe industrial accidents (e.g. Seveso, Minamata, Exxon Valdez). Policies were developed accordingly and ecotoxicology became an important part in environmental and ecological risk assessment. Unlike approaches driven by analytical chemistry that mostly quantify chemicals, ecotoxicological tests integrate toxic signals and thus it has been proposed to add toxicity-based criteria to the currently existing policies for the meaningful evaluation of the environmental hazard (Manusadzianas *et al.*, 2003; Põllumaa *et al.*, 2004; Kahru and Põllumaa, 2006). While risk assessment for human health concerns one species, environmental risk assessment should ideally consider millions of species, with different morphology, physiology, and habitat. As no single test or species shows uniform sensitivity to all chemical compounds, a battery of biotests with different sensitivity profiles is often used to assure a more adequate evaluation of the situation. Due to the complexity of ecosystems the ecotoxicological hazard assessment is more informative/predictive if the battery involves organisms of different trophic levels (Blaise, 1998; Blinova, 2000; Kahru *et al.*, 2000, 2008; Manusadzianas *et al.*, 2003; Kahru and Põllumaa, 2006).

In Europe, a further need for ecotoxicological testing has come with a new policy concerning safety of chemicals. Called the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), it was proposed by the European Commission in 2001 (CEC, 2001) and entered into force on June 1, 2007 (EC, 2006). REACH replaced approximately 40 different regulations with a single, streamlined one, as

well as created the European Chemicals Agency (ECHA). With REACH a single system for both new and existing industrial chemicals was created and the burden of proof for demonstrating the safe use of chemicals was transferred from EU member state authorities to the chemical industry. The main aims of REACH are to:

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

According to REACH, by the year 2018, all substances manufactured or imported in quantities of more than one tonne per year must have basic ecotoxicological information including short-term toxicity testing on crustaceans (preferred species Daphnia, OECD, 2004) and growth inhibition on aquatic plants (algae preferred, OECD, 2011). In addition, short-term toxicity testing on fish (OECD, 1992) is required in the next tonnage level (>10 tonnes per year). The chemicals are classified according to the response of the most sensitive of these three species. The three organism groups (crustaceans, algae, fish) represent different trophic levels of the aquatic food web, all of which have to be protected. The number of chemicals that require ecotoxicological assessment by 2018 is a matter of debate with estimations spanning from 30 000 to more than 100 000 substances in total (Pedersen *et al.*, 2003; Rovida and Hartung, 2009)·. This suggests that even in the best case scenario the task will be difficult to accomplish with traditional methods. Currently only a few hundred chemicals are toxicologically evaluated in Europe each year, which means there are not enough lab resources nor isn't it considered ethical to use as many laboratory animals as required by the traditional approach. The gap is expected to be filled with alternative methods, including computational toxicology and quantitative structure-activity relationships (QSARs) in particular.

REACH is broad in its scope and covers comprehensively most of the traditional environmental pollutants such as organic chemicals or heavy metals, however, there are new potential threats to health and environment that were originally not included in the regulation. Potentially the

biggest new class of pollutants are synthetic nanoparticles that are already used in consumer products as well as industry in rapidly increasing quantities. According to some predictions the production will reach approximately 2.5 million tonnes per year by 2025 (Robichaud *et al.*, 2009). Toxicity of nanoparticles is one of the most actively researched areas and discussions have started within the REACH competent authorities and its subgroup on nanomaterials on how REACH applies to nano size materials (EC, 2008a). Before any specific provisions are introduced into the law nanomaterials are still expected to be regulated by REACH because they are covered by the definition of a chemical "substance". The general obligations therefore apply as for any other substance, regardless of its size, shape or physical state. Thus, it follows that under REACH and the new Classification, Labelling and Packaging Regulation (EC, 2008b) manufacturers, importers and downstream users have to ensure that their nanomaterials do not adversely affect human health or the environment.

In conclusion, regulatory testing of ecotoxicological hazard of chemicals, the following test species in different food-web levels are recommended: fish (OECD guideline 203), Daphnia (OECD guidelines 202, 211), algae (OECD guideline 201). These aquatic organisms are also often used for water quality monitoring and hazard assessment of wastewaters since they respond to the presence of most types of pollutants in a predictable manner. As shown in Crane *et al.,* (2008), Kahru *et al.*, (2008) and Kahru and Dubourguier (2010) the types of test species and biological endpoints used within standard environmental hazard assessment frameworks are generally appropriate also for nanoecotoxicological research.

2.2. Microalgae in ecotoxicology

As primary producers, planktonic microalgae are a key component of food chains in aquatic ecosystems. Many species serve directly as a food source for zooplanktonic organisms, which are subsequently consumed by other invertebrates, fish or birds. Changes in the structure and productivity of the algal community may induce direct structural changes in the rest of the ecosystem and/or indirectly affect the ecosystem by affecting water quality (Nyholm and Peterson, 1997). It is therefore crucial to assess the toxicity of chemicals to algae as the pollution is likely to end up in water bodies *via* industrial or household waste.

As described above, results of toxicity tests with algae are part of the basic information required for the evaluation of environmental hazard of chemicals as recommended internationally by the Organization for Economic Cooperation and Development (OECD) and demanded in legislation such as REACH in EU, Toxic Substance Control Act in U.S.A and others. In addition, algae are often included among the species used in biotest batteries for hazard assessment of chemically contaminated wastes and leachates.

A number of studies have found algae more sensitive to chemicals than fish (Weyer *et al.*, 2000; Hutchinson *et al.*, 2003; Kahru and Dubourguier, 2010). According to Kahru and Dubourguier (2010) algae and crustaceans were also the most sensitive environmentally relevant species for synthetic nanoparticles. This implies that reliable algal toxicity data may help to reduce the number of fish needed for regulatory toxicity testing (Jeram *et al.*, 2005). The same tendency has been observed by Hutchinson *et al.* (2003) for active pharmaceutical ingredients (APIs): for 73 of the 91 APIs, the algal median effect concentration (EC50) and daphnid EC50 values were lower than or equal to the fish LC50 data. Thus, for approximately 80% of these APIs, algal and daphnid acute EC50 data could have been used in the absence of fish LC50 data to derive PNEC (predicted-no-effect concentration) water values. Analogously, Jeram *et al.* (2005) evaluated the acute toxicity for fish, daphnids and algae data from New Chemicals Database of the European Chemicals Bureau. Analysis of the sub-set of data (496 compounds) with precise L(E)C50 values for both algae and daphnia test results available showed that 401 out of the 496 substances acute algal EC50 and daphnid EC50 values were lower than or equal to the fish LC50 data meaning that in only in 91 (18.3%) cases fish was the most sensitive species. Hoekzema *et al.* (2006) also evaluated toxicity data sets for 507 compounds, including agrochemicals, industrial chemicals, and pharmaceuticals from their internal database and showed that in 188 (90%) of the 208 cases for which a complete data set was available, the median effect concentration for algae or daphnids was lower than the LC50 for fish. Therefore, Hutchinson *et al.* (2003) suggested that the current regulatory requirement for fish LC50 data regarding APIs should be succeeded by fish acute threshold (step-down) test data, thereby achieving significant animal welfare benefits with no loss of data for PNEC estimates. The above described research has recently yielded modification of the OECD guideline 203 on acute toxicity testing of chemicals using fish (OECD, 2008). Accord-

ing to this modified guideline, the fish test would be performed only at one concentration, the lowest between the EC50 concentrations obtained with previous testing with algae and daphnia. When fish would be more sensitive than algae and daphnia, testing with fish would be continued at lower concentrations (step-down). Currently, revised OECD guideline 203: fish, acute toxicity test involving the threshold approach, that takes into consideration EC50 values from relevant algae and acute invertebrate (e.g., daphnia) tests, is in the review phase.

2.2.1. Test formats and choice of species

Algal toxicity tests were used already in 1910 (Allen and Nelson, 1910), but the first standardized assay with freshwater algae was developed in the 1960s (Skulberg, 1964). It was used to assess the nutrient status of surface waters and the eutrophication potential of effluents entering water bodies. After the natural plankton was removed from the water sample, cultured algae were inoculated and the algal biomass yield after 1 or 2 weeks was used as the endpoint. This type of test using the algae *P. subcapitata* was introduced by Skulberg (Skulberg, 1964) and further developed by the U.S. Environmental Protection Agency (U. S. EPA, 1971) as the "algal assay bottle test" protocol (AAP test), which has been adopted worldwide. Other test protocols were later developed based on the AAP method in which the endpoint was growth inhibition, quantified either as reduced yield (biomass) or as reduced specific growth rate. Alternatively, photosynthesis was measured as ^{14}C assimilation (Steemann-Nielsen *et al.*, 1969; Hutchinson *et al.*, 1980) or as oxygen evolution (Kusk, 1978, 1981; Turbak *et al.*, 1986) or by short-term changes in chlorophyll flourescence (Rehnberg *et al.*, 1982; Wong and Couture 1986; Samson *et al.*, 1988). Other endpoints used for toxicity assays include inhibition of nutrient uptake (Nyholm, 1991) and changes in morphology (Soto *et al.*, 1979), pigmentation, or cellular components such as carbohydrates, lipids and proteins (Thompson and Couture, 1991).

Most standard algal toxicity tests that are currently used for regulatory purposes (US EPA , OECD, ISO) measure algal growth inhibition as the toxicity endpoint, i.e. the effects of a toxicant are investigated using a rapidly growing algal population in a nutrient-enriched test medium during an exposure period of 3 to 4 days. Either cell density is monitored directly by cell counting during the test, or other parameters which are

related to biomass are recorded. As a result, growth rate- or biomass-based EC50s, NOECs and LOECs can be calculated.

Although most standard algal bioassays that have been used for regulatory purposes are similar in design, small differences in all aspects of the testing procedure may have contributed to the large variability in test results obtained for individual species. Further standardization helps to eliminate factors that cause this variation, however only the variability that depends on the test method *per se* can be eliminated in this way. The variation in sensitivity arising from factors which modify the toxicity of chemicals also under natural conditions should not be eliminated because it reflects the validity of toxicity estimates. The algal toxicity tests proposed in various guidelines employ single algal species as a representative of the phytoplankton, which means that the variation in sensitivity related to the algal species becomes a central issue. For example, in a comparison where the toxicity of $K_2Cr_2O_7$, $CuSO_4$, $ZnSO_4$ and three pesticides were tested with seven algal species, including one blue-green alga, the EC50 values spanned 5 orders of magnitude. *P. subcapitata* was ranked as the most sensitive, whereas *Chlorella kessleri* was the least sensitive species (Rojíčková and Maršálek, 1999). *P. subcapitata* was in all cases more sensitive than the average sensitivity of all species to a compound. The sensitivity pattern of the studied blue-green alga *Synechococcus leopoliensis* stood out as different from those of the green algal species. Indeed, it has been shown that the main differences in cellular physiology between *Cyanophyta* and *Chlorococcales* are toxicologically relevant (Wängberg and Blanck, 1988).

In a comprehensive study using 27 pesticides, *Navicula pelliculosa* was generally the least sensitive species and *S. capricornutum* the most sensitive, even though none of the four algal test species used was consistently the most sensitive (Alexander and Hughes, 1993). Based on these and other studies it appears that when comparisons are made between species which are suitable for toxicity testing due to simple culturing methods, high growth rates and availability in culture collections, *P. subcapitata* stands out as one of the most sensitive.

Due to differences in algal sensitivity, the toxicity from one algal species cannot be directly extrapolated to another. The best solution to cover a wide sensitivity range of these organisms would probably be a species battery approach where all major algal taxonomic groups are repre-

sented (Lewis, 1995). Still, for legislative purposes a single algal species is preferred and thus the use of *P. subcapitata* or *Scenedesmus subspicatus*, as recommended in the OECD, EPA and ISO guidelines, is justified. Species that grow slowly and are more demanding in terms of culture requirements such as blue-green algae and diatoms are used only rarely. Since these groups of algae are ecologically important and due to the large inter-species variation in response to the same toxicant, the representatives of blue-green algae and diatoms should also be implemented in ecotoxicity assessment.

2.2.1.1. Taxonomy and distribution of *Pseudokirchneriella subcapitata*

P. subcapitata (Figure 1) is a planktonic species living in freshwater ponds, lakes and rivers. Cells in cultures are solitary except during cell division, occurring occasionally confluent to form few-celled clusters enveloped by a delicate and colourless mucilage. Cells have a helical shape, usually semi-circularly curved in the vegetative phase, while the twisting in old cultures could be one and a half turns. The diameter of the 154 - 360° arc ranges from 4.8 to 10.8 µm, width from 1.6 - 4.4 µm and depth/width

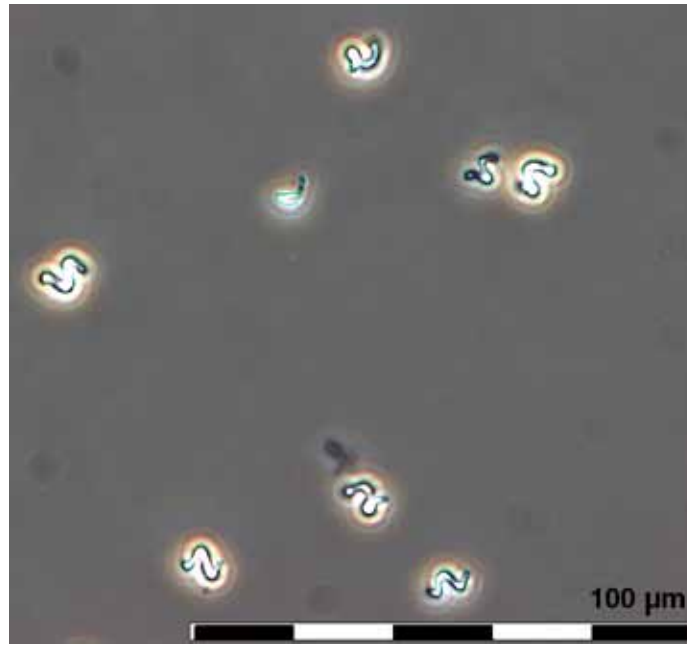

Figure 1. *Pseudokirchneriella subcapitata* cells under phase-contrast microscope.

ratio from 1.7 - 4.1. The chloroplast is parietal and devoid of pyrenoid. Reproduction is by division of the mother cell into 2, 4 or 8 autospores (Nygaard *et al.*, 1986).

The taxonomy of *P. subcapitata* is complex as several names have been and are still being used in parallel. The species was first described by Printz in 1914 as *Selenastrum capricornutum* (Printz, 1914). Subsequently in 1953 Korschikov renamed it *Kirchneriella subcapitata* (Korshikov, 1953) but the original name was still widely used at least until 1986 when Nygaard, *et al.* (1986) decided it belongs to the genus *Raphidocelis* (Hindak, 1977, 1990) and renamed it *R. subcapitata*. In 1988 Hindák established a genus called *Kirchneria* to accommodate those *Kirchneriella* species not known to have pyrenoids (Hindak, 1988). *Kirchneriella subcapitata* became the type species of this new genus and was named *Kirchneria subcapitata*. However, the name Kirchneria turned out to be already in use for other plants and so, in 1990, Hindák replaced it with *Pseudokirchneriella* (Hindak, 1990). There is some doubt in the justification for separating genera based on the presence or absence of a pyrenoid and thus some scholars still recognize the name *Krichneriella* (John, 2002). In fact, most papers in the field of algology use the latter version. To complicate things further, *Ankistrodesmus subcapitatus* (Korshikov) is sometimes mentioned as a taxonomic synonym (basionym) of *Raphidocelis subcapitata*.

The taxonomic classification according to Korshikov (1990) is as follows:

Phylum: *Chlorophyta*
Classis: *Chlorophyceae*
Ordo: *Chlorococcales*
Familia: *Chlorellaceae*
Subfamilia: *Ankistrodesmoidae*
Genus: *Pseudokirchneriella*
Species: *Pseudokirchneriella subcapitata*

Most, if not all strains of *P. subcapitata* used in the labs worldwide are progeny of the *S. capricornutum* isolated from River Nitelva, Akershus, Norway, by Olav Skulberg in 1959 (Norwegian Institute for Water Research (NIVA) -CHL 1). These include:

University of Toronto Culture Collection (UTCC) – *P. subcapitata* UTCC 37
American Type Culture Collection (ATCC) – *P. subcapitata* ATCC 22662

Culture Collection of Algae in The University of Texas at Austin (UTEX) - *S. capricornutum* UTEX 1648
Culture Collection of Algae and Protozoa (CCAP), Cambridge, England *S. capricornutum* CCAP 278/4

P. subcapitata is often described as a common species in planktonic environments and representative of both oligothrophic and eutrophic freshwater aquatic systems (Blaise, 1986), yet literature on its distribution is scarce. The published phytoplankton studies have revealed that this species was one of the most abundant species among *Chlorophyceae* in the river Ter in Spain (Sabater, 1987) and has been isolated/determined from the Botanical Garden of Copenhagen, Denmark (Nygaard *et al.*, 1986), Banglang Reservoir, Thailand (Ariyadej *et al.*, 2004), several water bodies in Romania (Momeu *et al.*, 2006; Porumb and Costică, 2008); lake Ca' Stanga, Italy (Tavernini *et al.*, 2009); lake Manzala, Egypt (El-Karim, 2008); Ogelube Lake, Nigeria (Biswas and Nweze, 1990.); Bulgarian coastal wetlands (Stoyneva, 2000); lake Kuusijärvi, Finland (Lahti *et al.*, 2008); ponds near Rostock, Germany (Alkhalaf *et al.,* 2009.) and lake Võrtsjärv, Estonia (Timm, 1973). Obviously, the phytoplankton studies where *P. subcapitata* has not been found greatly outnumber the ones referenced above.

2.2.2. Experimental factors affecting algal growth

The results of algal growth inhibition tests are affected by the experimental conditions such as the type of nutrient medium, test solution volume, light intensity, temperature and pH.. The effects of these experimental factors on the toxicity of the compounds analyzed have been reported on numerous occasions and a difference of one to two orders of magnitude in the results is not unusual. Several of the more important experimental factors are discussed below.

2.2.2.1. Light

Algae use light energy for growth and therefore uniform and sufficient illumination is essential for the growth inhibition test. Algal growth rate increases with increasing light intensity up to a level where light saturation is reached. That level depends on the species, temperature and nutritional status of the algae and is relatively high for green algae such as *P. subcapitata* (Nyholm and Källquist, 1989). When the temperature

is near optimum, the light saturation level will be higher. In the case of turbid samples or very dense cultures, shading by particles or the algal cells themselves may reduce effective light intensity. This can be overcome by mixing since algae can utilize short flashes of light. If, however, all light is adsorbed in the culture, linear growth will take place instead of exponential growth and toxic effects, unless the inhibition is near 100%, will not be detected. In addition, the chlorophyll content of algal cells depends on light intensity, being higher in lower illumination as the cells adapt to the light (Steemann-Nielsen *et al.*, 1962). This has to be considered when cholophyll content of the algal culture is used as a test endpoint. In order to avoid variation it is best to use low cell densities, sufficient mixing, small volumes (short light path) and light intensities higher than the saturation level. Still, lower than saturation level illumination is recommended by the guidelines due to practical reasons: it is difficult to remove the excess heat that may be generated by high intensity lamps. Thus, a light intensity between 4.4 and 8.9 klux is recommended by the OECD and 4.3 klux by the U.S. EPA (for *P. subcapitata*) which both remain below saturation level (Nyholm and Källquist, 1989). Novel, cooler light sources like light emitting diodes (LED-s) can be employed to overcome this problem. Interestingly, exponential growth has been achieved with LED-s at much lower light intensities compared to fluorescent tubes (Michel and Eisentraeger, 2004). LED-s have also the potential to provide completely uniform and specific light conditions when placed next to each sample, however, for general purposes fluorescent tubes are currently more practical. In order to keep the light intensity deviation below ±15% of the average over the incubation area a relatively simple setup may be recommended, which consists of a transparent table that carries test vessels and is illuminated with fluorescent tubes from below.

2.2.2.2. Temperature

It has been shown for a number of freshwater species that the growth rate/temperature relationship has three main parts. At lower temperature there is a near-linear or -exponential enhancement of growth rate with rising temperature; at intermediate temperature, a relative insensitivity of rate to temperature; at higher temperature, a decline of growth rate with temperature (Butterwick *et al.*, 2005). *P. subcapitata* can grow in a temperature range of 6 to 33 °C with an optimal temperature of approximately 28 °C. The growth rate is highly dependent on temperature

near the recommended testing range of 21-24 ºC and it is therefore important to ensure uniform temperature among sample vessels. Most processes of the algal metabolism are temperature-dependent, and the response to toxic substances is likely to be temperature-dependent as well. However, the studies published so far do not provide clear evidence as to how the toxicity of chemicals to algae depends on temperature.

Even though temperature and light intensity are generally considered as two independent factors, they are in fact closely related. It has been shown for a number of freshwater microalgae that the growth-rate response to light intensity is a function of temperature (Dermoun and Chaumont, 1992; Talbot *et al.*, 1991)·. This implies that lower light intensities are feasible at lower temperatures, thus reducing the growth rate and in addition helping to maintain stable pH (see below). The vast majority of toxicity studies using *P. subcapitata* employ the recommended 24±2 ºC as the algal growth temperature and lower temperatures are used only in rare cases (Hartmann *et al.*, 2010).

2.2.2.3. pH

The pH and buffering capacity of the OECD test medium is determined by its carbonate system. The conversion of CO_2 to carbohydrates by photosynthesis is pH neutral (Arensberg *et al.*, 1995):

$$nCO_2 + nH_2O \rightarrow (CH_2O)_n + nO_2 \quad (1)$$

but if the CO_2 mass transfer across the air/water interphase into the culture medium is smaller than the CO_2 utilization rate, bicarbonate is converted to CO_2 and hydroxide ions:

$$HCO_3 \rightarrow OH^- + CO_2 \quad (2)$$

and the pH increases as a result. This can be partly counteracted by using ammonium as the nitrogen source, as its utilisation creates hydrogen ions:

$$NH_4^+ \rightarrow H^+ + \text{algal-N} \quad (3)$$

However, in order to maintain stable pH, high cell densities have to be avoided. This can be achieved by constant shaking and using high surface/volume ratio in the test flasks that facilitates CO_2 mass transfer.

Also, it is possible to reduce the demand for CO_2 by reducing the initial cell densities to values between 5 x 10^3 and 10^4 cells/ml as recommended by the OECD (OECD, 2011). This way the pH increase can be kept smaller than the 1.5 units permitted by the OECD guideline. In addition, shortening of the duration of the test from 3 days to 2 days has been proposed as a way to keep pH stable (Mayer *et al.*, 1998). A lower testing temperature may help as well, but is not advocated as the effect of lower temperature on test results is unknown. A further option to overcome the carbon dioxide limitation is by using aerated cultures supplied with carbon dioxide-enriched air. This has been the method of choice when testing has to be carried out in a closed system, e.g. when testing volatile compounds (Mayer *et al.*, 2000). When the medium is enriched with carbon dioxide, care should be taken to avoid a decrease in pH. This can be achieved by increasing the bicarbonate content in the medium. Carbon dioxide equilibrates between the medium and the gas phase and pH can be calculated from:

$$pH = pk_{a1} + \log ([HCO_3^-]/[CO_2]_{aq}) \quad (4)$$

where pk_{a1} is the acidity constant of carbonic acid (pk_{a1} = 6.352). However the effects of enriched buffer on toxic responses and the test alga have not been carefully studied and the enriched carbonate buffer may result in increased ionic strength and lower test sensitivity (Brack and Rottler, 1994) .

2.2.2.4. Test medium

An essential component of algal growth inhibition tests is the nutrient-enriched medium which is a mixture of micro- and macronutrients. As the test endpoint is the inhibition of the algal growth, the composition of the medium may have a strong effect on the test results. For example, Adams and Dobbs (1984) compared the constituents in several media and evaluated their effect on the test results. The toxicity of aminothiazole was an order of magnitude less in the OECD medium compared to Bold's basal medium (Bold, 1942). Similar results have been reported for other chemicals and nutrient media (Vasseur and Pandard, 1988; Millington *et al.*, 1988). Factors like pH, hardness, chelators and major cations have been found to impact toxicity results. Concentration of chelators may be particularly important when testing heavy metals. While small quantities of chelators like ethylenediaminetetraacetic acid (EDTA) are necessary in

order to keep trace elements bioavailable, it has been demonstrated that EDTA greatly decreases the toxicity of metals due to chelation (Debelius *et al.*, 2009). For this reason, algal toxicity tests are sometimes carried out without EDTA in the test medium(Lin *et al.*, 2005). However, the algae may excrete organic metabolites that act as chelators and therefore a small amount of a chelating agent is preferred in order to have a defined and more stable chelating capacity of the medium. On the other hand, the use of natural water with unknown but site-specific chelating capacity would help to raise the predictive utility of the phytotoxicity data.

2.2.2.5. Quantification of algal biomass/growth

The most common response endpoints used in algal growth inhibition tests are growth rate and final yield (biomass or cell density at the end of the exposure period). While the yield based parameters may be required to fulfil specific regulatory requirements in some countries, the average growth rate during the whole exposure time is preferred as a more consistent measure of toxicity (OECD, 2011). Analysis of experimental results from International Standards Organization's ring tests (Hanstveit, 1982) showed that tests based on final yield were generally more sensitive (by as much as a factor of 2) compared to those based on growth rate. Nevertheless, from a theoretical point of view, growth rate is a better response variable than biomass, since it is less dependent on particular test system parameters (Nyholm, 1985). Also, from a standpoint of environmental hazard classification of industrial chemicals, the algal growth inhibition would be the driving force (i.e. more sensitive than crustaceans or fish) for the classification regardless of the choice between these two endpoints (Weyers and Vollmer, 2000).

Effects on biomass are usually measured indirectly by either counting algal cells in various types of counting chambers or using automated cell counters, or by means of optical density or fluorescence measurements. A conversion factor between the measured indirect parameter and biomass should be known. After the biomass/growth rate values for different samples have been calculated a dose-response curve can be plotted and fitted to the appropriate probit, logit or Weibull models, and from that the EC50 or other parameters can be obtained.

It should be mentioned that stimulatory effects are often recorded at low concentrations of toxicants, a phenomenon known as hormesis (Ceder-

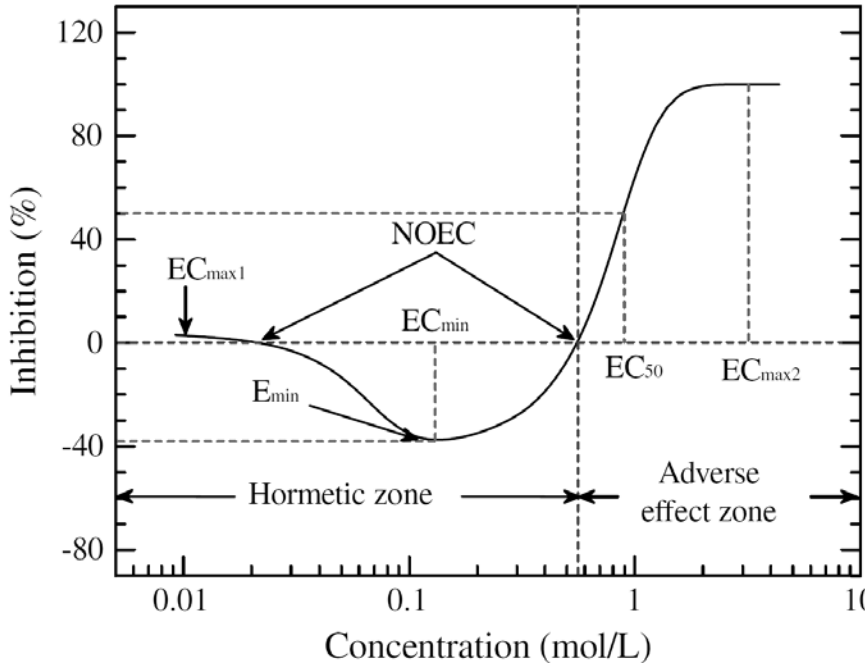

Figure 2. The J-shaped dose-response depicting hormesis. From Qin *et al.*, 2010, with permission.

green *et al.*, 2007). Formerly this stimulatory part of the dose-reponse curve was typically ignored and still there is no consensus about how to deal with such responses while calculating toxicity parameters. The curve-fitting may be difficult with traditional multiple linear regression methods due to small number of datapoints near the lower end of the dose axis as the emphasis is usually in the EC50 area. Recently proposed support vector regression methods that can cope with small samples may prove suitable for modeling hormetic dose-response with limited data (Qin *et al.*, 2010).

2.2.3. High throughput algal testing

Originally, the algal growth inhibition test was performed in Erlenmeyer flasks using a culture volume of 100 ml, a requirement that is still in the US EPA algal toxicity test protocol, only currently the flasks can be of any volume between 125 and 500 ml as long as the same size is used throughout a test and the test solution volume does not exceed 50% of the flask volume. Test vessels as large as that are quite impractical, as a lot of space is needed for incubation because of the flasks in three rep-

licates per concentration. The volume of toxic samples is relatively big, which might be problematic for laboratory staff and for waste disposal. In addition, the measurement of the algal biomass in each flask can be time consuming. Therefore, attempts have been made to convert the test to a microplate scale which would also allow automation. Blaise *et al.* (1998) have reviewed the more than 50 articles published on microplate testing. They conclude that while some studies confirm interprocedural comparability with flask-tests, notable differences are found in experimental variables and toxicity responses have been shown to vary accordingly (Blaise *et al.*, 1998). Later, Eisentraeger *et al.* (2003) have demonstrated with a set of 4 organic and 5 inorganic chemicals that results of the fluorometric microplate growth inhibition assay are highly comparable to those obtained with the fluorometric Erlenmeyer flask growth inhibition assay using algae *Desmodesmus subspicatus*. In another study comparing herbicide toxicity to several species of algae, including *P. subcapitata*, a good correlation was found between results from microplate and flask assays, but the toxic responses were smaller in microplates (Pavlic *et al.*, 2006) However, glass vessels are preferred in the US EPA, ISO and OECD guidelines, (even though "other chemically inert material" is permitted according to OECD; OECD, 2011) and stable pH may be difficult to achieve using microplates (Arensberg *et al.*, 1995). In addition, at least when using 96-well plates, the culture volume may not be sufficient for sampling and analytical determinations during incubation. Thus, a miniscale algal toxicity test using 20 ml glass scintillation vials has been proposed (Arensberg *et al.*, 1995). By means of constant shaking of the vials a highly stable pH can be maintained throughout the test. This setup seems to combine regulatory compliance and high throughput, especially when complemented with an efficient method to assess the biomass.

2.3. Ecotoxicology of heavy metal polluted soils

Heavy metal emissions from metal production, fossil fuel combustion, mineral fertilizers and other sources have created a central environmental issue. In fact, by mobilizing several times more heavy metals into the biosphere than released through natural weathering processes, the mankind has become the most important factor in the global biogeochemical cycling of heavy metals (Nriagu and Pacyna, 1988). A large proportion of the global heavy metal emissions is accumulated in soils and sediments due to their high sorptive capacity. This has created a concern in indus-

trialised countries, as the soils may soon become overloaded with toxic metals at the current rate of input.

Generally, the environmental risk assessment of heavy metal polluted soils is based on the total concentration of metals determined by chemical analysis. The degree of contamination is evaluated by comparing the estimated concentrations with specific threshold values. However, this approach does not account for the mobility and bioavailability of metals, which is important for understanding the ecological and health impacts of metal contamination (Kahru *et al.*, 2005). In this respect, toxicity assays are a valuable tool for evaluating the hazard of heavy metals in soil. Also, in contrast to chemical analyses, they detect the effects of multiple contaminants simultaneously. Soil toxicity tests that are recommended by the OECD include assays with earthworms, (OECD, 1984a), bacteria (OECD, 2000a; OECD, 2000b) and plants (OECD, 1984b). The earthworms (*Eiesnia fetida*) as well as collembola (*Folsomia candida*) tests have been shown to be relatively insensitive to heavy metals (Spurgeon *et al.*, 1994; Sandifer and Hopkin, 1996). In addition, the tests with earthworms and plants are work and space consuming, and thus small volume solid-phase bacterial tests, such as the Solid-phase Microtox test (Kwan and Dutka, 1992) have been suggested as an alternative. Analogously, a mini-scale algal toxicity assay using *P. subcapitata* has been developed (Arensberg *et al.*, 1995) and used for the analysis of soil suspensions. It turned out that in the case of soils contaminated with polyaromatic hyrdocarbons, the soil suspensions were several thousand times more toxic to *P. subcapitata* than respecive elutriates (Baun *et al.*, 2002).

In cooperation with Institut Superieur d'Agriculture (Lille, France) the ecotoxicological properties of topsoils sampled from a large, heavily polluted area surrounding two neighbouring lead and zinc smelters (Metaleurop, Union Minère) in Northern France were studied in our lab. Although *P. subcapitata* are known to be one of the most sensitive test organisms to heavy metals, the extracts of these metal-polluted did not inhibit their growth. Therefore it was considered important to introduce a soil suspension test with algae, which could be sensitive and reliable for ecotoxicological mapping of heavy metal polluted soils. Indeed, a major complicating factor in soil ecotoxicology is that most of the polluting substances are bound to soil particles but may become bioavailable upon direct contact of soil particles and organisms (Kahru *et al.*, 2005).

2.4. Ecotoxicology of metal oxide nanoparticles

Nanotechnologies, including the production and use of nanoparticles (NP), have become a significant priority in many countries. NPs are defined as natural or synthetic particles with one dimension smaller than 100 nm. Natural NPs include colloidal humus (Ryan and Elimelech, 1996; Lead and Wilkinson, 2006) and ultrafine particles in atmospheric emissions Englert, 2004). In the environment, NPs are regularly created as either weathering byproducts of minerals, as biogenic products of microbial activity, or as growth nuclei in super-saturated fluids (Wigginton *et al.*, 2007). Synthetic NPs can be inorganic nanopowders of metal oxides and metal salts like CdS (quantum dots) or carbon based like fullerenes, carbon nanotubes, dendrimers (Nel *et al.*, 2006).

Although the currently available information regarding production volumes is scarce and uncertain. Based on market study reports and data on industry websites it can be concluded that metal oxide NPs are already manufactured in large scale for both industrial and household use. It has been estimated that 60,000 tonnes of titanium dioxide and 10,000 tonnes of zinc oxide NPs are produced per year worldwide (Aitken *et al.*, 2006; Gottschalk *et al.*, 2010), which makes them probably the most important nano-materials in terms of production volume as well as potential environmental impact. Nano-TiO_2 is used as a UV-blocking agent in sunscreens (Popov *et al.*, 2005), in photocatalytic drinking water treatment (Theron *et al.*, 2008) and will likely be used in the manufacture of the next generation solar cells (Seo *et al.*, 2011). Zinc oxide NPs are similarly used as UV-filters in sunscreens (Serpone *et al.*, 2007), but also as a starting material for electronics applications, transparent UV-protection films and chemical sensors (Meulenkamp, 1998). Another important application of NPs is in biocidal coatings as nano-size particles have enhanced bactericidal properties compared to respective bulk materials. For example copper oxide and silver NPs are being incorporated into textile fibers and other polymers due to their biocidal properties (Khan *et al.*, 2011; Gunawan *et al.*, 2011). In addition copper oxide NP suspension (nanofluid) has excellent thermal conductivity for it to be used as a heat transfer fluid in machine tools (Chang *et al.*, 2005).

At nanosize range, the properties of materials differ from bulk materials of the same composition, mostly due to the increased specific

surface area and reactivity. The properties that make them attractive for commercial purposes, such as the ability to absorb ultraviolet light or biocidal effects, may also create risks for human health and the environment. Due to the current commercial development of nanotechnology, the occupational and public exposure to NPs as well as their potential release in the environment is expected to increase dramatically in the coming years. Therefore, the safety of synthetic NPs has become a worldwide issue. Although there is already remarkable amount of toxicological information concerning NPs available (obtained at various biological levels from *in vitro* cell cultures to *in vivo* studies on rodents), ecotoxicological data on NPs are just emerging. Kahru and Dubourguier (2010) have evaluated the existing literature data on toxicity (L(E)C50 values) of synthetic NPs. They found 77 effect values, mostly for nano TiO_2 (31%), C_{60}-fullerene (18%), nano ZnO (17%), nano Ag (13%), SWCNTs and nanoCuO (both 9%). Organism-wise, 33% of the data concerned crustaceans, 27% bacteria, 14% algae and 13% fish. Throughout, algae and crustaceans (daphnids) were the most sensitive and thus probably the most vulnerable organism groups to NP exposure. As stressed in the above mentioned review, in the case of some ecotoxicological publications there is a concern related to the way the harmful properties are discussed. For example, some papers claim that a compound/nanomaterial is toxic or even very toxic without indicating the concentration/dose that causes these adverse effects. However, for a meaningful (eco)toxicological profiling of NPs, quantitative toxicity data are required. EC50 values are the most robust values for the estimation of the dose-response (Isnard *et al.*, 2001) and median EC50 values are usually employed for the QSAR analysis (Cronin *et al.*, 2003).

There is vast experience in testing and developing standardized protocols for bulk/soluble chemicals that have resulted in recognized guidelines (ISO, OECD, US EPA). Usually, these testing methods cannot be copied blindly for testing NPs, as they differ from bulk chemicals and thus practical information for handling poorly soluble, turbid or colored chemicals and/or environmental samples, is helpful (OECD, 2000). For example gold and cobalt-ferrite particles have been found to flocculate and shield light too efficiently for algal testing (Núñez García *et al.*, 2010). Thus, assays initially designed for turbid environmental samples such as suspensions of soils and sediments, may find use in nanotoxicological research.

Although the NPs differ from bulk chemicals and show specific biological and environmental effects, the comparison of their toxicities shows some common tendencies. As mentioned above, algae and crustaceans were the most sensitive species to synthetic NPs studied so far and therefore the step-down approach of OECD 203 seems to be reasonable also for evaluating their toxicity to fish. Like for soluble chemicals, bioavailability remains a key factor for the hazard evaluation of synthetic NPs, regardless of the route of exposure and the mechanisms of action (Kahru and Dubourguier, 2010). Indeed, according to the most recent risk assessment exercise (Aschberger *et al.*, 2011), the main risk of NPs to the environment is expected from metals and metal oxide NPs, especially for algae and *Daphnia* due to exposure to both particles and ions that may leach from the particles.

2.5. Ecotoxicology of anilines and phenols in the context of REACH

The benefits of modern industrialized society are for a large part possible thanks to chemistry and chemical engineering. However, chemicals can also create a negative impact on human health and the environment when their production and use are not properly managed. Environmental contamination of organic aromatic chemicals is widespread as a result of the industrial applications ranging from their use as pesticides, solvents to explosives and dyestuffs. In addition, contamination with aromatic compounds can occur due to incomplete combustion of fossil fuels and the degradation of agricultural chemicals and industrial products such as pesticides, dyes and plastics (Latorre *et al.*, 1984; Hartter, 1985; Spain, 1995; Mantha *et al.*, 2001; Stolz, 2001; Liu *et al.*, 2002; Bhatkhande *et al.*, 2003). Among these substances, the derivatives of phenols and anilines are of major concern. For example, chlorophenols figure among the largest groups of compounds on the US Environmental Protection Agency list of priority pollutants, because of their resistance to degradation, toxicity, and their potential to accumulate into living organisms, soil and sediment. Chlorophenols are produced as by-products from the bleaching of pulp with molecular chlorine (Keith and Telliard 1979), and have been deliberately synthesised as wood preservatives. Anilines and azo-derivatives form the basis of much of the synthetic dye industry, part of which is lost in industrial effluent (Harvey *et al.*, 2002). These discharges are of environmental concern because of the stability of the dyes, which have been developed to withstand extremes of light and

temperature and exposure to detergents and microbes. Moreover, many azo derivatives of anilines are decomposed into potential carcinogenic amines under anaerobic conditions after discharge into the environment. For example, 4-chloroaniline (4-CA) which is both hematotoxic and genotoxic, is a known degradation product of a variety of substituted phenylurea compounds used mainly as herbicides, as intermediates in the synthesis of aniline dyes, and as accelerators and antioxidants in the rubber industry (Ellenhorn and Barceloux 1988).

The environment is thus regularly exposed to phenols and anilines through their use in a wide number applications. Phenol derivatives are nearly ubiquitous pollutants in all aquatic and terrestrial ecosystems (Zhao *et al.*, 2009). Because most of these chemicals have adverse effects on living organisms, their ecotoxicity and toxicity have been extensively studied (Cronin and Schultz, 1996; Könemann and Musch, 1981; Lu *et al.*, 2003) (Devillers, 2004; Liu *et al.*, 2006). However, despite the number of studies and data available, the information requirements for REACH are still largely unmet. It appears that a significant proportion of anilines and phenols, that have been used in volumes of more than 10 tonnes per year, lack important aquatic toxicity data (PAPER III). In order to close this information gap, REACH encourages the use of alternative methods, such as QSARs. Anilines and phenols should be a suitable group of chemicals to assess the validity of this approach: they are structurally similar and cover a wide range of physical, chemical and biological properties.

2.5.1. Quantitative structure-activity relationships (QSARs) in ecotoxicology

A QSAR is a mathematical model (usually a statistical correlation) relating one or more quantitative parameters that can be derived from chemical structure to a quantitative measure of a property or activity (e.g. a (eco)toxicological endpoint). The parameters used in a QSAR model are called (molecular) descriptors. Historically the first and still by far the most widely used descriptor is hydrophobicity or the octanol/water partitioning of a chemical, characterised by the respective coefficient (K_{ow}), which is nowadays itself often determined using a QSAR. Other descriptors are related for example to reactivity (molecular orbital energies), charge distribution or the shape of the molecule. The most common techniques for developing QSARs are regression analy-

sis, neural networks and classification methods. Examples of regression analysis include ordinary least squares, multiple least squares and partial least squares. Examples of classification methods are discriminant analysis, classification trees and distance based methods of similarity analysis. QSARs are typically based on experimental toxicity data wihich is divided into a training set which used for model development and a test set that can be used to verify the predictions.

According to the REACH legislation there is an obligation to carry out vertebrate testing only as a last resort, and to consider all other options first (Article 25 (1) REACH; EC, 2006). This includes the need to gather all existing information on physico-chemical, toxicological and ecotoxicological properties of a substance, including information generated by QSARs and chemical grouping methods. In addition, the sheer number of chemicals that have to be characterised in terms of toxicity by the deadlines set forth in the legislation necessitates the use of fast and cost-effective QSAR methods.

For the purposes of REACH the results of QSARs may be used instead of testing when the following conditions are met (ECHA, 2008):

1. results are derived from a QSAR model whose scientific validity has been established,
2. the substance falls within the applicability domain of the QSAR model,
3. results are adequate for the purpose of classification and labelling and/or risk assessment, and
4. adequate and reliable documentation of the applied method is provided.

According to the OECD (Worth *et al.*, 2005), for a QSAR model to be scientifically valid, its endpoint, algorithm and domain of applicability have to be properly defined. The domain can be described using the chemical structure (functional groups and their arrangement) or physicochemical properties (range and coverage) of the molecule. In addition, the biological/toxicological domain of the model should be described. It means that the mode(s) of action and the range of activity predicted by the QSAR should be known. Probably the most widely used method for assigning the mode of action to organic chemicals is the Verhaar scheme (Verhaar *et al*, 1992). It is based on fish (guppy, *Poecilia reticulata*) toxicity data and divides chemicals into four classes: non-polar narcotics,

polar narcotics, reactive chemicals and specifically-acting chemicals. In addition a fifth group may be used for substances that do not belong to any of the above described four classes. Each class is defined by certain structural features of the molecule. Narcosis (i.e. non-polar and polar narcosis) is the least specific but most important mode of toxic action in ecotoxicology since approximately 70% of all organic industrial chemicals are estimated to act *via* narcosis in acute exposures (1–14 days) (Bradbury *et al.*, 2003). Narcotic effects are believed to arise from the ability of a compound to interact with cellular membranes. The toxic potency of both non-polar and polar narcotic chemicals correlates strongly with their hydrophobicity and may be modeled using K_{ow} as a descriptor (Veith *et al.*, 1990; Verhaar *et al.*, 1992), however using toxicity data for a number of species, the polar narcotics have been shown to be 5–10 times more toxic than non-polar narcotics with the same K_{ow} (Vaal *et al.*, 1997). Currently the Verhaar scheme is used not only for fish toxicity but for aquatic toxicity in general.

Despite extensive studies in the field of QSARs, according to the analysis of endpoint study records submitted to ECHA for the 1504 dossiers of

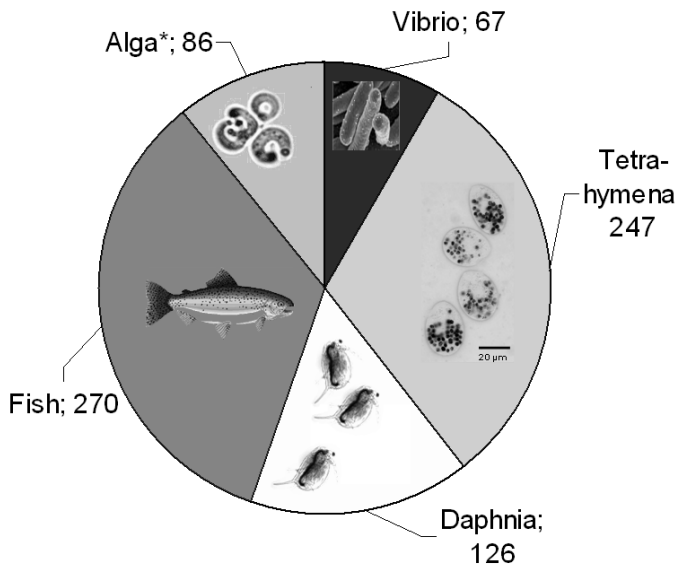

Figure 3. Bibliometry of peer-reviewed papers published on QSARs from 1991 to april 2011 according to ISI Web of Science. Numbers and area sizes refer to the quantiy of papers for each keyword, *-wildcard character. Modified from supplementary material of PAPER III

phase-in substances[1] at or above 1000 tonnes/year only about 1% of the information requirements of chemicals was met using QSARs (ECHA, 2011).

In the field of ecotoxicity of aquatic organisms bibliometric analysis shows that currently most of the QSARs have been developed using toxicity data on fish (34%) and protozoa *Tetrahymena* (31%), followed by *Daphnia* (16%) and the bacterium *V. fischeri* (8.4%). Surprisingly few QSARs (10.8% of the total) have been developed on algal data given that algal assay is obligatory for the registration of the chemicals and also REACH. Thus, seemingly, QSARs are developed upon availability of the published toxicity data even if the toxicity test used for creation of these data is not a regulatory one such as photobacterial luminescence inhibition assays as well as protozoan growth inhibition test (Kaiser and Devillers, 1994; Dimitrov *et al.*, 2003)[,]. For example, there has been a systematic effort to produce consitent *Tetrahymena pyriformis* toxicity data using the same growth impairment assay (Schultz, 1997), which has yielded a TETRATOX database with data for more than 2,100 industrial organic compounds of which more than 1,500 have been published. The situation is quite the opposite for algal toxicity as there is no consistent dataset with more than 100 values (Netzeva *et al.*, 2007). In addition, algal test results vary considerably due to the use of many different algal species and methods (see PAPER III). It is therefore important to develop methods for the generation of homogenous algal toxicity data that could be used in chemical safety assessment.

[1] Phase-in substance is a substance that was produced or on the market before REACH came into force and is subject to transitional arrangements if pre-registered.

3. AIMS OF THE STUDY

For the implementation of the EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) policy, a large group of chemicals (around 100 000) needs to be assessed for toxic and ecotoxic properties during this decade. This is an enormous challenge to European chemical industry and thus needs support from the scientists in providing toxicity information for chemicals obtained with regulatory ecotoxicity tests such as the algal growth inhibition assay as well as promoting the development of Quantitative-Structure-Activity-Relationship (QSAR) approaches.

Accordingly, the aims of this study were:
Using algae *Pseudokirchneriella subcapitata* as model organisms, to develop an algal toxicity assay that complies with the OECD 201 guideline, could also be applied to colored and particulate samples and would be easily scalable, in order to

1. analyze the toxicity of natural heavy metal polluted soils;

2. investigate the toxicity of ZnO, TiO_2 and CuO nanoparticles;

3. study of the toxicity of anilines and phenols.

4. MATERIALS AND METHODS

4.1. Toxicity testing with algae *Pseudokirchneriella subcapitata*

The OECD 201 algal growth inhibition test guidelines (OECD, 2011) were followed: exponentially growing algal cultures were exposed to various concentrations of the test substance under controlled conditions and the cell concentration of the control culture increased at least 16 times during 3 days. Also, the algal biomass measurements were performed at 24, 48 and 72 h as described in the OECD method. The algal stock culture for inoculation was taken from the commercial test system Algaltoxkit F (MicroBioTests Inc., Nazareth, Belgium) which uses the strain CCAP278/4 (from CCAP in Oban, Scotland, United Kingdom). The substances under investigation were incubated with *P. subcapitata* at 24 °C±1 °C for at least 72 h in 20-ml glass incubation vials containing 5 ml (9 ml when reading optical density from the vial, see below) of algal growth medium that was prepared according to the OECD 201 protocol. The vials, that were capped with air-permeable stoppers, were shaken on a transparent table and constantly illuminated from below with fluorescent tubes (Philips TL-D 38W aquarelle, Figure 4). The setup was a modifcation the method developed in Technical University of Denmark (Kusk, 2002, personal communication), and allowed simultaneous incubation of up to 136 samples. Soil and nanoparticle assays were run with all samples in three replicates and four controls, while assays with anilines/phenols with duplicate samples and four controls. Initial algal cell count was 10000 cells/ml as determined by counting under microscope in the Neubauer haemocytometer. Algal biomass was measured either by

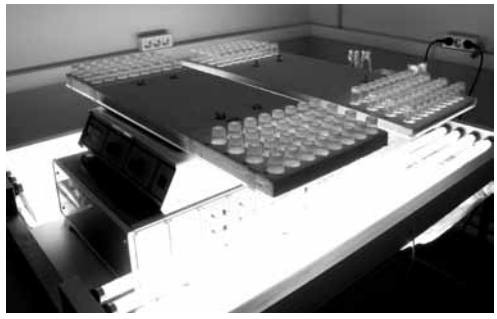

Figure 4. Setup of the algal growth inhibition test.

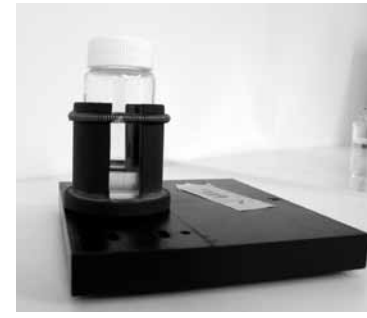

Figure 5. Vial (20 ml) holder for direct optical density measurement from the culture vessel

optical density at 682 nm directly from the incubation vials using a specially made vial holder for the spectrophotometer (Jenway 6300, Jenway Ltd., Essex, UK), or, in the case of turbid soil and nanoparticle samples, by chlorophyll fluorescence (see 4.1.1).

4.1.1. Test for turbid samples

In order to measure algal biomass from turbid soil samples and nanoparticle suspensions that interfere with optical density measurement a simple chlorophyll extraction protocol was developed. Dimethylsulfoxide/acetone has been used previously for the extraction of chlorophyll (Mayer *et al.*, 1997), however, in order to reduce the sampling volume and increase throughput, a microplate-compatible solvent was needed as acetone dissolved the microplate. Thus, 200 µl of ethanol was added to each 50 µl algal culture sample on a 96-well plate and the plate was shaken for 3 h in the dark (Figure **6**). Thereafter the fluorescence was measured with a microplate fluorometer (excitation 440 nm, emission 670 nm; Fluoroscan Ascent, Thermo Labsystems, Finland) using black microplates. The soil samples and metal oxide suspensions did not fluoresce under these conditions and their light absorbances at these excitation and emission wavelengths were below detection limit (Multiskan Spectrum, Thermo Electron Corp., Finland). During the exponential growth phase, the chlorophyll fluorescence (RFU) correlated linearly with cell density determined by counting in Neubauer hemocytometer (cell density (cells/ml)=RFU×2287000; R^2=0.9649).

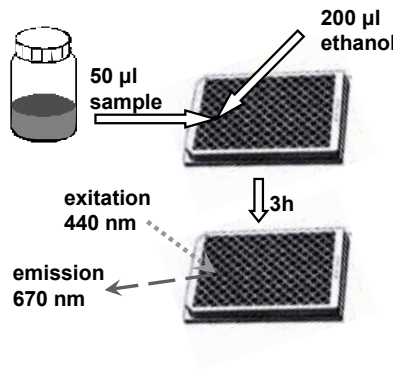

Figure 6. Exctraction and fluorometric quantification of algal chlorophyll.

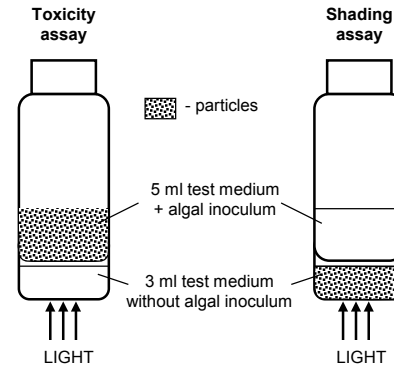

Figure 7. Two-compartment glass test vials for the assessment of the shading effect of particles. From PAPER II.

In order to evaluate the shading effects of particle suspensions, special double layer glass vials were constructed by sealing a smaller vessel (cut from a similar vial) under the 20 ml test culture vessel (Figure 7). The lower vessel was filled with 3 ml of algal growth medium but not inoculated with algae. For shading controls the same amount of metal oxide particles than in the respective test vessel was added to the lower vessel.

4.2. Sampling and characterisation of soils

Topsoils that were sampled from a large, heavily polluted area surrounding two neighbouring lead and zinc smelters (Metaleurop, Union Minère) in Northern France were chosen randomly from the soil bank of Institut Supe´rieur d'Agriculture (Lille, France). The soils were dried, and stored at room temperature. All soil chemical analyses were performed by the Laboratoire d'Analyses des Sols (INRA; Arras, France), using the metal solubilization protocol described in Sterckeman *et al.* (2000). Total soil Zn concentration was determined by flame atomic absorption spectroscopy (AAS), Cd and Pb concentrations by AAS with electrothermal atomization. The concentrations of Cd, Pb, and Zn in the water extracts of soils were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES).

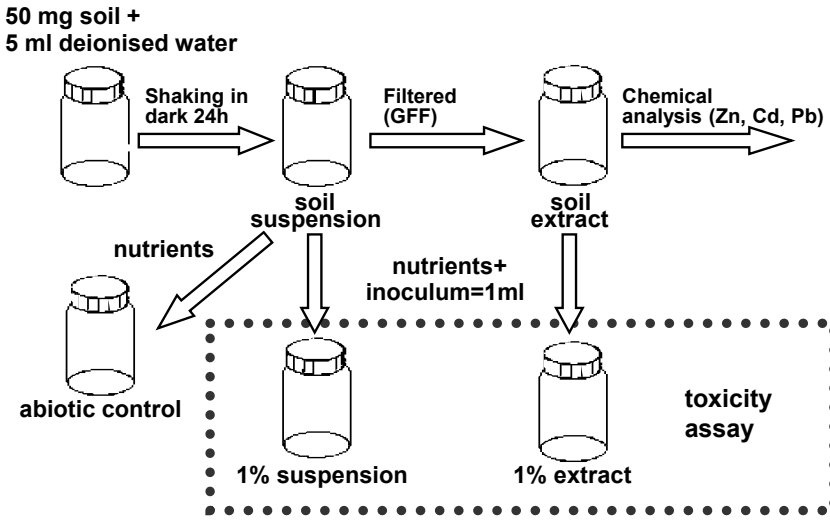

Figure 8. Preparation of soil suspensions and extracts.

The soil–water suspensions (1:99 w/v) were prepared by shaking the soil with MilliQ water for 24 h at room temperature (Figure 8). The particle-free extracts were obtained by filtering the suspension through a glass fiber filter (d=0.45 μm) and used for toxicity testing (soil-free extracts) as well as for ICP-AES. The soil:water ratio given above (1% soil suspension) was chosen as a compromise between the expected toxic concentrations and adequate light conditions for the growth of algae.

For the preparation of soil samples for dose–effect analysis of heavy metals a heavily metal-polluted soil, designated as "TREF" (soil 10, Table I in PAPER I) was mixed with soil designated "T017", which had otherwise similar physico-chemical properties but contained substantially lower concentrations of heavy metals (soil 9, Table I, in PAPER I). The stepwise dilution of soil 10 with soil 9 resulted in eight samples with different levels of heavy metals (see 5.2.2). The heavy metal concentrations in the mixed soil suspensions were calculated from the total concentration of metals in soils TREF and T017 and the ratios used for the mixing of soils. The water extracts of the soils (see above) were analyzed for Cd, Pb, and Zn using ICP-AES (Table II in PAPER I).

4.3. Chemicals

Nanosized metal oxides were purchased from Sigma-Aldrich, the particle sizes were specified by the manufacturer as follows: TiO_2: 25-70 nm; ZnO: 50–70 nm; CuO: 30 nm (mean). Bulk TiO_2 was purchased from Riedel-de Haën, ZnO from Fluka and CuO from Alfa Aesar. The 58 anilines and phenols from Sigma-Aldrich, Fluka, Merck, Acros-Organics and TCI Europe were 95% pure (52 chemicals more than 98% pure). All stocks were prepared in algal medium, and nano and bulk metal oxide suspensions were ultrasonicated prior to use for 30 min (Techpan Type UM-2, Poland). No co-solvents were used. If necessary, poorly soluble chemicals were dissolved by shaking the solutions overnight.

4.4. Statistical methods

The Log-normal model in the REGTOX software for microsoft Excel (Vindimian, 2005) was used for the calculation of toxicity parameters (EC and NOEC) and their 95% confidence intervals. One way analysis of variance (ANOVA) was used to determine statistical significance of the differences between values. The level of significance was accepted

at P≤0.05. Prism 5 (GraphPad Software Inc. www.graphpad.com) was used for calculations of algal growth rate and statistical significance of correlations.

4.5. Collection of previously published toxicity data

For the comparison of experimental toxicity values to previously published algal toxicity values for the studied anilines and phenols, toxicity data was collected from the US EPA ECOTOX (http://cfpub.epa.gov/ecotox/) database and from published papers. US EPA ECOTOX database was searched using Advanced Database Query and results (LC50, LD50, EC50, ED50, IC50, ID50) were exported to a Microsoft Excel spreadsheet. The toxicity values were corrected according to original concentration units and duplicate entries were removed. Roughly as many toxicity values as from the ECOTOX database were obtained from published papers that were identified using Google Scholar, Science Direct and ISI Web of Knowledge.

5. RESULTS AND DISCUSSION

5.1. Development of the algal growth inhibition assay for turbid samples

When using the algal growth inhibtion assay for coloured and/or particle-containing samples there are two technical factors that have to be accounted for: light intensity and biomass quantification. Samples that are not fully transparent adsorb the light energy needed for algal growth and may therefore appear toxic due to this shading effect. Secondly, the particulate material in the culture medium interferes with cell counting as well as optical density measurement. In the current thesis, a simple method to measure the biomass by the fluorescence of chlorophyll was developed (4.1.1, Figure 6.). Compared to the "whole water extract fluorescence" method presented by Mayer *et al.*, (1997), that uses acetone with DMSO for chloropyll extraction, ethanol was used as a solvent and the fluorescence was measured using a microplate fluorometer. Despite 30% lower fluorescence intensity compared to acetone/DMSO (Mayer *et al.*, 1997), the method compares favourably with the standard optical density measurement of biomass in terms of both sensitivity and linearity (R^2=0.997 compared to optical density; Figure.1 in PAPER I). In contrast to *in vivo* measurements where only about 1% of the absorbed light is re-emitted as fluorescence (Kirk, 1994), the extraction with ethanol uncouples chlorphyll from the photosynthetic apparatus and a much stronger signal is produced. In addition, ethanol is compatible solvent to be used on microplates.

Nevertheless, the chlorophyll content of algal cells is an indirect measurement of biomass and may depend on sevaral factors including the light intensity during the test, being higher in lower illumination as the cells adapt (Steemann-Nielsen *et al.*, 1962). The effect of light intensity on cellular chlorophyll concentration was investigated by covering the test vessel bottoms to a different extent with aluminum foil. As seen on Figure **9**, the chlorophyll content varied depending on light intensity and therefore the light conditions should be kept identical in all samples and controls. In the case of the soil suspension test it means there should be an identical amount of soil in samples and controls. The soil concentration used in the present experiments (1%) resulted in a light intensity of about 25 relative units in the test vessel. On the other hand, it is advisable to use light intensities above saturation level when testing

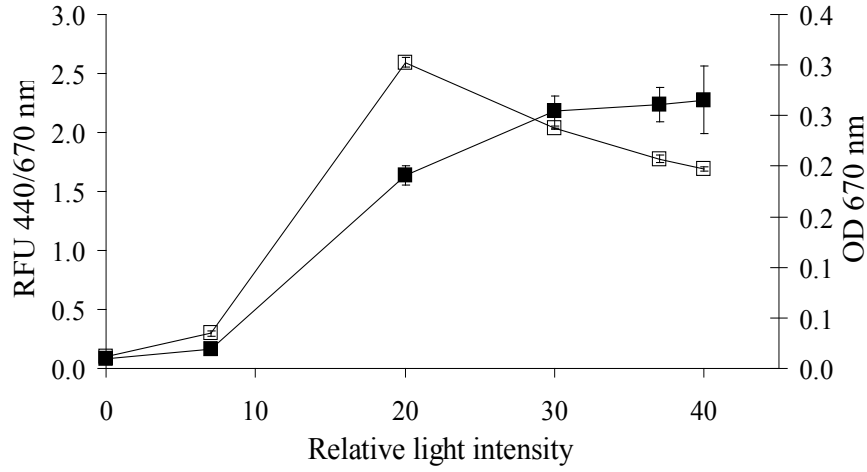

Figure 9. Chlorophyll fluorescence (in relative units RFU; open symbols) and optical density (OD; filled symbols) of the suspensions of algae *Pseudokirchneriella subcapitata* grown for 64 h at different relative light intensities. From PAPER I.

turbid samples, in order to reduce variation between samples due to different amount of shading.

In order to study the shading and toxic effects separately, the chorophyll exctraction method was combined with two-compartment vials that were designed on purpose (Figure 7). This way the amount of light-absorbing substance in the assay can be varied, while still accounting for possible shading effects. This setup was further successfully used for the analysis of turbid suspensions of metal oxide nanoparticles (5.3).

The developed algal toxicity assay complies with the OECD 201 guideline, even exceeding the requirements for pH stability and variation beween replicate control cultures.

5.2. Toxicity of heavy metal polluted soils

5.2.1. Stimulation of growth by soil suspensions

Heavily polluted soils from the area surrounding two lead and zinc smelters in Northern France were studied (4.2). Eight natural soils of the same type (carbonated sandy loam) with different levels of heavy metals were selected (Table I, in PAPER I). Seven of these eight samples

contained Cd and Zn in concentrations exceeding the permitted limit values for soils according to EU directive 86/272/EEC. However, when *P. subcapitata* was grown in 1% soil suspensions as well as in their respective particle-free extracts, no growth inhibition was observed compared to the control. In fact, in some exctracts and especially in all eight soil suspensions studied, the algae grew much better than in the standard medium, despite the fact that *P. subcapitata* is very sensitive to heavy metals. Still, there was some tendency toward a reduction of the stimulatory effect in the suspensions of the more polluted samples (Figure 3 in PAPER I). This suggested there could be a stimulatory effect of additional nutrients provided with all the tested soils as well as an adverse effect of the particle bound metals in the case of the more polluted soils.

In order to study whether the stimulation of algal growth could be a result of the supplementary macronutrients extracted from the soils, the least contaminated soil T314 (soil 11 in Table I of PAPER I) was used as a model. In addition to the 1% suspension and extract, a solution composed of 50 mg/l of NH_4Cl, 38.9 mg/l of $NaHCO_3$ and 7.4 mg/l of KH_2PO_4, that was supposed to simulate the amounts of nitrogen, phosphorus, and carbonates present in T314, as well as 2- and 5-fold concentrated OECD algal media were used for the cultivation of algae. However, the added minerals failed to enhance the growth of algae compared to the standard medium, and the growth stimulation was seen only with the actual soil suspension (Figure 4 in PAPER I). In parallel, all the above samples were spiked with 0.1 mg/l of Zn^+. As expected, the growth was inhibited by added Zn^+ in all mixtures, although the algal biomass in the spiked soil suspension marginally exceeded that in the clean (not spiked) algal medium (Figure 4 in PAPER I).

5.2.2. Dose-response analysis

In order to separate the stimulatory effect of soil components from the inhibitory effect of heavy metals, two soils with similar physico-chemical properties but different levels of heavy metals were mixed in 8 different proportions, as explained above (4.2). The relatively clean soil, designated "T017", contained (dry weight) 1.49 mg/kg Cd, 71 mg/kg Pb and 123 mg/kg Zn, while the respective concentrations in the relatively polluted soil, designated "TREF", were 20 mg/kg Cd, 1050 mg/kg Pb, 1390 mg/kg Zn. 1 % suspensions and respective exctracts were prepared from each soil mixture and used in the algal assay. This way the algae were exposed

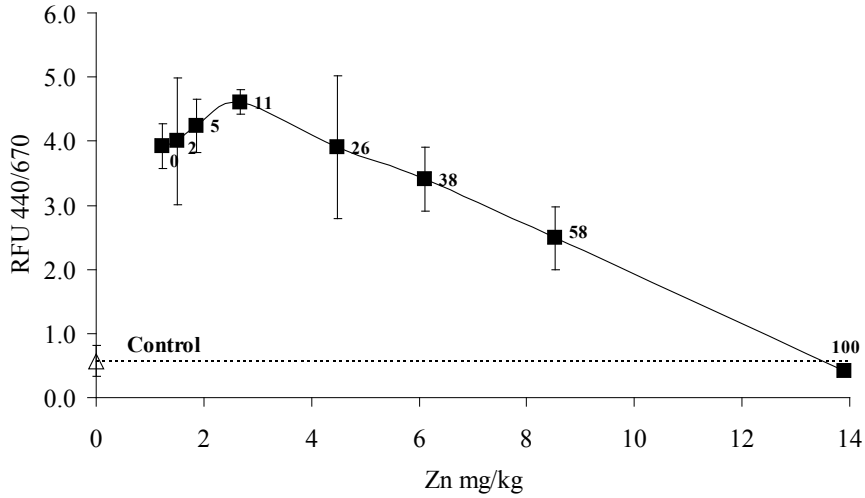

Figure 10. Chlorophyll content in algal cultures (in relative units, after 72 h growth in 1 % soil suspensions of soil mixtures with different heavy metal contents. Labels indicate percentage of polluted soil „TREF" in the mixture. Zn concentration shows the level of metal pollution. Modified from PAPER I.

to a constant amount of soil particles, but to steadily changing heavy metal concentrations. The algal growth with the soil extracts did not differ from growth in the clean medium (not shown), however, in the case of the soil suspensions, a clear dose-response was seen (Figure 10).

Compared to the control medium (dotted line on Figure 10), stimulation of algal growth was observed in all soil suspensions except the most polluted one (100% TREF; 13.9 mg/L of Zn in the test; Figure 10). For example, the biomass of algae after 72 h growth with the clean soil (0% TREF), exceeded the biomass in the soil-free medium by about 8 times. Samples containing between 2% and 11% of TREF stimulated the growth of algae even more than the clean 0% sample, which may be a manifestation of hormesis, a phenomenon of the stimulatory effect of low toxicant levels (Calabrese and Baldwin, 2001). However, beginning with a value of 6.1 mg Zn/l (38% TREF, Figure 10) in the soil suspension, a dose-dependent inhibition of the stimulation of algal growth indicated that the particle-bound metals had an adverse effect on algal growth. This suggests that the ecotoxicity analysis of soils should be carried out using a clean control soil with the same poperties instead of a mineral medium. However, it may be difficult to achieve in practice, unless the pollutant gradients form the point source are studied and the soil type does not change within the gradient.

5.2.3. Water extractability of metals

The analysis of the Zn, Cd, and Pb concentrations determined from extracts of the 1% soil suspensions showed that the water extractability of metals was quite low and similar regardless of the metal content in the soil (Table II in PAPER I). The higher the total amount of metals in the soil, the lower their relative extractability. For example, for soil suspensions with Zn concentrations between 6 and 14 mg/l, the water-extractable fraction of Zn was about 10%, whereas practically all Zn (80%) was water extractable from the suspension containing 1.2 mg/l of Zn. The water-extracted concentrations of Cd and Pb were too low to expect an inhibitory effect on *P. subcapitata*, however, the concentration of Zn in the soil extracts exceeded its EC50 value for *P. subcapitata* by as much as 10-fold (0.6 –1.4 mg/l compared to EC50 of 0.1 mg/l), which was inconsistent with the apparent lack of toxicity in the exctracts. This discrepancy could be explained by humic substances present in the soil exctracts that may have reduced metal toxicity. Koukal *et al.*, (2003) have demonstrated that the toxicity of Cd and Zn to *P. subcapitata* was significantly reduced in the presence of humic acids as the metals partitioned to the humic fraction where they were not bioavailable. In fact, their results suggested that humic acids reduced Cd and Zn toxicity in two different ways: (1) by decreasing the amount of free metal ions *via* complexation, and (2) by adsorbing onto algal surfaces and thereby shielding the cells from Cd and Zn ions.

There appears to be no direct correlation between leached metals and total metal concentrations in soil (Sheehan *et al.*, 2003, Kahru *et al.*, 2005). A later study in the Netherlands showed correlations between total and available Cd, Pb and Cu when soil parameters such as pH and clay content were taken into account, but the correlations were valid only for specific areas, it was difficult to predict metal availability for one area using relationships obtained for another (Van Gestel, 2008). Similarly to our results, the abovementioned study also revealed that despite high total metal concentrations (at times exceeding Dutch Intervention Values), available metal concentrations measured in the pore water were low. At the same time, elevated levels of Cd, Cu and Pb were seen in soil invertebrates. Thus, for proper risk assessment both the fraction of metal that may be leached into the aqueous phase as well as the fraction bound to soil particles, but still bioavailable upon direct contact with living organisms, should be evaluated.

5.3. Toxicity of ZnO, CuO and TiO_2 nanoparticles to algae

The stock suspensions of the studied metal oxide nanoparticles (NP-s) were turbid, apparently due to particle aggregation. TiO_2 and ZnO stocks became homogenous (milky) and lost their sediment only after sonication, whereas the dark CuO NP suspension was not visibly affected by ultrasound. In order to clarify whether these opaque suspensions could influence algal testing results by decreasing the intensity of available light due to shading, special double-layer vials were employed (Figure 7.). The analysis of the algal growth data showed that the shading effects were insignificant even for the most opaque TiO_2 suspensions (Figure 2 in PAPER II). Further toxicity tests were therefore carried out in regular culture vessels. The resulting EC50 values of the particle suspensions are shown in Table **1**.

The zinc oxide particles were the most toxic and their toxicity did not depend on particle size (the difference between nano and bulk formulations was insignificant). Copper oxide NPs could similarly be classified as "very toxic" while the nano titanium dioxide EC50 was only slightly below the 10 mg/l level that is considered "toxic" in terms of hazard classification. Copper oxide and titanium dioxide bulk formulations were clearly less toxic than the respective NPs. Our investigation showed that the toxicity was caused by mainly two factors: bioavailability of metal ions and adsorption of NPs to the algal cells.

Table 1. Toxicities of ZnO, CuO and TiO_2 particles and Zn and Cu salts to *Pseudokirchneriella subcapitata*, adapted from PAPER II.

	72h EC50		Aquatic toxicity ranking*
	mg metal/l	mg metal compound/l	
Nano ZnO	0.042	0.052	Very toxic
Bulk ZnO	0.037	0.046	Very toxic
$ZnSO_4$	0.042	0.104	Very toxic
Nano CuO	0.71	0.89	Very toxic
Bulk CuO	11.6	14.5	Harmful
$CuSO_4$	0.02	0.05	Very toxic
Nano TiO_2	5.83	9.73	Toxic
Bulk TiO_2	35.9	59.9	Harmful

*- based on metal oxide, 7th amendment 92/93 to Directive 67/548/EEC; <1 mg/l: very toxic; 1 - 10 mg/l: toxic; 10 - 100 mg/l: harmful

5.3.1. Metal bioavailability

ZnO particles as well as bulk ZnO inhibited the growth of *P. subcapitata* at already very low concentrations (<0.1 mg/l) and the total inhibition of algal growth was observed already at 0.16 mg Zn/l for both types of particles (data not shown). Interestingly, the $ZnSO_4$ toxicity to algae was the same as for particles (Table 1), suggesting the role of metal ions leaching from the particles in the overall toxicity of ZnO particles. Previous work in our laboratory with recombinant Zn-sensor bacteria demonstrated that up to 97% of Zn in both nano and bulk ZnO was in the bioavailable fraction when the concentration of particles was below 1 mg/l (Heinlaan *et al.*, 2008). The fact that microscopy did not reveal particle aggregation in the algal cultures with added ZnO is another indication that most of the ZnO may have been dissolved at these low concentrations. Our ZnO toxicity data are close to that obtained by Franklin *et al.* (2007) who showed *P. subcapitata* 72h EC50 values of 0.063 mg Zn/l for bulk and 0.068 mg Zn/l for nano ZnO. Using a dialysis membrane with a pore size of about 1 nm (permeable to Zn ions but not to ZnO particles) it was shown that both nano and bulk ZnO suspensions yielded similar dissolved Zn concentrations (Franklin *et al.*, 2007). Therefore the most likely mechanism of growth inhibition by ZnO is the toxicity of soluble zinc that leached from the particles. The same was true for ZnO toxicity to diatoms in seawater, only the toxic concentrations were higher. For example Wong *et al.* (2010) observed an EC50 of 4.6 mg/l for a diatom *Thalassiosira pseudonana*. A conclusion reached in a recent risk assessment was that in all available studies, it was shown that the toxicity of ZnO was mediated and caused only by leached Zn^{2+} ions. (Aschberger *et al.*, 2011). Since the ZnO dissolution has been reported to depend on particle size (Meulenkamp, 1998a), the toxicity should also be influenced by the particle size, especially when studied in a less sensitive assay where toxic effects appear at higher concentrations.

Similarly to experiments with zinc oxide suspensions, there were no visible aggregates present in the algal culture supplemented with copper oxide. Of the concentrations tested, complete inhibition of growth occurred at 6.4 mg Cu/l with NPs but at 25.6 mg Cu/l with bulk CuO (see PAPER II) When the incubation was extended several days beyond the standard 72 h, growth was observed in case of some initially toxic nano CuO concentrations (1.6 mg Cu/l of nano CuO). Soluble copper is a well known biocide and is used for antifouling in

marine paints, where the released copper ions prevent attachment of organisms to the vessel. However, on entry to the water column free copper is quickly complexed to carbonates, hydroxides and bound by organic ligands, that reduce its toxicity to the marine species (Tubbing *et al.*, 1994). Indeed, the bioavailability of copper, rather than the total copper present, is the primary determinant of toxicity as in the case of several toxic heavy metals (Campbell, 1995). In our experiments, the bioavailable fraction of Cu in the test medium was determined using the recombinant bacterial and yeast sensors. It turned out, based on both the bacterial and yeast Cu-sensor data, that the concentration of bioavailable copper was similar for all tested copper formulations (bulk CuO, nano CuO, $CuSO_4$), when the formulations were applied in equally toxic concentrations (Figure 4C in PAPER II). For example, at EC50 concentrations, the bioavailable copper was estimated to be 0.014–0.025 mg/l regardless of the type of copper compound tested. This indicates that similarly to nano and bulk ZnO, CuO toxicity to *P. subcapitata* was also caused by the soluble fraction of copper. Higher toxicity of nano CuO can be explained by its 141-fold higher bioavailability compared to bulk CuO at equitoxic concentrations. Other toxic effects of CuO NPs were not witnessed in the present study. In the case of procaryotic blue-green algae *Microcystis aeruginosa*, CuO NPs with a size less than 5 nm have been shown to enter the cells (Wang *et al.*, 2011). They also showed that NPs gathered at certain locations inside the cells, formed ROS, damaged DNA and finally affected membrane integrity as well as growth of algae. The CuO NP toxicity was enhanced by Suwannee River fulvic acid that may have increased Cu^{2+} dissolution from CuO NPs.

It is interesting to note that the results of the nano CuO study in PAPER II already seem to have practical consequences: toxicity of CuO NPs has been considered a serious limitation for the applications of copper nanofluids (Ebrahimnia-Bajestan *et al.*, 2011.).

5.3.2. Entrapment of algal cells by TiO_2

As titanium is not soluble in water, the inhibition of algal growth at relatively low concentrations has to have other reasons. and thus the TiO_2 toxicity cannot be mediated by solubilized metal ions. We observed in our experiments that the TiO_2 particles aggregated and adsorbed onto algal cells. The adsorption was more pronounced in the case of nano-

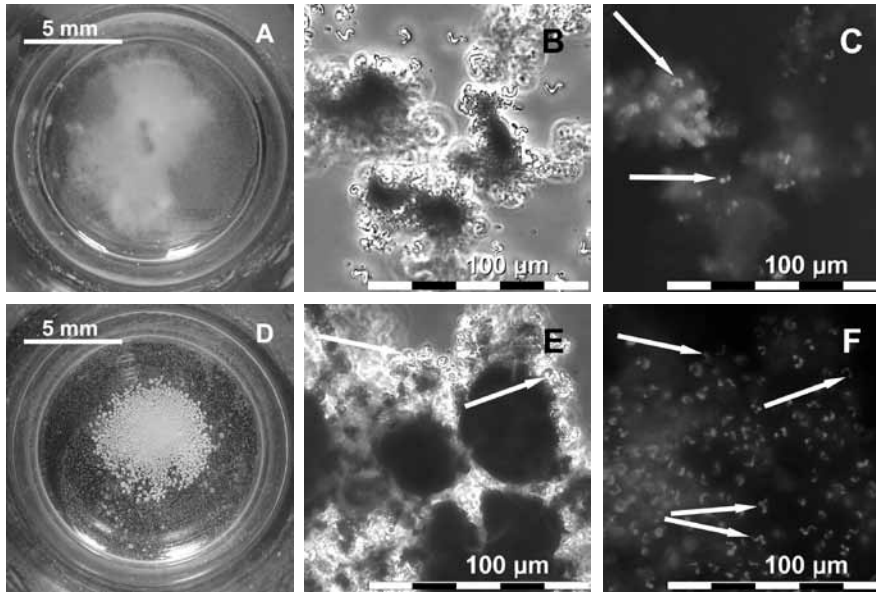

Figure 11. Aggregates of bulk and nano TiO_2 particles in *P. subcapitata* culture. Aggregates of bulk TiO_2 (A,B,C) and nano TiO_2 (D,E,F) in test medium, as visible to the naked eye (A,D), in phase contrast microscopy (B,E) and fluorescence microscopy (C,F). Arrows indicate algal cells. Adapted from PAPER II.

TiO_2 particles which formed large aggregates that entrapped most of the algal cells (Figure 11) and probably contributed to the toxic effect of nano TiO_2. The entrapment of algal cells could interrupt the cell function both physically and chemically. The adsorbed particles can block light as well as the movement of electrolytes and metabolites across the cell membrane. As the mass per unit volume of TiO_2 is at least 3 times higher than for the algal cells it limits their ability to remain suspended in the water column. Chemical interactions are most likely caused by reactive oxygen species since TiO_2 is a photocatalyst . When the TiO_2 NPs are irradiated with a light at wavelengths shorter than its bandgap energy, e.g., 3.2 eV

or 387nm for anatase, electron/hole pairs will be generated and migrate to its crystalline surfaces where reactive oxygen species (ROS) can be generated (Nagaveni *et al.*, 2004). ROS may initiate lipid peroxidation in the cell membrane which is detrimental to the cells. The lipid peroxidation of algal cell membrane due to TiO_2 NPs has recently been demonstated by Metzler *et al.* (2011). Interestingly, the extent of lipid peroxidation (registered by the amount of malondial-

dehyde produced) increased with decreasing initial algal population. One probable explanation was that with decrease of population density, the TiO_2 to cell ratio increased, leading to more serious damage to algal cells.

In our experiments, the presence of algal cells enhanced the aggregation of TiO_2 NPs (Figure 11), a phenomenon that has been demonstrated also for *Scenedesmus sp*. and *Chlorella sp*. (Sadiq *et al*., 2011). Also, the algal DNA tends to leak out of the cells upon contact with TiO_2 NPs. This has been shown for *P. subcapitata* (Metzler *et al*., 2011) and the blue-green algae *Anabaena variabilis* (Cherchi *et al*., 2011). A TEM analysis in the latter study suggested possible disaggregation of nano TiO_2 aggregates when in close contact with cells, potentially as a result of excreted DNA that may serve as a biodispersant. These interactions, as well as the concentration-dependent aggregation of TiO_2 NPs observed by Hartmann *et al*., (2010), could hamper the analysis of the concentration–response relationship in algal toxicity assays. However, based on the results so far, nano-TiO_2 can be considered of moderate concern compared to other NPs, mostly because of its low ecotoxicity (Aschberger *et al*., 2011). However, due to its wide use in commercial products and one of the highest production volumes among NPs, it can reach the environment through many diffuse sources and the concern is mainly based on the potential high environmental exposure.

5.4. Toxicity of anilines and phenols to *Pseudokirchneriella subcapitata*

The study of anilines and phenols was carried out in order to facilitate hazard evaluation/classification of industrial chemicals as required by REACH. In addition to providing new toxicity data, the aim was to investigate whether the algal toxcity data produced in a consistent way, i.e. using the same setup in a single lab, would facilitate development/validation of QSAR models. For that, a set of structurally similar 28 anilines and 30 phenols was selected (Figure 12, Table 2).

The EC50 values of *P. subcapitata* 72-h growth inhibition were experimentally determined for all 58 compounds. Despite the fact that the analyzed molecules were structurally similar, the EC50 values spanned two orders of magnitude ranging from 1.43 mg/l (3,4,5-trichloroaniline) to 197 mg/l (phenol)(Table 2).

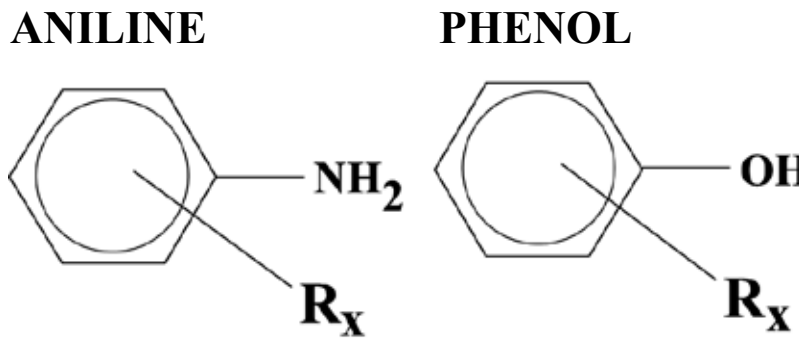

Figure 12. General formula of the studied compounds. R stands for -Cl, -CH_3 or -CH_2-CH_3, x is 1,2 or 3.

The toxicity of the studied compounds was dependent on the type (chloro-, methyl-, ethyl-), number (mono-, di-, tri-) and position (*ortho-, meta-, para-*) of the substituents. As a rule, the higher the number of substituents the higher the toxicity. The chloro-substituted molecules were generally more toxic than alkyl-substituted ones. Consistent substituent position effects were observed in the data. For almost all tested chemicals the substituent in the para-position tended to increase toxicity whereas most of the ortho-substituted congeners were the least toxic (Table **2**). The position effects in the case of anilines and phenols with a single substituent is illustrated on Figure 13. The same rules applied for structures with a higher number of substituents, so that molecules with substituents in both ortho positions turned out to be much less toxic than ones without ortho substituents (e.g. EC50 of 2,6-dimethylaniline was 107 mg/l, while EC50 of 3,4-dimethylaniline was 7.3 mg/l; Table 2). In order to verify whether these position effects can be found in previously published toxicity data, a thorough literature/ database search was performed (see 4.5). Algal toxicity data existed for 31 substances in our list of 58 (118 values in total), however, only 10 substances had data obtained in compliance with the OECD guidelines. In addition, the published toxicity values were highly variable, in some cases spanning several orders of magnitude, depending on testing methodology and data source (Figure 14). However, the published toxicity data generally followed the same pattern as our experimental data (filled symbols on Figure 14), escpecially for chemicals with more than two or three published EC50 values (see also Figure 1 in PAPER III). Also, better agreement with our experimental values was seen for data obtained using the same

Table 2. Toxicity of the studied 28 anilines and 30 phenols to *Pseudokirchneriella subcapitata* based on growth rate. Adapted from PAPER III.

Aniline	Abbreviation	EC50 mg/L	Phenol	Abbreviation	EC50 mg/L
aniline	A	54.20	phenol	P	197.00
2-chloroaniline	2-CA	39.10	2-chlorophenol	2-CP	51.80
3-chloroaniline	3-CA	26.90	3-chlorophenol	3-CP	11.50
4-chloroaniline	4-CA	3.55	4-chlorophenol	4-CP	31.40
2,3-dichloroaniline	2,3-DCA	6.75	2,3-dichlorophenol	2,3-DCP	10.90
2,4-dichloroaniline	2,4-DCA	3.96	2,4-dichlorophenol	2,4-DCP	8.13
2,5-dichloroaniline	2,5-DCA	16.50	2,5-dichlorophenol	2,5-DCP	3.68
2,6-dichloroaniline	2,6-DCA	23.20	2,6-dichlorophenol	2,6-DCP	16.10
3,4-dichloroaniline	3,4-DCA	2.50	3,4-dichlorophenol	3,4-DCP	2.19
3,5-dichloroaniline	3,5-DCA	4.39	3,5-dichlorophenol	3,5-DCP	2.10
2,3,4-trichloroaniline	2,3,4-TCA	3.55	2,3,4-trichlorophenol	2,3,4-TCP	4.16
-	-	-	2,3,5-trichlorophenol	2,3,5-TCP	2.26
-	-	-	2,3,6-trichlorophenol	2,3,6-TCP	8.05
2,4,5-trichloroaniline	2,4,5-TCA	3.14	2,4,5-trichlorophenol	2,4,5-TCP	7.57
2,4,6-trichloroaniline	2,4,6-TCA	4.94	2,4,6-trichlorophenol	2,4,6-TCP	5.64
3,4,5-trichloroaniline	3,4,5-TCA	1.43	-	-	-
2-methylaniline	2-MA	109.00	2-methylphenol	2-MP	127.00
3-methylaniline	3-MA	26.90	3-methylphenol	3-MP	145.00
4-methylaniline	4-MA	42.70	4-methylphenol	4-MP	57.60
2,3-dimethylaniline	2,3-DMA	30.80	2,3-dimethylphenol	2,3-DMP	48.10
2,4-dimethylaniline	2,4-DMA	39.40	2,4-dimethylphenol	2,4-DMP	19.30
2,5-dimethylaniline	2,5-DMA	70.60	2,5-dimethylphenol	2,5-DMP	32.50
2,6-dimethylaniline	2,6-DMA	107.00	2,6-dimethylphenol	2,6-DMP	41.60
3,4-dimethylaniline	3,4-DMA	7.34	3,4-dimethylphenol	3,4-DMP	32.00
3,5-dimethylaniline	3,5-DMA	27.80	3,5-dimethylphenol	3,5-DMP	27.20
-	-	-	2,3,5-trimethylphenol	2,3,5-TMP	13.50
-	-	-	2,3,6-trimethylphenol	2,3,6-TMP	14.20
2,4,6-trimethylaniline	2,4,6-TMA	20.30	2,4,6-trimethylphenol	2,4,6-TMP	9.64
2-ethylaniline	2-EA	49.20	2-ethylphenol	2-EP	31.40
3-ethylaniline	3-EA	14.20	3-ethylphenol	3-EP	40.30
4-ethylaniline	4-EA	8.82	4-ethylphenol	4-EP	21.90
2,6-diethylaniline	2,6-DEA	41.50	-	-	-

species (*P. subcapitata*) and testing conditions as in our experiments. This suggested that the observed effects were real and further analysis was needed to provide explanations.

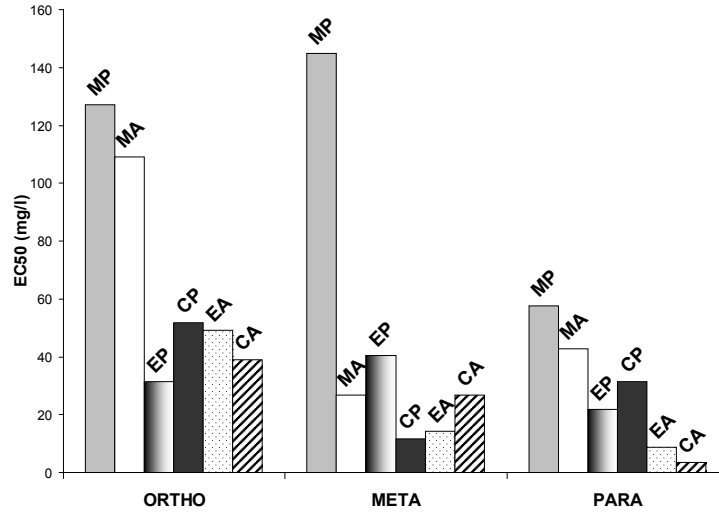

Figure 13. Effect of substituent position on the toxicity (EC50, mg/l) of substituted anilines and phenols to algae *Pseudokirchneriella subcapitata*. Data are plotted from and abbreviations are explained in Table 2.

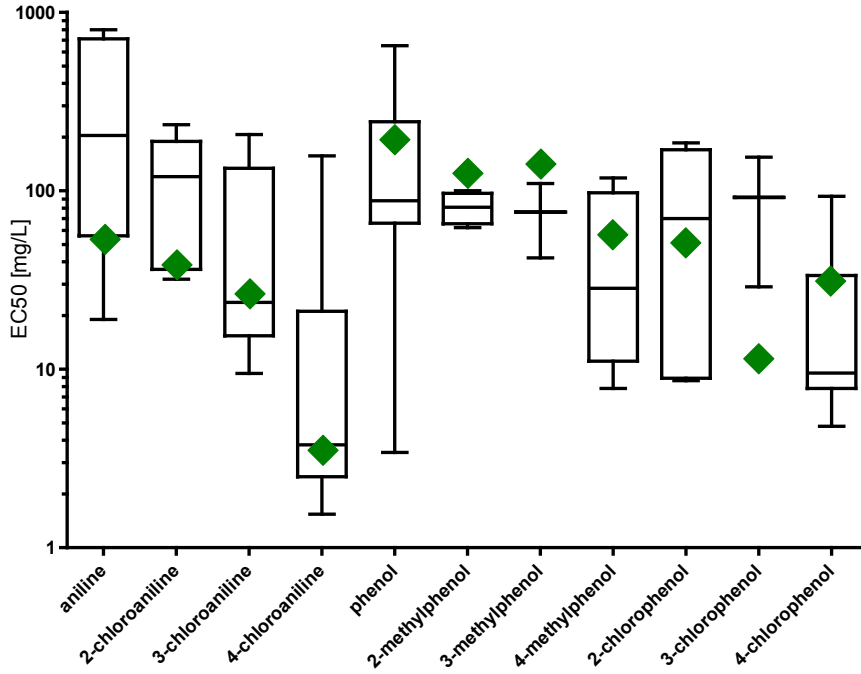

Figure 14. Example of variation in *Chlorophyta* toxicity data EC50 (mg/1) (exposure time 1–4 days) from US EPA ECOTOX database and published papers on a boxplot. Experimental toxicity data from this Thesis for *P. subcapitata* (see also Table 2) are shown as filled symbols. Adapted from Figure 1 in PAPER III.

5.4.1. Mode of toxic action and QSAR modeling

Based on the widely used Verhaar classification (see 2.5.1) all the 58 tested anilines and phenols (Table **2**) belong to class 2 of polar narcotic chemicals, which means that their toxicity should be proportional to hydrophobicity. In addition, several QSARs for anilines and phenols have been constructed using only hydrophobicity as a descriptor. For example the QSARs provided by the US EPA ECOSAR program, which are used for regulatory purposes, belong to this category. In order to elucidate the feasibility of this approach the toxicity was plotted against hydrophobicity. To comply with QSAR conventions, the negative logarithm of EC50 values was plotted against the logarithm of octanol/water partitioning coefficients (experimentally determined values obtained from the literature) as shown in Figure 15. Apparently, the toxicity of phenols was reasonably well described by a simple hydrophobicity-based relationship (solid line on upper panel of Figure 15, R^2=0.85), and the QSAR prediction based on the ECOSAR algal toxicity model for phenols was also close to the experimental data (Figure 15, dotted line on upper panel).
A more complicated picture was seen for anilines (Figure 15, lower panel), with up to 30- fold difference in toxicity for chemicals with the same hydrophobicity. This means that the toxicity could not be predicted using hydrophobicity alone and the ECOSAR model for aromatic amines, which is based on a training set of only 4 chemicals, performed particularly poorly. Other common descriptors used for QSARs such as the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) energies that express chemical reactivity, acid dissociation constant (pKa) that describes the proportion of ionized molecules at a given pH and a list of others describing molecular shape and polarity did not improve the correlation with toxicity (data not shown). In addition, the experimental toxicity data (Table 2) were compared to the algal toxicity

QSAR prediction from the Danish (Q)SAR database, which is being developed for regulatory use (ECB, 2005). The database does not disclose the models, other than stating that the particular algal values are predicted using a Multicase model based on a training set of 476 chemicals (obtained using *P. subcapitata* and some other algal species; ECB, 2005). Apparently, this model uses other descriptors in addition to hydrophobicity, but still failed to predict our experimental toxicity data (predicted *versus* observed R^2 = 0.166, Table 3 in PAPER III).

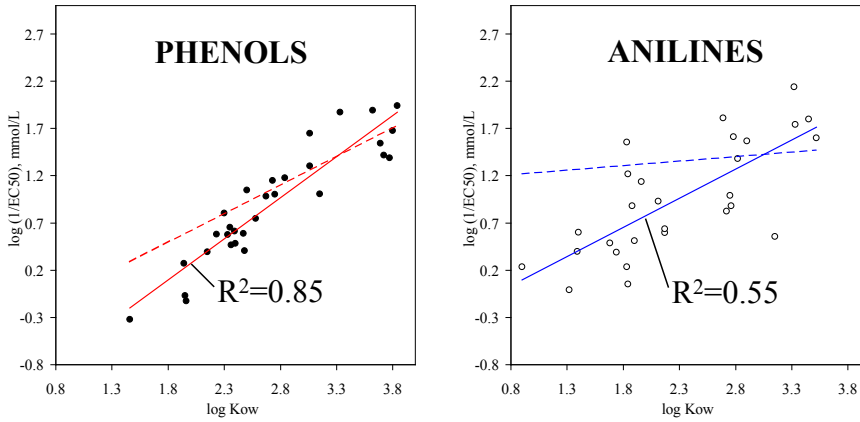

Figure 15. Experimental 72h *Pseudokirchneriella subcaptiata* toxicity data from Table 2 plotted against hydrophobicity, linear correlations (solid lines) and ECOSAR models (dotted lines). Adapted from PAPER III.

Interestingly, some of the substituent effects described above for anilines and phenols (see 5.4) have been observed earlier for chloro- and nitrophenols (Argese *et al.*, 1999). Their toxicity to many organisms appears to be due to their tendency to affect energy production in cells by inhibition of the electron transport or by destroying the electrochemical proton gradient built up across the membrane. The latter mode of toxic action is referred to as uncoupling of oxidative phosphorylation. It has been demonstrated for chlorophenols with vesicles of mitochondrial membranes (Argese *et al.*, 1995) as well as using the chromatophores of the purple photosynthetic bacterium *Rhodobacter sphaeroides* (Escher *et al.*, 1996). It is postulated that the proton gradient is neutralized by both a phenolate anion and a heterodimer that forms by association (i.e., hydrogen bonding) of a phenolate and a phenol species inside the membrane. The formation of such a heterodimer is hindered by chlorine substituents in ortho-position of the phenol. Consequently, chlorophenols that have two chlorine atoms in ortho positions should be particularly poor uncouplers. In addition to the steric hindrance to heterodimer formation the ortho substituents also interfere with delocalization of charge, reducing the mobility of a phenolate species in the nonpolar lipid bilayer. Localization of the charge makes it more difficult to accommodate the molecule in a nonpolar environment and strengthens its interaction with the polar groups in the membrane, which also decreases mobility. Notably, there does not appear to be information on the substituent position effects on biological activity for other types of phenols as well as for

anilines. Thus, data obtained in the current thesis suggests there may be additional structure-dependent mechanisms of action.

It can be concluded that even for relatively simple organic molecules, such as substituted anilines, it is difficult to predict toxicity based on structural similarity alone. Several mutually not exclusive mechanisms may be at play and triggered by small changes in substituent position. Further studies are needed to clarify the toxicity mechanisms of anilines and phenols.

6. CONCLUSIONS

According to REACH, by the year 2018, from 30 000 to 100 000 substances in total must have basic ecotoxicological information, including data concerning growth inhibition of aquatic plants (algal test OECD 201 preferred). In addition to above 'counted' industrial chemicals, a new class of complicated compounds – synthetic nanoparticles – already used in various consumer products needs evaluation for health and environmental effects. These tasks are a challenge for the industry as well as for the scientific community.

In this thesis, using algae *Pseudokirchneriella subcapitata* as model organisms, a growth inhibition assay was developed that complies with the OECD 201 guideline, can be also applied for toxicity evaluation of colored and particulate samples and is easily scalable. The applicability of the developed toxicity assay was evaluated on suspensions of heavy metal-polluted soils, turbid suspensions of metal oxide nanoparticles as well as organic chemicals. Specifically:

- Investigation of the toxicity of natural heavy metal polluted soils (sampled around the metal smelters) to algae showed that despite high levels of Zn in the soil elutriates that should have been toxic to algae, these soil eluatriates were not inhibiting the growth of algae, probably due to decreased bioavailability of heavy metals. When the suspensions of the same soils were analysed, they also stimulated algal growth compared to mineral medium. However, using suspensions mixed from clean and polluted soils in increasing ratios, soil-bound metals showed a dose-dependent inhibitory effect on algal growth, revealing the importance of relevant reference/control sample: preferably the clean soil with the same properties as the tested polluted soils.

- Evaluation of the toxicity of ZnO, TiO_2 and CuO nanoparticles on algae indicated that ZnO and CuO NPs were 'very toxic' as their 72 h EC50 values were 0.052 mg/l and 0.89 mg/l, respectively. According to algal toxicity data the TiO_2 NPs could be classified as 'toxic' (EC50 9.7 mg/l). TiO_2 NPs were about 6-fold more toxic to algae than the respective bulk formulation, probably due to the entrapment of algae in the particle aggregates observed during the testing. Based on nominal concentrations, CuO NPs were 16-fold more toxic than respective bulk formulation of CuO but toxic effect

of ZnO NPs and bulk ZnO occurred practically at the same nominal concentrations. The analysis of the solubilised Zn and Cu showed that the observed toxicity of ZnO and CuO was attributable to soluble metal ions leaching from the metal oxide particles. The effects of CuO nanopaticles to algae were investigated for the fist time.

- Testing and analysis of the toxicity of 58 congeneric substituted anilines and phenols to algae focused on supporting the environmental hazard classification for REACH. A consistent dataset was generated with 48 new experimental EC50 values that can be used for hazard classification and QSARs. Despite the fact that the analyzed molecules were structurally similar, the EC50 values spanned two orders of magnitude ranging from 1.43 mg/l (3,4,5-trichloroaniline) to 197 mg/l (phenol). The toxicity of the studied compounds was dependent on the type (chloro-, methyl-, ethyl-), number (mono-, di-, tri-) and position (ortho-, meta-, para-) of the substituents. As a rule, the higher the number of substituents the higher the toxicity. The chloro-substituted molecules were generally more toxic than alkyl-substituted ones. Consistent substituent position effects were observed in the data. For almost all tested chemicals the substituent in the para-position tended to increase toxicity whereas most of the ortho-substituted congeners were the least toxic. Thus, new correlations between toxicity and molecular structure of the studied chemicals were described, and it was shown that the relationship between toxicity and hydrophobicity is different for anilines and phenols.

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SUMMARY IN ESTONIAN

Vetikad *Pseudokirchneriella subcapitata* kemikaalide ja sünteetiliste nanoosakeste keskkonnaohtlikkuse hindamisel

Sissejuhatus

Vetikad on põhilised fotosünteesivad organismid vee-keskkonnas ning globaalselt olulised, kuna umbes pool Maa primaarproduktsioonist ning atmosfääris leiduvast hapnikust on pärit fütoplanktonist. Seetõttu on oluline välja selgitada praeguste ja võimalike uute keskkonnaohtude mõjud vetikate kasvule. Samas, kuna vetikaid on laboris lihtne kasvatada ja kuna nad on tundlikud paljudele kemikaalidele, on nad heaks tööriistaks keskkonnatoksikoloogias.

Euroopas võeti 2007. aastal vastu uus kemikaale reguleeriv määrus REACH (*Registration, Evaluation, Authorisation and Restriction of Chemicals*), mis on mõeldud inimeste ja keskkonna kaitseks kemikaalide võimalike kahjulike mõjude eest. REACH näeb ette kemikaalide keskkonnaohtlikkuse hindamise, sealhulgas toksilisuse hindamise vetikatele kõigi nende kemikaalide puhul, mida toodetakse või imporditakse Euroopa Liitu rohkem kui üks tonn aastas. Osadel juhtudel võib vetikatestidega saadud toksilisuse andmeid kasutada loomkatsete asemel, hoides kokku raha ja katseloomi. Toksilisuse osas tuleb uurida ka potentsiaalseid uusi keskkonnamürke, nagu näiteks sünteetilised nanoosakesed, mida kasutatakse üha rohkem laiatarbekaupades. Sealjuures tuleb suhtuda kriitiliselt biotestide sobivusse selleks otstarbeks, kuna testid on üldjuhul välja töötatud lahustuvate ainete, mitte tahkete osakeste jaoks.
Käesolevas töös töötati välja vetikate *Pseudokirchneriella subcapitata* kasvu inhibitsiooni test, ning kasutati seda raskemetallidega saastunud muldade, metallioksiidide nanoosakste ning tööstuslikult oluliste aniliinide ja fenoolide toksilisuse uurimiseks.

Metoodika

Põhiline meetod, mida antud töö raames täiustati ja kasutati oli OECD 201 suunistele vastav vetikate kasvu inhibitsiooni test. Reprodutseeritavuse tõstmiseks ning samaaegselt uuritavate proovide arvu suurendamiseks viidi test läbi väikestes 20-ml viaalides, mida loksutati

pidevalt läbipaistval, luminofoorlampidega altpoolt valgustatud plaadil. Selline süsteem tagas ühtlased valgustingimused kõigis viaalides ning pidev loksutamine võimaldas hea gaasivahetuse ja seega ühtlase pH kogu 72-tunnise testi vältel. Täiustati meetodit vetikate kasvu hindamiseks optilise tiheduse abil, mõõtes seda otse viaalist. Tahkeid osakesi (muld, nanoosakesed) sisaldavate proovide analüüsiks töötati välja klorofülli ekstraktsioonil ja ekstrakti fluorestsentsi mõõtmisel põhinev kasvukiiruse määramine. Halvasti valgust läbilaskvate proovide analüüsiks valmistati topeltpõhjalised viaalid, et eristada varjutuse võimalikku mõju ainega kokkupuutel tekkivast mõjust vetikate kasvule.

Tulemused ja arutelu

Töö tulemusena täiustati vetikate kasvu inhibitsiooni testi nii, et see sobis nii lahustumatute proovide analüüsiks kui ka suure arvu kemikaalide testimiseks. Testi rakendati kolmes uuringus.

1) Raskemetallidega (Zn, Cd, Pb) saastunud muldade uuring (artikkel **I**) näitas, et vaatamata kõrgetele Zn kontsentratsioonidele mulla veeekstraktides, mis pidanuksid olema vetikale mürgised, oli *P. subcapitata* kasv neis võrreldav kontrolliga. Puuduva toksilisuse põhjus võis olla humiinainetes, mis sidusid tsinki ja muutsid selle vetikale kättesaamatuks. Mullasuspensioonid, mis sisaldasid tunduvalt suuremaid raskemetallide kontsentratsioone kui ekstraktid, stimuleerisid vetikate kasvu võrreldes kontrolliga. Samas, kui testiti puhtast ja metallidega saastatud mullast valmistatud segusid, ilmnes mullaosakestele seotud metallide doosist sõltuv kahjulik mõju vetikate kasvule. Mullaproovide toksilisuse hindamisel tuleks võrdluseks kasutada samade füüsikalis-keemiliste omadustega puhast mulda.
2) ZnO, TiO_2 ja CuO nano-osakeste uuring (artikkel **II**) näitas, et ZnO ja CuO nano-osakesed oli "väga toksilised", st nende 72-tunni EC50 väärtused olid vastavalt 0,052 mg/l ja 0,89 mg/l (vastavalt direktiivile 67/548/EEC on kemikalide klassifikatsioon alljärgnev: EC50 <1 mg/l: väga toksiline; 1 - 10 mg/l: toksiline; 10 - 100 mg/l: kahjulik). Vetika kasvu inhibitsiooni põhjal võib TiO_2 nanoosaksesed liigitada "toksiliseks" (EC50=9,9 mg/l). TiO_2 nano-osakesed olid vetikatele umbes 6 korda toksilisemad kui vastavad mikro-osakesed, kusjuures nende toksilisus tulenes osakeste seondumisest vetikarakkudele. Nominaalsete kontsentratsioonide põhjal olid CuO nano-osakesed 16 korda toskilisemad kui vastavad mikro-osakesed, samas kui ZnO

puhul olid ühtviisi väga mürgised nii nano-osakesed kui ka mikro-osakesed. Lahustunud, bioloogiliselt kättesaadava Zn ja Cu kontsentratsioonide analüüs näitas, et ZnO ja CuO osakeste toksilisus tulenes dissotsieerunud metalli-ioonide mõjust. Kirjeldatud töö oli esimene CuO nano-osakeste toksilisuse uuring vetikatega.

3) 58 kongeneerilise aniliini ja fenooli toksilisuse analüüs (artikkel **III**), eesmärgiga toetada REACH-i seisukohast olulist kemikaalide keskkonnaohu klassifikatsiooni. Kasutades *P. subcapitata* kasvu inhibitsiooni testi, genereeriti väikese katseveaga andmestik, mis sisaldas 48 seni avaldamata EC50 väärtust, mida saab kasutada nii riski klassifitseerimiseks, kui ka struktuur-aktiivsussõltuvuste (QSAR) väljatöötamiseks. Vaatamata asjaolule, et analüüsitud molekulid olid struktuurselt sarnased, erinesid EC50 väärtused kahe suurusjärgu võrra, jäädes vahemikku 1,43 mg/l (3,4,5-trikloroaniliin) kuni 197 mg/l (fenool). Uuritud ainete toksilisus sõltus asendajate tüübist (kloro-, metüül-, etüül-), arvust (mono-, di-, tri-) ja asendist (orto-, meta-, para-). Reeglina kaasnes suurema asendajate arvuga suurem toksilisus. Kloro-asendatud molekulid olid üldiselt toksilisemad kui alküülrühmadega asendatud analoogid. Andmetest selgusid ka asendajate positsioonist tulenevad efektid, nii näiteks suurendas asendaja para-asendis peaagu kõigi ainete toksilisust, samas kui orto-asendusega analoogid olid kõige vähem toksilised. Näidati ka, et hüdrofoobsuse ja toksilisuse seos on aniliinide ja fenoolide puhul erinev.

Järeldused ja vajadus edasiseks uurimiseks

Vetikate kasvu inhibitsoonil põhinev ainete toksilisuse hindamise test, mida antud töös täiustati, on sobiv ka vees lahustumatuid osakesi sisaldavate proovide hindamiseks ning võimaldab väikese töökuluga analüüsida suurt hulka proove. Metoodika olulisust tõstab asjaolu, et seoses REACH-määrusega tuleb aastaks 2018 hinnanguliselt kuni 100 000 kemikaali varustada keskkonnaohtlikkust kirjeldava informatsiooniga, sealhulgas vetikate kasvu inhibitsiooni andmetega. Arvestades uuritavate kemikaalide suurt arvu, tuleb kasutusele võtta toksilisuse hindamise arvutuslikud meetodid, sealhulgas struktuur-aktiivsus sõltuvused. Edasised uuringud aniliinide ja fenoolide toksilisuse mehhanismide väljaselgitamiseks on vajalikud arvutusliku toksikoloogia edukamaks rakendamiseks sellist tüüpi kemikaalide puhul.

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I

PUBLICATIONS

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TOXICITY TESTING OF HEAVY-METAL-POLLUTED SOILS WITH ALGAE *SELENASTRUM CAPRICORNUTUM*: A SOIL SUSPENSION ASSAY

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Toxicity Testing of Heavy-Metal-Polluted Soils with Algae *Selenastrum capricornutum*: a Soil Suspension Assay

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ABSTRACT: A small-scale *Selenastrum capricornutum* (*Rhapidocelis subcapitata*) growth inhibition assay was applied to the toxicity testing of suspensions of heavy-metal-polluted soils. The OECD 201 standard test procedure was followed, and algal biomass was measured by the fluorescence of extracted chlorophyll. The soils, which contained up to (per kilogram) 1390 mg of Zn, 20 mg of Cd, and 1050 mg of Pb were sampled around lead and zinc smelters in northern France. The water extractability of the metals in suspensions (1 part soil/99 parts water w/v) was not proportional to the pollution level, as extractability was lower for soil samples that were more polluted. Thus, the same amount of metals could be leached out of soils of different levels of pollution, showing that total concentrations of heavy metals in soil (currently used for risk assessment purposes) are poor predictors of the real environmental risk via the soil–water path. Despite high concentrations of water-extracted zinc (0.6–1.4 mg/L of Zn in the test), exceeding by approximately 10-fold the EC_{50} value for *S. capricornutum* (0.1 mg Zn/L), 72-h algal growth in the soil extracts was comparable or better than growth in the standard control OECD mineral medium. The soil suspension stimulated the growth of algae up to eightfold greater than growth using the OECD control medium. Growth stimulation of algae was observed even when soil suspensions contained up to 12.5 mg Zn/L and could not be explained by supplementary nitrogen, phosphorous, and carbonate leached from the soil. However, if the growth of algae in suspensions of clean and polluted soils was compared, a dose-dependent inhibitory effect of metals on algal growth was demonstrated. Thus, as soil contains nutrients/supplements that mask the adverse effect of heavy metals, a clean soil that has properties similar to the polluted soils should be used instead of mineral salt solution as a control for analysis of the ecotoxicity of soils. © 2004 Wiley Periodicals, Inc. Environ Toxicol 19: 396–402, 2004.

Keywords: zinc; lead; cadmium; bioavailability; ecotoxicity; *Rhapidocelis subcapitata* (*Selenastrum capricornutum*); risk assessment

INTRODUCTION

Soil is a very efficient filter for most pollutants occurring in the terrestrial environment. For example, most heavy metals and PAHs sorb to a soil matrix, becoming less mobile and thus less toxic or nontoxic to living organisms (Welp and Brümmer, 1998; Bispo et al., 1999). Thus, for soil biota, a risk assessment should be based on not only water-mobile but also on matrix-bound pollutants, which can be assessed only in solid-phase assays, not in soil aqueous extracts. The OECD ecotoxicity tests for soils include assays with earth-

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TABLE I. Characterization of soils used in this study

No.	Sample[a]	Cd	Pb	Zn	Sand	Clay	Silt	Organic Matter	$CaCO_3$	pH
		(mg/kg dwt)			(g/kg dwt)					
	PLV[b]	1–3	50–300	150–300						
1	T13	2.5	118	207	254	150	596	28.6	2	8.0
2	TB2011	4.6	213	310	244	201	555	23.8	4	8.1
3	TMEPP1	5.0	276	324	323	165	512	25.3	4	8.2
4	TM2008	5.5	286	367	408	191	401	23.7	6	8.2
5	TM2005	3.3	139	417	377	168	455	26.8	7	8.2
6	TB2010	5.3	145	548	442	184	374	29.7	4	8.1
7	TB2001	6.4	105	741	242	205	553	25.0	5	8.1
8	TM2002	9.4	163	1250	301	150	549	32.4	6	7.8
9	T017	1.49	71	123	390	162	448	26.7	4	8.3
10	TREF	20	1050	1390	236	209	555	32.4	3	7.9
11	T314	1.53	58	161	390	162	498	22.9	6	7.9

[a] All samples belong to the same group according to their soil properties (Franqis et al., 2004).
[b] PLV—permitted limit values in soil according to EU directive 86/272/EEC (mg/kg).

worms (OECD, 1984a), bacteria (OECD, 2000a, 2000b), and plants (OECD, 1984b). As the tests with earthworms and plants are work- and space consuming, small-volume solid-phase microbiotests, for example, the solid-phase Microtox test (Kwan and Dutka, 1992) and solid-phase Flash assay (Lappalainen et al., 1999), which are both luminescent photobacterial assays, have been introduced. A miniscale algal toxicity assay using *Selenastrum capricornutum* has been worked out (Arensberg et al., 1995) and used for the analysis of soil suspensions (Baun et al., 2002). A solid-phase biotest using soil algae was recently developed by Burhenne et al. (1999).

In our previous study on soils heavily polluted with metals where total concentrations reached (per kilogram dwt) about 1200 mg for Zn, about 650 mg for Pb, and about13 mg for Cd, the toxicity of the water extracts of soils (soil–water ratio 1:9 w/v) to the protozoan *Tetrahymena thermophila*, the photobacterium *Vibrio fischeri*, the crustacean *Thamnocephalus platyurus,* and the alga *Selenastrum capricornutum* was practically absent as the concentrations of metals in these extracts (mg/L) were lower than the sensitivity of all the tests except algae that were sensitive to heavy metals at the sub–parts per million level (Kasemets et al., 2003). Thus, the lack of toxicity of the soil water extracts that was observed for algae in the above-mentioned study was unexpected. However, PAH-contaminated soils have been found to be several thousand times more toxic to *S. capricornutum* as suspensions than as elutriates (Baun et al., 2002). For the alga *Chlorococcum infusionum* the remarkable impact of particle-bound toxicity was also shown for Sb (Hammel et al., 1998). Therefore, we found it very important to introduce a soil suspension test for *Selenastrum capricornutum* that could be sensitive and reliable for ecotoxicological mapping of heavy-metal-polluted soils. In this article we describe a small-scale algal soil suspension toxicity test adapted from the method developed at the Technical University of Denmark (Kusk, 2002, personal communication), and essentially complying with OECD Guideline for Testing of Chemicals for the 72-h algal growth inhibition test (OECD, 1984c).

MATERIAL AND METHODS

Sampling of Soils and Chemical Analysis of Heavy Metals

Eleven topsoils (Table I) sampled around lead and zinc smelters in northern France were chosen from the soil bank collected for the agrochemical mapping of soils by Laboratoire Sols et Environnement, (ISA; Lille, France). The soils were dried, shipped, and stored at room temperature. All soil chemical analyses were performed by the Laboratoire d'Analyses des Sols (INRA; Arras, France). Briefly, calcination at 450°C and digestion with a mixture of hydrofluoric acid (HF) and perchloric acid ($HClO_4$) were performed essentially as described in Sterckeman et al. (2000) for the total solubilization of heavy metals. Total concentration of Zn in the soil was determined by flame AAS, and the total concentrations of Cd and Pb were determined by AAS with electrothermal atomization. The concentrations of Cd, Pb, and Zn in water extracts of soils (for the preparation of extracts, see below) were determined using ICP-AES. Other analyses were performed according to AFNOR or ISO methods: pH in water (ISO 10390), granulometry by wet sedimentation (AFNOR X31-107), organic matter by oxidation with dichromate (AFNOR X31-109), and carbonates by the HCl method (ISO 10693).

TABLE II. Characterization of soil mixtures used for the dose-effect analysis of heavy metals and toxicity of Cd, Pb and Zn for *Selenastrum capricornutum* in standard OECD assay

Designation[a]	TREF (%)	Cd in 1% Soil Suspension (mg/L)	Water-Mobile Cd (mg/L)	Pb in 1% Soil Suspension (mg/L)	Water-Mobile Pb (mg/L)	Zn in 1% Soil Suspension (mg/L)	Water-Mobile Zn (mg/L)
A	0.00%	0.015	0.0065	0.71	0.022	1.2	1.03
B	2.25%	0.019	0.0057	0.93	0.025	1.5	0.63
C	5.06%	0.024	0.0072	1.2	0.036	1.9	0.78
D	11.39%	0.036	0.0062	1.8	0.033	2.7	0.76
E	25.63%	0.063	0.0057	3.2	0.040	4.5	0.82
F	38.44%	0.086	0.0070	4.5	0.038	6.1	0.63
G	57.67%	0.12	0.0067	6.4	0.057	8.5	0.86
H	100.00%	0.20	0.0083	10.5	0.056	13.9	1.36
EC_{50} for *Selenastrum capricornutum* (72 h growth inhibition assay, mg/L)[b]			0.04		0.07		0.1

[a] Soils A–H were obtained by mixing soils TREF and T017 (soils *10* and *9,* Table I).
[b] Data taken from Kasemets et al. (2003). $ZnCl_2$, $CdCl_2$, and Pb acetate were used as standards.

Extraction of Soils with Water for Toxicological and Chemical Testing

The soil–water suspensions (1:99 w/v) were prepared by shaking the soil with MilliQ water for 24 h at room temperature. The particle-free extracts were obtained by filtering the suspension through a glass fiber filter ($d = 0.45$ μm) and used for toxicity testing (soil-free extracts) as well as for the determination of water-extracted heavy metals by ICP-AES. The soil:water ratio given above (1% soil suspension) was chosen as a compromise between the expected toxic concentrations and adequate light conditions for the growth of algae.

Preparation of Soil Samples for Dose–Effect Analysis of Heavy Metals Bound to Soil Particles

A heavily metal-polluted soil TREF (soil 10, Table I) was mixed with soil T017, which had similar properties but contained substantially lower concentrations of heavy metals (soil 9, Table I) and also served as a negative control soil. The stepwise dilution of soil 10 with soil 9 resulted in eight samples with different levels of contamination (designated as samples A–H, Table II). All the samples were tested in the algal growth inhibition assay as 1% soil suspensions. Soil–water extracts were analyzed in parallel.

The heavy metal concentrations in 1% suspensions of soils A–H used for the toxicity testing (Table II) were calculated from the total concentration of metals in soils 9 and 10 and the ratios used for the mixing of soils. The water extracts of the soils (see above) were analyzed for Cd, Pb, and Zn using ICP-AES (Table II). Thus, in all soil suspensions, the tested algae were exposed to a constant amount of soil particles (and therefore similar shading of light should be expected), but to steadily changing heavy metal concentrations.

Toxicity Testing with Algae *Selenastrum capricornutum*

The algal culture and stock solutions for the preparation of growth media were taken from the commercial test system AlgalToxkit F (MicroBioTests Inc., Nazareth, Belgium).

The soil–water suspensions and extracts were supplemented with mineral nutrients and incubated with *Selenastrum capricornutum* at 23°C ± 2°C either for 48 or 72 h. Incubation for 48 h was used when exponential growth ceased before 72 h (see also Arensberg et al., 1995). Then 20-mL glass incubation vials were shaken on a transparent table constantly illuminated from below with white fluorescent lamps. The test volume was 5 mL. The test was run in three parallels for the samples and in six parallels for controls. Algal growth was followed by optical density at 670 nm (OD_{670nm}) and/or by fluorescence of ethanol-extracted chlorophyll. For the extraction of chlorophyll 200 μL of ethanol was added to 50 μL of algal suspensions in 96-well microplates. After the addition of ethanol the plates were shaken for 3 h in the dark, and the fluorescence was measured (excitation 440 nm, emission 670 nm, Fluoroscan Ascent, Thermo Labsystems, Finland). The fluorescence of the extracted chlorophyll was used to estimate algal biomass as the presence of soil particles in the soil suspension assays interfered with the optical density measurements. Fluorescence of noninoculated blanks was subtracted from the results. For particle-free test samples (soil–water extracts), the correlation between optical density and fluorescence of the algal suspensions was very good ($R^2 = 0.997$; Fig. 1).

However, the chlorophyll content of algal cells depends on light intensity, being higher in lower illumination (Steemann Nielsen et al., 1962). We investigated the effect of light intensity on chlorophyll concentration by covering the test vessel bottoms with aluminum foil to various extents. As seen on Figure 2, the chlorophyll content varied depending on light intensity, and thus for the toxicity testing the

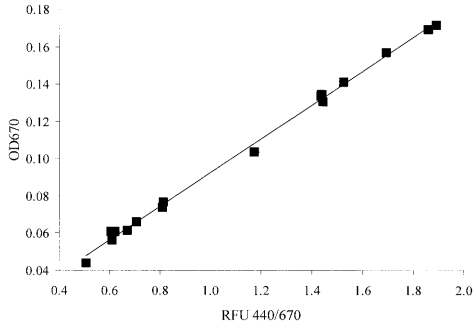

Fig. 1. Algal chlorophyll content (measured as relative fluorescence units, RFU) versus optical density (OD_{670}) of algal suspensions in the soil–water extracts.

algae should be incubated in identical light conditions in all samples and controls. For the soil suspension test this means there must be identical amounts of soil in the samples and controls. The soil concentration used in our experiments (1%) resulted in a light intensity between 20 and 30 relative units in the test vessel.

Preparation of Macroelement Mixture for Analysis of the Effect of Soil Nutrients on Algal Growth

Soil T314 was chosen as a model because of its low contamination level and the similarity of its properties with those of the other analyzed soils (Table I). The concentrations of nitrogen, carbonate, and phosphorus for the mixture were calculated taking into account the physicochemical data for this soil (data not shown except for $CaCO_3$; Table I). The form of the salts in the macronutrient mix was the same as that in the OECD standard medium for algae. Thus, a mixture consisting of 50 mg/L of NH_4Cl, 38.9 mg/L of $NaHCO_3$, and 7.4 mg/L of KH_2PO_4 was prepared as a substitute of 1% soil suspension of T314 (soil 11; Table I), designated as T314 simulation mixture.

RESULTS AND DISCUSSION

Water Extractability of Heavy Metals

The concentrations of Zn, Cd, and Pb were determined from the extracts made from the 1% suspensions of soils A–H by ICP-AAS (Table II). The analysis showed that the water extractability of metals was quite low and similar whatever was their content in the soil. Thus, the higher was the total amount of metals in the soil, the lower proved their relative extractability. For example, if the concentration of Zn in the soil suspension was 6–14 mg/L, the water-extractable fraction was about 10%, whereas practically all Zn (80%) proved water extractable at 1.2 mg/L level of Zn in the soil suspension (Table II). Thus, the same amounts of metals could be leached out of soils of different pollution levels, showing again that the total concentrations of heavy metals in soil (currently used for risk assessment purposes) are poor predictors of the real environmental risk via the soil–water path. Although the water-extracted concentrations of Cd and Pb were too low to expect an inhibitory effect on *Selenastrum,* the concentration of Zn in the water extracts exceeded the EC_{50} value for Zn as much as 10-fold (Table II), and thus Zn was considered the major toxic metal in this study.

Comparison of Algal Growth in Soil–Water Suspensions and Extracts of the Soils

Eight metal-polluted soil samples of different pollution levels but of the same type, that is, carbonated sandy loam as shown by soil analysis (Table I, soils 1–8), were studied. Seven of eight samples contained Cd and Zn in concentrations exceeding the values of the permitted limits for soils according to EU directive 86/272/EEC (Table I).

S. capricornutum algae were grown for 48 h in 1% soil–water suspensions as well as in their respective particle-free extracts. Water-extracted heavy metals were not chemically analyzed. Figure 3 shows the fluorescence of extracted chlorophyll from algal suspensions after cultivation for 48 h in the soil–water suspensions and soil-free extracts. The growth of algae in soil extracts was comparable or even slightly better than in the OECD standard mineral salts medium (hereafter referred to as the OECD control medium). Surprisingly, the soil suspensions—even the most polluted ones—stimulated the algal growth up to eightfold greater than in the OECD control medium (Fig. 3),

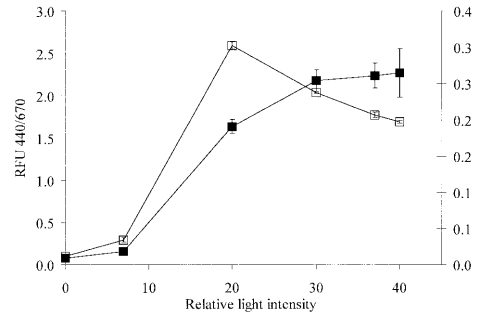

Fig. 2. Chlorophyll content (open symbols; measured as relative fluorescence units, RFU) and optical density (filled symbols) of the suspensions of algae *S. capricornutum* grown on standard OECD medium for 64 h in different light intensities. The test was run in three replicates.

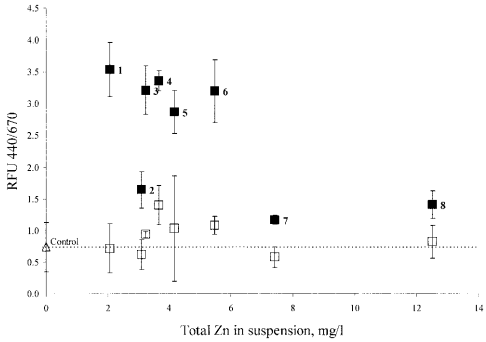

Fig. 3. Chlorophyll content after algal suspensions for 48 h (measured as fluorescence) in the soil suspensions (soil content 1%) of different pollution levels (filled symbols) and their respective water extracts (open symbols). The pollution level of soil is indicated by the content of Zn in the test suspension (plotted on *X* axis) calculated from data presented in Table I. The fluoresnce of algal suspension in the soil-free control (OECD medium) is indicated by triangle and dotted line. The test was run in three replicates.

although *S. capricornutum* algae are very sensitive to heavy metals (Table II). The stimulatory effect of the suspensions of polluted soils was surprising, as we expected strong inhibition of algal growth because of the influence of particle-bound metals such as PAHs. A strong toxic effect of particle-bound PAHs on algae *S. capricornutum* was found by Baun et al. (2002).

However, our data (Fig. 3) showed there was some tendency toward a reduction of this stimulatory effect in the more polluted samples (soils 7 and 8). Thus, there could be a stimulatory effect in all tested soils because of soil nutrients masking the potential dose-dependent inhibitory effect of heavy metals. As this tendency was observed only in the soil suspensions, not in the corresponding water extracts, it strongly suggests the adverse impact of particle-bound metals.

Effect of Macronutrients Extractable from Soil on Growth of Algae

To study whether the stimulation of the growth of algae by soil could be a result of the supplementary macronutrients extracted from the soils, soil T314 (soil 11, Table I) was used as a model. This soil had a low contamination level and had properties similar to those of previously analyzed soils. The 1% soil suspensions and their respective extracts (with and without OECD growth medium components) were analyzed in parallel. In addition to the soil, a mixture composed of nitrogen, phosphorus, and carbonates in the same amounts as in T314, the 1% suspension of soil, was used in parallel to soil suspensions and extracts (see the Material and Methods sections). Also, twofold- and fivefold-fortified OECD standard media were used for the cultivation of algae, that is, all the components of the standard medium (including trace elements) were added at a level two- and fivefold larger than in the standard OECD medium. The water-extracted concentrations of the metals were not determined. In parallel, all test samples were spiked with 0.1 mg/L of Zn (72-h EC_{50} value for *S. capricornutum*; Table II). Growth of algae was evaluated by measuring the algal biomass (fluorescence of extracted chlorophyll) after 48 h of cultivation.

Figure 4 shows that the OECD medium suited the growth of algae well, whereas the twofold- and fivefold-fortified OECD media were not optimal. Also the 1% suspension prepared from the T314 soil, and in addition, its particle-free extract without OECD mineral salts did not support algal growth. From a comparison of the concentrations of N, P, and carbonate in the OECD standard medium (containing 15 mg/L of NH_4Cl, 50 mg/L of $NaHCO_3$ and 1.6 mg/L of KH_2PO_4) and in the T314 "simulation" mix, it can be concluded that, theoretically, by the addition of 1% of the T314 soil into the OECD medium, the concentration of nitrogen in the growth medium increased 1.3-fold, the concentration of phosphorus 5.4-fold, and the concentration of carbonate 1.8-fold in comparison to the soil-free control. However, the growth of algae in the T314 extract with OECD components added (normal test condition used in this study) was comparable to that of the OECD control, and the soil suspension (OECD components added) again showed a stimulatory effect on the growth of algae com-

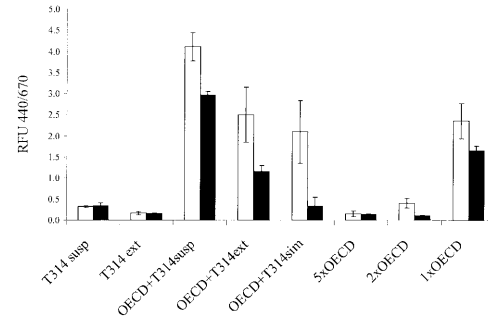

Fig. 4. The effect of supplementary N, P, and carbonate potentially originating from the soil on the growth algae in OECD 201 standard medium. As a model, 1% suspension and respective extracts of soil T314 (Table I) were analyzed. The biomass of algae was evaluated by the fluorescence of chlorophyll extracted from the algal suspensions after 48 h of growth (open columns). In parallel, all test samples were spiked with 0.1 mg/L of Zn (filled columns). The test was run in three replicates.

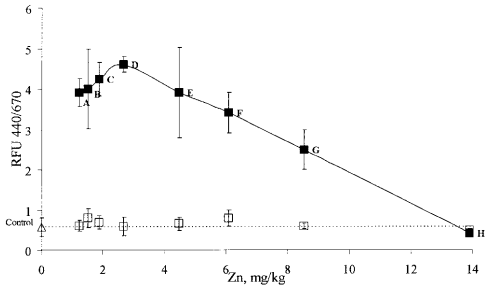

Fig. 5. Chlorophyll content of algal suspension after growth for 72 h in 1% soil suspensions (filled symbols) and the respective water extracts (open symbols) of soil mixtures with different heavy metal contents. The fluorescence of algae in OECD control medium is indicated by triangle and dotted line. The pollution level of soil suspensions is indicated by their content of Zn (plotted on *X* axis, mg/kg) calculated from data presented in Table II. The concentrations of Cd and Pb in the soil suspensions (mg/kg) and concentrations of Zn, Cd, and Pb in respective water extracts (mg/L) are presented in Table II. The test was run in three replicates.

pared to the OECD medium. The growth of algae in the OECD medium was the same as with the OECD medium with the T314 simulation mixture. Thus, according to our results, in addition to the macronutrients N, P, and carbonate, there should be additional substances in the soils stimulating algal growth. 0.1 mg/L of Zn inhibited the growth of algae from 0% to 84% (Fig. 4).

Comparison of Algal Growth in Soil–Water Suspensions and Extracts: Dose–Effect Analysis

To distinguish between the stimulatory effect of normal soil components and the inhibitory effect of heavy metals, polluted and clean soils were mixed (see Material and Methods section) in different proportions, and the growth of algae in polluted soil suspensions was compared to the growth in clean soil suspensions (Fig. 5). As the soil:water ratio for preparation of the suspensions was identical for all samples, the influence of the shading of the light by soil particles was excluded. Compared to the growth of algae in the soil-free OECD control medium (designated by a dotted line), stimulation of algal growth was observed in all soil samples except the most polluted one (sample H; 13.9 mg/L of Zn in the test; Fig. 5). For example, the biomass of algae after 72 h of growth (measured by the chlorophyll fluorescence of the cell suspension) in the clean control, soil A, exceeded by about 8 times the biomass of algae in the soil-free OECD control medium (designated by a horizontal dotted line in Fig. 5). Thus, obviously, the growth of *S. capricornutum* was highly stimulated by different substances leached out from the soil matrix. Samples B, C, and D, although containing more heavy metals than clean soil A, stimulated the growth of algae even more than sample A (Table II and Fig. 5). The latter could be a manifestation of hormesis, a phenomenon of the positive effect of low toxicant levels that has been demonstrated for many organisms and chemicals (Calabrese and Baldwin, 2001). However, beginning with a value of 6.1 mg Zn/L (sample F; Table II and Fig. 5) in the soil suspension, the clear dose-dependent inhibition of the stimulation of algal growth (if compared to the clean control, soil A) indicated that the particle-bound metals had an adverse effect on algal growth. The adverse effect of particle-bound metals was confirmed by a lack of a dose-dependent response of algae in particle-free soil–water extracts: although the concentration of Zn in the soil–water extracts was relatively high (0.6–1.4 mg/L), exceeding the EC_{50} for algae (0.1 mg Zn/L) up to 14-fold, the growth of algae in soil extracts was comparable to the growth of algae in the OECD mineral medium (Fig. 5).

CONCLUSIONS

1. The water extractability of the metals was not proportional to the pollution level, being lower for more polluted soil samples. For example, if the concentration of Zn in the extracted soil suspension was 6–14 mg/L, the water-extractable fraction was about 10%, whereas practically all Zn (80%) proved to be water extractable when the level of Zn in the soil was 1 mg/L. Thus, the same amounts of metals could be leached out of soils of different pollution levels, showing again that the total concentration of heavy metals in soil (currently used for risk assessment purposes) is a poor predictor of the real environmental risk via the soil–water path.
2. The water-extractable toxic hazard of soils heavily polluted with Zn, Cd, and Pb was evaluated using the growth inhibition assay of the algae *Selenastrum capricornutum*. Chemical analysis showed that only Zn was present in the water extracts of the soil (extraction ratio 1+99) in concentrations exceeding the EC_{50} value for algae (0.1 mg Zn/L) and thus could cause the inhibitory effect. Although the concentration of Zn in the soil–water extracts was relatively high (0.6–1.4 mg/L), the growth of algae in the soil extracts was comparable to that in the OECD mineral medium (control). The latter could be explained by (i) sorption of heavy metals on humic particles present in the extracts and a respective decrease in the bioavailable fraction and/or (ii) additional nutrients present in the soil extract compensating for (masking) the adverse effect of heavy metals.
3. The soil suspensions (even those containing as much as 12.5 mg/L of Zn, 1.6 mg/L of Pb, and 0.09 mg/L of Cd

in the test medium) did not inhibit but instead stimulated algal growth compared to the OECD control medium. The presence of the supplementary macronutrients N, P, and carbonate extractable from the soil did not explain this stimulatory effect. Thus, there should be additional substances in the soils stimulating algal growth.

4. As in many other test organisms, there could be several reasons for the apparent lack of toxicity of the metal-polluted soil suspensions to *S. capricornutum*. In fact, the net effect of (polluted) soils on algal growth could be the net result of several negative and positive effects: (i) Heavy metals in soil and even in soil extracts are not totally bioavailable (for example, they may be bound to humus particles or colloids). (ii) Algal growth is stimulated by different substances present in the soil, resulting in compensation for the toxic effects caused by heavy metals. (iii) The higher stimulatory effects of suspensions compared to the extracts could be explained by the additional leaching of nutrients during algal growth.
5. Finally, comparison of the growth of algae in suspensions mixed from clean and polluted soils revealed that soil-bound metals have a dose-dependent inhibitory effect on algal growth above a certain level of contamination. Thus, as soil often contains nutrients/supplements that mask the adverse effect of heavy metals, clean soil with properties similar to those of the polluted soils should be used as a control instead of mineral salt solution for analysis of the ecotoxicity of soils. In practice, that could be very difficult to achieve unless the pollutant gradients from the point source are studied, and the soil type within this gradient is not different.

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TOXICITY OF NANOPARTICLES OF CUO, ZNO AND TIO_2 TO MICROALGAE *PSEUDOKIRCHNERIELLA SUBCAPITATA*.

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Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*

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ABSTRACT

Toxicities of ZnO, TiO_2 and CuO nanoparticles to *Pseudokirchneriella subcapitata* were determined using OECD 201 algal growth inhibition test taking in account potential shading of light. The results showed that the shading effect by nanoparticles was negligible. ZnO nanoparticles were most toxic followed by nano CuO and nano TiO_2. The toxicities of bulk and nano ZnO particles were both similar to that of $ZnSO_4$ (72 h EC50 ~0.04 mg Zn/l). Thus, in this low concentration range the toxicity was attributed solely to solubilized Zn^{2+} ions. Bulk TiO_2 (EC50=35.9 mg Ti/l) and bulk CuO (EC50=11.55 mg Cu/l) were less toxic than their nano formulations (EC50=5.83 mg Ti/l and 0.71 mg Cu/l). NOEC (no-observed-effect-concentrations) that may be used for risk assessment purposes for bulk and nano ZnO did not differ (~0.02 mg Zn/l). NOEC for nano CuO was 0.42 mg Cu/l and for bulk CuO 8.03 mg Cu/l. For nano TiO_2 the NOEC was 0.98 mg Ti/l and for bulk TiO_2 10.1 mg Ti/l. Nano TiO_2 formed characteristic aggregates entrapping algal cells that may contribute to the toxic effect of nano TiO_2 to algae. At 72 h EC50 values of nano CuO and CuO, 25% of copper from nano CuO was bioavailable and only 0.18% of copper from bulk CuO. Thus, according to recombinant bacterial and yeast Cu-sensors, copper from nano CuO was 141-fold more bioavailable than from bulk CuO. Also, toxic effects of Cu oxides to algae were due to bioavailable copper ions. To our knowledge, this is one of the first systematic studies on effects of metal oxide nanoparticles on algal growth and the first describing toxic effects of nano CuO towards algae.

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1. Introduction

Metal oxide nanoparticles are already manufactured in large scale for both industrial and household use, yet hold even greater promise for future applications. For example titanium dioxide nanoparticles are already used as UV-blocking agent in sunscreens (Popov et al., 2005), in photocatalytic water purification (Hagfeldt and Gratzel, 1995) and will likely be used in the new generation of solar cells (Usui et al., 2004). Zinc oxide nanoparticles are a starting material for electronics applications, transparent UV-protection films and chemical sensors (Meulenkamp, 1998) as well as UV-filters in sunscreens (Serpone et al., 2007). Copper oxide nanoparticles have potential to replace noble metal catalysts for carbon monoxide oxidation (Zhou et al., 2006) and CuO nanoparticle suspension (nanofluid) has excellent thermal conductivity for it to be used as a heat transfer fluid in machine tools (Chang et al., 2005). In the form of manufacturing and household waste the metal oxide nanoparticles are likely to end up in natural water bodies.

While the novel properties of nanoparticles are increasingly studied, little is known of their interactions with aquatic organisms (Kahru et al., 2008). In aquatic risk assessment algal growth inhibition assay is widely used (Radix et al., 2000;

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Blinova, 2004). *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and *Rhapidocelis subcapitata*) is considered a model organism for freshwater algae and is widely used in the OECD algal growth inhibition test (Organisation for Economic Co-Operation and Development, 1984) as well as in US EPA green algae growth inhibition test (US Environmental Protection Agency, 1996). This species has also proven to be very sensitive to heavy metals (Blinova, 2004; Kahru et al., 2005). However, only few studies have investigated nanoparticle toxicity to algae. Hund-Rinke and Simon (2006) have studied 25 nm and 100 nm TiO_2 particles in regard to their toxicity to the green algae *Desmodesmus subspicatus* and found the smaller particles to be more toxic (72 h EC50 of 25 nm particles was 44 mg/l, for 100 nm particles the EC50 was beyond the tested range, >50 mg/l). They also investigated the shading effects by shining light through TiO_2 suspensions in a separate culturing plate on top of the plate with algae. In their setup the shading turned out to be insignificant. TiO_2 particles have also been tested with the algae *P. subcapitata*. In a study with "fine" (~99% TiO_2 core with ~1% Al surface coating, median particle size ~380 nm according to dynamic light scattering) and "ultrafine" particles (median particle sizes of ~140 nm; 90 wt.% TiO_2, 7% alumina, and 1% amorphous silica) there was little difference in toxicity: EC50 values were determined to be 16 mg/l for "fine" and 21 mg/l for "ultrafine" particles (Warheit et al., 2007).

Recently Franklin et al. (2007) have studied the toxicity of ZnO nanoparticles to *P. subcapitata* while also determining the concentration of dissolved Zn ions derived from ZnO. The toxicity of ZnO particles as well as $ZnCl_2$ was found to be essentially due to dissolved Zn. Also Heinlaan et al. (2008) studied the toxicity of ZnO nanoparticles to crustaceans *Daphnia magna* and *Thamnocephalus platyurus* as well as to bacteria *Vibrio fischeri* and showed that their toxicity was due to dissolved Zn ions as proved with recombinant Zn-sensor bacteria.

The aim of the current investigation was to estimate the toxicity of ZnO, TiO_2 and CuO nanoparticles to algae *P. subcapitata*. In order to clarify the respective roles of particle size and of metal oxide solubility, bulk formulations of metal oxides as well as $ZnSO_4$ and $CuSO_4$ were used as controls. Recombinant metal-sensing microorganisms (bacteria and yeast) were used to quantify the bioavailable fraction of copper released from CuO particles. To our knowledge, this is the first toxicity study of CuO (nano)particles on algae.

2. Materials and methods

2.1. Chemicals

Nanosized metal oxides were purchased from Sigma-Aldrich, the particle size specified by the manufacturer being 25–70 nm for TiO_2, 50–70 nm for ZnO and 30 nm (mean) for CuO. Bulk TiO_2 was purchased from Riedel-de Haën, ZnO from Fluka and CuO from Alfa Aesar. Stock suspensions of nano and bulk metal oxides were prepared in algal medium immediately before each experiment (TiO_2: 640 mg/l; ZnO: 10 mg/l; CuO: 100 mg/l). Before use they were ultrasonicated for 30 min (Techpan Type UM-2, Poland). It has been reported that ultrasonication is not effective in breaking down ZnO nanoparticle agglomerates (Zhang et al., 2006). However, the nano-ZnO and nano-TiO_2 stock suspensions became homogeneously turbid and without visible sediment after sonication. The appearance of nano- and bulk-CuO suspensions did not change: nano CuO suspension remained more homogeneous and much darker than the bulk formulation. Stock solutions of $ZnSO_4$*7-H_2O (Sigma-Aldrich) and $CuSO_4$ (Alfa Aesar) were prepared without sonication.

2.2. Toxicity measurements with algae *P. subcapitata*

In general, the OECD 201 algal growth inhibition test guidelines (OECD, 1984) were followed: we used exponentially growing algal cultures that were exposed to various concentrations of the test substance under controlled conditions and the cell concentration of the control culture increased at least 16 times during 3 days. Also, the algal biomass measurements were performed at 24, 48 and 72 h as described in the OECD method. The algal stock culture for inoculation was taken from the commercial test system Algaltoxkit F (MicroBioTests Inc., Nazareth, Belgium). The substances under investigation were incubated with *P. subcapitata* at 24 °C±1 °C for at least 72 h in 20-ml glass incubation vials containing 5 ml of algal growth medium (OECD, 1984). The vials were shaken on a transparent table and constantly illuminated from below with Philips TL-D 38W aquarelle fluorescent tubes (enhanced irradiation between 400 and 500 nm). All assays were run in three replicates with initial algal cell count of 10000 cells/ml and algal biomass was measured by chlorophyll fluorescence as described in Aruoja et al. (2004). Briefly, 50 μl culture samples were transferred to a 96-well plate, 200 μl of ethanol was added to each sample and the plate was shaken for 3 h in the dark. Thereafter the fluorescence was measured with a microplate fluorometer (excitation 440 nm, emission 670 nm; Fluoroscan Ascent, Thermo Labsystems, Finland). The metal oxide suspensions did not fluoresce under these conditions and also their light absorbances at these excitation and emission wavelengths were below detection limit (Multiskan Spectrum, Thermo Electron Corp., Finland). During the exponential growth phase, the chlorophyll fluorescence (RFU) correlated with cell density determined by counting in Neubauer hemocytometer (cell density (cells/ml) = RFU × 2287000; R^2 = 0.9649). Phase contrast as well as fluorescence micrographs were taken with Olympus CX41 microscope equipped with DP71 camera.

In order to evaluate the shading effects of particle suspensions, special double layer glass vials were constructed by sealing a smaller vessel (cut from a similar vial) under the 20 ml test culture vessel (Fig. 1). The lower vessel was filled with 3 ml of algal growth medium but not inoculated with algae. For shading controls the same amount of metal oxide particles than in the respective test vessel was added to the lower vessel.

2.3. Quantification of bioavailable fraction of Cu with recombinant microbial sensors (bacteria and yeast)

The bioavailable fraction of Cu was determined in the test suspensions of bulk and nano CuO with both sensors. *Escherichia coli* MC1061(pSLcueR/pDNPcopAlux) was used essentially as described by Heinlaan et al. (2008), except bacteria were

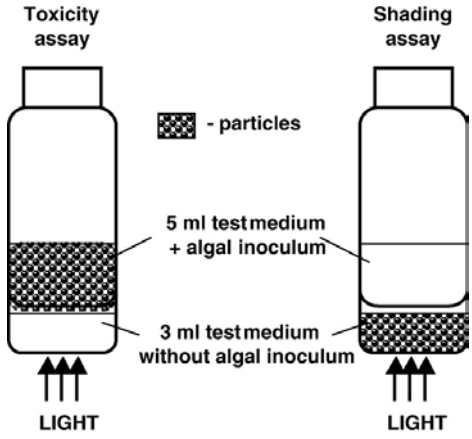

Fig. 1 – Two-compartment glass test vials for the assessment of the shading effect of particles.

incubated in the standard algal growth medium supplemented with casaminoacids (5 g/l) and glucose (2 g/l). Analysis with yeast sensor (Cu-sensing *Saccharomyces cerevisiae* BMA64-1A (pSALluc-skl) (Leskinen et al., 2003) and a control strain *S. cerevisiae* BMA61-1A(pRS316luc) (Michelini et al., 2005) was performed as described in Leskinen et al. (2003), except using malt extract media (Lab M) for yeast cultivation. Briefly, 50 μl of the sensors or controls and 50 μl of the sample suspensions were mixed and incubated in 30 °C for 1 h. After the addition of the bioluminescence reaction substrate (100 μl of D-luciferin in 0.1 M Na-citrate buffer, pH 5.0), the luminescence was measured. $CuSO_4$ was used as a standard for 100% bioavailable Cu.

2.4. Statistics

The Log-normal model in the REGTOX software for Microsoft Excel (Vindimian, 2005) was used for the calculation of toxicity parameters (EC and NOEC) and their confidence intervals. One-way analysis of variance (ANOVA) was used to determine statistical significance of the differences between values. The level of significance was accepted at $P \leq 0.05$. In the figures, values are drawn as mean ± standard deviation and in the table, EC and NOEC values are reported with 95% confidence intervals.

3. Results and discussion

3.1. Shading effect of the metal oxide particle suspensions

As aqueous suspensions of nanoparticles are opaque and thus may inhibit the growth of algae also by absorption of light, the special constructed vials for incubation of algae allowed to differentiate the true inhibitory effect of metal oxide from the light shading effect.

In control experiments, there was no significant effect on the 72 h growth of algae regardless of the concentrations of ZnO and CuO (see Fig. 2A,C; dotted lines). This also applies for TiO_2 even if the suspensions of TiO_2 were relatively opaque

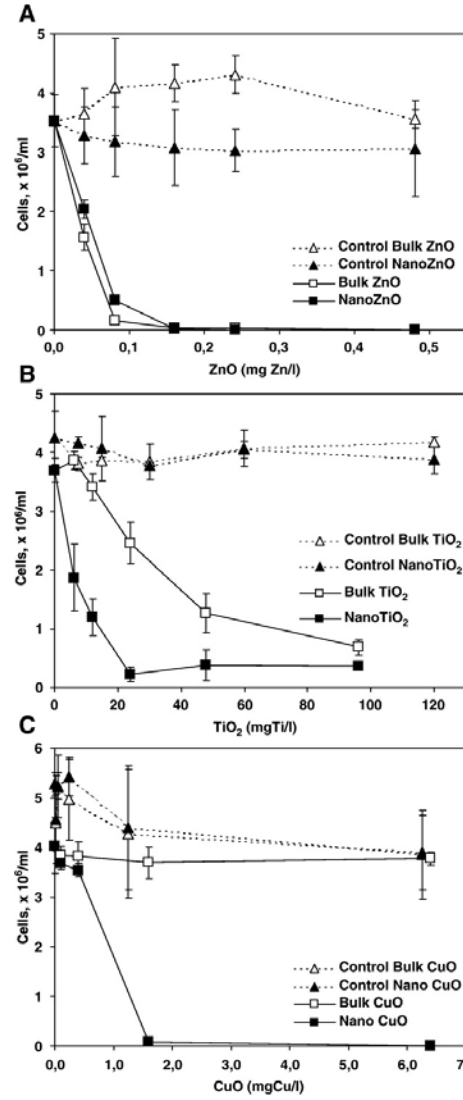

Fig. 2 – Toxic and shading effects of ZnO (A), TiO_2 (B) and CuO (C) suspensions on 72 h biomass of *Pseudokirchneriella subcapitata*. Data for nano metal oxide suspensions are shown as filled symbols, for bulk metal oxide suspensions as open symbols. In control experiments (dotted lines), bulk or nano metal oxides were added to the lower compartment of the test flask (see Materials and methods). Values are reported as mean of 3 replicates ± standard deviation.

Table 1 – The toxicity of metal oxide aqueous suspensions, $CuSO_4$ and $ZnSO_4$ to algae *Pseudokirchneriella subcapitata* (72 h growth inhibition)

Substance	EC50[a]			EC20[a]			NOEC[a,b]		
	mg metal/l	95% confidence interval		mg metal/l	95% confidence interval		mg metal/l	95% confidence interval	
$ZnSO_4$	0.042	0.010	0.122	0.010	0.001	0.075	0.005	0.0002	0.0693
Bulk ZnO	0.037	0.031	0.041	0.025	0.016	0.035	0.020	0.011	0.032
Nano ZnO	0.042	0.036	0.049	0.023	0.017	0.031	0.017	0.011	0.025
Bulk TiO_2	35.9	31.4	41.7	15.6	12.0	19.3209	10.1	7.10	13.2
Nano TiO_2	5.83	3.75	7.58	1.81	0.637	2.82	0.984	0.263	1.74
$CuSO_4$	0.020	0.016	0.031	0.016	0.014	0.027	0.014	0.012	0.025
Bulk CuO	11.55	8.64	18.9	9.10	7.28	16.1	8.03	6.58	14.9
Nano CuO	0.710	0.556	1.092	0.504	0.439	0.931	0.421	0.373	0.852

[a] Mean of 3 replicates.
[b] No-observed effect concentration.

upon preparation. Indeed, there was no shading effect on algal growth even in the case of the higher TiO_2 concentrations (Fig. 2B) due to the settling within 24 h of large TiO_2 clumps allowing sufficient light to pass though (see paragraph on TiO_2 below).

3.2. *Effect of metal oxide particles on algal growth*

Main toxicity parameters usually included in risk assessment, i.e. EC50, EC20 and NOEC are given in Table 1.

3.2.1. *ZnO*

During our experiments the starting pH was 8.0 and decreased by 0.4 units or less during the whole incubation period (up to 120 h). It has been shown (Heijerick et al., 2002) that Zn toxicity to *P. subcapitata* in standard OECD test medium increased with increasing pH but was virtually constant between pH 7.5 and 8.0. Thus there is likely no significant pH interference on our data.

As illustrated by the growth curves in Fig. 3A, ZnO nanoparticles as well as bulk ZnO were toxic to *P. subcapitata* already at very low concentrations (<0.1 mg/l) and the total inhibition of algal growth was observed already at 0.16 mg Zn/l for both types of particles (data not shown). Differences due to particle size were small with slight tendency for bulk ZnO to be more inhibitory (Fig. 3A). However, the 72 h EC50 values of nano and bulk ZnO (0.042 and 0.037 mg Zn/l, respectively) were not statistically different (Table 1 and Fig. 4A). Our toxicity data are close to data obtained by Franklin et al. (2007) who showed 72 h EC50 values for the same algal species to be 0.063 mg Zn/l for bulk ZnO and 0.068 mg Zn/l for nano ZnO. No aggregation of ZnO particles was observed during the assay possibly because most of the ZnO was dissolved at these low concentrations. Recombinant sensor measurements for Zn^{2+} were not performed in the current study. However, previous work in our laboratory with recombinant Zn-sensor bacteria demonstrated that between 69 and 97% of Zn in both nano and bulk ZnO particles was in the bioavailable fraction at concentrations below 1 mg/l (Heinlaan et al., 2008). Indeed, EC50 values of ZnO particle suspensions and $ZnSO_4$ (calculated on metal basis) were similar (Fig. 4A, Table 1). Consequently, the toxicity of ZnO nanoparticles should be attributed to dissolved Zn. Similar conclusions were drawn recently by Franklin et al. (2007) who, using a dialysis membrane with a pore size of about 1 nm (permeable to Zn ions but not to ZnO particles), showed that both nano and bulk ZnO suspensions yielded similar dissolved Zn concentrations.

3.2.2. TiO_2

Compared to both ZnO formulations, suspensions of TiO_2 were of remarkably lower toxicity to *P. subcapitata*. In the case of nano TiO_2 the lag phase was about 24 h longer compared to control with all tested concentrations starting from 24 mg Ti/l (Fig. 3B), while there was no detectable growth at the highest tested concentration of 380 mg Ti/l (data not shown). In the case of bulk TiO_2 the effect on lag phase duration started from 48 mg Ti/l (Fig. 3B) and was less pronounced, some growth was detected even at 380 mg Ti/l (not shown). As a result, nano TiO_2 appeared more toxic than the bulk form under our test conditions. The 72 h EC50 values for nano TiO_2 and bulk TiO_2 suspensions were 5.8 mg Ti/l and 35.9 mg Ti/l, respectively (Fig. 4B and Table 1).

Both nano and bulk TiO_2 formed aggregates during incubation (Fig. 5A and D). Algal cells were visible in phase contrast microscopy and also confirmed by their red fluorescence. In the case of TiO_2 nanoparticles (Fig. 5E and F), large aggregates were observed that entrapped almost all algal cells, whereas the cultures with bulk TiO_2 (Fig. 5B and C) always contained also free algal cells in addition to cells entrapped in small TiO_2 aggregates. This suggests that TiO_2 nanoparticle aggregates reduced the light available to the entrapped algal cells and thus inhibited their growth. Huang et al. (2005) have shown that *P. subcapitata* cells adsorbed onto their surface TiO_2 nanoparticles and carried 2.3 times their own weight in TiO_2 particles. The kinetics and the extent of nano TiO_2 adsorption on algae were highly dependent on pH, the maximum adsorption occurring at pH 5.5. Also, the flocculation of algae was observed (Lin et al., 2007). TiO_2 nanoparticle aggregation behavior strongly depended not only on pH but also on ionic strength. Also, cationic and anionic species or the presence of humic acids affected the stability of TiO_2 suspensions (Ottofuelling et al., 2007).

It has thus been suggested (Baveye and Laba, 2008) that aggregation of TiO_2 nanoparticles may have toxicological implications, which may result in very dissimilar biological activity. Another possible mechanism of toxicity of TiO_2

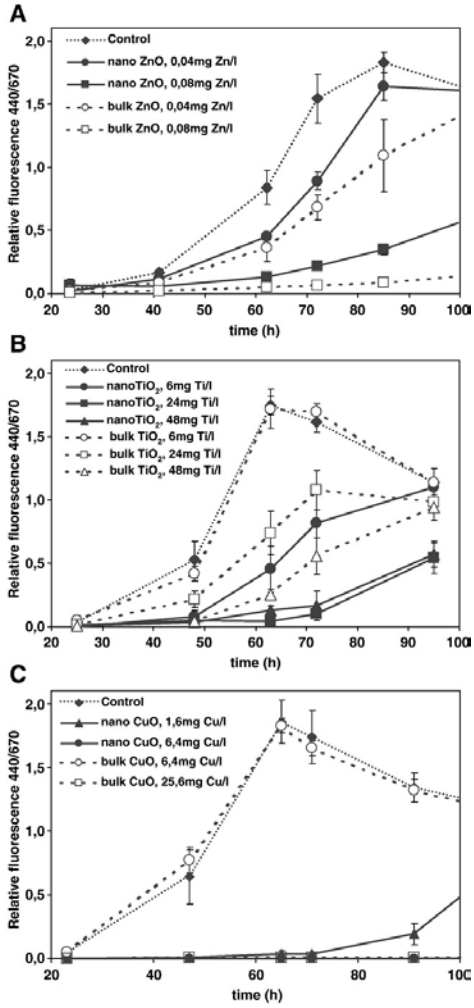

Fig. 3 – Growth kinetics of *Pseudokirchneriella subcapitata* in the presence of ZnO (A), TiO_2 (B) and CuO (C). Values are reported as mean of 3 replicates ± standard deviation.

suspensions may involve generation of hydroxyl radicals by TiO_2 nanoparticles due to visible light. TiO_2 nanoparticles in combination with UV light (370 nm) have been shown to inactivate algae *Anabaena*, *Microcystis*, and *Melosira* (Kim and Lee, 2005) and have been shown to destroy the cell surface architecture of blue-green algae *Chroococcus* sp. (Hong and Otaki, 2005). Toxicity of TiO_2 towards bacteria in the presence of light is well documented (Maness et al., 1999; Wei et al., 1994; Armelao et al., 2007) and appears to be mediated mostly by photochemical reactive oxygen species (ROS) generation. However, TiO_2 nanoparticles have shown toxicity to *Bacillus subtilis* and *E. coli* in the dark (Adams et al., 2006). Analogously, Reeves et al. (2008) have shown in fish cells *in vitro* that hydroxyl radicals were generated by TiO_2 nanoparticles also in the absence of ultraviolet light. Thus, the ROS-mediated toxic effects of TiO_2 particles towards algae observed in our experiments cannot be excluded. In addition to the above-mentioned mechanisms of toxicity, TiO_2 nanoparticles can adsorb Zn and P from algal growth medium and thus limit the availability of these nutrients to algae (Kuwabara et al., 1986).

3.2.3. *CuO*

In our experiments, CuO nanoparticles were clearly more toxic towards algae than the bulk form: the 72 h EC50 values being 0.71 and 11.55 mg Cu/l, respectively (Table 1). There were no

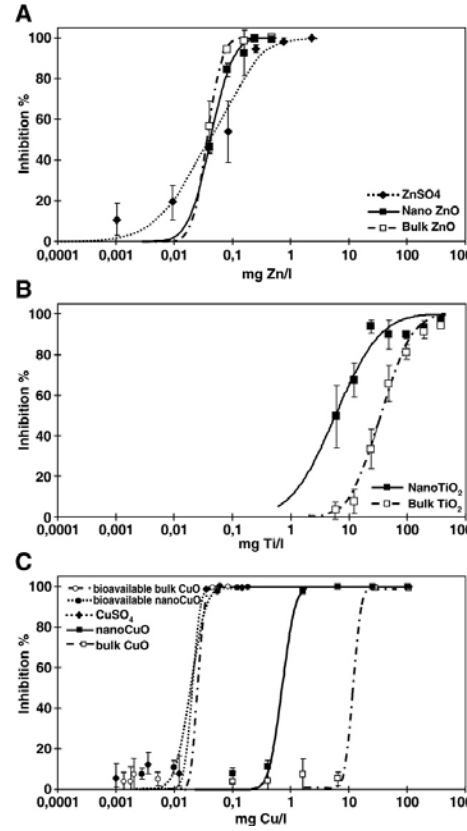

Fig. 4 – Dose response of *Pseudokirchneriella subcapitata* to metal oxide particles and respective metal salts. Bioavailable solubilized copper from (nano) CuO suspensions in algal growth medium was quantified using recombinant yeast Cu-sensor (see Materials and methods). Experimental data were analyzed with Regtox software (Vindimian, 2005). Values are reported as mean of 3 replicates ± standard deviation.

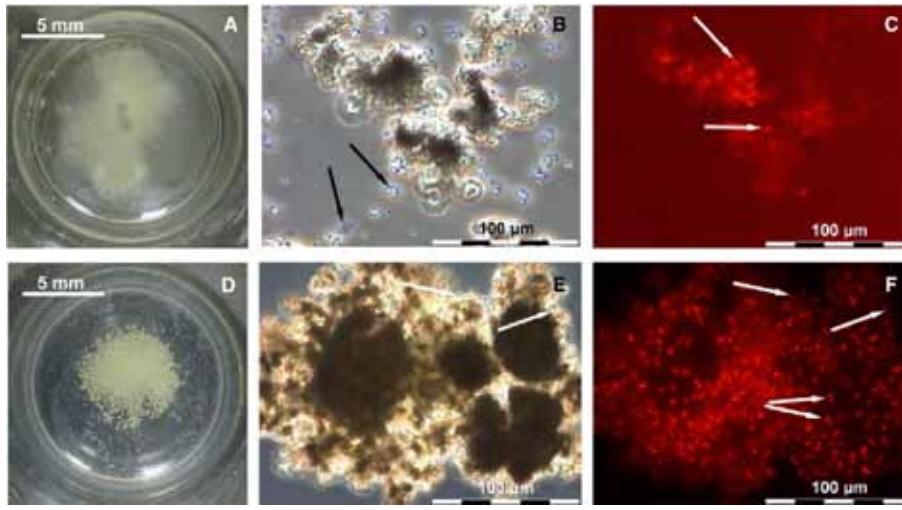

Fig. 5 – Aggregates of bulk and nano TiO_2 particles in *Pseudokirchneriella subcapitata* culture. Aggregates of bulk TiO_2 (A,B,C) and nano TiO_2 (D,E,F) in test medium, as visible to the naked eye (A,D), in phase contrast microscopy (B,E) and fluorescence microscopy (C,F). Arrows indicate algal cells.

visible aggregates present in the growth medium. Of the concentrations tested, complete inhibition of growth occurred at 6.4 mg Cu/l with nanoparticles but at 25.6 mg Cu/l with bulk CuO. When the incubation was extended several days beyond the standard 72 h, growth was observed in case of some initially toxic nano CuO concentrations (1.6 mg Cu/l of nano CuO) (Fig. 3C).

Copper salts have been used as biocides for a long time, but their use has been limited in recent years due to concerns of heavy metal contamination. The short-term toxic effect of copper is used for antifouling in marine paints, free copper ions released preventing attachment of organisms to the vessel. However, on entry to the water column free copper is quickly complexed to carbonates, hydroxides and bound by organic ligands, reducing its effect on the marine species (Tubbing et al., 1994). Indeed, the bioavailability of copper, rather than the total copper present, is the primary determinant of toxicity as in the case of several toxic heavy metals (Campbell, 1995). The bioavailability of copper is affected by different abiotic and biotic components within the aquatic environment (Meyer, 2002). Models of bioavailability and of toxicity of copper ions have been proposed (Di Toro et al., 2001; De Schamphelaere et al., 2003). Solubility of copper oxide in water has been extensively studied in various pH and

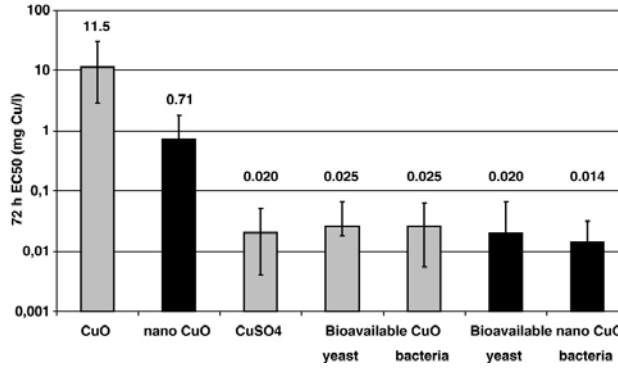

Fig. 6 – EC50 values (72 h algal growth inhibition) of nano CuO, bulk CuO and $CuSO_4$ and bioavailable copper in suspensions of CuO and nano CuO in algal growth medium at respective EC50 values. The bioavailable copper was quantified using recombinant bacteria and yeast. Values are reported as mean of 3 replicates ± standard deviation.

temperature conditions (Palmer et al., 2005). However, no data and no model concerning bioavailability of copper oxide have been published yet.

In our experiments, the bioavailable fraction of Cu in the test medium was determined using the recombinant bacterial and yeast Cu-sensors. Comparison of bioavailable copper in bulk CuO and nano CuO suspensions at concentrations in algal growth medium that inhibited the growth of algae by 50% showed that bulk CuO was remarkably less bioavailable: bulk CuO (EC50=11.55 mg Cu/l) and nano CuO (EC50=0.71 mg Cu/l) yielded practically similar bioavailable concentrations of copper (0.014–0.025 mg /l) measured with both microbial sensors (Fig. 4). Thus, at EC50 level (Table 1 and Fig. 6), 25% of copper from nano CuO was bioavailable differently from bulk CuO (only 0.18% bioavailable copper) showing that nano CuO was 141-fold more bioavailable (based on microbial sensor data).

As both microbial sensors showed analogous bioavailable copper concentrations, only bioavailability data obtained with the yeast Cu-sensor data are presented in the dose–effect curves on Fig. 4C. The bioavailable EC50 values of copper oxides were not significantly different from the EC50 of $CuSO_4$ (0.02 mg Cu/l; Table 1 and Fig. 6). Thus, the toxicity of bulk and nano copper oxides was due to the solubilized bioavailable fraction, most likely Cu^{2+} ions.

The copper bioavailability values are similar to those found in a study of copper nanoparticle toxicity on fish (Griffitt et al., 2007). When those nanoparticles were exposed to zebrafish, 6 to 12% of Cu became soluble (0.25 mg/l particles yielded 0.03 mg/l of soluble copper; 1.5 mg/l yielded 0.09 mg/l), which did not explain the Cu nanoparticle toxicity. In fact, the so-called "copper nanoparticles" manufactured by Quantum-Sphere Inc. (2007) (QSI-Nano® Copper particles) and used by Griffitt et al. (2007) have a copper oxide shell which forms between 10 and 70% of the particle mass. The exact CuO content of these particles is not specified by the manufacturer, which makes it difficult to calculate the total Cu content in the suspension.

4. Conclusions

To our knowledge, this is one of the first systematic studies on effects of metal oxide nanoparticles on algal growth and first for description of toxic effects of nano CuO towards algae.

Zinc oxides were equally toxic in bulk and nano formulations. The other studied metal oxides, TiO_2 and CuO, were remarkably more toxic to algae as nanoparticles. The most toxic of the nano metal oxides was nano ZnO followed by nano CuO and nano TiO_2, nano CuO and ZnO being toxic to algae already at sub mg/l concentrations. The shading of light by (nano)particles was not contributing to the overall toxic effect. Toxicity of ZnO and CuO was attributable to soluble metal ions originating from the metal oxide particles. At these low concentrations, both zinc oxides were totally soluble. CuO nanoparticles were more soluble and more toxic than bulk CuO. However, on basis of bioavailable copper, toxicities of both copper oxides were similar to that of $CuSO_4$ showing that the toxic effect of both copper oxides was solely due to copper ions. In case of TiO_2, entrapping of algal cells in aggregates of nano TiO_2 may play a major role in toxicity to algae *P. subcapitata*.

Lastly, this study again demonstrates that solubility is a key issue in the toxicity of metal containing (nano)particles, at least for organisms that *a priori* are not internalizing particles. Thus, the solubility properties reported as N/A (not available or not applicable) in MSDS (Material Safety Data Sheet) published for nano zinc oxide and nano copper oxide (Sigma-Aldrich, 2006, 2007, respectively) should be addressed.

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TOXICITY OF 58 SUBSTITUTED ANILINES AND PHENOLS TO ALGAE *PSEUDOKIRCHNERIELLA SUBCAPITATA* AND BACTERIA *VIBRIO FISCHERI*: COMPARISON WITH PUBLISHED DATA AND QSARS

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

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ABSTRACT

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

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1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

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

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

2.3. Acute bioluminescence inhibition assay with V. fischeri

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

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

2.4. Statistical methods

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

Table 1
Characteristics of the studied chemicals.

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

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

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

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

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

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

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

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

or

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

and

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

2.5. Previously published toxicity data

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

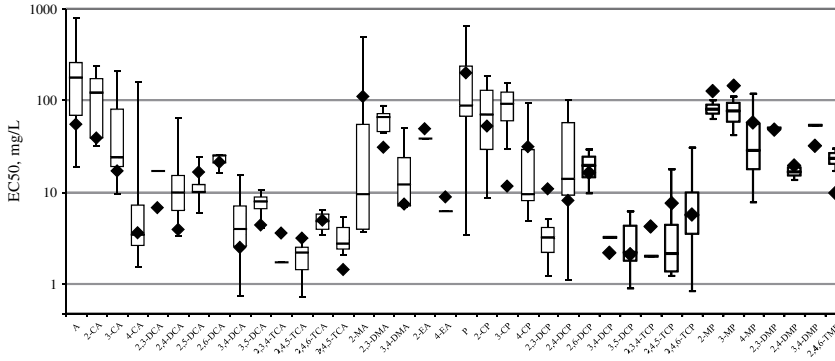

Fig. 1. Variation of *Chlorophyta* toxicity data EC50 (mg L^{-1}) (exposure time 1–4 d, median values) from US EPA ECOTOX database and published papers (see Table S1) on a boxplot. Experimental toxicity data from this study for *Pseudokirchneriella subcapitata* from Table 2 are shown as filled symbols.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

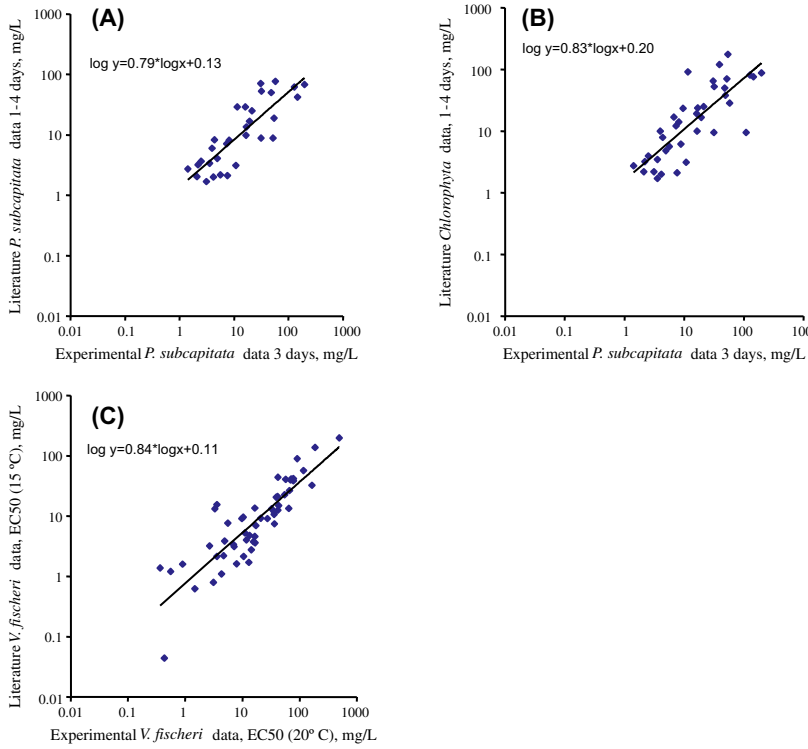

Fig. 2. Literature *versus* experimental data **A** – *Pseudokirchneriella subcapitata* toxicity data (1–4 d, median values) plotted against experimental data, $^{**}R^2 = 0.71$, $p < 0.01$; **B** – *Chlorophyta* toxicity data (1–4 d, median values) plotted against experimental data, $R^2 = 0.64$, $n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

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

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

3. Results and discussion

3.1. Experimental toxicity data

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

3.2. Experimental versus published data

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

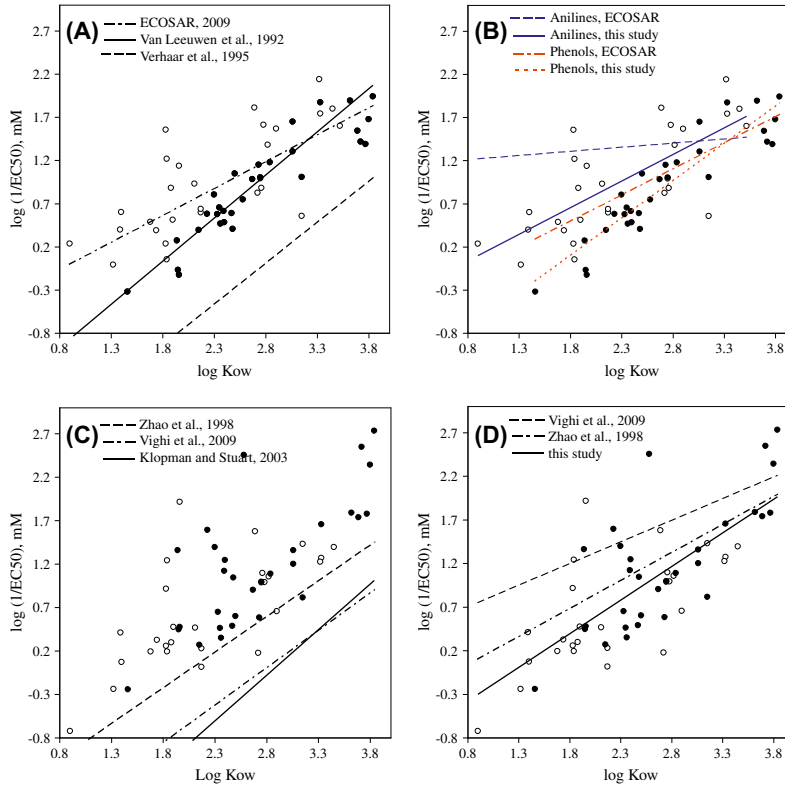

Fig. 3. Comparison of the experimental toxicity data with QSAR predictions. A and B – *Pseudokirchneriella subcapitata* (72-h EC50, mM), C and D – *Vibrio fischeri* (15-min EC50, mM), filled symbols depict phenols and open symbols anilines, equations for the lines are presented in Table 3.

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

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

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

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

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

3.3. Experimental data compared to QSAR-predicted toxicities

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

3.3.1. Algae: models versus experimental data

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

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

3.3.2. Bacteria: models versus experimental data

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

3.4. Classification based on environmental hazard

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

Table 4
Classification of the studied chemicals.

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

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

[a] Abbreviations are explained in Table 1.

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

[c] EC, 1967.

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

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

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

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

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

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

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

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

4. Conclusions and outlook

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

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

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Appendix A. Supplementary material

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

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