Acute toxicity to *Daphnia magna* in river water; Investigating mitigation and bioavailability of pure cationic surfactants and mixtures with SPME

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Summary

Many surfactants are evaluated according to REACH and a key component in exposure assessment is fate, which is influenced by various factors in the environment that can strongly reduce the toxicity observed in the laboratory. Toxicity is e.g. mitigated by their tendency to interact with natural organic matter (NOM) via hydrophobic interactions, but also electrostatically. Thus to determine the toxic potential of a surfactant, a quantification of the freely dissolved concentration, i.e. the bioavailable fraction, is necessary. AkzoNobel Surface Chemistry AB in Stenungsund, Sweden, supported this study with the aim to investigate the bioavailability and thereby the true acute toxicity of seven pure cationic surfactants and mixtures to *Daphnia magna* in river water using Solid-Phase Micro Extraction (SPME). A method where polyacrylate-coated fibers are added to the acute immobilization test (OECD 202) and the amount of sorbed surfactant on the fibers is directly proportional to the freely dissolved concentration. The most toxic substances in this study were hexadecylamine+2EO and didodecyldimethylammonium bromide, whereas the least toxic substances were Ethomeen C/12 and dodecylamine+2EO. Toxicity is increasing for primary fatty amine ethoxylates with the chain length increasing from 12 to 16 carbon atoms, caused by an increasing hydrophobicity within the molecule. Sorption increases with increasing amount of NOM but the mitigating effect is substance specific due to different sorption affinities and varies between 0.9 and 31.3 in this study. A general mitigation factor cannot be used, as the true toxicity will be either over- or underestimated. Different sorption affinities of individual mixture components to NOM also affects the composition of Ethomeen C/12, hence the mixture toxicity. The predicted mixture toxicity is overestimated with Concentration Addition in all test media but the overestimation decreases with increasing amount of NOM due to the altered composition.

Sammanfattning

Många tensider utvärderas enligt REACH och en viktig del i exponeringsbedömningen är ämnets öde, som påverkas av olika faktorer i miljön som till stor del kan minska observerad toxicitet i laboratoriet. Toxiciteten kan t.ex. mildras genom deras benägenhet att interagera med naturligt organiskt material (NOM) via hydrofoba interaktioner, men även elektrostatiska. Så för att bestämma den potentiella toxiciteten hos en tensid krävs en kvantifiering av den fritt lösta koncentrationen, det vill säga den biotillgängliga fraktionen. Denna studie stöddes av AkzoNobel Surface Chemistry AB i Stenungsund, Sverige, i syfte att undersöka biotillgängligheten och därmed den sanna akuta toxiciteten av sju rena katjoniska tensider och blandningar på *Daphnia magna* i flodvatten med Solid-Phase Micro Extraction (SPME). En metod där polyakrylatbelagda fibrer tillsätts i det akuta immobiliseringstestet (OECD 202) och mängden sorberad tensid på fibrerna är direkt proportionell mot den fritt lösta koncentrationen. De giftigaste ämnena i denna studie var hexadecylamine+2EO och didodecyldimethylammonium bromide, medan de minst giftiga ämnena var Ethomeen C/12 och dodecylamine+2EO. Toxiciteten ökar för primära fettaminetoxylater då kedjelängden ökar från 12 till 16 kolatomer, som orsakas av ökad hydrofobicitet inom molekylen. Sorptionen ökar med ökande mängd NOM men den mildrande effekten är ämnesspecifik på grund av olika sorptionsaffiniteter för NOM och varierar mellan 0.9 och 31.3 i denna studie. En generell mildringsfaktor kan inte användas eftersom den sanna toxiciteten kommer då antingen att över- eller underskattas. Olika affinitet för sorption till NOM för enskilda blandningskomponenter påverkar även sammansättningen av Ethomeen C/12, därmed blandningens toxicitet. Den predikterade blandningstoxiciteten överskattas med Concentration Addition i alla testmedier men överskattningen minskar med ökad mängd NOM till följd av den förändrade sammansättningen.

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1. Introduction

1.1 Surfactants

A wide range of products and applications used by consumers and industry of today's society contain **surf**ace-**act**ive **a**ge**nts**, or shortly surfactants. The use ranges from primary production processes to enhancing the quality of finished products, hence surfactants appear in products such as motor oils, pharmaceuticals, cosmetics, detergents, drilling muds and flotation agents and in recent decades also in electronic printing, magnetic recording, biotechnology, microelectronics and viral research (Rosen and Kunjappu, 2012). However, about 54% of the use is in different household products, including detergents, fabric softeners, cosmetics and sanitizers (Banat et al., 2000; Rust and Wildes, 2008). In 1993, the annual world production of synthetic surfactants amounted to 7.2 million tons (Di Corcia, 1998) and in 2008, the annual production was 13 million tons (Reznik et al., 2010) and was expected to increase by 2.8% annually until 2012 and 3.5-4% thereafter (Acmite, 2010).

Synthetic surfactants are economically important chemicals (Ying, 2006) and the main reason for this is their ability to modify surface and interfacial properties between liquids, solids and gases. These properties reside in their amphiphilic character, i.e. they generally contain a hydrophobic (nonpolar) tail and a hydrophilic (polar, charged or uncharged) head (fig. 1). The chemical structure of surfactants are not restricted to the simple schematic illustration shown in figure 1 but varies widely, which gives them their different characteristics (Holmberg et al., 2003).

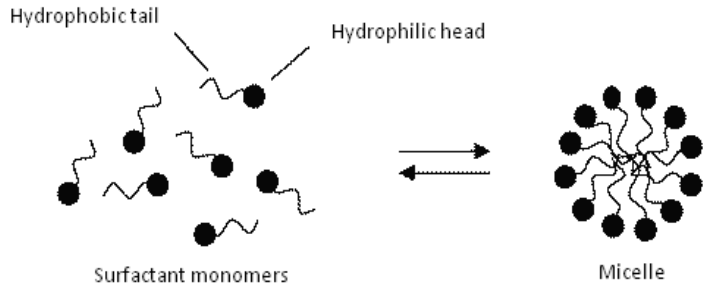

Figure 1. A schematic illustration of surfactant monomers and a micelle.

When a surfactant with an amphiphilic structure is dissolved in an aqueous solution, they prefer to migrate to surfaces or interfacial regions. This is because the hydrophobic group is incapable of hydrogen bonding and thus disrupts the normal water structure. As a consequence, the interfacial tension or surface tension of the system is increased, which is defined as the interfacial free energy per unit area of the boundary between two different phases (Holmberg et al., 2003; Rosen and Kunjappu, 2012). By orientation of the hydrophilic group towards the aqueous phase and the hydrophobic groups away from it, the interfacial tension is reduced and the normal water structure is restored. Hence, surfactants are concentrating at the interfaces separating immiscible phases (Haigh, 1996) and by lowering the interfacial tension of the medium in which it is dissolved, two different media or interfaces are able to mix or disperse readily as emulsions in water or other liquids (Holmberg et al., 2003).

Surfactants are present as monomers when dissolved in an aqueous solution at low concentrations. As the concentration of surfactant increases, the interface will eventually be saturated. At higher concentrations micelles will be formed, i.e. aggregation of surfactants (fig. 1), when the hydrophobic groups are oriented towards the center of the micelle and the hydrophilic groups towards the aqueous phase. This aggregation occurs at a surfactant concentration called the critical micelle concentration (CMC) (Holmberg et al., 2003) and varies with surfactant structure and solution chemistry, e.g. temperature, presence of electrolytes and various organic compounds. In general, the CMC decreases as the hydrophobic character of the surfactant increases and when electrolytes are present (Haigh, 1996). Concentrations above the CMC enables surfactants to solubilise more of a hydrophobic organic compound compared to what would dissolve in water alone (Haigh, 1996; Roberts, 2000), thus reducing the interfacial tension that has increased due to the presence of organic compounds (Holmberg et al., 2003).

Surfactants are represented in different forms but normally classified according to the presence of formally charged groups on the hydrophilic moiety. These different types include cationic, anionic, non-ionic and zwitterionic surfactants (Holmberg et al., 2003). Even though each surfactant have unique properties and characteristics some common characteristics can be attributed to each class. A*nionic* surfactants bears a negative charge, usually due to a sulphonate or sulphate group, and they are used in detergents due to their detersive action and efficiency to remove particulate soils. This benefit is possible due to the fact that anions are not prone to sorb to negatively charged substrates, such as particulate soils, thereby hindering redeposition of undesirable soils on fabrics' etcetera (Rosen and Kunjappu, 2012). Anionic surfactants are also the largest surfactant class, with approximately 60% of the world production, due to their ease and low cost of manufacture (Holmberg et al., 2003). *Non-ionic* surfactants are the second largest surfactant class and contain no ionic constituent and are thus compatible with charged molecules, e.g. ionic surfactants that result in beneficial associations. They have also very low sensitivity to water hardness and pH, which makes them very useful in liquid and powder detergents and to stabilize oil-in-water emulsions (Holmberg et al., 2003; Rosen and Kunjappu, 2012).

Cationic surfactants bears a formal positive charge and thus adsorbs strongly onto most substrates in the environment, e.g. metals, minerals, plastics, fibres, cell membranes etcetera, which are generally negatively charged. This changes the surface properties and makes a hydrophilic surface behave as if it was hydrophobic and vice versa, and thus impart special characteristics to the surface. Cationic surfactants are the third largest surfactant class and are used as conditioning agents in fabric softeners and hair care products, as corrosion inhibitors of metals in fuel and lubricating oils and as anticaking agents in fertilizers. The smallest surfactant class is *zwitterionic* surfactants which may have both positively and negatively charged moieties within the same molecule. They have their optimal surface activity around neutral pH, hence they are used in personal care products (shower gels, foam baths, shampoos, etc.) for their mildness and skin compatibility. They are often used together with anionic or non-ionic surfactants to enhance properties such as foam or detergency (Holmberg et al., 2003; Rosen and Kunjappu, 2012).

Differences in the nature of the hydrophobic group, which generally consists of long-chain hydrocarbon residues, are also important for the properties and the characteristics of surfactants but less pronounced than for the hydrophilic group. These structures includes differences in the length of the alkyl group, branching and unsaturation, presence of an aromatic nucleus, polyoxypropylene or

polyoxyethylene and perfluoroalkyl or polysiloxane groups (Rosen and Kunjappu, 2012). Surfactants are produced from petrochemical (synthetic) and/or oleochemical (renewable) feedstocks. The petrochemical feedstocks are mainly derived from crude oil and converted to different surfactant intermediates whereas oleochemical feedstocks are commonly derived from plant oil (palm and coconut), plant carbohydrates (sorbitol, sucrose and glucose) and animal fat (tallow) (Holmberg et al., 2003; Rust and Wildes, 2008).

1.2 Environmental fate – what is bioavailable?

Considering the widespread use and high consumption of surfactants and due to the fact that they are mainly used in household products, such as laundry detergents, fabric softeners and hair care products, they will be discharged to sewage treatment plants or directly to surface waters (Ying, 2006). Inevitably, aquatic organisms are exposed to different types of surfactants and their degradation products at various concentrations in different environmental compartments. The total surfactant concentration in wastewater may reach 10 mg/L in areas where it is extensively used, although the aqueous concentration are below a few tens of µg/L (WHO, 1996). Some reported concentrations for cationic surfactants, such as ditallow dimethylammonium chloride (DTDMAC), are 37 µg/L in river water, 334 µg/L in influent wastewater and 28 µg/L in effluents from sewage treatment plants (Wee, 1984), 60 µg/L in surface waters (Versteeg et al., 1992) and up to 5870 mg/kg in dry treated sewage sludge (Fernandez et al., 1996). Alkyltrimethylammonium compounds have measured concentrations ranging from 361 to 6750 mg/kg in sediments, where the highest concentration was observed in samples affected by effluents from wastewater treatment plants (Lara-Martín et al., 2010). Dimethyldiesterarylammonium chloride have been measured in effluents from wastewater treatment plants up to 503 µg/L (Barco et al., 2003).

Given a high enough concentration and a sufficient length of time, a chemical and/or its metabolites that come into contact with an organism and react at an appropriate target site(s) will elicit an adverse response or toxic effect. The effect is concentration-dependent and this relationship (fig. 2) varies with the chemical and species of organism. To express and measure the toxicity of a certain chemical to aquatic organisms, different end points are used, e.g. the median effect concentration (EC50). EC50 is the concentration estimated to produce a certain effect, e.g. immobility, in 50% of a test population over a specific time period (Rand et al., 1995).

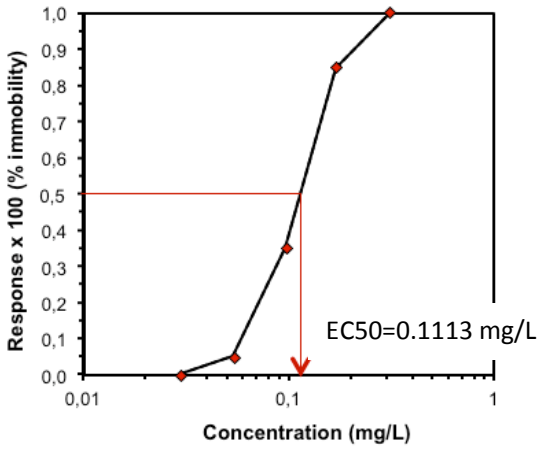

Figure 2. A typical form of the concentration-response relationship.

To improve the protection of human health and the environment, all chemical substances that are produced within or imported to the European market above 1 ton per year has to be assessed for its intrinsic properties. This is according to the European legislation of chemicals, REACH (Registration, Evaluation, Authorisation and restriction of Chemicals), which entered into force 1 June 2007 (Europa.eu, 2011). Ecotoxicological information is gathered through exposure and effect assessments where tests are performed with standard test organisms from at least three different trophic levels (algae, D*aphnia* and fish). The organisms are

exposed to the substance during a short (hours to a few days) or a longer (generally several days or weeks) period of time to evaluate potential hazardous properties and possible acute or chronic effects (ECHA, 2011). The freshwater micro crustacean *Daphnia magna* is included in the ecological risk assessment and used in acute immobilization tests because they are a primary food source for many fish species and convert phytoplankton and bacteria into animal protein, thus an ecologically important species (Cooney, 1995). They have also been shown to be the most sensitive species to some detergent chemicals according to Lewis and Suprenant (1983).

The mechanism of action of surfactants is widely believed to be narcotic, i.e. the toxicity is dependent on the ability of the surfactant to partition from the aqueous environment into lipid membranes of aquatic organisms (Rosen et al., 2001). Two different narcosis mechanisms have been recognized and are based on log P_{ow} (P=octanol/water partition coefficient) (Roberts and Castello, 2003) or log K_{mw} (membrane-water partition coefficient) (Robert and Castello, 2003:a). The first is general narcosis developed by Könemann (1981) where the substance act by a non-specific mechanism and is generally as toxic as their hydrophobicity indicates, i.e. a baseline toxicity. The second is polar narcosis, developed by Saarikoski and Viluksela (1982), and accounts for polar contributions to binding to membranes as the predicted baseline toxicity is generally lower than the observed (Roberts and Costello, 2003:a). Toxicity is also related to bioavailability, which is the freely available fraction of the surfactant that possibly can cross an organism's cellular membrane from the medium surrounding the organism (Semple et al., 2004). Cationic surfactants are found to be more toxic than anionic surfactants, and anionic surfactants are more toxic than non-ionic surfactants. In general, toxicity increases with an increase in the length of the hydrophobic group and decreases with branching (Rosen and Kunjappu, 2012). EC50 values below 1 mg/L after a 48 h test with *D. magna* and 96 h test with fish and algae are considered to be toxic (Holmberg et al., 2003).

Aquatic toxicity data are available for surfactants on different organisms, although the toxic effects are more evaluated for anionic, e.g. linear alkylbenzene sulphonic acid (LAS), and non-ionic surfactants, e.g. alcohol ethoxylate (AE), according to Ivankovic and Hrenovic (2010). For cationic surfactants, aquatic toxicity data is available but less evaluated for their environmental fate and toxic effects. Different quaternary ammonium compounds (QAC) exposed to several fish species have reported EC50-48h values ranging between 0.49 and 8.24 mg/L (Singh et al., 2002). Alkyltrimethylammonium compounds exposed to *D. magna*, such as cetyl trimethylammonium chloride have LC50-48h (lethal concentration) ranging between 0.025-0.05 mg/L (Lewis and Suprenant, 1983), whereas dodecyl-, tetradecyl- and hexadecyl trimethylammonium bromide have reported EC50-24h of 0.37, 0.091 and 0.058 mg/L, respectively by Sandbacka et al. (2000) and 0.38, 0.14 and 0.13 mg/L, respectively by García et al. (2001). García et al. (2001) also showed that substitution of a benzyl group for a methyl group appears to slightly increase the toxicity to *D. magna* and reported EC50-24h values of 0.13, 0.13 and 0.22 mg/L for dodecyl benzyl dimethyl ammonium bromide, tetradecyl benzyl dimethyl ammonium chloride and hexadecyl benzyl dimethyl ammonium chloride, respectively. Arquad 2C-75 have reported LC50-96h for fish ranging between 0.26 and 0.787 mg/L, an LC50-48h of 0.295 mg/L for crustacean and EC50-72h for algae ranging between 0.06 and 0.386 mg/L (ECHA, 2012: CAS 68391-05-9). Clearly, the effect concentration is below 1 mg/L for the most sensitive species *D. magna* and these values are all based on nominal concentrations, except the highest mentioned toxicity data for Arquad 2C-75 on algae.

However, the risk assessment of the surfactant is to a large extent based on these laboratory studies where the tested chemical is dissolved in a pure liquid media (OECD, 2004; van Wijk et al., 2009) and the effect is then extrapolated to the real environment (TGD, 2003). A key component in exposure assessment is fate, i.e. the concentration, transport, transformation and disposition of a surfactant (Lyman, 1995), and that is influenced by various factors in the aquatic environment that can strongly reduce the toxicity observed in the lab (Haigh, 1996; Alexander, 2000). Due to physical and chemical properties of the surfactant, such as the molecular structure and the nature of structural groups (amphiphilic structure), they have a tendency to form aggregates and a propensity to interact with natural particles (Jones-Hughes and Turner, 2005). This will reduce their toxicity, i.e. mitigate their effect.

Thus, sorption to natural organic matter (NOM) is an important property to consider regarding surfactants as they can at low concentrations in natural water exists in either or both the dissolved and the sorbed phase (Lyman, 1995). NOM is a complex mixture of compounds with different particle sizes that can be separated into particulate, colloidal and dissolved fractions. Their functional groups are diverse and have a broad range of interaction with surfactants, hence controls bioavailability and toxicity. Humic acid is one of the most abundant components of the colloidal fraction of NOM (Koopal et al., 2005), considered to be structured polyelectrolytes with an amphiphilic character (Guetzloff and Rice, 1994) and soluble in aqueous solutions in a wide pH range and thus easily transported in the aqueous environment (Koopal et al., 2004).

The impact of sorption is included in the environmental risk assessment for hydrophobic nonpolar chemicals where the organic carbon/water partition coefficient (K_{oc}) or the octanol/water partition coefficient (K_{ow}) can be used to describe the sorption to organic matter and subsequent reduced bioavailability (TGD, 2003; van Wijk et al., 2009). However, the sorption of cationic surfactants to natural organic matter is not only described by hydrophobic interaction and measured values are therefore necessary (TGD, 2003). Depending on the aqueous properties, such as pH, salinity, temperature and amount of suspended material (Rand et al., 1995), different sorption mechanisms are potentially involved for ionic surfactants, such as ion exchange, ion pairing and hydrophobic bonding (Jones-Hughes and Turner, 2005; Rosen and Kunjappu, 2012). The hydrophobic chains of cationic surfactants binds to the organic fraction of suspended matter and of humic acid through van der Waals forces, whereas the positively charged nitrogen group binds electrostatically to the negatively charged binding sites of the sorbents, hence both hydrophobic and electrostatic attraction are involved (Koopal et al., 2004; van Wijk et al., 2009). Surfactants differ in their hydrophobicity as well as how much that is charged at a specific pH. The hydrophobic binding of surfactants to substrates are assumed to concur with the equilibrium partition theory, whereas the electrostatic interaction (ionic) is governed by other parameters not included in this theory (Thomas et al., 2009).

To account for sorption of cationic surfactants a quantification of the freely dissolved concentration is necessary as this determines the toxic potential of a surfactant (Rufli et al., 1998) and not the surfactants that are strongly sorbed to colloidal phases. It is the freely dissolved concentration that controls evaporation, sorption, precipitation, biodegradation, bioconcentration and toxicity (Rico-Rico et al., 2009) and a quantification of this provides information about the bioavailability and thus the potential risk of cationic surfactants in the environment. The freely dissolved concentration is measured with the method Solid-Phase Micro Extraction (SPME). It is a sampling technique with polyacrylate-coated fibers that utilize the ion-exchange capacity of the fibers to sorb chemical

substances. The fibers are equilibrated for 24 hours in a test vessel and the concentration of chemicals on the fibers is directly proportional to the freely dissolved concentration by applying a compound specific fiber-water partitioning coefficient (K_{fw}). The SPME method began with hydrophobic compounds and in recent years, the application of SPME has extended and includes also more polar and ionized compounds. Difficulties with the calibration of SPME for ionic organics are that the partitioning is influenced by the solution chemistry (pH, salinity, type of counter ions, etc.) (Rico-Rico et al., 2009) and that they have an affinity to the test container. With optimized experimental conditions, SPME calibration isotherms have been made for anionic and non-ionic surfactants and all of them were linear at concentrations below their critical micelle concentration (CMC) (Droge et al., 2007; Rico-Rico et al., 2009). At last, a few cationic surfactants have been tested and evaluated with this technique (Chen et al., 2010).

Furthermore, the number of chemicals produced in today's society is increasing and to perform ecotoxicological tests on all of them are expensive, time consuming and raise questions about ethics. Predictions of their environmental behaviour, effect and fate by a model is thus necessary. Development of alternative hazard assessments are e.g. promoted by REACH (2006). Surfactants are present as pure individual substances but also as mixtures of e.g. different carbon chain lengths and structural groups, and these variations are numerous. Instead of testing every possible mixture combination, the mixture toxicity can be predicted if the toxicity and the concentration of the individual substances within the mixture are known.

One concept is Concentration Addition (CA) where the concentrations of the single substances are added to yield the toxicity of the mixture. This predictive model is applied to substances believed to have a similar mode of action described by Porsbring (2009). At first, each single substance in the mixture is scaled to a common effect level, i.e. a toxic unit (TU) (see appendix A). The TU of a single substance is the ratio between their concentration in the mixture and their effect concentration (e.g. EC50) when tested individually. Addition of the single TUs gives the TU of the mixture and the mixture conforms to CA when the TUs are equal, i.e. 1. However, if the addition of the single TUs will be less than 1, their joint toxicity is greater than additive and a lower mixture concentration than expected by CA is required to provoke an effect. Conversely, less than additive if the TU of the mixture is higher than 1.

With all this in mind, the toxicity of surfactants are obvioulsy affected by several factors in the aquatic environment. Therefore, a quantification of the bioavailable fraction with SPME is necessary to describe their true toxicity and the focus is on cationic surfactants. The study was performed in collaboration with AkzoNobel Surface Chemistry AB, in Stenungsund Sweden and Arnhem, the Netherlands. AkzoNobel is a multinational chemical corporation headquartered in Amsterdam, the Netherlands, which supplies industries and consumers worldwide with decorative paints, performance coatings and specialty chemicals. They have operations in more than 80 countries and employs around 55 000 people. Their approach is to find innovative solutions and sustainable answers to customers (AkzoNobel, 2012). REACH put a greater responsibility on the companies to evaluate substances and thus increase the competitiveness of the chemicals industry within European Union (Europa.eu, 2011). Therefore, AkzoNobel's main objective is to develop more environmentally friendly surfactants.

1.3 Tested cationic surfactants

Fatty amines and their derivatives are examples of cationic surfactants, produced either from synthetic or renewable feedstocks, where AkzoNobel Surface Chemistry is the world's leading supplier. These cationic surfactants are based on alkyl groups ranging from carbon chain lengths C_8 to C_{22}, with C_{12} to C_{18} chain lengths the most predominant (AkzoNobel, 2012). Available physico-chemical properties for the seven tested surfactants are found in appendix B.

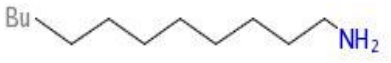

Figure 3. Chemical structure of dodecylamine.

Primary, secondary, tertiary alkyl amines and their salts ($RNH_3^+X^-$) are uncharged and insoluble in water at a high pH and therefore, not strictly cationic (Holmberg et al., 2003). *Dodecylamine* (abbreviated C12) is a pure primary fatty amine with a C_{12} carbon chain length (fig. 3). It has a pKa of 10.63 and is cationic at a pH below this value. Primary alkyl amines sorb strongly to solid phases by van der Waals forces and ionic interactions (e.g. ion pair formation and cation exchange). Dodecylamine is used for manufacturing of primary alkyl amines, formulation of fuel additives, lubricants, coating agents for fertilizer and products in textile industry, production of ethoxylates of primary alkyl amines, amine derivatives, amides, as metal corrosion inhibitor, antistatic agents and rubber additive and flotation agent in mining industry (ECHA, 2012:a).

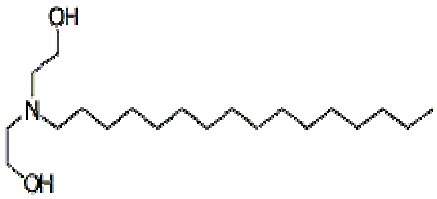

Figure 4. Chemical structure of hexadecylamine +2EO.

The amine can be ethoxylated to yield an ethoxylated amine. These surfactants can be cationic or non-ionic, depending on the degree of ethoxylation and on the pH at which they are used. They are considered as cationic surfactants when the pH is low enough to provide the ionic form. Ethoxylated amines are water-soluble over a large pH range due to the fact that the ethoxylation degree mainly governs the hydrophilic character of the fatty amine (Holmberg et al., 2003). *Dodecylamine +2EO*, *hexadecylamine +2EO* (fig. 4) and *octadecylamine +2EO*, abbreviated C12+2EO, C16+2EO and C18+2EO respectively in this report, are pure primary fatty amine ethoxylates (PFAEO). They have two ethoxylates attached to the amine and an alkyl chain length of C_{12}, C_{16} and C_{18} carbon, respectively. They have a pKa of 8.6 (Chen et al., 2012) and is therefore cationic under the test conditions in this study. *Ethomeen C/12* is a mixture of different fatty acid chain lengths, mainly C_{12} and C_{14} (appendix B), with two ethoxylates attached to the amine. The pKa is 8.8, hence it is cationic under test conditions in this study. It is used in applications as pigment processing additives and as thickening agents in polar solvents (AkzoNobel, 2011). It is also used in cosmetic products, cleaning and care products, lubricants and greases, plastic articles and as corrosion protection (ECHA, 2012:b).

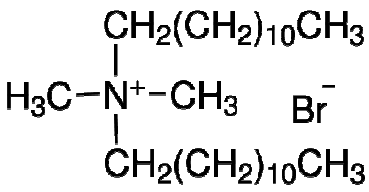

Figure 5. Chemical structure of didodecyldimethylammonium bromide.

Quaternary ammonium compounds (QAC) contain a positively charged nitrogen atom linked to four alkyl or aryl substituent's and the positive charge is permanent, regardless of pH (Rosen and Kunjappu, 2012). *Didodecyldimethylammonium bromide* (abbreviated DDAB) has two alkyl chains with C_{12} carbon respectively, and two methyl groups attached to the amine. The

counter ion is bromide (fig. 5).

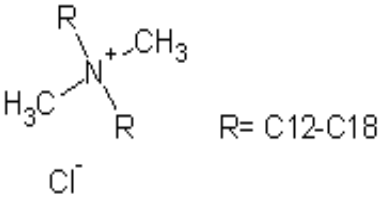

Figure 6. Chemical structure of Arquad 2C-75.

Arquad 2C-75 has two hydrophobic hydrocarbon chains, with carbon chain lengths varying from C_{12} to C_{18} respectively, but mainly C_{12} and C_{14} (AkzoNobel, 2012:a). The other two substituents are methyl groups. They are all linked to a positively charged nitrogen atom (fig. 6). It is used in industrial settings and by professional workers for treatment of minerals, application and manufacture of metal treatment products, coatings (organic solvent-borne, water-borne, solvent-free products and powder coatings), manufacturing of washing and cleaning products, cosmetic products and application of agricultural and agro products. The use by consumers is mainly by application of cosmetic products (ECHA, 2012:c).

1.4 Aim

The aim of this project was to investigate the bioavailability and thereby the true acute toxicity of pure cationic surfactants and mixtures to *Daphnia magna* in river water using the SPME technique.

1.5 Hypothesis and questions

The hypothesis is that the toxicity of these surfactants is to a large extent determined by their hydrophobicity due to a narcotic mechanism of action. Additionally, toxicity is also influenced by their ability to also interact electrostatically with biological surfaces due to their cationic charge.

- Is there a difference between nominal and measured concentrations? If so, why?
- Which of the tested cationic surfactants is the most and least toxic ones, and why?
- What is the mitigation factor for these surfactants? Is it the same mitigation for all cationic surfactants, i.e. is it possible to use a standard mitigation factor?
- How does carbon chain length affect toxicity? Is the response only a function of alkyl chain length?
- How does the degree of ethoxylation affect toxicity?
- Is there a difference between single substances and mixtures regarding toxicity? Does the cationic mixture conform to the predictive model Concentration Addition?

2. Materials and methods

2.1 Literature search

A literature search were performed for all the tested surfactants at the website of European Chemicals Agency (ECHA, 2012) to gather physico-chemical and ecotoxicological information, by searching on the individual CAS numbers. Aquatic toxicity data was also obtained at the ECOTOX database (U.S. EPA, 2012). The scientific databases Web of Knowledge, Scopus, Sciencedirect and Google Scholar were used to gather available scientific information about the tested surfactants and related surfactants. The same databases were also used to find information about surfactants and their environmental fate for the introduction.

2.2 Experiments

Experiments were performed during nine weeks from February to April 2012 at AkzoNobel Ecotoxicology and Environmental Testing lab in Arnhem, the Netherlands. The focus was on documenting and studying the bioavailability and the acute aquatic toxicity of pure cationic surfactants and mixtures. The used methods were the *Daphnia sp*. Acute Immobilization test (OECD 202) and the SPME technique (Solid-Phase Micro Extraction).

2.2.1 Chemicals

Surfactants, fibers and NOM

Dodecylamine (C12), purity ≥99.5% and Didodecyldimethylammonium bromide, purity ≥98% from Fluka Chemie GmbH, Switzerland. Arquad 2C-75, Dodecylamine (pure) +2EO, Hexadecylamine (pure) + 2EO, Octadecylamine + 2EO (pure) and Ethomeen C/12 from AkzoNobel Surface Chemistry AB, Stenungsund, Sweden. Polyacrylate coated SPME fibers (30 µm: FSA110170 and 7 µm: FSA110124 5, 15) from Polymicro Technologies, Phoenix, Arizona US (www.polymicro.com). Humic acid (EC: 215-809-6, CAS: 1415-93-6) from Sigma-Aldrich.

Test medium

The tests were performed in four different test media. First test medium was Dutch Standard Water (DSW), having a pH of approximately 8.2, and conductivity between 550 and 650 µS/cm. It contains per liter of de-ionized water: $NaHCO_3$ [100 mg], $CaCl_2{\cdot}2H_2O$ [200 mg], $MgSO_4{\cdot}7H_2O$ [180 mg] and $KHCO_3$ [20 mg]. Second test medium was DSW with humic acid (HA) [20 mg/L] added, other characteristics are the same as previous DSW. Third test medium was river water (HD) containing suspended matter [2.4 mg/L] and humic acid with a conductivity of 283 µS/cm and a pH of 7.8. Fourth test medium was HD water with DSW salts added to achieve a conductivity between 550 and 650 µS/cm. It contains per liter of HD water: $NaHCO_3$ [50 mg], $CaCl_2{\cdot}2H_2O$ [100 mg], $MgSO_4{\cdot}7H_2O$ [90 mg] and $KHCO_3$ [10 mg]. The dissolved oxygen and pH was measured and adjusted, if necessary, to achieve an oxygen concentration >7 mg/L and a pH of 8.2 (±0.2).

Culture medium

Culturing media for *D. magna* were M4. It is based on concentrated stock mineral salt solutions supplemented with vitamins. It was prepared by adding the stock solutions to de-ionized water preferably one day before the animals were introduced. The vitamins were added to the culture medium immediately before use. Following salts with final concentration in mg/L were used in M4: $CaCl_2{\cdot}2H_2O$ [293.8], $MgSO_4{\cdot}7H_2O$ [123.3], $NaHCO_3$ [64.8], KCl [5.8], $MnCl_2{\cdot}4H_2O$ [0.36], LiCl [0.31], RbCl [0.071], $SrCl_2{\cdot}6H_2O$ [0.152], $CuCl_2{\cdot}2H_2O$ [0.017], $ZnCl_2$ [0.013], $CoCl_2{\cdot}6H_2O$ [0.010], H_3BO_3 [2.86], NaBr [0.016], KI [0.0033], Na_2SeO_3 [0.0022], $FeSO_4{\cdot}7H_2O$ [0.9955], $Na_2EDTA{\cdot}2H_2O$ [2.5], $Na_2MoO_4{\cdot}2H_2O$ [0.063], NH_4VO_3 [0.0006], $NaSiO_3{\cdot}9H_2O$ [10], $NaNO_3$ [0.274], KH_2PO_4 [0.143] and K_2HPO_4 [0.184]. Following vitamins with final concentration in mg/L are included in M4: thiamine hydrochloride (B_1) [0.075], cyanocobalamine (B_{12}) [0.001] and biotin [0.00075].

D. magna were also cultured in HD water with the following vitamins and final concentration in mg/L in HD water: thiamine hydrochloride (B_1) [0.075], cyanocobalamine (B_{12}) [0.001] and biotin [0.00075]. Culture medium, both M4 and HD, were renewed twice a week, every Tuesday and Friday.

2.2.2 Sampling and characterization of river water

The natural surface water used as test medium and culture medium is river water (abbreviated HD from Heveadorp). It is sampled from a specific sample location in Heveadorp at Fonteinallee,

Doorwerth (Gelderland) with GPS coordinates: 51° 58' 10.29" N, 5° 48' 9.35" E (appendix C). The sample point is situated in a ground water protection area under management from water company Vallei Eem and the Gelderland province. There is no agriculture in the area and therefore no concerns regarding pesticide use in the area. The water source has also been analysed for dissolved heavy metals but there are no cause of concerns regarding this. The water is described of exceptional quality and diverse in flora and fauna. The river water (HD) has a total suspended solids-particulate matter (TSS) concentration of 2.4 mg/L. It was measured by filter 1 litre of river water through a 45 µm filter with known weight, then placed in the oven at 105°C for 24 hours and then weights the filter again. The total organic carbon (TOC) concentration is 2.21 mg/L. The conductivity is 283 µS/cm and has a pH of 7.8. The Ca^{2+} concentration is 34.3 mg/L (see appendix C).

2.2.3 Acute toxicity test with *Daphnia magna*

The toxicity tests for all tested substances were performed according to Organisation for Economic Co-operation and Development Guideline 202 (OECD, 2004), a *Daphnia* sp. acute immobilisation test with exposure duration of 48 hours. The acute toxicity to *Daphnia magna* is usually expressed as the median effective concentration for immobilization. This is the concentration, which immobilizes 50 % of the animals in a test batch within a period of continuous exposure (EC50). Furthermore, the concentration causing no significant immobility (NOEC) and the lowest concentration causing significant immobility in comparison to the control was determined (LOEC), if possible.

Test species

The test animals used in the acute toxicity tests were *D. magna* (water flea), taken from a stock cultured in M4 and HD water. They were grown in 3 L beakers covered with glass plates and contained about 2.5 L medium and the room temperature was between 18 to 23 °C. They were fed with 2 ml of algae (*Chlorella vulgaris, Pseudokircherinella subcapitata* or *Scenedesmus subspicatus*) six days per week and received feed equivalent to approximately 0.1 mg carbon per daphnia per day. The animals used in the test were less than 24 hours old and obtained from parent animals aged between 2 and 4 weeks. The day before the start of the test, the suitable group of test animals were sieved in the afternoon (around 4.00 p.m.) to remove the juveniles. On the day of the test, the same group were sieved in the morning (around 8.00 a.m.) again and the juveniles were collected in a dish with dilution water. Animals cultured in M4 were collected in DSW and animals cultured in HD were collected in HD water.

Test procedures

The test was performed as a static test for 48 hours with a light regime of 16 hours of ambient light and 8 hours of darkness. A total of 20 animals divided into 4 batches of 5 animals in 200 ml of test medium were tested at each concentration and in the control. Those animals that were not able to swim within 15 seconds after gentle agitation of the test vessel were considered to be immobile and were recorded. The number of animals being trapped at the surface was determined. These animals were not regarded as immobile. The test vessels were not aerated during the test and the animals were not fed. Glass beakers (test vessels) were covered with glass after introducing daphnia in them. The test was inspected at 0, 24 and 48 hours.

Preparations of solutions/suspensions of the test substance

All test substances were soluble in water (specifications for each test substance, see appendix D). A stock solution of approximately 100 mg/l was prepared by loading approximately 0.0100 gram of the

test substance, weighed out on an analytical balance and then filled up to the appropriate volume (100 ml) with de-ionized water to achieve a 100 mg/L stock solution. The solution was then stirred or sonicated whilst on ice (if required) for maximum two minutes until a homogenous solution was formed. The pH was checked and adjusted with sodium hydroxide (1 M) or hydrochloric acid (1 M) if required to approximately 8.2.

Test concentrations

To minimise contamination from previous tests, all glassware were rinsed in methanol and de-ionized water prior to be used in the new test. Preliminary tests (range finding) were conducted for all the tested substances with the following standard concentrations: 0.01, 0.1, 1.0 and 10 mg/L to determine the range of concentrations for the definitive test. 100 ml of test solutions divided in two test vessels and 200 ml of test medium (control) divided in four test vessels with 5 daphnids in each were used.

For the definitive test, test solutions were prepared on the day of the test in 200 ml volumetric flasks by diluting the stock solution in test media to achieve five test concentrations in a geometric series with a separation factor not exceeding 2.2. The highest test concentration resulted in 100 per cent immobilisation and the lowest test concentration resulted in no observable effect, compared to the control. Controls containing only test medium was also included in the test.

Determination of dissolved oxygen, pH and temperature

The dissolved oxygen and pH were determined in the test vessels and adjusted, if necessary, before the start (t=0h) of the test in the highest and lowest test concentrations and in the control. It was also determined at the end of the test (t=48h). The temperature was also measured at the beginning and at the end of the test.

2.2.4 Bioavailability test with Solid-Phase Micro Extraction (SPME)

Preparation of SPME fibers

The polyacrylate (PA) coated fibers used in the tests had a glass core of 110 µm diameter and a thickness of either 7 (mainly ionic interaction) or 30 µm (mainly hydrophobic interaction), depending on the test substance (table 1). Gloves were used to avoid contamination of the fibers while cutting them in a length of 3.4 centimetres. Subsequently, they were activated by heating them up in GC Oven 8000 series (Fisons instruments) with a helium flow of 30 ml/min and a temperature of 120°C for at least 16 hours, then changed to a temperature of 60°C for at least two hours. After heating, they were placed in a vial with de-ionized water for minimum 24 hours before they were used in the test.

The CEC for the 7 µm PA fiber is much higher than for the 30 µm PA fiber (Chen et al., 2010), thus used for the surfactants that are always positively charged (Arquad 2C-75) and where the pKa of the substance is much higher than at the tested pH (dodecylamine). DDAB (QAC) were tested with the 30 µm due to a misunderstanding, but it is still possible to measure it based on hydrophobicity although with less sensitivity. The 30 µm fiber were tested with the remaining substances and have a higher affinity of the neutral species than for the ionized (cationic) species. Still, the calibrated fiber-water isotherm at a certain pH reflects the freely dissolved concentration at that pH. Below the pKa of the substance, which is the case for these substances, the 30 µm fiber extracts relatively much of the neutral species from the solution and equilibrates with this low concentration, but this is instantly

replenished by the speciation constant of the compound at the solution pH and still represent the total of freely dissolved cationic/neutral species at a given pH.

Test procedures

Approximately 24 hours after *Daphnia* immobility test start, four SPME fibers were added with tweezers into two of the four replicates for each test concentration, including the control, and left to equilibrate. After 24 hours, the SPME fibers were removed with tweezers, dried and placed in HPLC vials. Subsequently, they were cut in three pieces and 1 ml of a mobile phase (table 1) was added to each vial. Furthermore, 0.75 ml of the middle test concentration was transferred with a pipette to vials already containing 0.75 ml of leaching solution. The vials were closed and analysed with Liquid Chromatography/Mass Spectrometry (LC/MS).

Table 1. Mobile phases used for LC/MS and thickness of fibers used for each tested surfactant.

Mobile phase	Tested substances	Thickness of fiber (µm)
90:10 Methanol + 2% formic acid: H_2O + 2% formic acid	Dodecylamine	7
50:50 Methanol:H_2O + 0.65 ml TFA + 0.75 ml NH_3 + 1.15 ml CH_3COOH	Didodecyldimethylammonium bromide	30
	Arquad 2C-75	7
50:50 Methanol:2-propanol + 2 ml TFA + 2 ml NH_3 + 2.5 ml CH_3COOH	Dodecylamine +2EO	30
	Hexadecylamine +2EO	30
	Octadecylamine +2EO	30
	Ethomeen C/12	30

Preparations of solutions and calibration curves of the test substance

Calibration curve

A leaching solution was prepared with approximately 100 grams of $MgCl_2 \cdot 6H_2O$ weight into a 1 liter Erlenmeyer flask and then added 500 ml of methanol and 2-propanol, respectively. The content was shaken until the salt was dissolved completely. A stock solution of the test substance was prepared with leaching solution as dilution media. Test solutions for the calibration curve were prepared by diluting the stock solution in leaching solution to achieve eight concentrations in a range from 0 to maximum 1500 µg/L. Control containing only leaching solution was also included in the calibration curve (appendix E).

SPME calibration curve

A stock solution was prepared by loading an accurate amount of the test substance, weighed out on an analytical balance and then filled up with the appropriate volume with de-ionized water. Test solutions for the SPME calibration curve were prepared by diluting the stock solution in regular DSW and modified DSW (45 % of the salts added, see appendix C) to achieve eight concentrations ranging from 0 to maximum 1500 µg/L depending on the surfactant (appendix E). Two SPME fibers were added with tweezers into each beaker for each test concentration, including the control, and left to equilibrate. The fibers were removed after 24 hours using the same procedure as for the *Daphnia* immobility (toxicity) test. Furthermore, 0.75 ml of the test solutions in the test vessels was transferred with a pipette to vials already containing 0.75 ml of leaching solution. Subsequently, the vials were closed and analyzed with Liquid Chromatography/Mass Spectrometry (LC/MS).

2.2.5 Statistical and mathematical calculations

The software ToxCalc v5.0.23 was used to calculate EC50, NOEC and LOEC. The method Trimmed Spearman-Karber gave EC50 and the method Williams' test gave NOEC and LOEC. The raw data from the SPME analysis was treated in Microsoft Excel. TUs for the single substances and the mixture and the predicted EC50 for the mixture are calculated with the equations given in appendix A.

2.3 Problems encountered during the experiments

A few problems were encountered during the experiments. One was floating *daphnid's* due to a different surface tension in the test vessel compared to the culture medium. An internal test, following the OECD 202, was performed to see whether the temperature, test media or if the beakers were contaminated or not (methanol wash) could have an influence. Four different test media were used: M4, handmade DSW, tank DSW and HD water and the test was performed in three different rooms: *daphnia* test room, *daphnia* culture room and *lumbriculous* room, with different room temperatures. No significant results were obtained. Next hypothesis was if the test media was aerated too much because the *daphnid's* had air bubbles underneath the shell. Floating is not a huge problem in an acute toxicity test since the animals are not fed during the test and therefore, they do not have to swim around and eat algae to survive. In addition, the surfactant concentrates at interfacial regions and the surface where the floating *daphnid's* are and the acute toxicity is exerted anyway. However, the floating seemed to be caused by the use of plastic pipettes as they most likely released something into the water and thereby changed the surface tension. At higher test concentrations the number of floating *daphnid's* were less, probably because the surfactant lowered the surface tension. Problem was solved by using glass pipettes. Another problem was that the *daphnid's* cultured in M4 had a lower survival rate and was much smaller and more pale compared to the ones cultured in HD water. This resulted in high mortality in the DSW control during the tests and tests had to be repeated.

Problem was also encountered with the SPME fibers. Normally, 1-2 % of the chemical in the test vessel sorb to the fiber and therefore do not influence the toxicity to the test animals. The sorption of Ethomeen O/12 was around 30% and for C16+2EO it was around 10%. Toxicity was thus altered and the test was repeated with two additional beakers for each tested concentration (without test animals) where the SPME fibers were placed. See also discussion "problems with determination of truly dissolved concentration".

3. Results & discussion

3.1 Nominal and measured concentration

3.1.1 Relationship between nominal and measured concentration

The ratio between nominal and measured concentration is presented in appendix F and is nearly constant (linear) over the entire concentration range for all the substances where it was possible to measure the freely dissolved concentration. Hence low residual variability and the model fits the data very well. The lowest R^2 value (0.884) has C16+2EO as a single substance in Ethomeen C/12 (measured with the LC/MS) in the test media DSW+HA. A linear relationship indicates that the calibration curve (sorption isotherm) for the SPME fibers are effective over the concentration range, hence the sorption of the surfactant to the fibers is directly proportional to the freely dissolved concentration in the test vessels. A linear relationship also concludes that the concentration range is below the CMC for the substances.

Problems with determination of truly dissolved concentration

Nevertheless, the truly dissolved concentration measured with SPME fibers couldn't be determined for dodecylamine (C12) and octadecylamine +2EO (C18+2EO) in this study. Other cationic surfactants; Lilaflot D817M, hexadecylamine (C16), Armeen T, Ethomeen HT and Ethomeen O/12, were aimed to be tested but due to low or no sorption to the SPME fibers in the calibration curve they were not tested for their nominal toxicity either, although a range finding were performed on all of them. These substances are more hydrophobic and less water-soluble than other cationic surfactants tested in this study. Water solubility might be an explanation to why some substances have difficulties with SPME, although not confirmed in this study. However, Lilaflot D817M should be able to measure with SPME but the tested batch seemed to be old.

The sorption of truly dissolved dodecylamine to the SPME fibers in the calibration curve at an aqueous concentration up to 25 µg/L is not significant, and at an aqueous concentration of 75 µg/L it is slightly higher but still low. In the *Daphnia* immobility test, the sorption of truly dissolved dodecylamine to the SPME fibers at the lowest nominal concentrations (0.03-0.06 mg/L in DSW and 0.1 mg/L in HD) are also not significant and it is first when the nominal concentration is 0.12 mg/L in DSW and 0.2 mg/L in HD that the aqueous concentration corresponds to about 25 µg/L and thus possible to sorb onto the fiber. Therefore, the true toxicity (EC50) couldn't be calculated since the true effect range of dodecylamine is within this low concentration range. Low sorption in this study may be due to; 1) high sorption to the test vessels and/or *daphnid´s* instead of the SPME fibers, 2) different type of fibers compared to the ones used by Utrecht university, i.e. another type of activation might be necessary, 3) the test medium contains Ca^{2+} and other divalent cations which strongly competes with the ion-exchange affinity of the cationic species to the fiber, 4) the desorption volume, and 5) not applying a column in the LC/MS that separates the compound from the "noise" eluting from the fibers. However, the Utrecht University in the Netherlands have determined the aqueous detection limit for the application of SPME on dodecylamine to be 1.0 µg/L (Chen et al., 2012). Further tests with dodecylamine and SPME is necessary to make the working range of the SPME to cover the effect range of the substance, i.e. optimize the test conditions and

the analytics, so it is possible to fully evaluate the mitigating factor. The 30 µm fiber may be used instead, as it extracts the small neutral fraction but it is less dependent on the electrolytes in the solution, although dependent on the pH. One alternative to minimize loss of substance to other surfaces is to precondition the glassware with the test substance (Rufli et al., 1998).

Determination of a SPME calibration isotherm for octadecylamine +2EO (C18+2EO) resulted in a non-linear relationship. Above an aqueous concentration of approximately 65 µg/L for this substance, the SPME fiber is saturated and the fiber concentration analyzed with LC/MS reached a maximum value at approximately 30-40 µg/L. As a consequence, there will be an underestimation of the freely dissolved concentration of C18+2EO at higher aqueous concentrations. Hence, the measured concentrations and the true EC50 is going to be less reliable as the uncertainty around the truly dissolved concentration of C18+2EO is increasing with increasing aqueous concentrations.

3.1.2 Difference in nominal and measured concentration

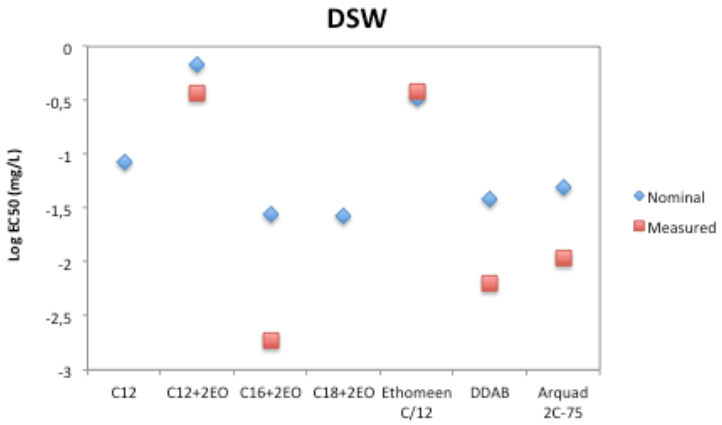

Figure 7. Nominal and measured concentration (EC50 in mg/L) in DSW for all tested surfactants. Measured EC50 is missing for dodecylamine (C12) and octadecylamine (C18+2EO) due to problems with the SPME (see section 3.1.1.).

The nominal and measured EC50 (mg/L) in DSW of all tested surfactants are presented in figure 7. A comparison between nominal and measured concentration in DSW, where no suspended matter or humic acid is present, clearly shows the "surface-acting" behavior of these substances. This difference is mostly due to their strong tendency to adsorb to pipettes, glassware, *Daphnia* and other surfaces during preparation of test concentrations and running of the test during 48 hours.

Table 2. Factor difference between nominal and measured EC50 in DSW for all tested surfactants.

Substance	Factor difference
Dodecylamine (C12)	-
Dodecylamine +2EO (C12+2EO)	1.856
Hexadecylamine +2EO (C16+2EO)	14.842
Octadecylamine +2EO (C18+2EO)	-
Ethomeen C/12	1.183
Didodecyldimethylammonium bromide (DDAB)	6.206
Arquad 2C-75	4.556

Furthermore, the difference is also distinctive between substances. The difference in nominal and measured concentration is smallest for Ethomeen C/12 and C12+2EO, whereas for C16+2EO, DDAB and Arquad 2C-75 the difference is larger (table 2). For Ethomeen C/12 the measured concentration is even higher than the nominal. One hypothesis regarding this difference in sorption is related to their hydrophobicity, i.e. increasing sorption with an increase in hydrophobicity of the molecule. An increase of carbon atoms in the alkyl chain length is related to an increased hydrophobicity, hence increasing sorption. It has for example been reported by Duman and Ayranci (2010) that tested several cationic surfactants and found that hydrophobic interactions appeared to determine the adsorption to activated carbon cloth (ACC), where an increase in carbon chain length were reflected in an increased sorption to ACC.

The hydrophobicity in this study is based on log K_{ow} (partitioning between octanol/water) modelled with U.S. EPI Suite (2011). An increased value reflects a higher hydrophobicity, hence a higher migration (or sorption) to surfaces or interfaces. The values for C12+2EO, C16+2EO and C18+2EO are 3.9, 5.86 and 6.85, respectively. The higher log K_{ow} of C16+2EO explains the higher sorption compared to C12+2EO in this study. Assuming the hypothesis is correct, the higher log K_{ow} of C18+2EO and the correlation should thus result in an even higher sorption compared to C16+2EO. Unfortunately, the measured concentration is missing for C18+2EO and the hypothesis cannot be confirmed in this study for these surfactants. The modelled log K_{ow} for DDAB is 6.62 and that surfactant also adsorb strongly to surfaces.

Ethomeen C/12 is a mixture of different carbon chain lengths, but mainly consists of C12+2EO (≥50%), and has a log P_{ow} value of 0.7 (AkzoNobel, 2011). It is a substance that show a weaker tendency to adsorb to surfaces in the same way as C12+2EO, compared to Arquad 2C-75 which has a log P_{ow} of 4.8. More data on nominal and measured toxicity in standard water for a larger set of cationic surfactants are necessary to be able to make any conclusions regarding hydrophobicity in this case.

3.1.3 Toxicity comparison between substances

The toxicity (EC50 in mg/L) of the seven tested surfactants in four different test media are presented in figure 8 based on nominal concentrations and in figure 9 for measured concentrations. The nominal concentrations required to immobilise 50% of the *D. magna* population after 48h of exposure (EC50) ranged from 0.026 to 1.61 mg/L whereas the required SPME derived aqueous (measured) concentrations (EC50-48h) ranged from 0.0019 to 0.87 mg/L.

Nominal concentration

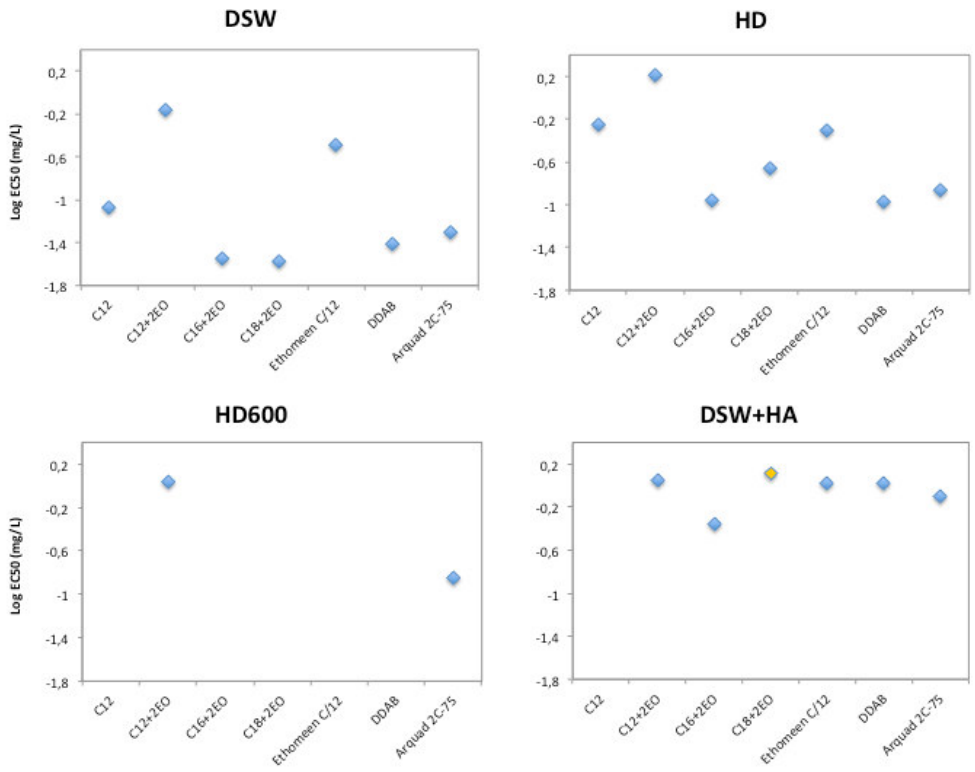

Figure 8. Nominal log EC50 (mg/L) for all tested substances in four different test media. The EC50 for C18+2EO in DSW+HA (orange) is an estimated value based on 60% mobile *daphnid's* at 1.25 mg/L. All surfactants are tested in in DSW and HD, some of them in HD600 and DSW+HA (see "test media").

Regarding nominal concentrations, the most toxic substance in DSW is C18+2EO (EC50=0.0264 mg/L), whereas the least toxic substance is C12+2EO (EC50=0.681 mg/L). In the test media HD, the most toxic substance is DDAB (EC50=0.107 mg/L) and the least toxic is once again C12+2EO (EC50=1.612 mg/L). Only two and five out of total seven surfactants were tested in HD600 and DSW+HA, respectively (explanation see discussion "test media"). In DSW+HA, the most toxic was C16+2EO (EC50=0.433 mg/L) and the least toxic was C12+2EO (EC50=1.131 mg/L), based on definitive results. If the estimated EC50 for C18+2EO (1.3 mg/L) is taken into account, it is thus regarded as the least toxic surfactant.

The nominal EC50 to *D. magna* in DSW is below 1 mg/L for all seven surfactants, in contrast to the test media HD and DSW+HA where the highest EC50 value is around 1.6 mg/L (in HD). Based on 95% confidence interval, the toxicity is not statistically different for C16+2EO, C18+2EO and DDAB in DSW and C16+2EO, DDAB and Arquad 2C-75 in HD. C12 and Ethomeen C/12 are also not statistically different in HD. In DSW+HA, the toxicity of C12+2EO, Ethomeen C/12 and DDAB is not statistically different whereas Arquad 2C-75 is not statistically different from Ethomeen C/12 and DDAB.

In conclusion, the least toxic substance regarding nominal concentration is C12+2EO, regardless of test media. Whereas the most toxic substance varies depending on test media, although C16+2EO

can be regarded as one the most toxic substance since the toxicity is not statistically different from C18+2EO in DSW and DDAB in HD and still the most toxic substance in DSW+HA.

Measured concentration

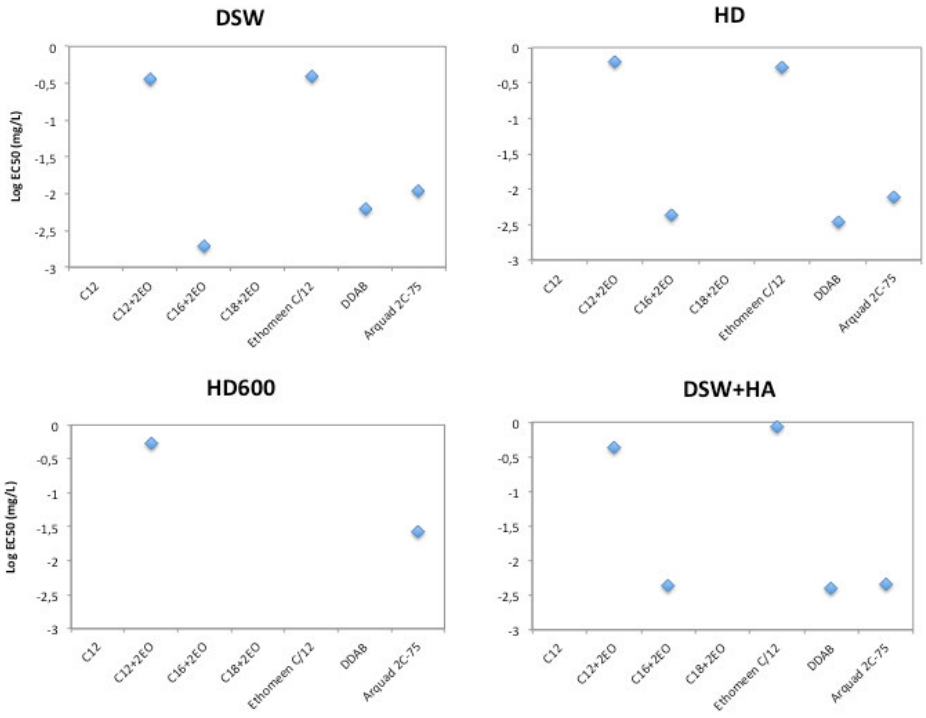

Figure 9. Measured log EC50 (mg/L) for five tested substances in four different test media. Measured concentrations are missing for C12 and C18+2EO due to problems with the SPME. All surfactants are tested in DSW and HD, some of them in HD600 and DSW+HA (see "test media").

Measured concentrations are missing for a few substances due to problems with the SPME (see "problems with determination of truly dissolved concentration"). Regarding the SPME derived aqueous (measured) concentrations (fig. 9), the most toxic substance in DSW is C16+2EO (EC50=0.0019mg/L) whereas the least toxic substance is Ethomeen C/12 (EC50=0.389 mg/L). In HD, the most toxic substance is DDAB (EC50=0.0034 mg/L) and the least toxic is C12+2EO (EC50=0.6327 mg/L). In DSW+HA, the most toxic is DDAB (EC50=0.0039 mg/L) and the least toxic is Ethomeen C/12 (EC50=0.874 mg/L). Only two surfactants were tested in HD600 and are thus the most and least toxic substances.

EC50 based on measured concentrations are below 1 mg/L for all tested surfactants that was possible to test with the SPME technique. However, the substances that had problems with the SPME had nominal EC50 values below 1 mg/L and thus regarded as toxic. Based on 95% confidence interval, the measured EC50 are not statistically different for Ethomeen C/12 and C12+2EO in DSW and HD; DDAB and Arquad 2C-75 in DSW; C16+2EO, DDAB and Arquad 2C-75 in DSW+HA and DDAB and C16+2EO in HD. Whereas the toxicity of all the other substances in the different test media are statistically different.

In conclusion, the most toxic substance to *D. magna* in this study regarding measured concentrations is C16+2EO, since it is not statistically different from DDAB in HD and DSW+HA. However, DDAB and

Arquad 2C-75 is also very toxic and the toxicity for Arquad 2C-75 seems to increase as the amount of humic acid increases since the EC50 values are statistically different in HD and DSW+HA. The least toxic substance is Ethomeen C/12, but C12+2EO is not very toxic either as it is not statistically different from Ethomeen C/12 in DSW and HD. From the literature search, few or no toxicity studies have been performed with these surfactants except for the tests that have been done for registration according to REACH. Furthermore, a majority of the available reported values are based on nominal concentrations.

The primary fatty amine, dodecylamine (C12), have a reported acute nominal EC50 to *D. magna* of 0.146 mg/L in freshwater (ref. 13 in appendix B), which is a factor 3.87 lower than the nominal value in HD in this study. The freshwater that was used had a concentration of 17.6 mg/L of suspended matter and a TOC of 5.9 mg C/L, i.e. a higher amount of NOM compared to the HD water used in this study. A higher mitigation of the reported value would thus be expected, i.e. a higher EC50, because when based on nominal concentrations, the variation in toxicity between substances in freshwater will differ due to e.g. their tendency to sorb to the available amount of NOM. However, pH and conductivity differ between these two test media and might explain the dissimilar result for these two studies.

Reported toxicity values for Arquad 2C-75 are higher than the data in this study, both for nominal and measured concentrations. The reported toxicity in HD water based on chronic nominal concentrations to crustacean is 1.15 mg/L (EC10) (ref. 32 in appendix B) whereas the toxicity based on acute measured concentrations to algae are varying from 0.148 to 0.386 mg/L (EC50-72h) (ref. 34 in appendix B). The reported chronic EC10 is a factor 8.5 higher than the acute EC50 in this study and the lowest reported EC50 to algae is a factor 19.2 higher than the measured EC50 to *D. magna* in this study (0.0077 mg/L) and the latter could be attributed to different sensitivity between species. Previous studies have shown that sensitivity between different species of invertebrates towards the same surfactant can differ up to 2300 times (Lewis and Suprenant, 1983) thus enhancing the different sensitivity between the two different species algae and *D. magna* towards Arquad 2C-75.

For DDAB, a nominal LC50 (24h) of 1.2 mg/L (U.S. EPA, 2012 cas: 3282-73-3) to crustacean in freshwater is also higher (a factor 11.2) than the nominal EC50 (48h) in HD in this study. The exposure duration differ and the characteristics of the freshwater (amount of NOM, water hardness, etcetera) is unknown and may explain the higher reported toxicity value. DDAB is the pure compound and the toxicity is similar to Arquad 2C-75 (mixture) since the mixture is mainly composed of this substance. The QAC are always positively charged and thus adsorbs rapidly and strongly to negatively charged substrates (Ying, 2006) and their high toxicity may be explained by a high electrostatical interaction with the membranes of aquatic organisms. Their relatively high hydrophobicity also contributes to the toxicity as a baseline toxicity (Könemann, 1981).

The primary fatty amine ethoxylates are less toxic than the QAC, except C16+2EO, and this could be attributed to a lower hydrophobicity. The reported data for Ethomeen C/12 are also higher than the toxicity data in this study, although not as much as for the QAC. Reported acute nominal EC50 to *D. magna* in standard water varies from 0.84 to 1.4 mg/L (AkzoNobel, 2012:b), a factor 2.6 to 4.3 higher than this study. The nominal EC50 in HD water in this study is more consistent with the reported chronic EC50 to *D. magna* of 0.405 mg/L (AkzoNobel, 2012:b). In comparison with algae, the reported acute nominal EC50 of 0.107 mg/L in HD (AkzoNobel, 2012:b) is lower than in this study, in contrast

to the QAC where it was the other way around. Once again, sensitivity towards different surfactants differ between species. The algae might be more sensitive towards PFAEO than QAC, which may be due to their lower hydrophobicity.

The evaluation of cationic surfactants according to REACH are performed on mixtures, which either consists of mainly short or longer alkyl chains. Thus, the toxicity of C12+2EO can partly be explained by the toxicity of Ethomeen C/12 and the toxicity is similar regarding measured concentrations. The higher toxicity of C16+2EO can be explained by another mixture, i.e. Ethomeen 18/16 (oleyl), which mainly consists of chain lengths of 16 and 18 carbon atoms.

Reported chronic nominal EC50 on *D. magna* for Ethomeen C/12 and Ethomeen 18/16 (oleyl) are 0.405 and 0.0463 mg/L, respectively (AkzoNobel, 2012:b), that is a higher toxicity with higher alkyl chain lengths and thus explaining the difference in toxicity between C12+2EO and C16+2EO. However, it is based on nominal concentrations. Toxicity based on measured concentrations are few, one reported acute EC50 to *D. magna* in standard water of Ethomeen 18/16 (oleyl) is 0.043 mg/L (AkzoNobel, 2012:b), which is a factor 22.6 higher than the measured EC50 for C16+2EO and a factor 8.5 lower than the measured EC50 for C12+2EO in this study. Ethomeen 18/16 (oleyl) is a mixture and a comparison with pure substances is not always straight-forward, which was seen for the measured concentrations of the single substances in Ethomeen C/12 in the test media DSW+HA (see "test media – Ethomeen C/12"). The higher reported measured EC50 of Ethomeen 18/16 (oleyl) could be due to a lower solubility of the longer alkyl chain lenghts, especially since there was problems with determination of measured concentrations of C18+2EO in this study. Thus, the true toxicity is more exerted by the shorter alkyl chain lengths, which is less toxic.

The toxicity of C16+2EO is similar to Arquad 2C-75 and DDAB regarding measured concentrations when the amount of NOM is increasing. However, the nominal concentrations indicate a higher toxicity of C16+2EO. The lower nominal EC50 in DSW+HA for C16+2EO compared to the QAC is most probably exerted by its hydrophobicity. The QAC are mitigated to a higher degree due to their stronger tendency to adsorb to the humic acid via ion-exchange compared to the PFAEO. Thus, the toxicity exerted by PFAEO is more based on hydrophobic interactions, whereas the toxicity of QAC is based on electrostatic interaction. At last, it can be concluded that it is difficult to compare nominal and measured concentrations since it obviously differs between substances due to sorption to surfaces or interfaces. Further tests with SPME and more surfactants are necessary to actually be able to compare them.

3.2 Test media

Two to four different test media are used in the tests and all tests are performed in DSW and HD water since these two different media were supposed to be comparable when measuring the truly dissolved concentration with SPME fibers. Due to problems with sorption and comparison of measured EC50 values, the conductivity was changed in the HD water by adding salts (abbreviated HD600) to be more similar to the conductivity in DSW. As a consequence, toxicity was altered and further tests were done without this test medium. Instead, a second calibration curve for SPME in DSW with a less amount of salts (abbreviated DSW modified) was used to get a better and more equal comparison between DSW and HD. Many previous toxicity studies have been made in standard water with purified humic acid (Chen et al., 2010; van Wijk et al., 2009; Ishiguro et al., 2007; Koopal et al., 2004), thus a fourth test medium (DSW with 20 mg/L commercial humic acid) was used to get

another reference point. Commercial HA is used as a surrogate for natural aquatic humic substances and accounts for almost 100% of the DOC (dissolved organic carbon) in those preparations, whereas the HA in natural waters only account for approximately 50 to 75 % of the total DOC. The mitigation factor are thus based on the detoxification in HD water, i.e. the real environment. The correspondent effect of DOM (dissolved organic matter) in natural water will otherwise be overestimated (Haitzer et al., 1998). As van Wijk et al. (2009) wrote "A good understanding of sorption in relation to toxicity is needed to understand the relevant mitigating effects for chemicals" .

3.2.1 Factor difference between different test media

The nominal EC50 of a cationic surfactant varies widely depending on the test media. Due to sorption to NOM naturally present in river water (HD) and added humic acid to DSW, the freely available concentration will be the same in all test media since that is the bioavailable fraction. Therefore, the measured EC50 will vary with a factor 2 maximum from measured EC50 in DSW for one substance. A factor 2 is chosen as an acceptable difference when measuring the freely dissolved concentration with SPME, based on experiments from previous investigations with SPME. The nominal and measured EC50 of one surfactant is represented in each figure below, where all the EC50 are related to the measured EC50 in DSW (which is set to 1). All EC50 values are presented in table 3 at the end of section 3.2.1.

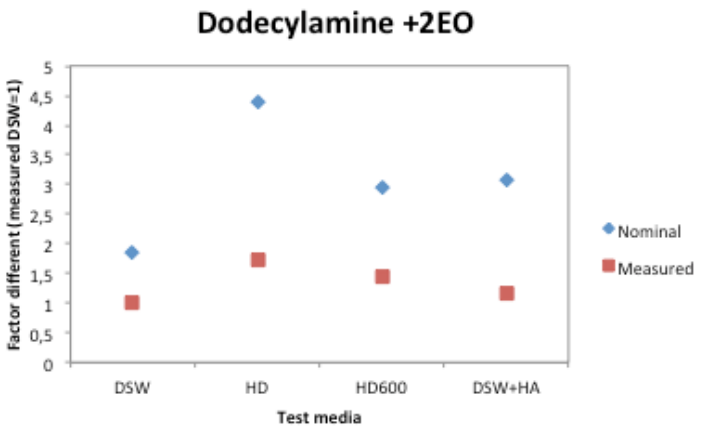

Figure 10. Nominal and measured EC50 for dodecylamine +2EO (C12+2EO), expressed as a factor different from measured EC50 in DSW.

For C12+2EO (fig. 10), the nominal EC50 varies with a factor of maximum 2.366 (DSW compared to HD) and the measured EC50 between the different test media varies with a factor of maximum 1.724 (DSW compared to HD). This is the only substance where the nominal EC50 is higher in HD than in DSW+HA, however the mitigation factor is still higher for DSW+HA (2.642) than for HD (2.548). Thus, the mitigation, i.e. relating the laboratory conditions to the real environment, for this substance is a factor of 2.5.

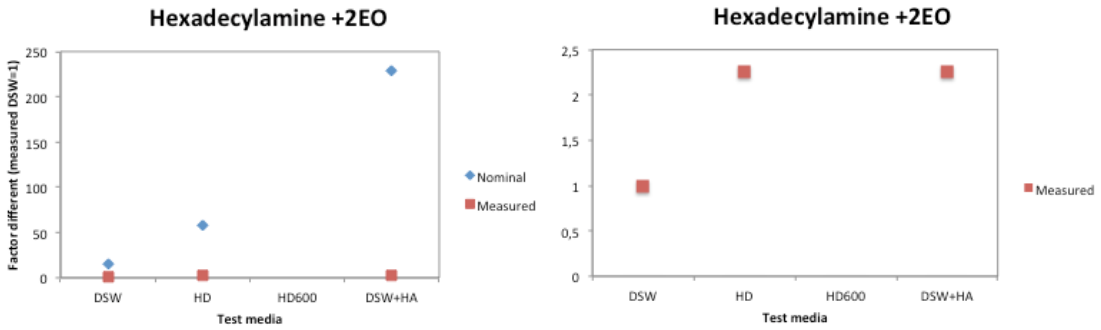

Figure 11. Nominal and measured EC50 for hexadecylamine +2EO (only measured EC50 in the graph to the right), expressed as a factor different from measured EC50 in DSW. The substance is not tested in HD600.

For C16+2EO, the variation in nominal EC50 between different test media is higher with a maximum factor difference of 15.369 between DSW+HA and DSW (fig. 11). The difference between the measured EC50 is also higher and varies with a factor of 2.263 for both HD and DSW+HA, compared to measured DSW (see graph to the right in fig. 11). This value is slightly higher than the accepted difference of a factor 2. Still, the effect of C16+2EO is mitigated with a factor of 100.791 in DSW+HA and about 25.884 in HD, compared to a factor of about 2.6 for C12+2EO. The higher measured EC50 in the test media HD and DSW+HA compared to DSW might be due to the stronger sorptive behaviour of C16+2EO to natural organic matter, because of the long hydrophobic alkyl chain. Thus, the truly dissolved concentration that can adsorb to the SPME fibers and to the organisms is lower, and subsequent toxicity is lower.

The detoxification increases as the amount of humic acid increases, as well as carbon chain length. García et al. (2006) found that the sorption to activated sludge largely increased as the carbon chain length of QAC increased from C_{12} to C_{16}, van Wijk et al. (2009) reported a decrease in toxicity with an increasing concentration of humic acid and Versteeg and Shorther (1992) reported that HA had a concentration-dependent mitigating effect that was more prominent on the longer alkyl chain lengths. Hence explaining the difference in mitigation factors between C12+2EO and C16+2EO. The mitigation factor for C16+2EO is 25.9, a factor 10 higher than for C12+2EO and this is mostly due to the longer alkyl chain.

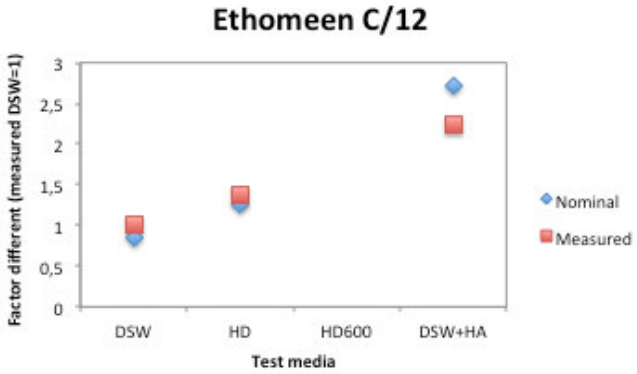

Figure 12. Nominal and measured EC50 for ethomeen C/12 as a mixture, expressed as a factor difference from measured EC50 in DSW.

The variation in nominal EC50 between different test media of Ethomeen C/12 is also lower (fig. 12), as it is for C12+2EO. The maximum variation is between DSW+HA and DSW with a factor 3.209. Ethomeen C/12 as a mixture have a measured EC50 in DSW+HA that is 2.246 times higher than the measured EC50 in DSW, compared to 1.362 in HD. Thus, in DSW+HA it varies more than the acceptable factor 2. Here, the measured EC50 for the mixture is based on addition of the measured concentration of the individual mixture components (see appendix B), since the analysis with LC/MS extracts the different carbon chain lengths that Ethomeen C/12 consists of and not the mixture as a whole. The mitigation factor is also low, the highest value of 1.207 is for DSW+HA. The corresponding value for HD is 0.919.

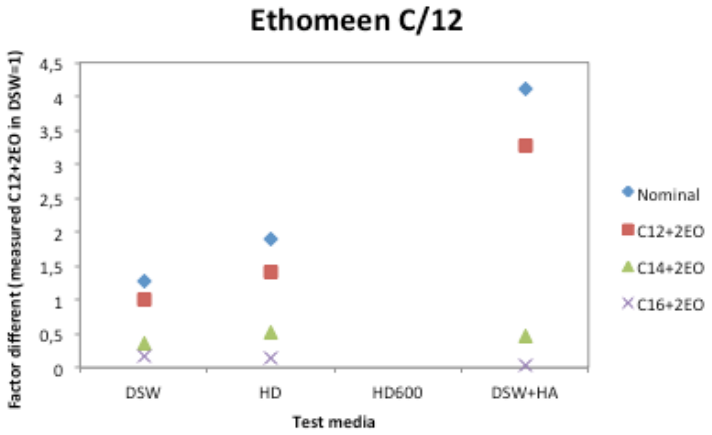

Figure 13. Nominal EC50 for Ethomeen C/12 as a mixture and measured EC50 for single substances present in the mixture, expressed as a factor difference from measured EC50 for C12+2EO in DSW.

The extracted concentration of each carbon chain length that Ethomeen C/12 consists of, recalculated to measured EC50, is presented in figure 13. The measured EC50 of the individual mixture components clearly shows that it is C12+2EO that give rise to the higher factor difference (3.264) between measured EC50 in DSW+HA and DSW. The fraction of each single substance in Ethomeen C/12 measured with LC/MS is presented in appendix B. The measured EC50 in HD and DSW+HA for C14+2EO are within a factor 2 different from the measured EC50 in DSW. Whereas for C16+2EO, the measured EC50 is within a factor 2 different in HD but a factor of 3.542 different in DSW+HA compared to DSW.

Ethomeen C/12 mainly consists of C12+2EO and thus explains why the measured EC50 for the mixture (fig. 12) is higher in DSW+HA, as it is in fig. 13. However, the reason to why the EC50 is higher is unclear. The measured EC50 for C12+2EO tested individually is within a factor 2 different in all test media (see fig. 10), whereas it is higher than a factor two for C16+2EO when it is tested individually in HD and DSW+HA (see fig. 11). Their presence in a mixture leads to an unexpected behavior. There might be some sort of interaction of C12+2EO or competition between the single substances in the mixture that causes this. One possible explanation is that C14+2EO and C16+2EO are stronger competitors, i.e. have a stronger adsorption affinity for the sorption sites on humic acid, fibers and the organisms. An increased adsorption affinity to clay and sediment with increasing alkyl chain length has been demonstrated by Droge and Hermens (2010) with alcohol ethoxylate homologues. This may thus confirm the indicated stronger sorption of C16+2EO to humic acid in DSW+HA since

that measured EC50 is 3.5 times lower than the measured EC50 in DSW, in contrast to the measured EC50 for C12+2EO that is 3.3 times higher in DSW+HA than in DSW. The measured concentration for Ethomeen C/12 is also based on addition of the concentrations of the single substances in the mixture, the higher truly dissolved concentration of C12+2EO in the mixture will thus result in a higher measured EC50 for the mixture in DSW+HA. If the concentration of NOM in HD were higher, a similar result might have been observed there as well. Further discussed under "single substances and mixture toxicity".

According to these data, the mitigation factor increases with increasing amount of NOM and alkyl chain length. The measured EC50 for C14+2EO is almost the same in all test media, whereas the measured EC50 for C16+2EO is decreasing from DSW to DSW+HA, indicating a stronger sorption to NOM. The mitigation factor for C16+2EO in DSW+HA is 87.967, compared to 9.695 for C14+2EO and 1.261 for C12+2EO. However, the mitigation is adjusted according to the entire mixture in the river water and is set to 0.9.

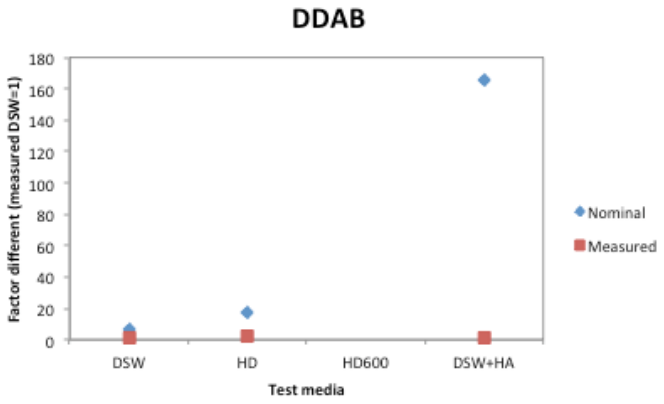

Figure 14. Nominal and measured EC50 for didodecyldimethylammonium bromide (DDAB), expressed as a factor different from measured EC50 in DSW. DDAB is not tested in HD600.

The nominal EC50 of DDAB (fig. 14) in DSW+HA is 26.719 higher than in DSW. Corresponding value for HD is 2.724. The measured EC50 is within a factor 2 different from DSW for both HD (1.853) and DSW+HA (1.615). The mitigation factor is 31.324 for HD and 267.872 for DSW+HA, thus the mitigation for this substance is 31.3.

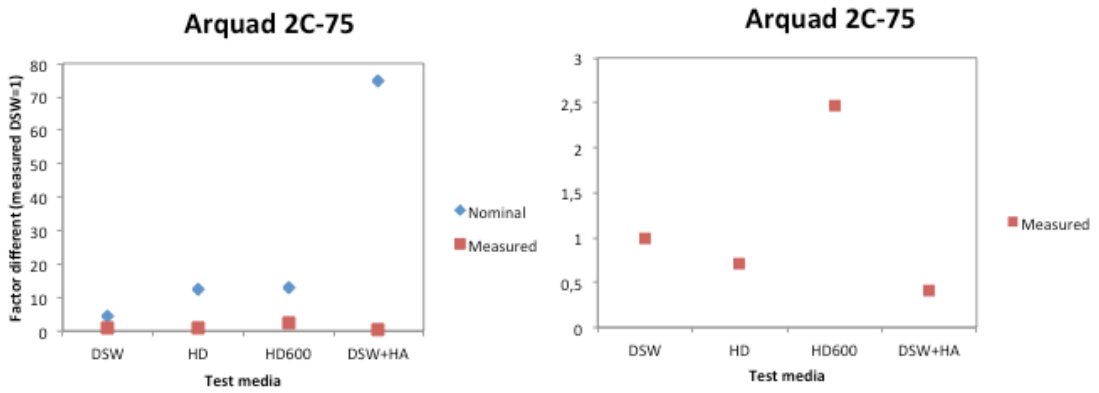

Figure 15. Nominal and measured EC50 for Arquad 2C-75, expressed as a factor different from measured EC50 in DSW.

For Arquad 2C-75, the nominal EC50 is highest for DSW+HA with a factor of 16.370 higher than DSW (fig. 15). For HD and HD600, the corresponding difference is a factor 2.752 and 2.874, respectively. Regarding measured EC50, the acceptable factor of maximum 2 difference is exceeded in HD600 (2.472) and DSW+HA (2.400), compared to HD where the factor is only 1.403 different from DSW (see graph to the right in fig. 15). The mitigation factor for the three different test media (HD, HD600 and DSW+HA) are 17.584, 5.296 and 178.978, respectively. A mitigation factor of 17.5 can be used for this substance. Arquad 2C-75 is also a mixture of different carbon chain lengths and the measured EC50 in the four different test media are here presented by the most dominant carbon chain length detected with LC/MS. Apparently, the bioavailable fraction of a mixture may not be similar in different test media due to changed fractions of individual mixture components as a result of their different sorption affinities, which may explain why the measured EC50 in DSW+HA is a factor of 2.4 lower than in DSW.

QAC and other fatty amine derivatives have an amphiphilic structure, thus have the potential for both hydrophilic and hydrophobic interactions with NOM. The most abundant component of NOM is HA and due to their amphiphilic structure, they play a major role in controlling the bioavailability, hence toxicity, of surfactants (Koopal et al., 2005). The type of sorption was not actually determined for the tested surfactants in this study, only the differences in nominal and measured concentrations in different test media. Two different binding mechanisms of cationic surfactants to organic matter have been observed. One is through van der Waals forces (hydrophobic interaction) between the apolar carbon chain of the surfactant and the organic fraction of suspended matter and humic acid, and the other is through electrostatic interaction, i.e. ion-exchange, of the positively charged nitrogen group to the negatively charged sites of humic acid (van Wijk et al., 2009). Cationic surfactants thus binds electrostatically to humic acid, whereas nonionic surfactants don't. Cationic surfactants also binds hydrophobically to humic acid and this is demonstrated with an increase in sorption with increasing alkyl chain length (Koopal et al., 2004), as can be seen for C12+2EO and C16+2EO. This may support the stronger sorption of the QAC (DDAB and Arquad 2C-75) as they are always positively charged compared to the PFAEO. Furthermore, according to van Wijk et al. (2009) the CEC of the sorbent is more important than the organic matter content as the CEC results in an additional electrostatic sorption, although not examined in this study.

According to this study, the mitigation of the cationic surfactants toxicity by sorption to NOM is substance specific. Previous tests performed by AkzoNobel in river water have used a standard mitigation factor of 10 for all substances when determining their true toxicity, i.e. the bioavailable fraction. Consequently, both over- and underestimation of their true toxicity have been done. The difference in mitigation factors varies from 0.9 to 31.3 in this study and is related to HD water with a TSS of 2.4 mg/L, a TOC of 2.21 mg C/L and a water hardness of 5.56 °dH. For risk assessment purposes, a standard mitigation factor for all surfactants may thus have serious implications.

Table 3. Nominal and measured EC50 with 95% CI in mg/L for all tested surfactants in different test media. A missing nominal or measured EC50 due to problems with SPME or not tested in that test medium at all, is denoted with (-). The nominal EC50 for C18+2EO in DSW+HA is an estimated value based on 60% mobile daphnid's at 1.25 mg/L.

Substance	DSW (mg/L)		HD (mg/L)		HD600 (mg/L)		DSW+HA (mg/L)	
	Nom. (95% CI)	Meas. (95% CI)	Nom. (95% CI)	Meas. (95% CI)	Nom. (95% CI)	Meas. (95% CI)	Nom. (95% CI)	Meas. (95% CI)
C12	0.0849 (0.0712-0.101)	-	0.566	-	-	-	-	-
C12+2EO	0.681 (0.578-0.804)	0.367 (0.302-0.446)	1.612 (1.481-1.755)	0.633 (0.579-0.691)	1.086 (0.941-1.252)	0.526 (0.433-0.640)	1.131	0.428
C16+2EO	0.0282 (0.0253-0.0316)	0.0019 (0.0017-0.0020)	0.111 (0.0945-0.131)	0.0043 (0.0030-0.0062)	-	-	0.433 (0.372-0.505)	0.0043 (0.0035-0.0054)
C18+2EO	0.0264 (0.0210-0.333)	-	0.217 (0.146-0.323)	-	-	-	*1.3*	-
Ethomeen C/12	0.329 (0.309-0.351)	0.389 (0.358-0.424)	0.487 (0.411-0.578)	0.530 (0.420-0.669)	-	-	1.056 (0.962-1.159)	0.874 (0.716-1.067)
Ethomeen C/12 *-C12+2EO*	0.329	0.257 (0.236-0.279)	0.487	0.360 (0.289-0.450)	-	-	1.056	0.837 (0.730-0.961)
Ethomeen C/12 *-C14+2EO*	0.329	0.0906 (0.0833-0.0984)	0.487	0.131 (0.100-0.171)	-	-	1.056	0.121 (0.104-0.142)
Ethomeen C/12 *-C16+2EO*	0.329	0.0425 (0.0391-0.0462)	0.487	0.0379 (0.0299-0.0481)	-	-	1.056	0.012 (0.0102-0.0141)
DDAB	0.0391 (0.0286-0.0535)	0.0063 (0.0037-0.0106)	0.107 (0.0825-0.137)	0.0034 (0.0020-0.0059)	-	-	1.045 (0.906-1.204)	0.0039 (0.0030-0.0050)
Arquad 2C-75	0.0492 (0.0442-0.0548)	0.0108 (0.0091-0.0128)	0.135 (0.124-0.148)	0.0077 (0.0064-0.0093)	0.141	0.0267	0.805 (0.708-0.917)	0.0045 (0.0034-0.0058)

3.2.2 Changed conductivity in river water (HD to HD600)

The conductivity in river water (HD) was changed after the first test with Arquad 2C-75, because of a higher measured EC50 value for HD than for DSW (not presented in this report). With an increased salt concentration (Na^{2+}, Ca^{2+} etc.) in the water, sorption to the fibers was expected to be lower due to competition between positively charged salt ions and surfactants (Chen et al., 2010) and thus give a better measurement on the freely dissolved concentration.

Nevertheless, the sorption to the fibers increased by adding salts to HD water, indicating a higher amount of freely dissolved surfactants in the water (see appendix C), hence increasing toxicity. For C12+2EO the nominal EC50 was increased with a factor of 1.485 by adding salts to HD water. The difference in nominal EC50 between HD and HD600 is statistically significant with EC50 values of 1.612 (1.481-1.754) and 1.086 mg/L (0.941-1.252), respectively. However, the difference in measured EC50 is not statistically significant, with EC50 values of 0.633 (0.579-0.691) and 0.526 mg/L (0.433-0.640), respectively. Thus based on measured concentrations, the alkyl chain length and not the cations seems to determine the toxicity of C12+2EO. In contrast, the difference in nominal EC50 between HD and HD600 is not statistically significant for Arquad 2C-75 with values of 0.141 and 0.135 mg/L (0.124-0.148), respectively. Whereas the difference in measured EC50 is, with values of 0.0077 (0.0064-0.0093) and 0.0267 mg/L, respectively. Here, the cation activity determines the toxicity of Arquad 2C-75 and since there are more competitive inorganic cations available in HD600, the toxicity of Arquad 2C-75 is thus lower (i.e. a higher EC50). A study by Hisano and Oya (2010) with a mixture of an anionic and a cationic surfactant at different fractions resulted in a decreased toxicity as the water hardness increased from 25 to 625 ppm. The mixture was assumed to be affected by the existence of metal ions with the result of a decrease in toxicity. The decreasing toxicity was not seen when the anionic surfactant was tested individually. This enhance the result of the decreased toxicity of Arquad 2C-75 in HD600.

Instead, the salts seems to have an effect on the sorption to particles, i.e. negatively charged clay, present in HD as they might be stronger competitors than the cationic surfactants. The bioavailability and thereby the sorption of C12+2EO and Arquad 2C-75 to the fibers are thus increased, as opposed to expectations. The factor difference in nominal and measured toxicity for C12+2EO is 2.548 in HD and 2.064 in HD600, indicating a stronger sorption of C12+2EO to particles in HD and to the fibers in HD600. For Arquad 2C-75, the corresponding values are 17.584 for HD and 5.296 for HD600. The values for Arquad 2C-75 are higher as it is always positively charged and more suspectible to competition of inorganic ions.

3.3 Degree of ethoxylation

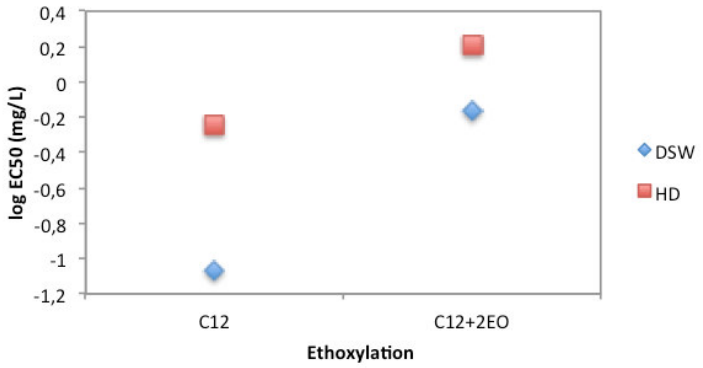

Figure 16. Nominal EC50 for dodecylamine and dodecylamine +2EO in DSW and HD.

Dodecylamine (C12) with no ethoxylates is compared with dodecylamine +2EO (C12+2EO) that has two ethoxylated groups attached to the amine (fig. 16). Based on nominal concentration, toxicity is decreasing when two ethoxylated groups are attached to the amine. The toxicity between the two substances is statistically different in both test media. C12 has an EC50 of 0.0849 mg/L (0.0712-

0.1011) in DSW, whereas C12+2EO has an EC50 of 0.6813 mg/L (0.5776-0.8037). For HD, the corresponding toxicity values are 0.5657 mg/L and 1.6121 mg/L (1.4813-1.7544), respectively. There is also a difference in sorption to suspended matter and humic acid between the substances. The difference in EC50 between DSW and HD is higher for C12 (factor 6.663) than for C12+2EO (factor 2.366).

The ethoxylation mainly governs the hydrophilic character of the fatty amine (Holmberg et al., 2003), thus makes C12+2EO more water soluble. A decreasing water solubility is reflected in an increasing biophilic character and as a consequence the molecule is more likely to adsorb on lipid membranes and disrupt different membrane functions (Singh et al., 2002) and this explains the higher toxicity of the less water soluble C12. Decreasing water solubility may also increase the sorption affinity for NOM present in HD and then explain the higher difference in sorption for C12. The pKa is also lower for C12+2EO (8.6 compared to 10.63 for C12), which may affect how much of the substance that is cationic under the actual test conditions. If the fraction cationic is lower, the sorption to negatively charged substrates in river water may decreases and the sorption may then be mainly based on hydrophobic interactions. Previous studies with alcohol ethoxylates (non-ionic surfactants) have shown a decrease in toxicity with an increase in EO units based on nominal concentrations (Hisano and Oya, 2010). Measured EC50 values are unfortunately missing for C12, hence a comparison between the freely dissolved concentrations is not possible. Further tests with SPME and primary fatty amines, as well as with ethoxylated groups attached, are necessary to determine how toxicity is altered.

3.4 Toxic response as a function of the alkyl chain length

The toxic response of *D. magna* as a function of the alkyl chain length is based on PFAEO C12+2EO, C16+2EO and C18+2EO. The nominal EC50 values are presented in figure 17 and the measured EC50 in figure 18. In general, the nominal EC50 is decreasing with increasing alkyl chain length, although deviations occur at longer alkyl chain length. Regarding measured concentrations, the results indicates an increasing toxicity with an increase in the number of carbon atoms in the alkyl chain.

Nominal concentration

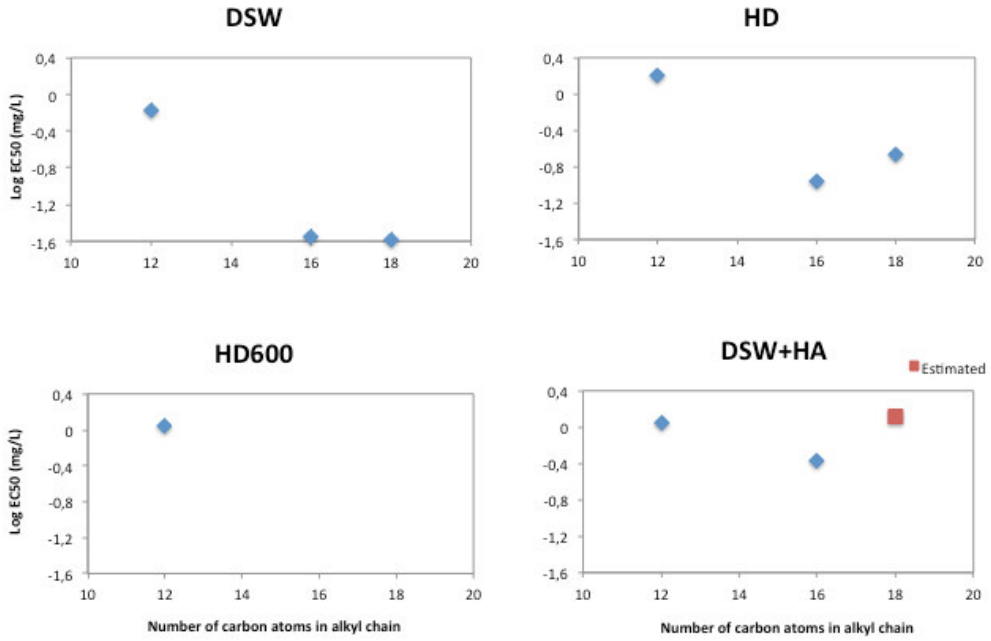

Figure 17. Nominal log EC50 (mg/L) for three cationic surfactants with different alkyl chain lengths. The EC50 in DSW+HA for octadecylamine +2EO is estimated, based on 60% mobile daphnids at 1.25 mg/L. HD600 was only used as test medium for dodecylamine +2EO.

The toxicity is increasing with an increase in the carbon chain length from C_{12} to C_{16}, based on both nominal and measured concentrations (see fig. 17 and 18). For C16+2EO and C18+2EO, the nominal EC50 is not statistically different in DSW with values of 0.0282 (0.0253-0.0316) and 0.0264 mg/L (0.0209-0.0333), respectively. However, the toxicity is statistically different in HD and DSW+HA with a decreasing toxicity from C_{16} to C_{18} with increasing amount of NOM. The EC50 values for C16+2EO and C18+2EO in HD are 0.1113 (0.0945-0.1311) and 0.2171 mg/L (0.1457-0.3233), respectively. The EC50 value for C16+2EO in DSW+HA is 0.433 mg/L (0.372-0.505) and for C18+2EO in DSW+HA it is estimated to 1.3 mg/L, based on 60% mobile daphnid's at 1.25 mg/L, but clearly shows that the toxicity is decreasing with increasing carbon chain length and amount of NOM. The relationship between C_{12} and C_{18} is not linear, it deviates after C_{16} and the reason for this is probably due to solubility problems of C18+2EO. The relationship between C_{12} and C_{16} seems to be linear, however, a conclusion about it cannot be made due to missing data for tetradecylamine (C14) +2EO.

Furthermore, sorption increases as the amount of NOM and the alkyl chain length increases. The factor differences between DSW and HD are 2.37, 3.95 and 8.22 for C_{12}, C_{16} and C_{18}, respectively. For DSW+HA the corresponding factor differences are 1.66, 15.4 and approximately 49.2. The factor difference between DSW and DSW+HA for C_{18} is based on an estimated toxicity value. This increasing sorption with increasing alkyl chain length is also seen for the factor difference between nominal and measured EC50 for C_{12} and C_{16}, with a factor 1.86 and 14.84, respectively, different for DSW and 2.55 and 25.88, respectively for HD. HD600 is not included since only C12+2EO is tested in this.

Measured concentration

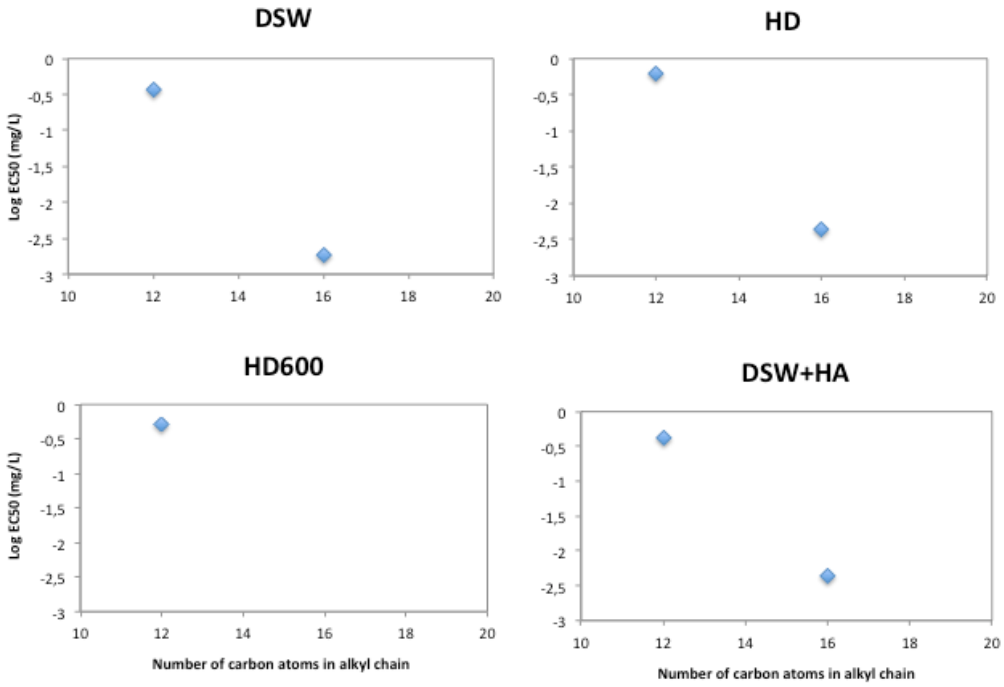

Figure 18. Measured log EC50 (mg/L) for two cationic surfactants with different alkyl chain lengths. Measured EC50 is missing for C18+2EO (see "test media") and only C12+2EO is tested in HD600.

Similarly to nominal concentrations, the measured EC50 is increasing with increasing carbon chain length, from C_{12} to C_{16} (fig. 18). Measured concentration is, unfortunately, missing for C18+2EO. The toxicity is statistically different in the three different test media between C_{12} and C_{16}. For EC50 values, see table 3, p. 26. Only C12+2EO is tested in HD600 with a result similar to HD. The linear relationship between C_{12} and C_{16} is uncertain due to missing data for C14+2EO, same argument as for nominal concentrations. In contrast to nominal concentrations, sorption is not increasing as the carbon chain length and amount of NOM increases. The factor difference between DSW and HD is 1.72 and 2.26 for C_{12} and C_{16}, respectively. The corresponding values between DSW and DSW+HA are 1.17 and 2.26, respectively. This is because the EC50 here is based on the freely dissolved concentration of C12+2EO and C16+2EO, hence the amount adsorbed to organic matter is excluded.

According to a QSAR model based on hydrophobic narcotic chemicals (general narcosis), an increased carbon chain length gives the molecule a larger hydrophobic fraction and toxicity is thus expected to increase (Könemann, 1981). A higher toxicity with an increase in the number of carbon atoms in the alkyl chain have been reported for zwitterionic surfactants on *D. magna* and *P. phosphoreum* (García et al. 2008), for cationic surfactants on *D. magna* and rainbow trout (Sandbacka et al., 2000), for nonionic surfactants on *D. magna* and fathead minnow (Wong et al., 1997) and for cationic and anionic surfactants on *B. calyciflorus* (Versteeg et al., 1997). The increasing toxicity was observed up to a chain length of 14 carbon atoms based on nominal concentrations. The trend is the same for measured concentrations as reported for anionic surfactants exposed to methanogenic microorganisms, *D. magna* and *P. promelas* (García et al., 2006:a), and for cationic surfactants on *P. subcapitata* (van Wijk et al. 2009) and *P. promelas* (Versteeg and shorter, 1992). Van Wijk et al.

(2009), Sandbacka et al. (2000) and Wong et al. (1997) reported a decreasing tendency after 14 carbon atoms, whereas Versteeg and Shorter (1992) reported an increase in toxicity up to a chain length of 16 and 18 carbon atoms for monoalkyl QAC. The alkyl chain length vs. toxicity relationship has also been reported for Ethomeen products with different carbon chain lengths. Reported nominal LC50 (96 h) to fish in standard water are 0.5-0.6 mg/L for Ethomeen C/12 and 0.2 mg/L for Ethomeen 14/12 (mainly C14+2EO) (AkzoNobel, 2012:b). The corresponding LC50 (96 h) on fish for Ethomeen 18/16 (oleyl) is 0.1 mg/L (AkzoNobel, 2012:b), but this is based on measured concentrations. Even if it is a measured concentration, it is only slightly lower than the nominal for Ethomeen 14/12.

This enhance the results of this study, which shows an increasing toxicity with increasing carbon atoms in the alkyl chain. However, the deviation occur at C_{16} in this study since C14+2EO is not tested. Van Wijk et al. (2009) also reported a decreasing toxicity with increasing humic acid concentrations, which is similar to this study when comparing the toxicity in HD and DSW+HA based on nominal concentrations. In addition, the sorption to substrates in the study by van Wijk et al. (2009) seemed to increase as the chain length increased from 10 to 18 carbon atoms, which is similar to this study. That is, the effect is mitigated to a larger degree with a longer alkyl chain. Koopal et al. (2004), Ishiguro et al. (2007) and van Wijk et al. (2009) reported that cationic surfactants binds to humic substances via both electrostatic and hydrophobic interaction. A longer aliphatic chain gives the molecule a stronger hydrophobic character (Ishiguro et al., 2007) and the stronger sorption of C16+2EO compared to C12+2EO is thus the result of their longer aliphatic tail. The same for C18+2EO compared to C16+2EO. In addition, the hydrophobicity of humic acid is increasing when long-chain surfactants adsorbs to them and thus influence further adsorption of cationic surfactants as well as other contaminants to humic acid (Koopal et al., 2004). Thus, the mitigation is increasing with increasing humic acid concentrations, as well as an increase in carbon chain length due to its higher hydrophobicity. The toxicity is also increasing with increasing carbon chain length but it seems to have a tendency to diminish after 14 carbon atoms according to previous studies, both for nominal and measured concentrations, and after 16 carbon atoms in this study based on nominal concentrations.

Furthermore, cationic surfactants are very toxic compared to anionic and non-ionic surfactants (Singh et al., 2002), and polar narcosis, i.e. polar contributions when binding to membranes (Saarikoski and Viluksela, 1982) might be necessary to take into account as the predicted baseline toxicity is generally lower than the observed for polar narcotics (Roberts and Costello, 2003:a). That is, the toxicity is probably not only governed by the length of the alkyl chain. The pKa of the substance together with the pH of the environment decides whether the substance is cationic or nonionic. The studied PFAEO have a pKa of about 8.6 (Chen et al., 2012) and tested at a pH of 8.2, thus cationic during lab conditions. However, the fraction of ionic species are supposed to be the same for C12+2EO, C16+2EO and C18+2EO and the charge (polar moieties) thus governs the toxicity exerted by the electrostatic interaction, whereas the alkyl chain length governs the toxicity exerted by hydrophobic interactions. Although the cationic part contributes to the sorption and the toxicity, a comparison of the sorption of primary fatty amine ethoxylates and subsequent toxicity, to aquatic organisms in this study are mainly driven by hydrophobic interactions and also explains why C16+2EO are more toxic than C12+2EO. In addition, the presence of NOM in the real environment reduces toxicity as it competes with *Daphnia* as substrate for sorption. This is seen in HD and DSW+HA compared to DSW. The additional electrostatic sorption of cationic surfactants to negatively charged

substrate is not considered in these QSAR calculations (van Wijk et al., 2009) and further enhance the need for measurements of the truly dissolved concentrations to determine the true toxicity.

In contrast to previous studies, García et al. (2001) didn't see any incremental increase in toxicity to *D. magna* and *P. phosphoreum* with increasing carbon chain length for monoalkyl QACs. It was attributed to a decreasing water solubility with increasing carbon chain length, with the result of lower bioavailability, hence lower toxicity. This might enhance the results in this study as the relationship between a chain length of C_{12} and C_{18} is not linear, with C18+2EO being less soluble than C16+2EO, hence lower bioavailability and toxicity. However, a decreasing water solubility is also related to an increased biophilic character of the molecule, and as a consequence it has a stronger tendency to adsorb onto lipid membranes of aquatic organisms and disrupt different membrane functions (Singh et al., 2002). Apparently, the water solubility of the molecule and subsequent toxicity has a mutual limit. Since nominal and measured EC50 values are missing for C14+2EO, this study can't confirm if there is an increase in toxicity from C_{12} to C_{16} or if the tendency decreases after C_{14}. Nor can this study see the measured EC50 to *D. magna* for C18+2EO, due to problems with the SPME, to fully evaluate the true toxicity and the relationship between alkyl chain length and toxicity.

3.5 Single substances and mixture toxicity

Chemicals are in these days tested for their intrinsic properties according to REACH which concerns substances on their own, in preparations and in articles. Development of new alternative hazard assessments are promoted (REACH, 2006) and since chemicals, e.g. surfactants, are not only present as single substances in the environment, but rather as mixtures, predictive mixture toxicity models can be used. Concentration Addition (CA) is a toxicity model for predicting mixture toxicity based on substances with a similar mode of action. Three substances; dodecylamine +2EO, hexadecylamine +2EO and octadecylamine +2EO, are tested individually to evaluate their nominal and measured concentrations. A mixture of these substances, Ethomeen C/12, is also tested and the concept of CA is applied to see whether it is possible to predict the toxicity of Ethomeen C/12 from the effect of the single substances.

Nominal concentration

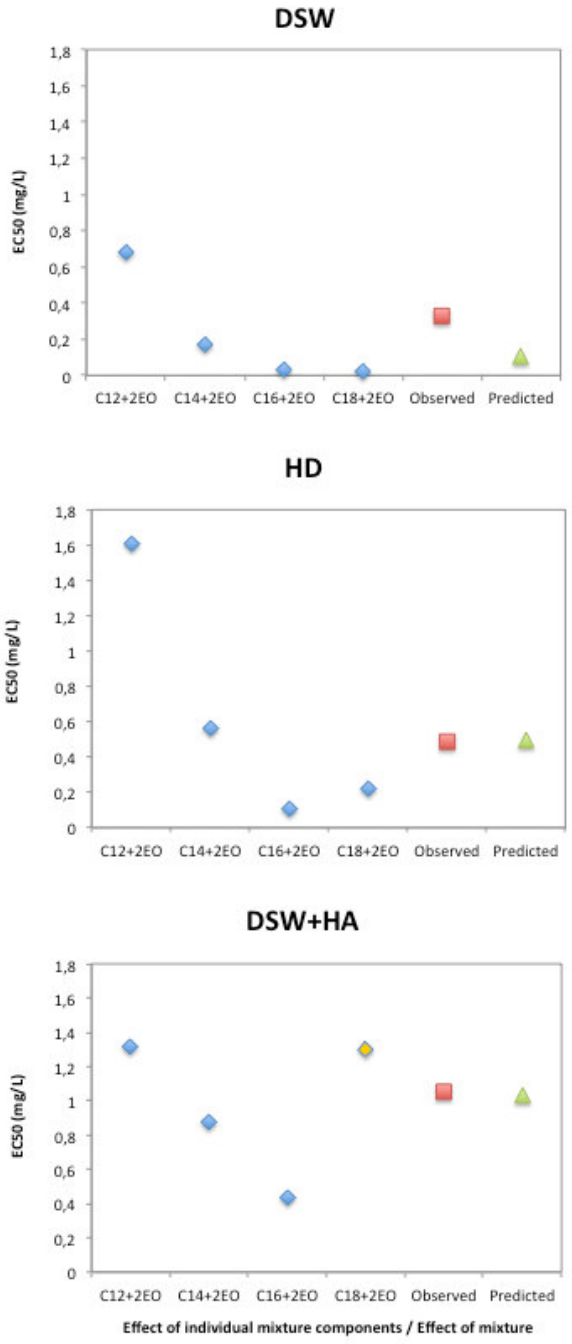

Figure 19. Nominal EC50 (mg/L) of individual mixture components and effect of the mixture, both observed and predicted. The EC50 for C14+2EO is calculated and for C18+2EO (orange) it is estimated based on 60% mobile *daphnid's* at 1.25 mg/L.

The nominal EC50 to *D. magna* of the single substances and the mixture, both observed and predicted, are presented in figure 19. The nominal EC50 of C12+2EO, C16+2EO and C18+2EO in the three different test media represents their effect when tested individually. Whereas the EC50 for C14+2EO in the three different test media is calculated from a linear relationship between the other three logarithmic EC50 values (see appendix A). The toxicity is increasing as the carbon chain length increases, although it decreases after C_{16} and as the amount of humic acid increases (see discussion "toxic response as a function of the alkyl chain length").

The nominal EC50 of Ethomeen C/12 is increasing as the amount of NOM increases as expected, both for observed and predicted toxicity. In the three different test media, the observed toxicity of the mixture is between the highest and lowest toxicity value of the single substances and this result is also expected. The toxicity is well predicted in HD and DSW+HA but is 3 times higher than the observed in DSW. The joint toxicity of the individual components in the test media DSW is thus less than additive and CA overestimate the mixture toxicity.

Measured concentration

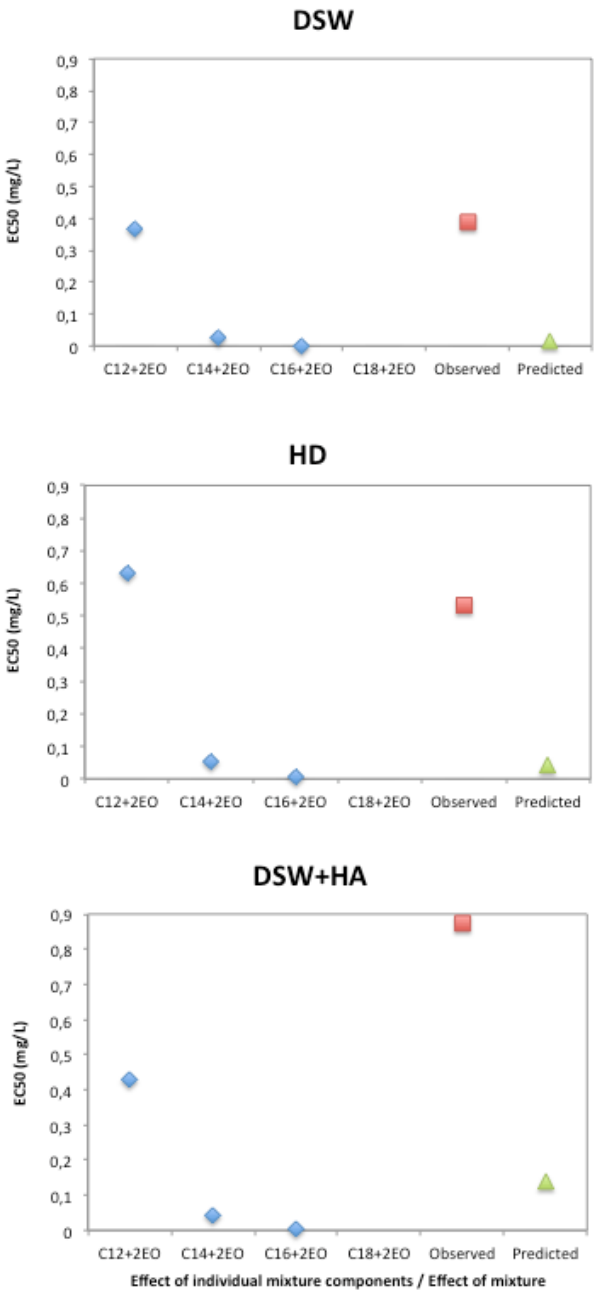

Figure 20. Measured EC50 (mg/L) of individual mixture components and effect of the mixture, both observed and predicted. The EC50 for C14+2EO is calculated. Measured EC50 is missing for C18+2EO.

The measured concentrations, i.e. the truly dissolved concentration that is bioavailable and have the potential to exert toxicity to *D. magna*, of the single substances and the mixture are presented in figure 20. The measured EC50 is missing for C18+2EO and the EC50 for C14+2EO is calculated from the linear relationship between the logarithmic EC50 values of C12+2EO and C16+2EO (see appendix A). The predicted toxicity of Ethomeen C/12 in the three different test media (DSW, HD and DSW+HA) are higher (23.8, 12.0 and 6.3 times, respectively) than the observed. As a consequence, the toxicity of Ethomeen C/12 is overestimated with CA in all test media when measured toxicity is considered. Meaning that the joint toxicity of the individual mixture components are less than additive and a higher mixture concentration than expected by CA is required to provoke the same effect as the sum of the individual mixture components. However, the overestimation decreases with increasing amount of humic acid.

Noteworthy is that the observed EC50 for Ethomeen C/12 in DSW+HA is higher than the highest EC50 value for the single substances (C12+2EO). This is partly due to the higher factor difference (2.246) from the measured EC50 in DSW (see "factor difference between different test media"), but could also be due to the analytics as the measured concentrations of Ethomeen C/12 is higher than the nominal in DSW and HD. The difference between the observed and predicted EC50 in DSW+HA would be smaller, if the factor were less than 2.

The prediction of the EC50 for Ethomeen C/12 is dependent on the knowledge of the mixture components and their individual fraction (Backhaus et al., 2003). According to the Certificate of Analysis for Ethomeen C/12 (AkzoNobel, 2012:c), the mixture consists of alkyl chain lengths varying from C_8 to C_{18} with different fractions. The lower alkyl chain lengths (C_8 to C_{10}) are excluded in the predicted mixture toxicity based on nominal concentrations, due to the unknown relationship between C_8 and C_{12}. If a linear relationship is expected from C_8 to C_{18}, the difference between observed and predicted mixture toxicity based on nominal concentrations are still the same. Their TUs are very low and do not contribute substantially to the mixture toxicity. The predicted nominal EC50 in all three test media is based on the weight fraction of each single substance in the mixture, i.e. no considerations is taken regarding sorption to NOM in HD and DSW+HA since those fractions are unknown for the longest alkyl chain (C18+2EO).

For mixture toxicity based on measured concentrations, only C_{12} to C_{16} are taken into account, both for observed and predicted toxicity. The truly dissolved concentration of the three detectable single substances within the mixture is measured with LC/MS in three different test media and the fraction of each is calculated from the total concentration and presented in table 4. When no NOM is present, i.e. in DSW, the measured fraction with LC/MS of C12+2EO, C14+2EO and C16+2EO are approximately 0.7, 0.2 and 0.1, respectively. Which is similar, except for the higher fraction of C12+2EO, to the determined mixture composition according to Certificate of Analysis (see appendix B) of Ethomeen C/12 used in the prediction of the nominal EC50 for the mixture. However, when NOM is present, i.e. in HD and DSW+HA, the measured fraction with LC/MS of C12+2EO, C14+2EO and C16+2EO changes.

Table 4. Fraction of the individual mixture components measured with LC/MS in three different test media.

	C12+2EO	C14+2EO	C16+2EO
DSW	0.70	0.20	0.10
HD	0.69	0.24	0.07
DSW+HA	0.87	0.12	0.010

Previous discussion about hydrophobicity is valid here, i.e. the longer alkyl chains have a higher sorption affinity to NOM and other surfaces due to a higher hydrophobicity (García et al., 2006), with the result that C14+2EO and C16+2EO are present at a lower fraction when there is a high amount of NOM in the water. Conversely, C12+2EO is present at a higher fraction. The toxicity predicted with CA based on measured concentrations is thus going to be largely exerted by the shorter alkyl chain length (C12+2EO) in presence of NOM since the longer alkyl chains have a lower fraction in the mixture. This is because the calculated TUs for the longer alkyl chains become smaller, compared to when their fraction is higher as it is in DSW. Meaning that when no NOM is present, the predicted mixture toxicity is more determined by the longer alkyl chain lengths as they will have a higher TU. Apparently that is not the case regarding observed mixure toxicity in DSW since that measured EC50 is a factor of almost 24 higher than the predicted EC50.

If the substances are acting with a known similar or dissimilar mechanism of action, any increase or reduction in the overall statistical uncertainty of the predicted mixture toxicity are thus, among others, largely governed by the ratio of the individual substances within the mixture. Furthermore, deviations from the prediction of the mixture toxicity may occur under environmental conditions due

to, e.g. physico/chemical interactions in or with the mixture. The predictive power of CA may also be reduced due to synergistic or antagonistic effects because of interferences of the mixture components (Backhaus et al., 2003). A limitation of the CA concept is thus that it is based on the fraction of the substances related to their individual effect concentrations in the mixture. Interactions with natural organic matter and different sorption affinities are not taken into account in this equation.

The mixture toxicity is overestimated in DSW based on both nominal and measured concentrations, but to a higher degree regarding measured concentrations. The predicted mixture toxicity in DSW based on nominal concentrations is 3 times lower than the observed, whereas the corresponding value based on measured concentrations is 23.8. This difference could be explained by lower individual EC50 values regarding measured concentrations and an increasing difference between nominal and measured concentrations with increasing alkyl chain length due to a stronger sorption affinity, which results in higher TUs for measured concentrations.

The observed measured EC50 of Ethomeen C/12 in the three different test media should be and are almost the same because the toxicity is based on the truly dissolved concentration, i.e. the bioavailable fraction that is believed to exert the toxicity. The toxicity predicted with CA should therefore also be the same as the observed in all test media. A factor 2 is an acceptable difference with SPME between the measured EC50 in DSW with other test media and could therefore be applied on the difference between the predicted measured EC50 as well. The difference are however a factor 2.7 and 8.5 higher for HD and DSW+HA, respectively, than the predicted measured EC50 in DSW. This could only be attributed to changed fractions of individual mixture compontents when NOM are present due to different sorption affinities, which mitigate their effect differently, and consequently, different predicted mixture toxicity.

CA is a concept based on the assumption that substances with a similar mode of action have an additive mixture effect, thus exchangeable with other substances that have the same TU as they have in a certain mixture. However, toxicity is not in general linearly related to molecular descriptors. The ecotoxicity of surfactants are typically increasing logarithmically with a linear increase in the alkyl chain length and applying the concept of CA is thus going to be largely governed by those mixture components that are most toxic. As a consequence, the mixture toxicity will be overestimated (Boeije et al., 2006) which is the case in this study when the predictive mixture toxicity is determined in DSW based on both nominal and measured concentrations, but also in HD and DSW+HA based on measured concentrations. The reason for this is that the presence of the most toxic substances is not reflected in the calculated average structure of the mixture. That is, the nonlinearity of the most toxic components impact is disproportionate to their molar abundance, whereas the calculation is (Boeije et al., 2006). CA also interprets that it is the overall binding to the target site that determines the effect and all organisms are susceptible to baseline toxicity since they all contain membranes (Porsbring, 2009). The combined effects of the components are estimated well with CA if they belong to this group of baseline toxicants (Könemann, 1981) or if they have an identical molecular mechanism of action (Backhaus et al., 2003).

There are no available literature data on comparison between nominal and measured concentrations, including toxicity, sorption and concentration addition on surfactants. Mixture toxicity studies in general contains mixtures between anionic/non-ionic/cationic surfactants, not

cationic/cationic. A study by Hisano and Oya (2010) with a mixture of an anionic and a cationic surfactant exposed to *D. magna* in standard water was less than additive as the TU were greater than or equal to 1. This result was in agreement with another study with binary and ternary mixtures of anionic, non-ionic and cationic surfactants, referred by Hisano and Oya (2010). It is also similar to this study.

Boeije et al. (2006) reported a measured EC50 value for a mixture of non-ionic Alcohol Ethoxylates (AE) that was 1.5 times higher than the EC50 predicted by CA, but due to variability in the analytical recovery, the measured concentration could be overestimated with the result that the mixture toxicity is actually more consistent with the CA than observed. Boeije et al. (2006) also referred to other studies which states that the CA model is valid for AE but also for other baseline toxicants. However, the toxicities of non-ionic surfactants are well predicted with the general narcosis equation (Roberts and Castello, 2003) and thus enhance why the CA model is applicable for AE. Cationic surfactants on the other hand, have been shown to act by a polar narcosis mechanism (Roberts and Castello, 2003) and may explain why the mixture of PFAEO do not conform to CA. However, the observed toxicity is lower than the predicted in this study and that should not be the case if they are polar narcotics. Other factors might then influence and affect the mixture toxicity.

Although predictive toxicity models are very useful when considering economy, time-efficiency, animal testing etcetera, in determining the toxicity of mixtures, the risk that they might over or underestimate the mixture toxicity is still there. Regarding registration of surfactants according to REACH, where the intrinsic properties of the surfactant are supposed to be evaluated, it is thus better to test the substance, i.e. the mixture itself, to minimize this risk. From an environmental risk assessment point of view, it is actually useless to test a specific mixture as the real environment consists of an infinite amount of different mixtures.

Concluding summary

The SPME method used in this study measures the truly dissolved concentration of surfactants in the water and that is the concentration believed to be bioavailable, thus have a potential to exert toxicity to aquatic organisms. The method is very useful as the total concentration of surfactants in natural water may be of less importance (Haitzer et al., 1998) when risk assessments are performed to predict the potential effect and environmental concentrations, hence the risk posed by them to aquatic organisms. However, the toxicity of these cationic surfactants to *D. magna* are probably greater than their hydrophobicity imply as a consequence of their ability to also interact electrostatically with biological surfaces. This study have only measured how much the effect is mitigated and not how, that is hydrophobically or electrostatically. The aquatic toxicity of a pure substance, e.g. one specific alkyl chain length attached to the amine, is assumed to be the same regardless of test media when it is based on the truly dissolved concentration. Whereas the composition of a mixture changes in different test media due to, e.g. different sorption affinities of the individual mixture components, and this is reflected in the truly dissolved concentrations. As a consequence, the toxicity is altered and more obvious in predictive toxicity models.

3.6 Further recommendations

This study examined the acute toxicity of one primary fatty amine, four primary fatty amine ethoxylates and two quaternary ammonium compounds. Further tests with these and other related cationic surfactants are necessary to fully evaluate the SPME method for cationic surfactants and to

be able to build a QSAR model for them regarding aquatic toxicity and bioavailability. One factor that seems to affect the SPME method is the water solubility of the cationic surfactants. The less water soluble it is, the more difficult it is. Water solubility of chemicals is a factor that matters for a QSAR based on log P (Könemann, 1981). If the substance is infinitely soluble in water the toxicity is not possible to predict with an equation baed on hydrophobicity, only slightly soluble substances is.

When further tests are done, a QSAR based on log P can be used to model the toxicity of primary fatty amines, PFAEO and QAC, especially in mixture toxicity studies, to determine if the tested cationics follow a general or polar narcotic mechanism of toxicity.

Long term toxicity test with *D. magna* and SPME should preferably be performed to se whether the relationship from short term to long term is linear or not. In addition, test should preferably also be performed on other organisms, e.g. algae and fish as the sensitivity differ between species. *Daphnia* is believed to be the most sensitivie species towards cationic surfactants (Lewis and Suprenant, 1983) based on nominal concentration. What would be the results if it is based on measured concentrations?

What is the molecular mechanism of cationic surfactants towards different species of organisms, i.e. how are the *daphnia*, algae and fish affected by cationic surfactants? In general, the toxicity of surfactants are indicated to be determined by their affinity to adsorb onto the cell membrane, mainly driven by nonspecific hydrophobic interactions, and their ability to penetrate the membrane bilayer (Rosen et al., 2001). The plasma membrane consists of lipids and mostly phospholipids, which also have an amphiphilic structure. Surfactants disrupts the hydrophobic interactions of the bilayer by binding to the hydrophobic region of transmembrane proteins and the hydrophobic fatty acid tails, thus forming protein-surfactant complexes and solubilizing the phospholipids (Alberts et al., 2004). Fish may thus be affected as the water is constantly pumped through the gills, whereas algae has a larger, negatively charged surface area and *D. magna* may be affected as they are filter feeders. If the adsorption to cell membranes is mainly driven by nonspecific hydrophobic interactions, what is then the difference in toxicity between e.g. non-ionic and cationic surfactants?

Limitations of this study

This study has focused on the nominal and measured EC50 values and all the comparisons within and between substances are based on this. Further studies, e.g. on mixture toxicity, should preferably look at the entire concentration range from EC1 to EC100 to better see any under- or overestimation of mixture toxicity when the concept of CA is applied.

Sorption to humic acid may also enhance mobility of surfactants in the soil and hasn't been considered in this study.

At last, the pKa of the primary fatty amine ethoxylates are low and the pH in the test is high, with the results that the molecule may not be entirely cationic. A determination of how much is cationic under the test condition could be necessary, at least when comparison between other cationic surfactants are made.

4. Conclusions

SPME fibers extracts the freely dissolved concentration of the tested surfactants in various test media and provides information about the bioavailability, thus the potential risk of cationic surfactants in the environment. Based on the results from the acute immobility test (OECD 202) and the SPME it can be concluded that sorption of the tested cationic surfactants to NOM in river water (HD) clearly has a mitigating effect, although substance specific, on the acute toxicity to *Daphnia magna*.

- The mitigation of each surfactant in different test media are determined and the difference between nominal and measured concentrations of cationic surfactants are due to their strong tendency to sorb to substrates via hydrophobic, as well as electrostatic interaction. The toxicity is governed by both of these interactions, however this study haven't examined how much each of these interactions contribute and how it may differ between surfactants.
- The most toxic substance regarding measured concentrations is hexadecylamine +2EO, although didodecyldimethylammonium bromide in HD and DSW+HA are not statistically different from C16+2EO. Furthermore, Arquad 2C-75 is also very toxic and the toxicity seems to increase as the amount of HA increases. The least toxic substance is Ethomeen C/12, together with dodecylamine +2EO as it is not statistically different in DSW and HD.
- Mitigation factors for cationic surfactants are substance specific and varies from 0.9 to 31.3 in this study. A standard mitigation factor for all substances will inevitably lead to either over- or underestimation of their true toxicity, depending on which surfactant it is.
- Toxicity is increasing with an increase from C_{12} to C_{16} in the alkyl chain for PFAEO, based on both nominal and measured concentrations, and it is related to an increasing hydrophobicity within the molecule. The tendency is decreasing from C_{16} to C_{18} regarding nominal concentrations probably due to a lower water solubility.
- An addition of two ethoxylated groups to dodecylamine results in a higher nominal EC50, both in DSW and HD, due to a higher water solubility of the molecule.
- The predictive toxicity model Concentration Addition overestimates the mixture toxicity of Ethomeen C/12 in Dutch Standard Water based on nominal and measured concentrations, the joint toxicity of the individual mixture components are thus less than additive. Regarding measured concentrations, the overestimation decreasas as the amount of NOM increases due to a changed composition of the mixture, i.e. the fraction of individual mixture components, caused by different sorption affinities. This in turn affects the predicted toxicity.

An overall conclusion is that the SPME method is a good technique to quantify the truly dissolved concentration of cationic surfactants, but further studies are necessary to properly evaluate the method for these kind of substances to be able to find mitigation factors. These acute tests only gives an explanation that the mitigation is substance specific for cationic surfactants and they may vary as the amount of suspended matter, humic acid and other sorbents in the aquatic environment varies from one place to another and over the year.

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