



Screening methods for aquatic toxicity of surfactants

Master of Science Thesis in the Master Degree Programme Materials and Nanotechnology

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Cover:

The picture published on the cover is a result from the *Hygrophila polysperma* aquatic plant test using Ethomeen C/15 in a concentration interval of 10 mg/l, 5 mg/l, 2 mg/l and 1 mg/l. The highest concentration is placed to the left where it is visible seen that the plant is highly affected. The picture is found on page 48, figure 20.

This thesis has been carried out in collaboration with AkzoNobel  in Stenungsund, Sweden, and partly at AkzoNobel in Arnhem, The Netherlands.

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Abstract

Aquatic toxicity is an important characteristic of all surfactants. Since the research and development at AkzoNobel develops substances as non-toxic as possible, it is of great importance to test products during the development stage of this process. For this purpose, screening methods for acute aquatic toxicity is vital to be able to predict standardized toxicity results or get an indication of substance toxicity. In this study, different screening methods have been evaluated to find, and suggest, suitable screening methods for aquatic toxicity.

Four screening methods have been evaluated; red beetroot bioassay; root elongation; the aquatic plant test and Microtox. The results from these tests were then compared and correlated to the highly standardized OECD methods; algae acute toxicity test (OECD 201), daphnia acute toxicity test (OECD 202) and fish acute toxicity test (OECD 203) in order to determine the validity of the tests. Since all the tests are normally performed in water and there are plenty of hydrophobic surfactants, the possibility of using solvent in these tests has also been evaluated.

To test surfactants from different families with different characteristics, several well-known surfactants were used during the evaluation process; Ethylan 1005; Ethomeen T/25, Ethomeen T/15, Ethomeen T/12, Ethomeen C/15, Ethomeen C/12, Arquad 2C-75, AG 6202 and Cocobenzylamin + 1EO.

Three different aquatic plants (*Hygrophilia polysperma*, *Cabomba Aquatica* or *Elodea Canadensis*) were evaluated as test species in the aquatic plant test. It was found that *Hygrophilia polysperma* is the most suitable plant since a clear visible result can be achieved and that it can be used to detect toxic surfactants (surfactants with a standard OECD EC50 result below 1 mg/l). It was also found that the results obtained during the test using *Hygrophilia polysperma* correlated well to the standard OECD 201 and 202 by ranking since the surfactants could be placed in almost the same toxicity order. When the effects of small amount of solvents were evaluated for the test species it was found that ≤ 1 % solvent not affects the toxicity result.

During the evaluation of Microtox it was found that it is an easy and fast method which gives toxicity results comparable to the ones obtained by standard OECD methods, especially OECD 202, since they place the surfactants in the same toxicity order. Thereby it is possible to state that Microtox and OECD 202 correlate by ranking. It was also found that a small amount of solvent (<0,5%) can be used in the Microtox test to improve the water solubility of hydrophobic surfactants. This amount is often enough to improve the solubility and create a homogenous solution because a very small amount of the toxicant often is tested.

Since it was found that *Hygrophilia polysperma* aquatic plant test and Microtox are working as environmental screening tools they are recommended to use. The test using the test species red beet root and root elongation are not working as environmental screening tools because agreement with OECD standard result and valid growth, respectively, not occurred.

Key words: Aquatic toxicity, screening test, OECD standard test, test species, surfactant

Abbreviations

CMC- Critical micelle concentration

Coco-Ethomeens (C-Ethomeens)- Ethoxylated coco alkyl amines

DMSO - Dimethyl sulfoxide

HCl- Hydrochloric acid

IPA-Isopropyl Alcohol

NOEC- No observed effect concentration

OECD - Organisation for Economic Co-operation and Development

LOEC- Lowest observed effect concentration

REACH- Registration, Evaluation, Authorisation and Restriction of Chemical substances

Tallow-Etomeens (T-Ethomeens)- ethoxylated tallow alkyl amines

WAF- Water Accommodated Fraction

Dictionary

Acute toxicity- the effect suffered by organisms from short-term exposure to toxic chemicals

Algae growth - the increase in cell concentration over the test period

Amphiphile - a chemical compound possessing both hydrophilic and lipophilic properties

Aquatic toxicity- degree to which a substance can damage a living or non-living organisms in an aquatic environment

Bioaccumulation- the accumulation of substances, such as pesticides, or other organic chemicals in an organism

Biodegradation (biotic degradation or biotic decomposition) - the chemical dissolution of materials by bacteria or other biological means

Biological indicators- species used to monitor the health of an environment or ecosystem

Biogenic substance - a substance produced by life processes. It may be either constituents, or secretions, of plants or animals. A more specific name for these substances is bio-molecules.

Biomonitoring- is the measurement of the body burden of toxic chemical compounds, elements, or their metabolites, in biological substances

Biomass-Cell concentration - the number of cells per ml

CMC- The concentration at where surfactants starts to aggregate (create micelles).

Contaminant- a minor and unwanted constituent in material, physical body, natural environment etc

Coco-Ethomeens-Surfactants from the Ethomeen family containing a coco hydrophobic chain

Chronic toxicity- effect suffered by organisms from long-term exposure to toxic chemicals

EC_x- the concentration at which x% of the test species is effected

Eutrophic- an environment that offers excess of nutrients

Growth rate- the increase in cell concentration per unit of time

High-performance liquid chromatography- a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture

Hypocotyl- is the stem of a germinating seedling, found below the seed leaves and above the root

Invertebrates- an animal without a backbone, for example daphnia

Inoculation- to introduce a test species in an environment for growth

Inoculum- the microorganism used in an inoculation

LC_x- the concentration at which x% of the test species is dead

OECD- the OECD provides a forum in which governments can work together to share experiences and seek solutions to common problems

OECD media- the notorious solution a test is preformed in for a fresh water standard OECD test

Oligotrophic- an environment that offers very low levels of nutrients

Tallow-Etomeens- surfactants from the Ethomeen family containing a tallow hydrophobic chain

Toxicology- is a branch of biology, chemistry, and medicine concerned with the study of the adverse effects of chemicals on living organisms. It is the study of symptoms, mechanisms, treatments and detection of poisoning, especially the poisoning of people.

Toxicity- the degree to which a substance can damage a living or non-living organisms

Toxicants- chemical hazardous to ecosystem health

Screening method Surfactant- an amphiphilic molecule

Standard OECD methods- highly standardized tests by OECD

Synthetic surfactants- surfactants produced in the laboratory

Parafilm- plastic paraffin film that is commonly used for sealing or protecting vessels

Uv-vis spectroscopy- refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region

Xenobiotic substance- a chemical which is found in an organism but which is not normally produced or expected to be present in it. It can also cover substances which are present in much higher concentrations than are usual. Specifically, drugs such as antibiotics are xenobiotics in humans because the human body does not produce them itself, nor are they part of a normal diet.

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Introduction

Synthetic surfactants are widely used over the world in household cleaning detergents, personal care products, textiles, paints, polymers, pesticide formulations, pharmaceuticals, mining, oil recovery and pulp and paper industries. The world production of synthetic surfactants amounts to 13 million tons annually [1] and are economically important products. Because of surfactants widespread use they can be potential toxicants when large quantity enters the environment, and since surfactants mainly enter the environment by wastewaters, aquatic toxicity and aquatic behavior are of major importance [2]. It is nowadays known that many surfactants used in the past were hazardous and with respect to that, irrespective of their intended use, product safety – including environmental protection – is of great importance for all new surfactants.

The mainly focus of this paper lies within aquatic toxicity of surfactants and how to test this characteristic with screening toxicity tests. There are different techniques to measure aquatic toxicity, both standard and screening, but the endpoint is often a LC50 (50% lethal concentration) or an EC50 (50% effect concentration). As the knowledge about surfactants toxicity grew different methods to test this were developed, and some later was standardized by *Organisation for Economic Co-operation and Development* (OECD), for example algae acute toxicity test (OECD 201), daphnia acute toxicity test (OECD 202) and fish acute toxicity test (OECD 203). Before a standardized test is to be done it is common to do a screening test. Thereby it is possible get an indication of the toxicity in a simpler and cheaper way.

The AkzoNobel aquatic toxicity standard methods testing are taking place in Arnhem, Netherlands. For a newly developed surfactant it can be both expensive and time consuming to send each sample to Arnhem and therefore AkzoNobel Research and development in Stenungsund is looking for a screening method which can give an indication of the aquatic toxicity at an early stage of product development.

During this work four screening methods were tested at the laboratory in Stenungsund. The aquatic toxicity screening results of these screening methods were then compared and, if a good result could be achieved, correlated to the OECD standard method.

Aim

The aim of this project is to find and suggest suitable screening methods to determine the aquatic toxicity of surfactants.

Limitations

- Only acute aquatic toxicity tests will be considered
- Four screening tests will be evaluated in the laboratory; Microtox, root elongation test, red beet root bioassay and the aquatic plant test.
- This four screening methods will be compared with the toxicity results for the OECD standard method 201,202, 203.
- The number of surfactants used to evaluate the methods will be limited by time.
- To get a proper evaluation it is desirable to use at least one non-toxic substance and one very toxic substance. Surfactants with different degree of water solubility will be evaluated.
- Two solvents were used to test if small amounts of solvent can be used in the tests to enhance solubility of hydrophobic surfactants, IPA and ethanol.
- Only pure substances and not blends of different surfactants will be tested.
- In order to compare the screening methods OECD standard results must be present. The surfactants that not have OECD standard results available (201, 202 and 203) will be tested in Arnhem if possible.

Theory

Toxicology

Paracelsus (1493-1541) was very clear when he stated that all things are toxic in to high concentrations. He was not perfectly right but toxicants must be defined both quantitatively and qualitatively since toxicity is dose-responsive. Therefore, a chemical might be a contaminant at one concentration and a toxicant at a different concentration because dosage makes a big difference. [3]

During the last 50 years biological indicators have become a critical element in defining the nature of environmental toxicants. Today they are designed on five experimental elements; the test species, the form of the sample, the test time, the endpoint (toxicity result), and the dose response.[3]

Aquatic toxicology

In aquatic toxicology exposure is of big importance. Contrary to mammalian toxicology, where the test organism often is administrated with the toxicant at a known internal dose directly via food or injection, exposure in aquatic environment is much more complicated. In aquatic toxicology tests the toxicant is instead dissolved in the test medium, which often is aquatic. The test organisms in the aquatic environment then have to build up an internal concentration of the test substance through the skin or the breathing organs (gills) from the aquatic environment to be affected. One of the major problems during aquatic tests is therefore how the concentration in the solution is related to the toxic effect. Because of this, toxicology in aquatic environments is often expressed as external concentration in the exposure medium, rather than as internal concentration of the test organism. Since the actual concentration of the test chemical together with the duration of exposure is of prime importance in determining whether an affect will occur or not, concentration and exposure time will be considered carefully during the test.[4]

Since concentration is a very important parameter during the test, it is important that it is maintained stable during the test period to be sure of the external dose. For insurance of concentration duration during a test it is recommended to make quantitative measurements in real time, for example with High performance-Liquid chromatography. Some test chemicals may be volatile chemicals, degradable chemicals, highly bioaccumulative chemicals or chemicals with low water solubility and poses great problems in practice, but still have to be tested. Therefore, various methods have been developed for exposing aquatic organisms to such substances in order to look for eco-toxicological effects. Three general types of toxicant delivery systems are used in toxicity testing:

- Static
- Renewal
- Flow- through

A static test is a test where the test organism is exposed to the same test solution for the whole test duration. A renewal test is also called a semi-static test and instead of keeping the test organism in the same solution they are periodically transferred to fresh solution. During a Flow-through test organism is exposed to a continuous- flow exposure system that, depending on the flow rate, continuously changes the test solution. This test set-up is very common for aquatic toxicity test with fish, especially if the toxicant is poorly soluble or volatile.[4,5]

Laboratory aquatic toxicity tests with test species as fish, invertebrates or algae are usually single-species tests in which the toxicity of a chemical is measured through mortality, decreased growth rate and lowered reproductive capacity, either by an acute toxicity test or a chronic toxicity test. [4,5] These tests have been highly standardized, by OECD, and are applied to a selected group of organisms. The toxicity results from these tests are reported to REACH in order to be able to register a new molecule. REACH handles the Registration, Evaluation, Authorization and Restriction of Chemical substances, which first letters can be read out in the name. [6,7].

The purpose of eco-toxicity testing is not to protect individuals in nature, but rather whole population and ecosystems. It is assumed that if the most of the species are protected, the ecosystem is protected. It is of course hard to draw the line where the ecosystem is preserved and by test a few species extrapolate the result to be sure that growth, survival and reproduction will proceed, but by test very sensitive aquatic organisms it is assumed that the most species are protected and thereby the ecosystem. The acute aquatic toxicity tests that are standardized by OECD are using algae (OECD 201), daphnia (OECD 202) and fish (OECD 203) as test species. These species are very sensitive (μl) and simulates a small ecosystem.[4]

The objective of acute toxicity testing is to determine the concentration of a particular chemical that will obtain a specific response or measurable endpoint from a test species in a relatively short period of time, 2-7 days. In chronic toxicity tests, on the other hand, effects are studied over a prolonged periods of exposure that lasts during an entire life cycle. Chronic studies have often longer test periods than acute tests but generally do not exceed a period equivalent to one-third of the time taken for a species to reach sexual maturity. Short-term toxicity and acute toxicity are not the same and can be explained by test with algae. Standard 96 h toxicity tests with algae are short-term chronic studies, because algae have relatively short life cycle. Acute exposure may lead to chronic effects.[4]

Acute toxicity has two general applications in environmental risk analyses. The first one is to determine acute toxicity. The objective of this determination is to measure the degree of biological response produced by an external particular level of chemical stimulus. The second type is to screen for toxicological effect which have the purpose to determine whether the chemical or solution being tested is biologically active, biological-available, with respect of the endpoint being measured. Screening tests often provide yes or no answers (toxic or nontoxic, mutagenic or no mutagenic etc) or an indication of the endpoint (toxicity result). [4]

When a chemical or mixture of chemicals is tested for acute aquatic toxicity a test organism (for example bacterium, plant or animal) is exposed to a concentration interval of the test substance to achieve a dose response curve, see Figure 1. From the dose response curve it is possible to determine the concentration where a certain percentage of the test species (X%) are dead (lethal concentration, LCX) or effected (effect concentration, ECX). Normally the concentration that causes 50 % of the test species to die or be effected is reported to the authorities, the LC50 (fish) or the EC50 (algae and daphnia) but other results can also be reported, for example the EC10. The lower the effect concentration, the more toxic is the tested substance.[4,8]

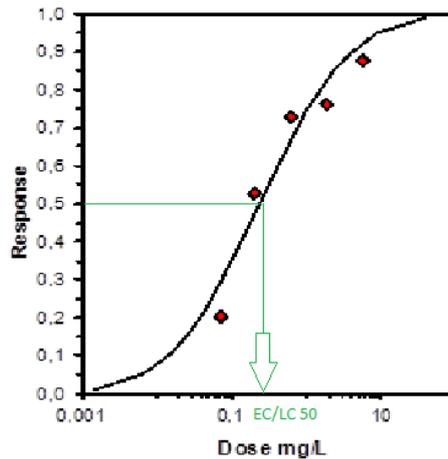


Figure 1, In this picture a dose response curve to calculate the concentration (dose) were the response (lethal or effect) is 50% (LC50 or EC50). The red dots illustrate a concentration prepared. It is seen in the picture that as the concentration of the toxicants gets higher the response gets higher. The picture can also be found in Appendix 3 where the result from the algae test is presented.

To achieve a linear approximation of the dose responses it is preferable that the concentrations tested are in a geometric series. First a range finding test is done to determine in what concentration interval the effect or death concentration is found. The range finding interval is often 1000 mg/l, 100 mg/l, 10 mg/l and 1mg/l but is determined dependent on the test substance predicted toxicity, if it is expected that the substance is very toxic the highest concentration can be replaced by a lower one. When the range finding test is done a definite test with a smaller concentration interval is done in the range where affect was detected. The factor in between the concentrations are instead of 10 often in between 2 or 3, for example if effect between 1mg/l and 10 mg/l in the range finding is found the definite test can be done in the following concentrations; 1 mg/l, 2 mg/l, 4 mg/l, 8 mg/l and 10 mg/l. [4,9,10]

Surfactants

Surfactants are molecules consisting of a hydrophobic and a hydrophilic part, this amphiphilic property is the main reason of surfactants big usage in industrial products. The hydrophobic part usually consists of mostly carbon (8 to 20 carbon atoms) that often is derived from hydrocarbons in fatty acids, paraffins, olefins or alkylbenzenes. [11, 12, 13] Since mostly all surfactants are soluble in water to some extent, surfactants can be divided into four groups that are characterized by the polar heads specific charge or non-charge. The groups are anionic (negatively charged), cationic (positively charged), zwitterionic (both positively and negatively charged) and nonionic (uncharged). Their chemical structure can vary widely and consist of many hydrophobic and hydrophilic parts and are because of that not restricted to the simple picture (Figure 2) below. [14] In view of their hydrophilic nature, surfactants tend to be water soluble to some extent. Depending on the head group and the surrounding environment, solubility varies from very soluble (e.g. some anionic surfactants) to insoluble (e.g. some cationic surfactants)[12,13]

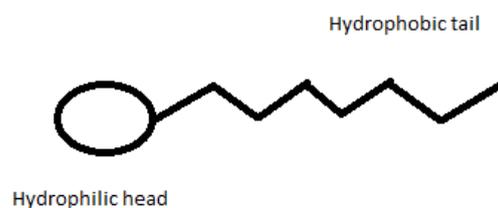


Figure 2, Illustration of a schematic surfactant.

When adding surfactants to a solution they will enrich at interfaces and because of their dual hydrophilic and hydrophobic nature lower the free energy (surface tension). At the interface, the hydrophilic part of the surfactant orients itself towards the aqueous phase and the hydrophobic parts orient itself away from the aqueous phase into the second phase. [11,13] Because of lowering of the surface tension, surfactants makes it possible to mix water with organic matter to different extents, dependent on the surfactant and the organic substance. [15]

When the interface (for example surface) in a solution is saturated with unimers (single surfactants), the surfactants will no further change the surface tension of the surfactant solution. If additionally surfactants are to the solution when the surfaces are saturated with unimers, the surfactants will aggregate to micelles to lower their free energy. [15,13,14,16] Every surfactant have a certain concentration where the micelles starts to form, the critical micelle concentration (CMC) (def. the concentration of surfactants above which micelles are spontaneously formed) which is dependent on the surrounding aqueous environment, for example salt content [16,17].

The CMC of a surfactant is also dependent on the hydrophobic tail and the hydrophilic head, a more hydrophobic surfactant results in a lower CMC value and a more hydrophilic head a higher CMC value. [15] Nonionic surfactants have in general lower CMC levels than anionic and cationic surfactants because they usually are not as pleasant in aqueous solutions due to the differences of the head group. [14] Depending on the surrounding media of the micelle, the surfactants can also lower their free energy by aggregate to either reverse (Figure 3a) or normal micelles (Figure 3b).

[12,13]. Not all micelles are spherical, as in Figure 3, primarily because of the ratio between the area of the head group and the volume of the hydrophobic tail group the micelles can also for example be rod-shaped and disk-shaped. [17] When a surfactant solution have a surfactant concentration above the CMC value, the solution gets different properties, it is for example in this situations the solution gets its solubilisation properties. [11]

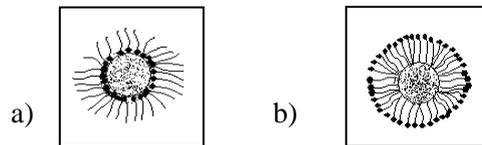


Figure 3 Representation on a) reversed micelle and b) normal micelle.

Anionic surfactants

Anionic surfactants are the most produced surfactant class (60% of world production). They are cheap to manufacture and are mainly used in detergent formulation. The polar head group often consists of a carboxylate, sulfate, sulfonate or phosphate and the hydrophobic chain often consist of an alkyl or alkylarye chain in the C12-C18 range. [15]

Nonionic surfactants

Non-ionic surfactants are the second largest produced surfactant class.[11] The polar head group is often consisting of polyether consisting of 5-10 oxethylene (EO) units.[15] Nonionic surfactants are characterized by higher hydrocarbon solubilizing power, weaker adsorption to charged sites, less toxicity to bacteria, poor foaming properties and compatibility with other types of surfactants. [16]

Cationic surfactants

Cationic surfactants are the third largest surfactant class and adsorbs strongly to most surfaces. Since the majority of all surfaces are negatively charge (metals, minerals, plastics, cell membranes etc.) the prime uses of cationic surfactants relate to their tendency to adsorb at these surfaces. The majority of cationic surfactants hydrophilic head group consists of amines or quaternary ammonium with a positively charged nitrogen atom. [15]

Zwitterionic surfactants

Zitterionic surfactants are the smallest surfactant class and are known to have excellent dermatological effects. The polar head group consists of a positive and a negative charge and the charge of the surfactant is pH dependent. The surfactant is cationic at low pH and anionic at high pH, which makes the properties of the surfactant change as the pH changes. [15]

Toxicity of surfactants

Synthetic surfactants toxicity for aquatic organisms was early discovered when a large number of fish was found dead in strongly contaminated waters. Since then, many studies have been done to determine surfactants toxicity for both land-living and aquatic-living organisms, mainly on fish, and many surfactants used today is not as toxic as they used to be thanks to research. [14]

Nowadays the acute toxicity on commonly tested species for the most common surfactants is well known for many common surfactants but the chronic effect is not as studied yet. [2, 18] Because

surfactants are highly consumed over the world, surfactants and their degradation product have to be considered carefully. [14]

Human toxicity of surfactants

Surfactants and their breakdown products have showed a generally low toxicity on land- living animals in laboratory test animals such as rats and guinea pigs, and the effect decreases as the molecular weight increases, probably due to lower adsorption in the intestine. An acute toxic effect is therefore not to be likely but a chronic effect, can however, be more possible since a regular dosage of a human is about 0.3-3 mg/l from drinking waters, detergents, toothpaste and food. However, the risk is not big since laboratory chronic tests (during 3 years) not showed any big changes but small effects on some small rodents. No inhalation effect on the lungs was neither discovered.[18] Studies published in the last decades suggest that acute tests with invertebrates may be used, instead of rats and other rodents, as screening methods for the assessment of the lethal toxicity of new chemicals to mammals and humans.[19]

Surfactants toxicity for aquatic organisms

The toxicity of a surfactant is dependent on the exposure time and the concentration as well as its surrounding aquatic environment. It has for example been shown that a toxicant is less toxic in marine-environment compared to fresh water environment. The surfactants biodegradation properties and the biodegradation products are also of importance as well as the bioaccumulation properties. [18] The toxicity of surfactants to an aquatic organism can therefore only be evaluated if the rate and completeness of their biodegradation, mainly by through microbial activity, is taken into account. [15]

Biodegradation is an important process to treat surfactants in raw sewages in sewage treatment plants, and it also enhances the removal of these surfactants in the environment, thus reducing their impact on the aquatic environment. Substances with high toxicity will generally not have any harmful effect on aquatic organisms if they are degraded sufficiently quickly. In modern day use surfactants in general are considered to have good biodegradation properties which mainly depends on the surfactants structure, and not the environment.[14,11] Because of this, the environmental ranking of a surfactant in the OECD guidelines is based on the values of ready biodegradation and aquatic toxicity.[15]

The surfactant concentrations in the environment (mainly aquatic environment) are normally below CMC, where the maximum aquatic toxicity for a surfactant is found.[14] Surfactants toxicity for aquatic organisms is mainly in the mg/l (ppm) range, that is 1-1000 mg/l, but normally the toxicity is between 1mg/l to 100 mg/l. For some sensitive species at sensitive stages in life, sensitivity goes below 0,1 mg/l, for example for young *Daphnia magna*. [19] A surfactant is considered toxic if the EC50/LC50 is below 1mg/l after 96h testing on fish and algae and 48 h on daphnia. Environmentally benign surfactants should preferably be above 10 mg/l. [11,15] As indicted before, sensitivity might depend on the organism group and their life stage, but of course, different aquatic organisms are differentially sensitive to the same surfactant as well as the cationic-, anionic-, nonionic and zwitterionic surfactants gives dissimilar reaction and sensitivity dependant on the structure. This has been proven in fish studies that fish toxicity is strongly dependent on the structure of the surfactant, as exemplified by structural isomers. [18] It is because the differences of the organism's sensitivity

for different chemicals, important that the most sensitive result are reported to the authorities to ensure preservation of the eco-system.

In general for surfactants it has been shown that fish and aquatic organism toxicity increases with the surfactants effective length of the hydrophobic chain. For non-ionic surfactants it has also been shown that the toxicity decreases with the increasing number of EO chains and in anionic surfactants, branching and an internally located hydrophilic group reduce the toxicity. [17,20] Non-ionic surfactants are more toxic than the anionic surfactants to for example for three aquatic organisms: gastropod *Physa acuta*, crustacean *Artemia salina* and alga *Rapidocelis subcapitata*, but both anionic and non-ionic surfactants are toxic to various aquatic organisms, but generally nontoxic to bacteria.[17]

Surfactants are more or less toxic to aquatic organisms due to that surfactants surface active properties acts at the contact surfaces between the water and organisms, for example intestines, gills and skin.[14, 21] Gill epithelial cells are therefore important candidates as *in vitro* models in aquatic toxicology. [20] Since water organisms often also have surface enlarged breathing organs that consist of thin tissue and cell membranes, they are likely to be affected, both because of changed surface tension and changes in permeability of surfactants and other substances. [18] Surfactant has to be taken up into an organism before it can elicit an effect and this processes and factors influencing uptake are relevant when assessing the environmental risk. [14]

Cationic surfactants sorbs strongly onto surfaces that are negatively charged, predominantly sludge, sand and cell membrane in aquatic environments [14], which not has been seen for anionic surfactants [13]. This phenomenon is predominantly depending on the charge of the hydrophilic head group. Because the cationic surfactants adsorb strongly to the surfaces of cells through a combination of hydrophobic and electrostatic interactions, this surfactant class are often more toxic than the other ones. [19] Because of surfactants tenancy, especially cationics, to absorb strong and fast to surfaces, they are particularly hard to toxicity test due to loss of concentration from the solution to the surface. If a very low concentration is tested a large percentage of the substance adsorbs to the surfaces and the effect of the test species in the solution is reduced due to loss of concentration. As a result a much lower concentration than expected is tested. [11]

Some surfactants are poorly soluble in water and are therefore very difficult to test for aquatic toxicity. This kind of surfactants can, instead of dissolve in the surrounding media, create particles. To test the aquatic chemical toxicity of this kind of substances it is important to improve the solubility for example by heating or ultra sonic division. If particles still is present the sample can be filtered and the surfactant quantity of dissolved substance can be measured. Instead of filtering the sample it is also possible to do a Water Accommodated Fraction (WAF). When a stock solution is prepared with a high amount of surfactant and different phases are observed a WAF sample (sample that are taken from the homogenous phase) can be taken. The amount of sample is measured quantitatively and the test of the surfactant can continue. Another way to improve solubility is to use organic solvent. Even though solvent never will be present at the concentration tested it is a way to achieve a higher test concentration, especially when other equipment and time consuming additional laboratory not are available. Many other problems can occur and are described in OECD Guidance document on Aquatic toxicity of difficult substances and mixtures. [22]

At the moment standard OECD tests for surfactants are carried out in OECD standard marine and fresh water. These testing medias are prepared in the laboratory and designed to ensure that the surfactants are tested at the same conditions (same amount of nutrition and ions present). These waters are a simulation of fresh and marine water in nature, but without dissolved organic matter from living species.[23,24] This organic dissolved organic matter plays an important role in for example river water because an enhanced amount of hydrophobic xenobiotic substances can be dissolved, it works like a natural solvent. It has been difficult to standardize river water since it is big differences in dissolved organic matter between oligotrophic and eutrophic waters, but will certainly be the case in the future. [25]

Tested substances and their properties

In the progress of selecting the surfactants to test many properties were of importance. It was important to select substances whose toxicity from different tests was known, to be able to characterize and compare the results from the screening methods with the standard OECD acute toxicity tests. Several surfactants from different families were selected, see Table 1.

Table 1, Tested surfactants and some important properties.

Surfactant	CMC	Appearance
Ethylan 1005	1,0 g/l	Liquid
Ethomeen T/12	0,01 g/l	Two phase (liquid -solid)
Ethomeen T/15	0,02 g/l	Liquid
Ethomeen T/25	0,03 g/l	Liquid
Ethomeen C/12	0,05 g/l	Liquid
Ethomeen C/15	0,04 g/l	Liquid
Arquad 2C-75	0,02 g/l	Liquid in 25 % IPA
Cocobenzylamin+1EO	N/A	Liquid
AG 6202	14 g/l	Liquid in 40 % water

The first one to be chosen was the non-ionic surfactant Ethylan 1005, with three EO-chains. This surfactant was used as the 50 % reference in the Red beet root bioassay test and is a non toxic alcoholetoxylat.

It was also interesting to study a product family to compare and observe influence of number of EO chains and length of hydrophobic chain. The tallow (T) chains consists longer hydrophobic tail (C16-C18) than the coco (C) chains (C12-C14). Ethomeen T/25 has 15 EO groups, Ethomeen T/15 and C/15 have 10 EO groups, and Ethomeen T/12 and Ethomeen C/12 have 2 EO groups.

AG 6202 is a non-ionic sugar surfactant and was tested in order to test a completely non toxic surfactant.

Arquad 2C-75 is a cationic surfactant and was tested because it is a very toxic product but also because it is important to find a way to characterize cationic surfactants as a group since they often are toxic. The product consists of 75% Arquad 2C and 25% IPA but the tested substance was 100% Arquad 2C.

Many surfactants are non-soluble in water, which causes difficulties to test in aquatic environments. To be able to solve this problem test with the non-water soluble surfactant Cocobenzylamin+1EO were done.

Toxicity table of the tested surfactants

In Table 2 below the available OECD standard results from the tested surfactants from algae (OECD 201), daphnia (OECD 202) and fish (OECD 203) are represented.

Table 2, In the table below of the OECD 201, 202 and 203 toxicity values are presented for the tested surfactants. These values are important to be able to compare the screening results and conclude if the method used is good or not. Nv means that a new test with a different value was done. (1) = sample was done in OECD fresh water. (2) = sample was done in OECD marine water.

Surfactant	Algae (1) ErC50 72h	Daphnia (1) EC50 48h	Fish (1) LC50 96h	Fish (2) LC 50 96h
Ag6202	306	>98	>310	558
Ethylan 1005	8,4	3,6	13	18,8
Ethomeen T/25	1,26	1,94	N/A	N/A
Ethomeen T/15	0,24	0,31	N/A	N/A
Ethomeen T/12	0,04	0,043	N/A	N/A
Ethomeen C/12	0,107	0,84	0,3	N/A
Ethomeen C/15	0,24	1,41	0,66	N/A
Arquad 2C-75	0,038	N/A	0,26	N/A
Cocobenzylamin + 1 EO	N/A	N/A	N/A	N/A

Most data is available from test carried out in fresh OECD media with daphnia and algae. Unfortunately, no toxicity value on the T-Ethomeens are available for fish tested in OECD media, and an comparison between the toxicity values from the tests carried out in OECD media will therefore be more difficult. It is also not possible to compare different species toxicity in different waters since the kind of aquatic environment effects the toxicity result. If a comparison between the toxicity of Ethylan 1005 to fish in fresh OECD media with marine OECD media is done, it can be seen than the toxicity is lower in marine waters. This is typically and it is therefore important to do toxicity comparisons for a specific species in one kind of water. As seen in the table as well, not many tests are performed in marine OECD media and the correlations to the screening methods will therefore be based on test done in fresh water OECD media.

The values of the tested surfactants, if the fresh water tests OECD 201 and 202 are compared, the sensitivity for daphnia is greater for AG 6202 and Ethylan 1005. Daphnia and algae are equally sensitive to the T-Ethomeens and algae are more sensitive to the C- Ethomeens. As seen in the table the values from OECD 201 and OECD 202 preformed in fresh OECD media, not differs that much and places the surfactant in almost the same toxicity order. AG 6202 is the least toxic one, followed by Ethylan 1005, according to all test results in the table above. The Ethomeen are placed in similar toxicity order, according to OECD 201 and 202. The only difference is that algae are equally sensitive to C/15 and T/15 but daphnia is more sensitive to Ethomeen T/15 than Ethomeen C/15 and swaps places between Ethomeen C/12 and Ethomeen T/15.

In order for a substance to be non toxic, all values from these tests have to be above 1mg/l. Because of this, all surfactants, besides Ethomeen T/25, Ethylan 1005 and AG 6202, are toxic.

Standard methods according to OECD guidelines

OECD have standardized many test in order to ensure that the test are preformed in the exact same way to guarantee a reliable result used for registration at REACH, the European Community Regulation on chemicals and their safe use. The tests described below are the ones relevant for this paper.

Algae acute toxicity testing, OECD 201

The aim of this test is to determine the effect of the test chemical on the growth of freshwater unicellular green algae and/or cyanobacteria. For test with green algae it is recommended that fast growing green algae are used (e.g. *Selenastrum capricornutum*, *Scenedesmus* or *Chlorella vulgaris*). Exponentially-growing cultures of the selected species prepared in OECD media are then exposed to various concentrations of the test substance over several generations under defined conditions. The inhibition of growth in relation to a control culture is determined over a fixed period (72 or 96h). The cell concentration in the control cultures should have increased by a factor of at least 16 within three days for the test to be valid. [26]

The mean value of the cell concentration for each test substance concentration and for the controls is plotted against time to produce growth curves and achieve the result.[26]

The results from algae can be presented as E_bC_n or E_rC_n . Toxicity to algae measured as growth inhibition is expressed as Effect Concentration (EC_n) values. The EC_n values are the concentrations of the test substance showing n% reduction in either growth (E_bC_n refers to the increase in cell concentration (i.e. biomass) over the test period) or specific growth rate (E_rC_n refers to the rate of increase in cell concentration per unit time over the test period) relative to the controls. In Europe the ErC_{50} is common and in USA EbC_{50} is common. Depending on the test results obtained, the Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) can also be determined. The LOEC is defined as the lowest tested concentration at which growth is significantly inhibited as compared to the control. The NOEC is defined as the highest tested concentration at which growth shows no significant inhibition relative to the control values and the tested concentration next lower than the LOEC.[27]

Daphnia magna acute immobilisation test, OEDC 202

The aim of this test is to determine the effect concentration (EC_{50}) of a test chemical on the test species daphnia magna. In this test the test chemical effect on the swimming capability of daphnia is tested in a range of concentrations prepared in OECD media. Certain concentrations result in certain percentages of daphnia being no longer capable of swimming (immobilized) after the test time (24h or 48h). For the test to be valid no more than 10 % of the daphnia should have been immobilized or trapped at the surface of the water. The test species should be *Daphnia magna*, or any other suitable daphnia species, not more than 24 hours old at the beginning of the test. The daphnias are cultured in the laboratory and at the test day they should be apparently healthy and with a known history.[23]

When the test is done the percentage immobility at the test time is plotted against concentration on logarithmic-probability paper. The EC_{50} for the appropriate exposure and the confidence limits ($p = 0.95$) is determined. [23]

Fish acute toxicity test, OECD 203

The aim of this test is to determine the concentration at which 50 % of the test species are dead (LC50). The test species is fish and there are several to choose from, for example Zebra fish, guppy or rainbow trout. The fish used in the test should be in good health and free from any apparent malformation and must be held in the laboratory for at least 12 days before the test. The chosen test species are then exposed to a range of concentrations preferable for 96 h. During the test mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 % of the fish (LC50). Fish are considered dead if there is no visible movement (e.g. gill movements) is seen when the caudal peduncle (where the tail fin is attached) is touched. [24]

In order for the test to be valid the mortality in the control should not exceed 10 % (or one fish if less than ten are used) at the end of the test and constant conditions should be maintained as far as possible throughout the test and, if necessary, semi-static or flow-through procedures should be used.[24]

The logarithm of the increasing percentage mortality for each exposure period (24, 48, 72, 96) is plotted against the logarithm of the concentration. The LC50 value for the appropriate exposure period and the confidence limits ($p = 0.95$) is determined. [24]

Lemna growth inhibition test, OECD 221

This test is one of a series of tests that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances. Plants of the genus Lemna ,Lemna gibba (in the US) and Lemna minor (in Europe and Canada), are allowed to grow as monocultures in different concentrations of the test substance over a test period of seven days. It is important to use a specific cloned culture to minimize genetic differences.[28]

The aim of the test is to quantify substance-related effects on vegetative growth over this period based on the number of leafs and evaluation of biomass (total frond area, dry weight or fresh weight). To quantify substance-related effects, growth in the test solutions is compared with the growth in the controls. The concentration that causes a specified percentage of growth inhibition (e.g. 50 %) is determined and expressed as the EC50. In addition, LOEC and NOEC may be statistically determined.[28]

Screening tests

Screening tests are done to get an indication of the toxicity and from this predict the OECD standard test results, for example by doing a range finding test. It is also possible from some tests to screen for toxicity by achieving a yes or no answer (yes- toxic, no- nontoxic).[4,25] The described screening tests below are the ones evaluated during this paper.

Root elongation test

The root elongation test is one of the standard tests that have been developed by the office of prevention, Pesticides and toxic substances, United States Environmental Protection Agency, for use in the testing of pesticides and toxic substances. The root elongation test is a root growth inhibition test that can use different kind of fast growing seeds as test species to calculate an EC10 or an EC50, for example *Cucumis sativus* (cucumber); *Lactuca sativa* (lettuce); or *Glycine max* (soybean).[29]

This test is intended to use in developing data on acute toxicity of chemical substances and mixtures but can also be used as a screening tool. The test is designed for water soluble test chemicals but if solubility problems occurs with non-water soluble surfactants, a solvent that is non toxic for plants can also be used in this method if necessary [29].

This test is a growth inhibition test that assumes that growth is dependent on the dosage of the toxicant. Because of this, the toxicant is tested at different concentrations to observe a difference in growth in a dose-response manner. The test procedure is very simple and not much equipment and laboratory space is needed. Seeds are put in an appropriate test plate in contact with toxicants prepared in a concentration interval. When 65 % of the control seeds have germinated and developed roots that are at least 20 mm long, which often are after 96 h, the test ends. The exposure period may be shortened if data suitable to establish the test solution concentration series for the definitive test can be obtained. When the test is done the roots that have elongated are measured, from the transition point between the hypocotyl and the root to the tip of the root, with a ruler. Means and standard deviations are then calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves. [29]

The purpose of the test is to determine the concentration-response curves for the tested surfactants in order to get an EC10 and EC50 result and their 95% confidence limits, for seed germination and root elongation.[29]

Aquatic plant test

This test was developed in order to fast and easy screen surfactants for acute toxicity. Simplicity, minimal preparation and ready-to-use for everyone were in mind when the method was established. Aquatic plants that have water as their natural environment were for that reason used as test species, but instead of study growth, which often is done in plant tests, visual appearance of the plant in the surfactant solutions was the purpose.

When growth or no growth is the endpoint of the test it is very important to give the plant the right nutrition and light source. Therefore, OECD media is not necessary to use for this test, since decay and visual appearance of the plant in the aqueous solution instead are studied. [28] There are also

other benefits not to use OECD media, algae growth is minimized and time consuming preparation is limited.

In the aquatic plant test a number of concentrations are prepared to study the dose response. One aquatic plant is put in each concentration prepared in an appropriate sized sample flask and left in regular desk light during the test time. The plant is cheap and bought from a regular pet store (Arken Zoo, Nordstan).

When the plants are put in the flasks the differences in appearance is evaluated. With appearance means visible changes, for instance changes in the solution or the plants color, loss of leaves or loss in freshness etc. The assumption within this method is that the more visual differences from the control is observed, the more toxic is the tested substance or mixture.

Since it is hard to tell when the plant is dead or have been affected a certain amount, without special equipment, it will be difficult to calculate an EC50. Even though the plants will gradually change due to increasingly dose response a dose response curve will be hard to construct since it is impossible at this point to know how much it has changed. The results will instead be presented as an effect interval from not affected to dead.

Microtox

Microtox is an established micro scale biomonitoring tool in environmental toxicology, see setup in Figure 5. It is an eco-toxicological screening method designed to detect aquatic toxicity, monitor changes in toxicity and predict toxicity results of other toxicity tests.[3,30] This screening test uses a luminescent marine bacterium, *Vibrio fischeri*, as its test species and is a unique bacterial bioluminescent inhibition assay. The *Vibrio fischeri* is a cloned culture which diminishes possible genetic differences, as well as thoroughly ensuring good quality control. Multiple Microtox toxicity tests of a compound have showed excellent replicability which probably is a result of the well-standardized organisms. Since the bacteria are freeze-dried under vacuum in vials, no culturing of the test medium is needed [3]. No pre-culturing is required since Microtox is available as ready to use, and because measurable light emission begins immediately after water activation of the lyophilized bacteria strain. [3] This method, that takes about 5% of the actual work involved in the standard procedures [16,31], is primarily used as a quick alternative to acute toxicity tests with fish (OECD 203) or daphnia (OECD 202) but because all test media and glassware are pre-packaged, standardized and disposable in Microtox it uses minimal quantity and the cost and toxic waste is reduced [4]. Both pure substances and blends of substances can be used to reveal synergetic effects.[32,34]

Even though the test species of Microtox is not as sensitive as *Daphnia* and algae, which detect toxic compounds earlier [31], it is recommended as a single test in the eco-toxicity screening phase. [33] Differences between the sensitivity of fish and Microtox are within one order of magnitude for some measured chemicals, for example for cadmium nitrate. [31]

At AkzoNobel in Stenungsund this method can, among others, be used to roughly estimate aquatic toxicity for different surfactants. The samples will in time before the test, be prepared in deionized water in the laboratory. As the samples are in produced and assembled in advance, distractions and inadequacies such as soil and sediment are avoided.

Since problems with solubility often can occur with surfactants, solvents to improve solubility may be necessary used to ease the process. Solvents, for example DMSO, acetone, methanol and ethanol may therefore be necessary to solubilise certain non-water-soluble products. First, however, controls with small amounts of solvents alone are tested in order to investigate the toxicity levels of the specific solvent. When the toxicity levels are known, it might be possible to test the non-water-soluble product with a small amount of solvent ($\leq 1\%$) and investigate the toxicity level of the product. All bio-monitoring species are sensitive to organic solvent toxicity, it is therefore very important to keep the amount below the Microtox reagent detectable toxicity level. [25]

The endpoint of the test is to screen for aquatic toxicity. The toxicity is expressed as the concentration causing 50% inhibition of luminescence (EC50) and the concentration which reduces light production by 10% (EC10) [31]. As the toxicants concentration increases, the bacterial light emission decreases in a dose-dependent manner [3] and according to the standard procedures the EC10 and EC50 values are determined by least square fitting of a line to the prohibit transformed percent inhibition of luminescence versus the log concentration points of the duplicate tests.[31] A illuminometer and supporting computer software with a standard log-linear model is used to calculate the result. All EC50 values and EC10 values are expressed as ppm or mg/l with 95% confidence interval. [3,34]

In order to get as good EC50 as possible it is preferable to do a range finding test were a different start concentrations are tested. When an appropriate start concentration is found the confidence range of the results should be as small as possible and the slope of the resulting curve should be close to one. It is also important to keep a good intensity of the control during the test.[32]

The picture below (Figure 4) shows how a desirable Microtox data report should look like. As the concentration gets higher the luminescent bacterium loses light production and a straight line is formed by the log-linear model if a suitable highest concentration of the dilution series was prepared. When the loss between the different concentrations are in the same range as in the picture below it is a good chance that the resulting curve looks like in Figure 4. I0 is the initial reading light intensity result (t=0) and IT is the light intensity result from the 15 min reading (t=T). As seen in Figure 4 the confidence limit is narrow as well as has a slope close to one. The control did not lose much intensity during the test, I0/IT, which also is desirable. [32,34]

MICROTOX DATA REPORT

FILE NAME: ETOH-1.K15

TEST DATE: _____

Investigator: _____

TEST TIME: _____

Sample Description:

Approved by: _____

Procedure: BASIC

Osmotic Adjustment:

Initial Concentration : 16 ppm

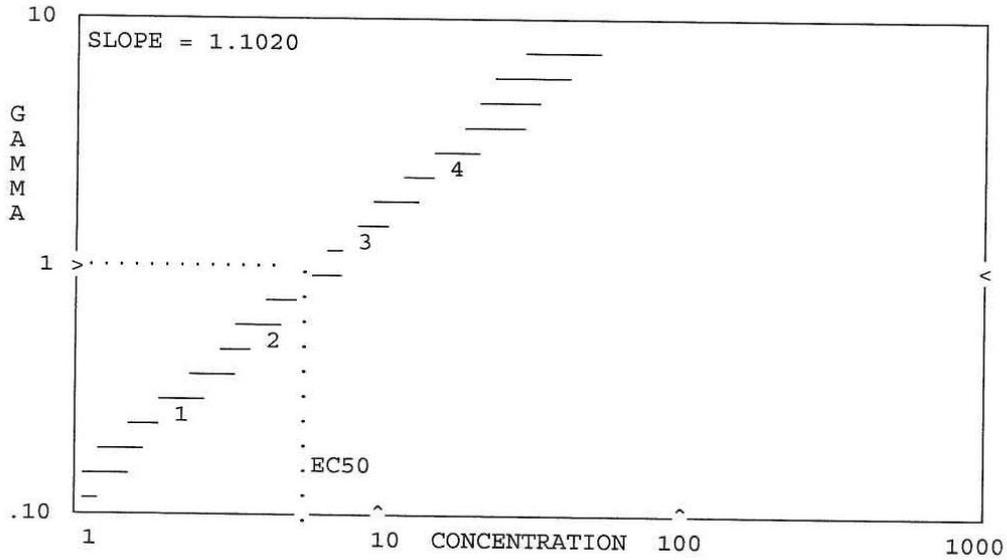
Dilution Factor : 2

Test Time: 15 minutes

Concentration Units: ppm

NUMBER	I0/IT	CONC.	GAMMA
1	109.51/ 90.88	2.0000	0.31151#
2	109.91/ 74.49	4.0000	0.60593#
3	107.78/ 48.33	8.0000	1.42721#
4	111.47/ 30.43	16.0000	2.98696#

CONTROL IT/I0 = 122.76/112.79
CORRECTION FACTOR = 1.0884



EC50 5.9421 (95% CONFIDENCE RANGE: 5.3327 TO 6.6212)

Used for calculations

Figure 4, This picture is an example of how a good Microtox data report can look like.



Figure 5, Microtox equipment present at AkzoNobel in Stenungsund.

Red beetroot bioassay

The red beet root bioassay is a screening toxicity method that uses red beetroots as test species in order to measure the cell disruption of the membrane by the released color (betanin) in a 0,1% (1g/l) surfactant solution. The released betanin from the vacuole in the red beet root cells are then measured with a Uv-vis spectrophotometer and compared with the controls. The outcome of this method is percentage of disrupted cells where the reference, 1% HCl in methanol, causes 100% cell disruption and the second type of reference, Ethylan 1005, causes 50% cell disruption. [35]

The assumption within this method is that the toxic substance affects the cell membranes in the red beet roots cells and the vacuole membrane where the color of the beet root is situated. This breakage causes release of the colored substance betanin, the more betanin that are released from the beet root cells the more toxic is the substance assumed to be.

The amount of disrupted cells is calculated as follows:

$$\frac{A_s}{A_{Ref}} \times 100\% = \% \text{ disrupted cells}$$

Where: A_s – the absorption of surfactant solution, A_{Ref} – the absorption of the reference solution; 1% HCl in methanol.[35]

Screening tests – Methods, results and discussion and conclusion

Four tests was tested as a eco-toxicity screening tool

- Root elongations test
- Microtox
- Red beet root bioassay
- Plant test

If a successful screening result could be achieved, the screening results were compared to the results from the OECD standard methods 201, 202 and 203. No quantitative measurements were done during these tests and the results are only good for screening, not registration.

Method

Root elongation test

Test procedure

When performing the root elongation test it is recommended to first start with a range finding test, in order to roughly estimate at which concentration interval the toxic effect lies in (for example 1g/l, 100mg/l, 10mg/l, 1 mg/l etc). When this test is finished and the effect interval is determined it is recommended to do a definite test. During the definite test the seed of each species tested should be exposed to at least 6 concentrations, instead of 4 during the range finding test, of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g. 2, 4, 8, 16, 32, and 64 mg/L).

A range finding test was done at the following concentrations; 1g /l, 100mg/l, 10mg/l and 1mg/l in deionized water. Four surfactants in for duplicates were tested at these concentrations; AG 6202, Ethomeen C/12, Ethomeen C/15, Ethomeen T/25. Additional four controls in deionised water were also included in the test.

Preparation of the stock solutions and range finding concentrations

To prepare the stock solutions 0,10 g were put in a volumetric flask of 100 ml and filled with deionized water. The stock solutions were left stirring until the surfactants were dissolved in the water. AG 6202, Ethomeen C/15 and C/25 are soluble in water and had transparent stock solutions, they became homogenous at once. The stock solution of Ethomeen T/12 was whitish but was considered homogenous after 2 hours stirring on a magnetic stirrer. When the stock solutions were done they were used to prepare the range finding concentrations. For the highest concentration the undiluted stock solution (1g/l) was used. To the second concentration 10 ml of the stock solution was put in a 100 ml volumetric flask and filled to the mark with deionized water. To the third concentration 1 ml of the stock solution was transferred to a 100 ml volumetric flask and filled with deionized water to the mark and to the fourth concentration 0,1 ml of the stock solution was put in a 100 ml volumetric flask and filled with deionized water to the mark.

A filter paper,9 cm wide, and approximately 10 seed were put in the Petri dish, without touching them in any way. The seeds were pored directly from the seed bag they were delivered in. When the filter paper and seeds were prepared and the Petri Dishes were marked with surfactant,

concentration and sample number, 5 ml of each surfactant concentration were poured in, see Figure 6. The Petri dishes were randomly placed in a dark box for 96 h, or when 65 % of the control seeds had germinated and developed roots that were at least 20 mm long. The roots were after 96 h measured with a ruler (mm), from the transition point between the hypocotyl and root to the tip of the root.

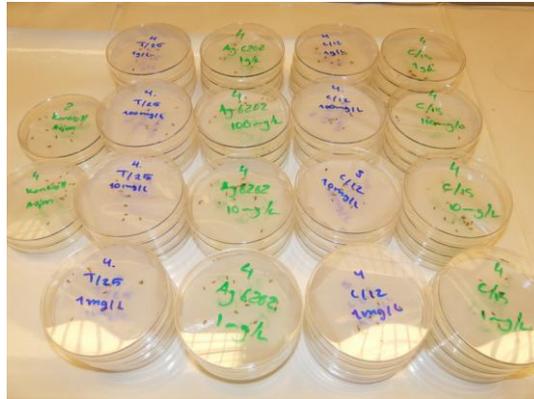


Figure 6, Picture of the Petri Dishes from the Root elongation test before incubation in dark for 96 h.

Aquatic plant test

Preparation of the stock solutions

During the aquatic plant test the following surfactants were evaluated; AG 6202, Ethylan 1005, Ethomeen T/25, Ethomeen T/15, Ethomeen T/12, Ethomeen C/15, Ethomeen C/12. All stock solutions were prepared at 1,0 g/l in tap water. Two solvents diluted in tap water were evaluated; ethanol and IPA and two mixtures of surfactant/solvent diluted in tap water were evaluated; Cocobenzylamin+1EO in IPA and Cocobenzylamin+1EO in ethanol. The stock solutions for Cocobenzylamin + 1EO 1g/l was prepared in 100% solvent (IPA or ethanol). All stock solutions were left stirring 2 hours until the foam had disappeared and a homogenous solution was formed.

Test species

Three plants that had water as their natural environment and not require soil or sand were tested; Hygrophilia polysperma, Cabomba Aquatica Aquatica and Elodea Canadensis, see Figure 7. The plants were after washing used as they were, no tissue was removed. To minimize variations it was important that the plants had the same history, looked green and fresh, were in the approximately the same sizes and had approximately the same number of leaves, for minimizing big surface differences. All three plants were considered sensitive plants that were expected to give a faster result than a non sensitive, more robust, plants. All three plants require no more than regular daylight, are supposed to be pleasant in the pH range 5-9 and regular room temperature. [36, 37]



Figure 7, the three aquatic plants tested in the plant test is seen in this picture, from the left; Cabomba Aquatica, Hygrophilia Polysperma and Elodea Canadensis.[37, 38, 39]

Test procedure

Five different tests were done to test different plants (test species) and dose response. Not all surfactants and solvents were included in all tests. The first test was done with two surfactants and more test substances were tested as the method was developed. Solvents were tested to study the solvent effects and the ability to test non water soluble surfactants, see test five. In table 3 all tests and the tested concentrations are represented.

No pH adjustments were done for any of the test solutions since the pH not was outside the optimum for the investigated plants (5-9).

When the test concentrations were ready the plants were carefully cleaned and put in the 500 ml sample flasks. The samples were then left under a fluorescent desk light for 96 h in room temperature without cap. The plants were photographed and the differences in the appearance of the plants were evaluated at 0, 24, 48, 72 and 96 h to conclude an effect interval. At least two controls were included in the study, see Figure 8. When unexpected root growth was detected during test three performed with Elodea Canadensis the test time was expanded to 7 days, as OECD 221, and the root growth was studied. The number of roots that had elongated was counted.



Figure 8, Reference for the first Hygrophilia polysperma test to the left and the reference for the first Cabomba Aquatica test to the right.

Microtox

Aqueous and organic samples were prepared in accordance with the basic dose response design; 1 control and 4 test concentrations in a 1:2 dilution series. Some samples may however require an extended range protocol using eight to ten dilutions and two controls, to improve the result. This was during the test not necessary.

Preparation of stock solution

When the stock solutions without solvent were prepared 0,5 g of surfactant (100%) were put into a 500 ml volumetric flask. After dissolving the surfactant in distilled water the volumetric flask were filled to the mark. All stock solutions were left stirring 2 hours until the foam had disappeared and homogenous solutions were formed. The following surfactants were tested; AG 6202, Ethylan 1005, Ethomeen T/25, Ethomeen T/15, Ethomeen T/12, Ethomeen C/15, Ethomeen C/12, Arquad 2C-75 and Cocobenzylamin+1EO. Cocobenzylamin + 1EO is not soluble in water and a homogenous stock solution could not be prepared in deionized water. To test oil soluble surfactants the stock solution was instead prepared with solvent. Four stock solutions with 1g/l Cocobenzylamin+1EO was prepared with Ethanol and IPA; 100% Ethanol, 50% Ethanol and 50% deionized water, 100% IPA, 50% IPA and 50% deionized water.

The bacterium is very sensitive to pH changes hence the stock solution were pH adjusted to 7, $3 \pm 0,5$.

Test procedure

Test tubes were placed in the chambers, A1 - A5 and B1 – B5 for the first sample, C1 - C5 and D1 - D5 for the second sample and E1- E5 and F1 - F5 for the third sample, see Figure 9. One test tube was placed in the reagent chamber.

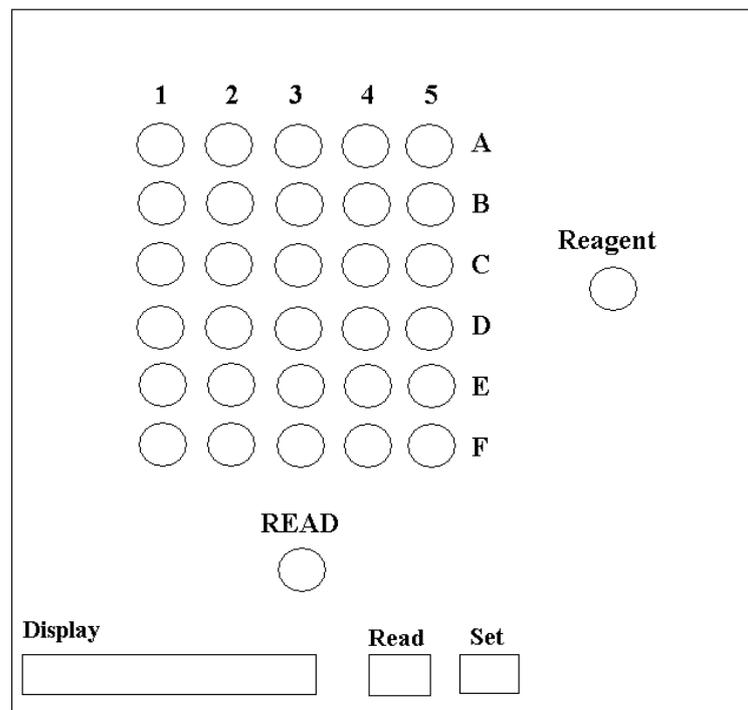


Figure 9, Schematic picture of the Microtox equipment from above.

1 ml Reconstitution-solution was immediately put in the test tube in the reagent chamber for adjustment to the appropriate temperature. The bacterium were taken from the fridge and put in the reconstitution solution in the reagent chamber by taking of the cap and pouring the pieces in the Reconstitution-solution. The test tube was mixed with a vortex mixer.

The dilution series needed for the dose response curve starts from the test tube A5, C5 and E5 were the highest concentrations are prepared. In this method a dilution factor of two was chosen.

Example: if the A5 test tube had a concentration of 4,5 ppm, the test tube of A4 had a concentration of 2,25 ppm, the test tube in A3 a concentration of 1,125 ppm and the test tube in A2 a concentration of 0,5625 ppm. The test tube in A1 is the control and only consists of dilution solution.

To start the test the concentrations in A5, C5 and E5 were prepared.

1 ml diluent were put in the A, C and E rows while 0,5 ml were put in B, D and F rows. The sample to be toxicity screened was put to A5, C5 and E5, see Figure 9. Depending on the concentration tested different amount of the stock solution prepared in deionized water was added, 0,25 ml osmotic adjustment (MOAS) was added and the test tube was filled with diluents up to 2,75 ml volume.

Example: Ethomeen T/25 is tested with a highest concentration of 45 ppm. 0,25 ml of 1 g/l stock solution; 0,25 ml MOAS; 2,25 ml diluents is added to A5. After the highest concentration is prepared the dilution can start.

When A1, C1 and E1 are set with the right concentrations they are properly mixed with a pipette. To make the 2:1 dilution 1 ml is transferred from A5 to A4 and properly mixed with the pipette. 1 ml is transferred from A4 to A3 and properly mixed with a pipette and 1 ml is transferred from A3 to A2 and properly mixed with a pipette. No sample is put in A5, which is the control. The same procedures are done for the sample rows C and E.

The computer was started and the file name, concentration and dilution factor were put in.

When the dilution series is done the bacterium is added. The bacterium is very sensitive and is recommended to be used no shorter that 20 minutes before and 2 hours after they have been put out of the fridge. 10 µl bacterium is added to the cells B1-B5, D1-D5, and F1-F5 with an automatic pipette and mixed with a vortex mixer.

When the bacterium is added the initial reading is done. Each test tube is read by putting it into the reading chamber and pressing the read button, starting from B5- B1, D5-D1 and F5-F1. The computer tells you to start and change test tube. After the initial reading is done 0,5 ml sample is transferred from A1 to B1, from A2 to B2, from A3 to B3, from A4 to B4 and from A5 to B5 and mixed with a vortex mixer. The same procedure is done for all rows. When exactly 5 min have passed from the initial reading the 5 min reading is done and when 15 min passed from the initial reading the 15 min reading is done. The procedure is the same as the initial reading.

The dose response curve is printed and the EC50 is read out.

Red beet root bioassay

Preparation of the stock solution

All stock solutions that were tested were prepared at 1,0 g/l in deionized water. Since no further dilution was done it was important to be as accurate as possible. First 0,5 g substance was put in a 500 ml volumetric flask, after that some deionized water was added and the solution was left stirring until a homogenous solution with no foam was formed. The flask was filled to the mark and left stirring until the test was to start. The following surfactants were tested; AG 6202, Ethylan 1005, Ethomeen T/25, Ethomeen T/15, Ethomeen T/12, Ethomeen C/15, Ethomeen C/12 and Arquad 2C-75. Two non surface active polymers; PEG-400 and Poly glycol AM/20 20, and one non surface active toxic substance (Formaldehyde) was also tested at 1 g/l.

Solutions with water and solvent were also prepared to test the red beet roots solvent effect and ability to test less water soluble substances. Since no further dilution of the 1 g/l stock solution is done it was not an option to prepare a non water soluble substance in 100% solvent. Cocobenzylamin+1EO was instead prepared in 10% IPA/10% Ethanol and 90% deionized water and 1% IPA/1% Ethanol and 99% deionized water. Samples with only solvent and no surfactant were also tested in the same concentrations to eliminate the solvent effect.

Test procedure

Ecologically cultivated beet roots were used as test species for the test in order to avoid all possible previous chemical impact. The roots were sliced in 2 mm thick uniform slices with a regular kitchen machine and punched into 1 cm diameter "tablets". The red beet root "tablets" were carefully washed several times for removal of betanin, released from the beets during the slicing and punching, and left in water in the refrigerator during night for removal of additional betanin from the destroyed cells. After preparation of the test species the stock solutions were prepared.

10 "tablets" were put in a Petri dish, 5 ml of a 0,1 % surfactant solution (Table 4) was poured in and the Petri dishes were moved to a thermostat, where they were incubated for 3h at 30°C (in this method one concentration is prepared instead of an concentration interval, all surfactants are tested at that same concentration). Duplicates of all substances were done. The weight of Petri plate with "tablets" and test solution were measured gravimetrically before and after incubation. After incubation 0,5 ml from each plate was mixed with 4,5 ml deionized water and an absorption of the solutions were recorded by Uv-Vis spectrometer at 535nm.

Table 4, List of tested substances used in the red beet root bioassay test.

<i>Substances tested</i>	<i>Concentrations</i>
<i>References</i>	
HCl in methanol (100%)	1 vol% (2,70 ml/100 ml)
HCl in water	1 vol% (2,70 ml/100 ml)
Ethylan 1005 in water (50%)	0,1 wt% (1,00 g/l)
<i>Surfactant solutions</i>	
Ethomeen T/25 in water	0,1 wt% (1,00 g/l)
Ethomeen T/15 in water	0,1 wt% (1,00 g/l)
Ethomeen T/12 in water	0,1 wt% (1,00 g/l)
Ethomeen C/15 in water	0,1 wt% (1,00 g/l)
Ethomeen C/12 in water	0,1 wt% (1,00 g/l)
Arquad 2C-75 in water	0,1 wt% (1,00 g/l)
Ag6202 in water	0,1 wt% (1,00 g/l)
	0,1 wt% (1,00 g/l)
<i>Non surface active polymer solutions</i>	
Poly glycol AM/20 20	0,1 wt% (1,00 g/l)
PEG - 400	0,1 wt% (1,00 g/l)
<i>Solvents</i>	
Ethanol in water	10 vol% EtOH in water
	1 vol% EtOH in water
IPA in water	10 vol% IPA in water
	1 vol% IPA in water
<i>Surfactant solutions with solvent</i>	
Cocobenzylamin + 1EO	1wt% substance (1,00g/l) and 10 vol% EtOH in water
	1wt% substance (1,00g/l) and 1 vol% EtOH in water
	1wt% substance (1,00g/l) and 10 vol% IPA in water
	1wt% substance (1,00g/l) and 1 vol% IPA in water
<i>Toxins</i>	
Formaldehyde	1wt% (1,00 g/l)

Results and discussion

Root elongation test

The measured seed growth in the range finding root elongation test were much lower than the average controls mean, found in the literature, which is about 22 mm, and what was recommended to stop the test at 96 h. Approximately one seed in each plate did root elongate a couple of millimeters (see Appendix 1) after 96 h. However, since not the recommended growth for the test was observed it was no point to proceed with a definite test and calculate EC50, mean or variance.

It is at this point unknown why growth during the root elongation test performed differs so much from the mean average from test done before, but there can be several reasons. One idea is that the filter papers that were used in the Petri Dish to ensure a growing environment for the seeds were toxic. Another idea why growth did not occur is that the seeds qualities were bad in some way. However, an evidence of that was that after 96 h, when the growth was studied, mould was detected in some dishes. The mould may have prevented growth in the present dishes but since mould not was seen in all dishes this cannot explain the bad result for the hole test.

Aquatic plant test

Since the aquatic plant test was performed for the first time, different plants were tested as appropriate test specie. Three different aquatic plants were tested; *Hygrophilia polysperma*, *Cabomba Aquatica* and *Elodea Canadensis*, see Figure 7.

During the tests the same type of sample flask with the same history to minimize differences within the samples were used. This was important because when the test is performed without quantitative measurements it is important to use as little differences in surface area as possible. The reason for that are a possible adsorption of surfactants and a loss of certain concentration on the additional surfaces, especially for cationics. Therefore aquatic plants which do not require soil (additional surface) are mostly preferable during this test.

Test 1

To detect visible changes the first test was done at high concentration interval (10g/l-0,1g/l), see Table 3.

Almost immediately after putting *Cabomba Aquatica* in the surfactant solution the sample solution of the highest concentration of Ethomeen C/15 became green, probably because the surfactant caused the plant to release chlorophyll. The effect was not a washing effect since all plants were washed before they were put in the test solution, to eliminate this. After 24 h the green solution was more yellow and after 48 h, see Figure 10 below, the solution was completely yellow. This color change is probably due to a chemical reaction with chlorophyll.

The *Cabomba Aquatica* plants in the Ethomeen T/12 solution had not the same fast response as the plants in the Ethomeen C/15 solution, however after 48 h the plant became browner and the solution changed its originally whitish color to more light green. The solutions in the highest concentration had also separated to two phases which is a problem when testing for toxicity response since the plant not will be exposed to the correct concentration. To avoid this problem the test should be performed at lower test concentrations, as in the remaining tests.

After 48 h all Cabomba Aquatica plants look affected, even though the solution did not turn green in all cases, see figure 10.



Figure 10, Picture from the first Cabomba Aquatica test , Ethomeen T/12 (1g/l) to the left and Ethomeen C/15 (1g/l) to the right with the control in the middle.

From the results of the first test with Hygrophila polysperma it is clear that the plants change color from green to brown after 48 h, see Figure 11, however the color of the solution was not affected. Hygrophila polysperma plant was therefore used in the next test since it is beneficial to have transparent solution, since it makes it easier when doing a visible judgment. It is also necessary to use a concentration interval since a gradual dose response is wanted.



Figure 11, Pictures from the first test using Hygrophila polysperma. As seen in the picture both plants are dead. The picture to the right is Ethomeen C/12 (0,1g/l) and the picture to the left is Ethomeen T/12 (0,1g/l). The picture in the middle is the reference.

Test 2

The second test was a continuation of the first Hygrophilia polysperma test. Additional concentrations in a more narrow interval were used.

When the plants were placed in the flasks some plants were too long and had a couple of leaves above the surface. These leaves were ignored during the test evaluation because they are not considered affected of the aquatic environment.

No immediate changes were seen. After 24 h some difference was detected; leaves at the higher surfactant concentrations had started to get dark brown in the edges. No visual color change was seen in the solution. In some concentrations the plant had many leaves at the water surface. These leaves were more effected than others, probably because surfactants are surface active and a higher concentration of surfactants is present. After 48 h the affect was even more visible and increased even more with time, especially for the Ethomeen C/15 solutions that were prepared at higher concentrations. Ethomeen C/15 was prepared at higher concentrations than Arquad 2C-75 since it is known that Arquad 2C-75 is more toxic, see Table 2. No affect was seen on the plants in any of the concentration with AG 6202. This result shows that AG 6202 has an EC50 over 100mg/l, which is in accordance with the OECD standard results.

After 96 h no more visual effect that would make a big difference for the screening result was seen. For some surfactants it would be possible to get a screening result faster but since some surfactants have a slower response (e.g. Arquad 2C-75 have a slower response than Ethomeen C/15) the test time is recommended to 96 h.



Figure 12, Picture of Arquad 2C-75 series with the highest concentrations from left to the right; 10mg/l, 5mg/l, 2,5mg/l, 1mg/l and 0,5 mg/l after 96 h.

As seen in picture 12 and 13, the affect on the plant is gradually changed with the concentration both in the case for Ethomeen C/15 and Arquad 2C-75, which is a covet dose response series for a visual affect. According to the obtained results the EC50 for the Arquad 2C-75 are between 1 and 2,5 mg/l, since all plants in the concentrations above 2,5 mg/l are considered dead. For Ethomeen C/15, Figure 13, EC50 is below 10 mg/l but higher than 1mg/l. As seen from the picture all plants in the solutions

with higher concentrations are dead. If more accurate result is needed concentrations between 10 mg/l and 1 mg/l in a more narrow interval has to be used (e.i. a definite test).



Figure 13, Picture of dose response for the Ethomeen C/15 series. The highest concentration is to the left and the lowest concentration to the right; 100 mg/l, 50 mg/l, 25 mg/l, 10mg/l, 1mg/l.

It is always recommended to use as sensitive species as possible, since the species with a high sensitivity provide faster results, which is the case for the OECD standard methods. Arquad 2C-75 is a very toxic surfactant and has an EC50 of 0,038 mg/l according to OECD 201. In order to get EC50 interval effect as close to 0,038 mg/l as possible, it was decided to use a more sensitive plant.

Test 3

Since *Hygrophilia polysperma* not gave as sensitive result as thought could be achieved, the third test was done with two different plants. Two species that were thought to give a lower dose response than the *Hygrophilia polysperma* plant was tested; *Cabomba Aquatica* and *Elodea Canadensis*.

To extend the test, six surfactants were evaluated in similar concentration intervals.

The third test did not have as visible results as test 2, where *Hygrophilia polysperma* was used, even though *Cabomba Aquatica* and *Elodea Canadensis* are regarded as more sensitive plants. The surfactant influence on *Cabomba Aquatica* resulted in a less fresh looking plant which more easily moved in the sample flask. This test gave an obscure visual effect for Arquad 2C-75 in 1 mg/l concentration solution. The drawback of the use of this plant is that the changes in appearance are not obvious to not experienced people.

In the test with *Elodea Canadensis* no direct change was seen. Some plants became brown but the results were very random reproducible compared to results obtained from the test with *Hygrophilia polysperma*. After 96 h root elongations were detected in some of the sample flasks. The test was therefore extended to 7 days and the growth was instead of visual effect studied, see test 4.

Therefore, *Cabomba Aquatica* or *Elodea Canadensis* are not recommended to be used as test species in the aquatic plant test since the visual appearance is vague.

Test 4

In this test growth and growth inhibition were studied since growth was detected in several flasks during test 3, who used *Elodea Canadensis* as test species.

After 7 days many plants had started root elongate, see table 5-10 and Figure 14. The test time was decided to 7 days since it is a common test time for plant growth, for example in the Lemna minor test (OECD 221). It was found that some *Elodea Canadensis* plants (especially in the lower concentrations tested and in the non toxic solutions) grew many roots. Some roots were a couple of mm and others up to 10-15 cm long. As seen in the table 8 the non toxic surfactant AG 6206 showed growth at all concentrations. Since the OECD measured EC50 for this substance is much higher than the concentrations tested this was expected, this is also the case for Ethylan 1005 that showed a similar result, see table 10. For Ethomeen C/15 it is very clear that the three highest concentrations inhibited growth, see table 5, which is the point of a growth test. For Ethomeen T/25 and T/15 the results is not as clear as in the Ethomeen C/15 case since the growth variation is bigger, see table 6 and 7. This phenomenon can be explained by possible lack of required light for good growth. During growth tests the light is very important and is an important requirements for example OECD 201 and OECD 221, where a light incubator is used. To solve the light problem of this test it might be enough to cover the sides inside a box with folia and have a fluorescent lamp inside the seal to reflect the light. This would be a simple light incubator that probably would be enough for screening.

One way to measure growth is to measure the roots. This was the point in the root elongation screening test but also one of the parameters when doing more standardized plant test for example in the Lemna test (OECD 221). Unfortunately this was not done in this test but would be good to do next time if a proper light source is to be used.

Table 5, Growth of roots in the samples prepared with tap water and Ethomeen C/15.

Ethomeen C/15					
Sample no	Concentration	Roots?	Sample no	Concentration	Roots?
101	10 mg/l	No	102	10 mg/l	No
103	5 mg/l	No	104	5 mg/l	No
105	2,5 mg/l	No	106	2,5 mg/l	N/A
107	1 mg/l	Yes	108	1 mg/l	Yes
109	0,5 mg/l	Yes	110	0,5 mg/l	Yes
111	0,1 mg/l	Yes	112	0,1 mg/l	Yes
113	0,02 mg/l	Yes	114	0,02 mg/l	Yes

Table 6, Growth of roots in the samples prepared with tap water and Ethomeen T/15.

Ethomeen T/15					
Sample no	Concentration	Roots?	Sample no	Concentration	Roots?
115	10 mg/l	No	116	10 mg/l	Yes
117	5 mg/l	Yes	118	5 mg/l	No
119	2,5 mg/l	Yes	120	2,5 mg/l	Yes
121	1 mg/l	Yes	122	1 mg/l	Yes
123	0,5 mg/l	Yes	124	0,5 mg/l	Yes
125	0,1 mg/l	Yes	126	0,1 mg/l	No
127	0,02 mg/l	No	128	0,02 mg/l	Yes

Table 7, Growth of roots in the samples prepared with tap water and Ethomeen T/25.

Ethomeen T/25					
Sample no	Concentration	Roots?	Sample no	Concentration	Roots?
87	10 mg/l	No	88	10 mg/l	Yes
89	5 mg/l	Yes	90	5 mg/l	No
91	2,5 mg/l	Yes	92	2,5 mg/l	Yes
93	1 mg/l	Yes	94	1 mg/l	Yes
95	0,5 mg/l	Yes	96	0,5 mg/l	Yes
97	0,1 mg/l	Yes	98	0,1 mg/l	No
99	0,02 mg/l	Yes	100	0,02 mg/l	Yes

Table 8, Growth of roots in the samples prepared with tap water and AG 6202.

AG 6202					
Sample no	Concentration	Roots?	Sample no	Concentration	Roots?
79	100 mg/l	Yes	80	100 mg/l	Yes
81	50 mg/l	Yes	82	50 mg/l	Yes
83	5 mg/l	Yes	84	5 mg/l	Yes
85	2,5 mg/l	Yes	86	2,5 mg/l	Yes

Table 9, Growth of roots in the samples prepared with tap water and purified Arquad 2C-75.

Arquad 2C-75					
Sample no	Concentration	Roots?	Sample no	Concentration	Roots?
129	5 mg/l	No	130	5 mg/l	No
131	2,5 mg/l	No	132	2,5 mg/l	No
133	1 mg/l	Yes	134	1 mg/l	No
135	0,5 mg/l	Yes	136	0,5 mg/l	Yes
137	0,1 mg/l	No	138	0,1 mg/l	Yes
139	0,02 mg/l	Yes	140	0,02 mg/l	Yes

Table 10, Growth of roots in the samples prepared with tap water and Ethylan 1005.

Ethylan 1005					
Sample no	Concentration	Roots?	Sample no	Concentration	Roots?
141	20 mg/l	Yes	142	20 mg/l	Yes
143	10 mg/l	Yes	144	10 mg/l	Yes
145	5 mg/l	Yes	146	5 mg/l	Yes
147	1 mg/l	Yes	148	1 mg/l	Yes
149	0,5 mg/l	Yes	150	0,5 mg/l	Yes



Figure 14, Picture of root growth for sample 143, Ethylan 1005 in a concentration of 10 mg/l prepared in tap water.

The Elodea Canadensis growth inhibition test has good potential to be a screening method for toxicity evaluation if further evaluation is done. This is because no OECD media is required for growth and on account of that, no visible algae growth was detected, but great growth in certain concentrations occurred. However, due to some irreproducible response, more experiments have to be performed before validity of the method will be approved.

Test 5

The fifth test was done with equal concentrations for all tested surfactants, 10 mg/l, 5 mg/l, 2 mg/l and 1mg/l, in order to characterize them by comparison. All available surfactants were tested to correlate as many substances as possible. Since Cabomba Aquatica or Elodea Canadensis did not give a clear visible result in test 3, this test was performed with the plant Hygrophyllia polysperma.

To investigate whether hydrophobic surfactants can be tested with the plant Hygrophyllia polysperma, solvents were also included in this test. IPA and Ethanol were used, since they are the most common solvents used in surface chemistry. The solvent concentrations that were tested were kept low for mainly two reasons. First of all it is necessary to minimize the solvent to be able to simulate nature in the best way, the second reason is to ensure that the test species are not additionally affected by the solvent. The level of solvent was also kept low because hydrophobic substances in general are more toxic than water soluble surfactants since their CMC is lower. Surfactants with low CMC will go faster to the surfaces, especially cationic surfactants, and cause a more toxic effect. It will for this reason not be necessary to test higher amounts of solvent than those tested. Since it is always recommended to use as little solvent as possible, and to prepare a stock solution with as much water as possible is therefore beneficial.

AG 6202, Ethylan 1005 and Ethomeen T/25, Figure 16, 18, 20, are non-toxic and did not affect the plants. These results are in a good agreement with the results obtained by standard OECD results. AG 6202 and Ethylan 1005 have an EC50 above or near 10 mg/l for the sensitive OECD species and since Hygrophyllia polysperma is not as sensitive, the results are credible. Ethomeen T/25 is according to the theory the least toxic one of the three T- Ethomeens tested which can be confirmed by this test since no effect was shown.

Ethomeen C/12 and Ethomeen T/12 are the most affected ones, see figure 22 and 23 below. Ethomeen T/12 and Ethomeen C/12 are two very toxic substances and the result is not unexpected even though Ethomeen T/12 is about 2 ½ times more toxic than Ethomeen C/12 according to the OECD methods 201 and 202. Arquad 2C-75 is also very toxic and is according to OECD 201 equally toxic to Ethomeen T/12. The results for Arquad 2C-75 are not as expected because according to this test it is slightly less

toxic than Ethomeen T/15. Ethomeen T/15 is also a toxic substance but Arquad 2C-75 should have affected the plant more. This can be a solubility affect since the stock solution for Arquad 2C-75 was turbid and the one of the tested surfactants in tap water that was the most difficult to dissolve. It might be good to use some solvent when testing this kind of surfactants to improve the solubility. The result is still ok since the plant is affected and the test gave an indication of that the substance is toxic.

The test also shows that plants exposed to Ethomeen T/15 is more affected than those exposed to C/15, see figure 19 and 21, which is in accordance with the daphnia test. The algae test results for these two substances are more equally but still it is possible that Ethomeen T/15 is more toxic than Ethomeen C/15 because surfactants with longer hydrophobic tail and lower CMC, see table 1, tend to be more toxic. They have equally amount of EO chains but Ethomeen T/15 have a tallow chain which is longer and there by more toxic than the hydrophobic tail of Ethomeen C/15, which is shorter.

IPA and Ethanol did not affect the plant and from this results it might be possible to say that the solvent effect at these concentrations can be neglected, see Figure 24- 27. This is very promising because it makes it possible to test more substances, not only the water soluble ones. In the test concentrations with solvent and Cocobenzylamin + 1EO effect was seen in the three first concentrations. The plants in the two first concentrations were dead and the third one was visible affected, especially in the series with ethanol solvent. Since an OECD acute toxicity result not is present for this substance it is hard to do a comparison but it is possible to say that the solution with surfactant had larger effect than the solvent in water is self.

No change was seen on the references, se Figure 15. Therefore it is possible to say that no changes due to light, temperature or nutrition occurred during the test time.

The results from the test were very clear and it was possible to tell in which interval the EC50 value is present. The Hygrophila polysperma plant test will always give lower toxicity results (compare to OECD standard methods) because the test species is not as sensitive, but with this methods test result it is possible to make a comparison between surfactants. It is also possible to establish a relation between this test and the OECD 201 and 202 tests if more definite tests based on the concentrations in test 5 are done. A visible affect interval as for Arquad 2C-75, see Table 11 below, would be requested in that case.

Table 11, comparison between EC50 effect interval achieved in the Hygrophila polysperma test and the EC50 result for OECD 201 and EC50 result for OECD 202.

Surfactant	EC50 interval Hyg. Test	EC50 mg/l, OECD 201	EC50 mg/l, OECD 202
Ag6202	>10mg/l	306	>98
Ethylan 1005	>10mg/l	8,4	3,6
Ethomeen T/25	>10mg/l	1,26	1,94
Ethomeen T/15	2-5 mg/l	0,24	0,31
Ethomeen T/12	<1mg/l	0,04	0,043
Ethomeen C/15	2-5mg/l	0,24	1,41
Ethomeen C/12	<1mg/l	0,107	0,84
Arquad 2C-75	2-2,5 mg/l	0,038	N/A

If a definite screening test would have been done the LOEC for Ethomeen T/15 and C/15 would be 2 mg/l and the highest concentration 5 mg/l. The concentrations for Ethylan 1005 and Ethomeen T/25 would have been higher and for AG 6202 much higher. The highest concentration for Ethomeen T/12 and Ethomeen C/12 would have been 1 mg/l. The effect concentration interval for Arquad 2C-75 is ok and not necessary to redo.

Even though a relation between OECD 201 and 201 and the Hygrophila polysperma plant test not could be established Hygrophila polysperma acute toxicity range finding test is recommended. This is mainly because it is a simple test and the results have been very promising. The purpose of a screening test can be a yes or no answer and that is what is achieved in this test. If a very simple test is to be done four concentrations as in test 5 can be prepared, and the outcome is yes or no. If no effect is seen on the 10 mg/l concentration (as for Ethylan 1005, AG 6202, Ethomeen T/25) it is a good chance that the surfactant or mixture is less toxic, a yes is achieved, and if an effect is seen in any of the concentrations prepared, a no is achieved (yes=nontoxic, no=toxic) This is possible to say since all the surfactants with an EC50 below 1 mg/l (OECD 201 and OECD 202) effected the plant below the test concentration 10 mg/l. In general when developing new surfactants, the surfactant is “environmentally approved” to produce if the EC50 is above 1 mg/l and has good properties in biodegradation (a surfactant with an EC50=1 mg/l is considered toxic) or has an EC50 over 10mg/l. If a no is achieved from the plant test using Hygrophila polysperma, this means that the EC50 probably is below 1mg/l, and the product tested not have required toxicity properties. This yes or no endpoint of a test is not always wanted but since it is very important to detect newly developed surfactants with EC50 below 1mg/l at R&D in Stenungsund, this is a good method.



Figure 15, Two of the references in test 5 after 96 h, no change can be detected.



Figure 16, The Ag6202 samples looked like the references in both series after 96 h in all concentrations. In this picture the first series (10mg/l, 5mg/l, 2mg/l, 1mg/l) is shown.



Figure 17, In this pictures the first Arquad 2C-75 series are shown (10mg/l, 5mg/l, 2mg/l, 1mg/l). The plants in the two highest concentrations are dead and the third concentrations are slightly affected in both series but more visible in the picture above, see the top of the sample 13.



Figure 18, Both Ethomeen T/25 series were unaffected as in the Ethylan 1005 and Ag6202 case. In the picture the first series is shown (10mg/l, 5mg/l, 2mg/l, 1mg/l).



Figure 19, The two series of Ethomeen T/15 in the concentrations 10mg/l, 5mg/l, 2mg/l, 1mg/l were equally effected. The two first concentrations are very affected and the third concentration is visible affected in both series but not dead. Several leaves have fall off and are at the bottom or at the surface. The lowest concentrations are in both cases not affected.



Figure 20, No visible affect was seen in the two Ethylan 1005 series. In the above picture the first series is shown (10mg/l, 5mg/l, 2mg/l, 1mg/l)



Figure 21, In this picture the second series of Ethomeen C/15 is shown (10mg/l, 5mg/l, 2mg/l, 1mg/l). The first two concentrations in both series are affected but the third and fourth ones are considered healthy.



Figure 22, In the Ethomeen C/12 test all plants in both series died. In the picture above the first series is represented (10mg/l, 5mg/l, 2mg/l, 1mg/l). As seen in the picture all leaves even in the lowest concentration had changed color and fall off.



Figure 23, As in the Ethomeen C/12 case all the plants in the Ethomeen T/12 concentrations died. Both series of Ethomeen T/12 showed the same results. In the picture above the first series of Ethomeen T/12 with the reference to the right is showed (10mg/l, 5mg/l, 2mg/l, 1mg/l, Ref)



Figure 24, As seen in the picture above the no affect was seen in the solvent reference with IPA. The concentrations used were; 10mg/l, 5mg/l, 2mg/l, 1mg/l in tap water.



Figure 25, The samples with Cocobenzylamin+1EO IPA showed clear visible affect in the three highest concentrations. The concentrations were 10mg/l, 5mg/l, 2mg/l, 1mg/l. The plants in the two highest concentrations are dead and the plant in the third concentration is visible affected, as seen in the picture the leaves are brown in the edges but not as effected in the higher concentrations. The lowest concentration was as healthy as the reference in tap water.



Figure 26, As seen in the picture above the no affect was seen in the solvent reference with Ethanol. The concentrations used were; 10mg/l, 5mg/l, 2mg/l, 1mg/l in tap water.



Figure 27, As in the Cocobenzylamin+1EO with Ethanol the samples with Cocobenzylamin+1EO IPA showed affect in the three highest concentrations. The concentrations used were 10mg/l, 5mg/l, 2mg/l, 1mg/l. The plants in the two highest concentrations are dead and the plant in the third concentration was not dead but visible affected. The lowest concentration was as healthy as the reference in tap water.

Microtox

For surfactants it is recommended to use the EC50 value from the 15 min reading, surfactants are big molecules and needs more than 5 min to affect the bacterium, the Microtox and OECD standard results are presented in the Table 12. [27]

Table 12, Toxicity values for Microtox compared to toxicity results for OECD 201, OECD 202 and OECD 203.

Surfactant	Highest concentration	EC50 15 minutes reading (mg/l)	EC50 mg/l OECD 201	EC 50 mg/l OECD 202	EC50 mg/l OECD 203
Ag6202	180 ppm	427,7313	306	>98	>310
Ethylan 1005	45 ppm	14,0389	8,4	3,6	13
Ethomeen T/25	45 ppm	13,6085	1,26	1,94	N/A
Ethomeen C/15	9 ppm	3,4347	0,24	1,41	0,66
Ethomeen C/12	6 ppm	2,1546	0,107	0,84	0,3
Ethomeen T/15	4,5 ppm	1,5060	0,24	0,31	N/A
Ethomeen T/12	4,5 ppm	1,4239	0,04	0,043	N/A
Arquad 2C-75	4,5 ppm	1,1994	0,038	N/A	0,26

As seen in the table above it is possible to see a toxicity difference between the different surfactants. Ag6202 is the least toxic one followed by Ethylan 1005, which is thereon the least toxic one, see table 16. After Ethylan 1005 Microtox places Ethomeen T/25 that is the least toxic among the Ethomeens, which is in accordance with the theory since it contains the most EO chains (15). According to Microtox the tallow- Ethomeens are more toxic than the coco-Ethomeens which also is in accordance with the theory since the tallow surfactants have a longer hydrophobic tail (lower CMC) and because of that often are more toxic.

Since the Microtox toxicity values of the tested surfactants are placed in the same order the as OECD 202 and 203 it is possible to say that the values correlate by ranking. This was expected since Microtox is developed to predict toxicity for essentially daphnia and fish testes. By comparing OECD 202 and Microtox is it possible to predict an OECD 202 for some groups of surfactants. The test species, *Vibrio Fisheri*, used in Microtox will always have lower sensitivity than the OECD standard methods and therefore the Microtox EC50 value will be divided with a certain value to predict for example daphnia toxicity. By dividing the achieved Microtox value by roughly 5 for Ag6202, Ethylan 1005 and Ethomeen T/25 the toxicity of daphnia can be predicted. For the toxic T-Ethomeen the bacteria is more sensitive than for the C-Ethomeens and therefore all the tested Ethomeens cannot be divided with the same value. The sensitivity is 2,5 times higher for C-Ethomeens in daphnia compared to *Vibrio Fisheri* and for the T-Ethomeens the lower the number of EO-chains, the higher the sensitivity. For daphnia the sensitivity of Ethomeen T/12 is 35 times higher and for Ethomeen T/15 the sensitivity is 7 times higher, compared to *Vibrio Fisheri*. This kind of prediction is not possible for OECD 203 since not as many OECD 203 values for the tested surfactants are present. It has been found in other Microtox tests that the differences between the toxicity results of fish and Microtox are about one order of magnitude for surfactants, however, since not many values are available that is in this case not possible to state.

If the Microtox result is compared to OECD 201 the surfactants is almost placed in the same order, OECD 201 places Ethomeen C/12 more toxic than Ethomeen T/15. However, it is difficult to predict if Ethomeen T/15 is more toxic than Ethomeen C/12 since the first one has more EO chains but longer

hydrophobic tail than the second one that has less EO chains but shorter hydrophobic tail. So, besides the fact that OECD 201 not places Ethomeen C/12 and C/15 in the same order, Microtox is in the accordance with OECD 201, that have results on all the tested surfactants, see table 16. Since Microtox and OECD 201 and 202 almost places the surfactants tested in the same toxicity order it is recommended to use Microtox as a screening tool.

When a screening test is done with Microtox it is recommended to test a surfactant of the same family, or with a similar structure, with a known EC50 at the same time. By doing that it is possible to do a toxicity comparison between the tested substances. Since it is shown that Microtox places the surfactants tested in the same order the comparison method, previously described, is a trustful screening tool.

Microtox tested with stock solutions prepared with solvents

To be able to test hydrophobic surfactants the effect of adding small amounts of solvents were tested. Two solvents were evaluated, ethanol and IPA. Methanol and DMSO were also mentioned in the literature but not evaluated because of environmental aspects.

Ethanol have according to Microtox an EC50 5 min of 8,5059 ppm and an EC50 15 min of 5,9421 ppm. Because of this result it is clearly that the concentration interval tested affected the bacterium. To see how the bacterium reacted from much lower concentration two tests were done; 4,5 ppm and 2,25 ppm as the highest concentrations. Both IPA and ethanol tested at 4,5 ppm (4,5ppm=25µl is 0,9% of 2,75ml) showed toxicity but when 2,25 ppm (2,25ppm = 12,5µl is 0,45% of 2,75 ml) was tested no toxicity was shown. However, this result shows that 1% solvent do affect the bacterium and 0,5% not affects the bacterium which not corresponds to the literature, that recommends that no more than 1% solvent should be used. However, it is desirable to use as small amount solvent as possible and to do so is good to prepare the stock solution with no more solvent then the necessary amount to achieve a homogenous solution.

Table 13, As seen in the table below the light intensity does not change with increased concentration (2,25; 1,125; 0,5625; 0,28125 ppm). I0 is the light intensity at t=0 and IT is the light intensity at t=T (5 or 15 min dependant on which reading is referred to).

Ethanol, 2,25 ppm, I0/IT		IPA, 2,25 ppm	
I0/IT, 5min	I0/IT, 15min	I0/IT , 5min	I0/IT , 5min
90,85/66,30	90,85/54,69	90,45/69,79	90,45/69,79
94,60/70,94	94,60/59,06	90,82/67,28	90,82/67,28
95,42/70,40	95,42/61,84	91,39/66,01	91,39/66,01
93,85/71,56	93,85/61,90	91,79/64,86	91,79/64,86

To be able to test a hydrophobic surfactant with Microtox the surfactant Cocobenzylamin+1EO was tested. Even though an OECD standard toxicity value not is present this is expected to be a toxic surfactant since it is a cationic surfactant and its CMC is very low. Because of this it was tested at the same concentrations as Ethomeen T/12 and Arquad 2C-75 (4,5 ppm). In order to keep the solvent content below 0,5% the stock solutions was prepared as 50% solvent and 50% water. The stock solution with ethanol became turbid, but was still considered homogenous, and the stock solution with IPA became transparent. When the stock solutions are prepared in this manner no more than

0,5 % (2,25 ppm) solvent is added in the highest concentration and the solvent effect can be neglected.

Both stock solutions of Cocobenzylamin+1EO prepared in 50% solvent and 50% deionized water showed that the surfactant was toxic. The results are similar and have almost the same confidence range, see table 14. It is possible that the sample containing IPA gives a more toxic result than the sample in ethanol because of the solvent used; IPA is a better solvent than ethanol for this surfactant since the stock solution was transparent.

Table 14, Comparison between the two prepared stock solutions (1:1 ethanol: water, 1:1 IPA: water) with Cocobenzylamin+1EO.

4,5 ppm 1:1 ethanol:water		4,5 ppm 1:1 IPA:water	
EC50 15 min (ppm)	Confidence range	EC50 15 min (ppm)	Confidence range
0,9023	0,5459-1,4914	0,6205	0,2136-1,8026

Some products also contain different amount of solvent to, for example Arquad 2C-75 that contains 75% surfactants and 25% IPA. To simplify the test procedure and be able to test a product that contains a small amount of solvent two tests were done; solvent free Arquad 2C-75 and Arquad 2C-75 with 25 % solvents. Both tests were done with 4,5 ppm surfactant concentration. The EC50 15 min for Arquad 2C-75 without IPA was 1,994 ppm and EC50 for Arquad 2C-75 with 25% IPA was 1,2606 ppm. The result shows that IPA not affected the results. Since 1,125 ppm solvent is lower than 1% solvent content in 2,75 ml this is in accordance with the literature.

Red beet root bioassay

During this test it was examined if surfactant affects the cell membrane of the red beetroot and causes betanin release. This toxic effect was examined in order to investigate if the results are comparable to the OECD standard methods. The reproducibility of the results, effect of solvents and color of the released betanin were studied.

During the measurement it was observed that almost all liquid had evaporated from the HCl reference solution, see Figure 28. Surprisingly, it was found that the weight has decreased 2,5-3 g. Furthermore, the amount had not decreased equally in the duplicated plates, which caused a difference in the results.

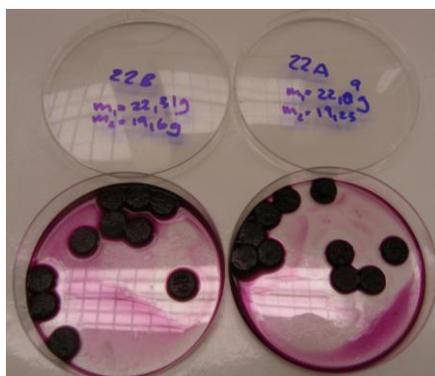


Figure 28, Sample of HCl in methanol after incubation. As seen in the picture the first reference lost 2,71 g and the second reference lost 2,87g.

It was found that unacceptably high (ex. A=2,882) absorption values from the reference have been obtained. According to Lambert Beers law it is known that Uv-Vis spectroscopic value is accurate if the absorbance of the measured solutions are between 0 and 1. No percentages of disrupted cells were because of this calculated, in Table 15 the results from the red beetroot test are presented.

Table 15. Red beet root bioassay test without parafilm

Test solutions	Absorption measured at 535 nm		Absorption mean	% Disrupted cells
HCl in methanol	2,882	N/A	2,882	
HCl in water	0,916	1,118	1,017	
Water	0	0	0	
Ethylan 1005 in water	0,793	0,723	0,758	
Ethomeen T/25 in water	0,592	0,486	0,539	
Ethomeen T/15 in water	0,370	0,380	0,375	
Ethomeen T/12 in water	0,181	0,183	0,182	
Ethomeen C/15 in water	0,729	0,846	0,788	
Ethomeen C/12 in water	0,603	0,627	0,615	
AG 6202 in water	0	0	0	
Poly glycol AM/20 20	0	0	0	
PEG – 400	0	0	0	
Ethanol in water (10%)	0	0	0	
Ethanol in water (1%)	0	0	0	
IPA in water (10%)	0	0	0	
IPA in water (1%)	0	0	0	
Cocobenzylamin + 1EO (10% ethanol)	0,182	0,152	0,167	
Cocobenzylamin + 1EO (1% ethanol)	0	0	0	
Cocobenzylamin + 1EO (10% IPA)	0,192	0,183	0,375	
Cocobenzylamin + 1EO (1% IPA)	0	0	0	
Formaldehyde	0	0	0	
<i>No dilution because of big color change</i>				
Arquad 2C-75 in water	0,192	0,128	0,160	

In order to minimize the effect of evaporation a parafilm was used to seal the space in between of Petri plate and Petri lead. In Table 16 the results from the test where parafilm was used are presented.

Four tests of HCl in methanol, four tests of Ethylan 1005 and double tests of the remaining other surfactant (Table 16) solutions were performed. Additional references were used to study the duplicity of the test. No weight changes were observed before and after incubation, see Figure 29.

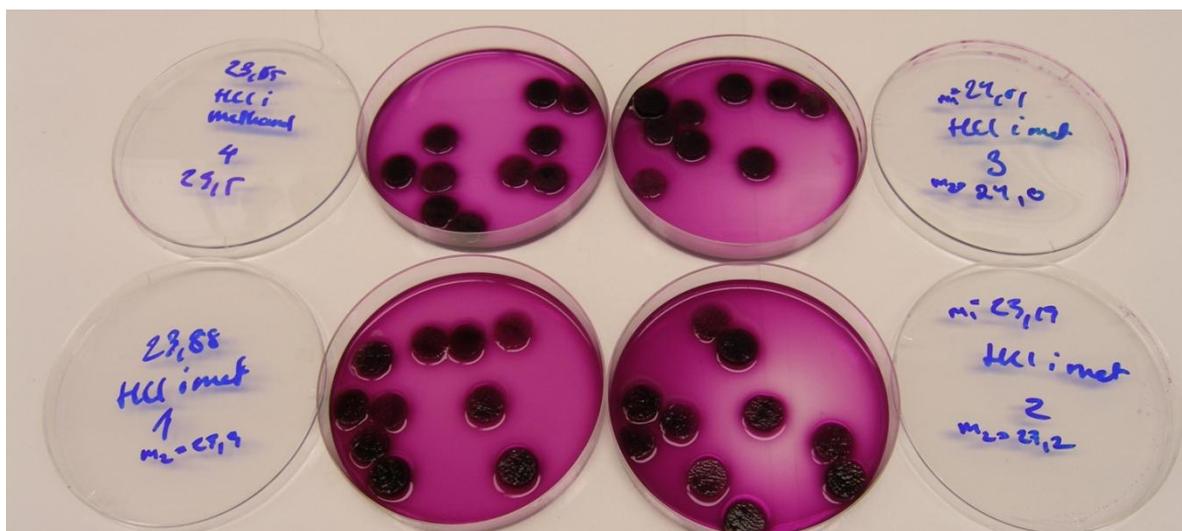


Figure 29, Picture of the reference samples prepared with parafilm, no weight changes was seen.

Table 16. Red beet root bioassay test results with parafilm.

Test solutions	Absorption measured at 535 nm				Absorption mean	% Disrupted cells
HCl in methanol	0,739	0,822	0,836	0,841	0,810	100 %
HCl in water	0,722	0,639	N/A	N/A	0,681	84 %
Water	0	0	N/A	N/A	0	0 %
Ag6202	0	0	N/A	N/A	0	0 %
Ethylan 1005	0,614	0,619	0,652	0,554	0,610	75 %
Ethomeen T/12	0,299	0,278	N/A	N/A	0,289	36 %
Ethomeen T/15	0,500	0,455	N/A	N/A	0,478	59 %
Ethomeen T/25	0,581	0,522	N/A	N/A	0,552	68 %
Ethomeen C/12	0,682	0,569	N/A	N/A	0,663	78 %
Ethomeen C/15	0,733	0,744	N/A	N/A	0,739	91 %

Even though methanol free samples did not decrease more than 0,5 g during the incubation it is recommended to use parafilm for all samples. As seen in table 16 above no Uv-Vis measurements were above one, after diluting all samples ten times, which is a requirement for the measurement to be valid. During the test it was seen that Ethylan 1005 not caused 50% cell disruption in neither of the tests, which was unexpected because it is also an requirement for the test to be valid. To minimize evaporation of methanol HCl in water was tested as a suitable replacement for HCl in methanol. It was seen that HCl in water not gave the same cell disruption as HCl in methanol. It is because of this result not possible to use HCl in water as reference instead of HCl in methanol.

What can be seen from Table 15 is that some of the tested substances and solvents not caused a betanin release (Absorbance =0). These substances are by this method assumed to be nontoxic. The results from AG 6202 was expected since a very high EC50 is reported, see Table 2. It was also found that the formaldehyde sample not caused any cell disruption.

During the experiments it was found that poorly soluble toxic surfactants give low or very low cell disruption value. This phenomenon can be explained by adsorption of the surfactants on the surface of the Petri plates or formation of particles which makes them bio-unavailable, since they were not properly dissolved in water. Therefore it was decided to investigate a possibility to run such

experiments in the presence of organic solvent. However the solvents were tested alone in order to find out if the solvents cause a cell disruption and if they would be used together with surfactants they would not give a wrong cell disruption value. It is important to note that neither the tests with ethanol or IPA caused cell disruption.

Solubility is a general problem with surfactant tested at concentrations higher than 10mg/l. Many substances for example Ethylan 1005, Ethomeen T/12, Ethomeen C/12 and Arquad 2C-75 are turbid but homogenous and other surfactants are not present in water at all. A substance that is almost insoluble in water (Cocobenzylamin + 1 EO) was tested with different amount of solvents (1% and 10%). It was found that the aqueous solution of the substance containing 10 % of organic solvent was not completely soluble. This might be the reason why such a toxic substance gives a very low cell disruption. It is probably not possible to use more solvent than 10% in the stock solution since no further dilution is done (no dilution series is done in this test as in the other tests). More solvent than 10 % in the final test plate will definitely cause problems for the bet root and is not recommended, even though no betanin release was seen.

Among the substances tested three color changes were noticed, see Figure 30. The references are more purple than the color of the Ethomeens and Ethylans that are more alike the original red beetroot color, the cationic surfactant (Arquad 2C-75) got orange. When analyzing the Ethomeens, Ethylan 1005 and HCl in water and methanol no problem was discovered because they have the same lambda maximum at 535 nm. It was more difficult to draw a conclusion from the cationic surfactant because the orange color has a maximum absorption at 607 nm wave length [33], were no reference is available.



Figure 30, Picture of the detected color changes during the test. The upper left picture shows the color of the Arquad 2C-75 solution after incubation. The picture up to the right shows the response of Ethomeen T/25 and the lowest picture shows the response of HCl in methanol.

The results obtained by the red beetroot bioassay, see Figure 30, are not in an agreement with the OECD results. The results from the red beetroot bioassay shows that Ethomeen T/12 is less toxic than Ethomeen T/15 and T/25 (Table 16); Ethomeen C/12 more toxic than Ethomeen T/12 and Ethomeen C/15 (Table 21); Ethomeen C/15 is as toxic as Ethylan 1005 (Table 16), according to the OECD

standard methods it should be the other way around. The red beet root bioassay result for AG 6202 showed no cell disruption which indicates that the substance is nontoxic and have a high EC50, which is in agreement with all the OECD standard methods in table 2.

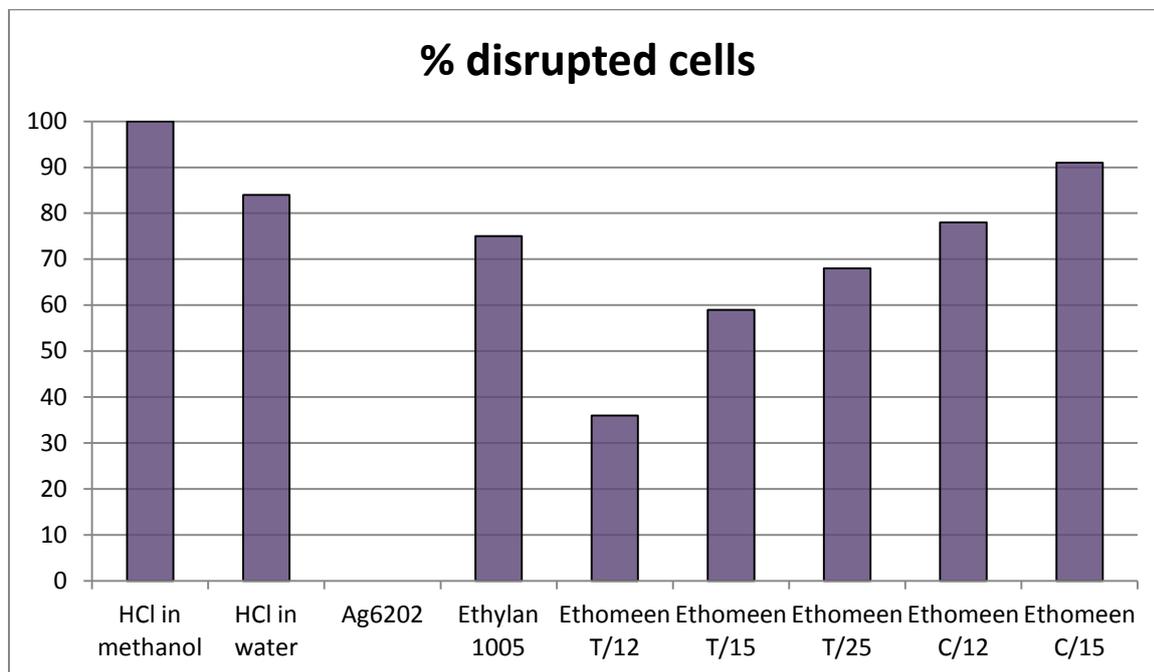


Figure 31, Diagram of red beet root bioassay results with parafilm. The percentage of disrupted cells are illustrated as staples.

According to all the data above the red root beet bioassay cannot be used as an easy, universal toxicity screening method as all the toxicity values, besides for AG 6202, not are in agreement with the OECD standard methods. The method also has problems to handle hydrophobic substances since these substances not show any toxic affect in this bioassay. In this method it is not possible to use solvent to solve this problem because the surfactant solutions tested are in to high concentration and to large amount of solvent would be needed to solubilise the hydrophobic surfactant. In that case to high toxicity value would be provided. This method is also very restricted is what kind of surfactants that can be used, quaternary surfactants (e.g. Arquad 2C-75) gives other type of color, which cannot be evaluated. It has also been shown that water soluble non surface active toxins (e.g. Formaldehyde) are according to the method non-toxic.

However it can be used for the fast evaluation of the surfactants which are easily soluble in water, since all non-toxic surfactants (AG 6202) gave no cell disruption.

Conclusion

Four different methods were examined as possible screening toxicity tests. These are: Aquatic plant test, Microtox, Red beet root bioassay and Root elongation. The Aquatic plant test was used for the first time.

Three different aquatic plants (*Hygrophilia polysperma*, *Cabomba Aquatica* or *Elodea Canadensis*) were used in the aquatic plant test. The plant test using *Hygrophilia polysperma* is recommended to use as a screening tool for toxicity because it was found during the test that it can be used to detect toxic surfactants (surfactants with a standard OECD EC50 result below 1 mg/l). It was also found that the results obtained during the test using *Hygrophilia polysperma* were in agreement to the standard OECD results. It is not recommended to use the aquatic plants *Cabomba Aquatica* or *Elodea Canadensis* instead of *Hygrophilia polysperma* as test species since the provided visible affect not is easy to detect.

Microtox is recommended as a screening tool because it was found that it is an easy and fast method which gives the toxicity results comparable to the ones obtained by standard OECD 201 and OECD 202 tests.

Since all the tests are normally performed in water and there are plenty of hydrophobic surfactants, the possibility of using solvent in the tests has been evaluated. It was found that small amounts of solvent improves water solubility of hydrophobic surfactants and do not affect the toxicity results in neither the *Hygrophilia polysperma* aquatic plant test (≤ 1 % solvent) or Microtox tests ($< 0,5\%$).

It was found during the progress of this paper that the red beet root bioassay and the root elongation tests cannot be used as universal screening tools for all types of surfactants. Red beet root bioassay is not recommended since the tested surfactants not is in agreement to the OECD standard methods results and nor is the root elongation test since not recommended growth occurred.

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Appendix 1

In this appendix the growth from the root elongation test is shown. Every table represents a Petri Dish and the number of seeds (approximately 10). Every green square in a seed that grew and the measured length in mm are filled in.

Controls

Control 1

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	2	-	-	-	-	-	-	-	-	-	-	-

Control 2

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	3	1	-	-	-	-	-	-	-	-	-

Control 3

No	1	2	3	4	5	6	7	8	9	10
L(mm)	-	-	-	-	-	-	-	-	-	-

Control 4

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	1	-	-	-	-	-	-	-	-	-	-	-	-	-

AG 6202

1g/l plate 1

No	1	2	3	4	5	6	7	8	9	10
L(mm)	4	3	-	-	-	-	-	-	-	-

1g/l plate 2

No	1	2	3	4	5	6	7	8	9
L(mm)	1	2	-	-	-	-	-	-	-

1g/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	3	2	-	-	-	-	-	-	-	-	-	-

1g/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-

100 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-

100 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10
L(mm)	10	-	-	-	-	-	-	-	-	-

100 mg/l plate 3

No	1	2	3	4	5	6	7	8	9
L(mm)	-	-	-	-	-	-	-	-	-

100 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10
L(mm)	1	2	-	-	-	-	-	-	-	-

10 mg/l plate 1

No	1	2	3	4	5	6	7	8	9
L(mm)	-	-	-	-	-	-	-	-	-

10 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	4	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	29	30	31	32	33	34	35	36	37	38	39	40	41	42
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	43	44	45	46	47	48	49	50	51	52	53	54	55	56
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	57	58	59	60	61	62	63	64	65	66	67			
	-	-	-	-	-	-	-	-	-	-	-			

10 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	3	1	-	-	-	-	-	-	-	-	-	-	-	-
	15	16	17	18	19	20	21	22	23					
	-	-	-	-	-	-	-	-	-					

10 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	1	-	-	-	-	-	-	-	-	-	-	-	-	-

1 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	7	-	-	-	-	-	-	-	-	-	-	-	-	-

1 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10
L(mm)	1	-	-	-	-	-	-	-	-	-

1 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	2	-	-	-	-	-	-	-	-	-	-

1 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11	12	13
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-

Ethomeen C/12

1g/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	-	-	-	-	-	-	-	-	-	-	-

1g/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1g/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15													
	-													

1g/l plate 4

No	1	2	3	4	5	6	7	8	9	10
L(mm)	-	-	-	-	-	-	-	-	-	-

100 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10
L(mm)	-	-	-	-	-	-	-	-	-	-

100 mg/l plate 2

No	1	2	3	4	5	6	7	8	9
L(mm)	1	-	-	-	-	-	-	-	-

100 mg/l plate 3

No	1	2	3	4	5	6	7	8
L(mm)	-	-	-	-	-	-	-	-

100 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	-	-	-	-	-	-	-	-	-	-	-

10 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	-	-	-	-	-	-	-	-	-	-	-

10 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10
L(mm)	-	-	-	-	-	-	-	-	-	-

10 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10
L(mm)	2	-	-	-	-	-	-	-	-	-

10 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16												
	-	-												

1 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	5	3	-	-	-	-	-	-	-	-	-

1 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	-	-	-	-	-	-	-	-	-	-	-

1 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10
L(mm)	4	2	-	-	-	-	-	-	-	-

1 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	7	3	-	-	-	-	-	-	-	-	-

Ethomeen C/15

1g/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15													
	-													

1g/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	-	-	-	-	-	-	-	-	-	-	-

1g/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	-	-	-	-	-	-	-	-	-	-	-

1g/l plate 4

No	1	2	3	4	5	6	7	8	9
L(mm)	-	-	-	-	-	-	-	-	-

100 mg/l plate 1

No	1	2	3	4	5	6	7	8	9
L(mm)	-	-	-	-	-	-	-	-	-

100 mg/l plate 2

No	1	2	3	4	5	6	7	8	9
L(mm)	7	-	-	-	-	-	-	-	-

100 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	29	30	31	32	33	34	35	36	37	38	39	40	41	42
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	43	44												
	-	-												

100 mg/l plate 4

No	1	2	3	4	5	6	7	8
L(mm)	3	-	-	-	-	-	-	-

10 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-

10 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	4	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16												
	-	-												

10 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10
L(mm)	-	-	-	-	-	-	-	-	-	-

10 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16												
	-	-												

1 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12	13
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-

1 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16	17	18	19									
	-	-	-	-	-									

1 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	4	14	4	-	-	-	-	-	-	-	-

1 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	3	-	-	-	-	-	-	-	-	-	-	-

Ethomeen T/25

1g/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12	13
L(mm)	2	3	-	-	-	-	-	-	-	-	-	-	-

1g/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11	12	13
L(mm)	2	2	-	-	-	-	-	-	-	-	-	-	-

1g/l plate 3

No	1	2	3	4	5	6	7	8	9	10
L(mm)	2	2	-	-	-	-	-	-	-	-

1g/l plate 4

No	1	2	3	4	5	6	7	8
L(mm)	-	-	-	-	-	-	-	-

100 mg/l plate 1

No	1	2	3	4	5	6	7	8	9
L(mm)	-	-	-	-	-	-	-	-	-

100 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	1	1	-	-	-	-	-	-	-	-	-

100 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10
L(mm)	1	3	3	-	-	-	-	-	-	-

100 mg/l plate 4

No	1	2	3	4	5	6	7	8	9
L(mm)	4	-	-	-	-	-	-	-	-

10 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	5	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16												
	-	-												

10 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	2	1	-	-	-	-	-	-	-	-	-	-	-	-

10 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	-	-	-	-	-	-	-	-	-	-	-

10 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	5	-	-	-	-	-	-	-	-	-	-

1 mg/l plate 1

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	2											

1 mg/l plate 2

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L(mm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	16	17	18	19	20	21	22	23	24				
	-	-	-	-	-	-	-	-	-	-				

1 mg/l plate 3

No	1	2	3	4	5	6	7	8	9	10	11	12
L(mm)	2	-	-	-	-	-	-	-	-	-	-	-

1 mg/l plate 4

No	1	2	3	4	5	6	7	8	9	10	11
L(mm)	4	-	-	-	-	-	-	-	-	-	-

Appendix 2

OECD tests – Methods, results and discussion and conclusion

Algae acute toxicity test, OECD 201

Test procedures

An adequate amount of OECD algae test medium was prepared for the test in an appropriately sized volumetric vessel. The medium, described by OECD is a perfect medium for algae to grow within. This medium had a pH of 8.0 after preparation and pH adjustment were therefore not necessary.

The OECD medium in the volumetric vessel was then sterilized by filter sterilization with a 0.45 µm filter to remove impurities. Adequate amounts of stock solution were added to the sterilized test vessels, 100 ml Erlenmeyer flasks, to achieve the desired test concentrations in 40 ml total volume. The test vessels were filled with medium up to 40 ml using a sterilized dispenser. The inoculums (algae) were added from an exponentially growing culture with a pipette. In addition 4 replicates of the control without test solutions were included. All Erlenmeyer flasks were then incubated for 96 h. The extinction in each Erlenmeyer flask was measured after 0, 72 and 96 hours. Test medium was used as a blank in the spectrophotometer to correct for media absorbance.

No chemical analysis for quantification of the test substance concentration was performed during the study. This had to be done for a GLP study but since this was non GLP no analytics was done.

Method

Four substances were tested in Arnhem;

- Ethylan 1005
- Ethomeen T/15
- Ethomeen T/25
- Arquad 2C-75

Preparation of the stock solution

Approximately 100 mg/l of test substance was prepared in test media for Ethomeen T/15 and Ethomeen T/25. The stock solutions were then agitated with a magnetic stirrer until a homogeneous solution was achieved. The test substance appeared to dissolve easily and the solutions were transparent. For Ethylan 1005 a stock solution of approximately 1g/l was prepared. The stock solution was found white and after agitated with a magnetic stirrer the solution were homogeneous. For Arquad 2C-75 a stock solution of approximately 100 mg/l was prepared. The substance was not easily dissolved and was left agitated with a magnetic stirrer for 2 h. The stock solution was then left for additionally 2 h mild stirring to minimize the foam. To be able to pipette an accurate amount stock it was diluted 10 times. The stock solution was after the treatment transparent. The definite concentrations for the four substances can be read out in table 4. The pHs for the three stock solutions were measured, see table 4. The appropriate volume of the test substance stock was then transferred to each of the 100 ml Erlenmeyer flasks to generate the concentrations in table 5,6 and 7.

pH measurements

pH are measured after the test solutions have been prepared and when the test is finished. It is important to stir carefully during the measurements because input of CO₂ can influence the pH. pH is corrected when delta pH > 0,5 from the blank test media to ±0,2 pH units from the pH of the blank test medium.

Pre-culture (testing)

To do an algae test the algae needs to be pre-cultured (grow in OECD media). This pre-culture is tested at the beginning of the test to ensure proper growth. A quantity of maximum 8 ml is transferred from a pre-culture to separate Erlenmeyer flasks containing new sterile medium (40 ml in a 100 ml Erlenmeyer flask with sterile NaHCO₃ via syringe with a filter of 0,2 µm). These new cultures are then incubated in the illuminated shaking incubator for at least 24h.

On the day of the test a review is made of which of the pre-cultures incubated for at least 24 h that has growth in the exponential phase (required to ensure good growth). The pre-culture is in the exponential phase if the extinction measured with the spectrophotometer at 436 nm in 4 cm cuvette is between 0,6 and 1,0. Prior to use the pre-culture is checked for its purity using the microscope.

Range finding test

Range finding tests are in general conducted at the standard concentrations of 0.1, 1.0, 10, and 100 mg/l to determine the definitive test concentrations. This is not always required but it can be hard to guess the concentrations for a definite test without the range finding procedure. The methodology for both range finding and definitive tests are the same.

The control vessels are prepared by dispersing 40 ml of the algae test medium into a 100 ml Erlenmeyer test vessels. A stock solution with appropriate concentrations is prepared in algae test medium. Based on the stock solutions concentrations a calculation is made to determine the volume of stock required in a total volume of 40 ml to achieve the desired test concentration. This volume is then transferred into the correct amount of test media so the volume in the Erlenmeyer flask equals 40 ml. This is repeated for all test concentrations adjusting the volume of stock and media as required.

After the previous preparation algae are added. The volume required is calculated using the most recent calibration curve in the appropriate algae logbook for the specific algae species. The number of cells is recommended in the relevant OECD guideline and is about 10⁴ cells/ml.

Absorbance in a clean 4 cm cuvette at the time 0 is then measured for all test vessels using the spectrophotometer set at 436 nm with test medium as the reference. The medium is after the measurement returned to the test vessel to keep the original volume.

The Erlenmeyer flasks are then sealed by means of sterile bugs and the test begins. The Erlenmeyer flasks are placed into the illuminated shaking incubator for the required test time. Every day it is important to randomize the vessels to minimize growth differences dependent on difference in light in the shaking illuminator.

Definitive test

After the range finding test it is recommended to do a definite test to get a more accurate test result.

In addition to the method described five samples should be taken at random and examined for purity and morphology at the end of the test with the microscope.

Determination temperature and light

During the test the temperature is measured continually by the laboratories temperature sensor and data logging system. An additional max/min thermometer may also be used as a backup if needed. Light intensity is measured at the beginning and the end of the test. Temperature was set at 23 °C in the light incubator and varied less than $\pm 2^{\circ}\text{C}$ throughout the test period. The light intensity in the light incubator was measured at the beginning of the test to ensure good light quality, the light intensity was measured at $107,8 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Table 17, Stock concentrations and pH measurement

Test substance	Concentration	pH
Ethylan 1005	1,045 g/l	8,2
Ethomeen T/15	0,121g/l	8,4
Ethomeen T/25	0,116 g/l	8,3
Arquad 2C-75	0,0109 g/l	8,3

Test concentrations

The concentration intervals are based on previous screening test results.

Table 18, Concentrations for Ethylan 1005

Concentration (mg/l)	Control	5,4	16,3	49	147	441
Add from stock (ml)	0	0,21	0,62	1,88	5,63	16,88
OECD (ml)	40	39,79	39,38	38,12	34,37	23,12

Table 19, Concentrations for Ethomeen T/15

Concentration (mg/l)	Control	0,07	0,20	0,61	1,83	5,49
Add from stock (ml)	0	24,1 μl	69 μl	0,21	0,63	1,89
OECD (ml)	40	39,98	39,93	39,79	39,37	38,11

Table 20, Concentrations for Ethomeen T/25

Concentration(mg/l)	Control	0,08	0,26	0,83	2,66	8,50	27,20
Add from stock (ml)	0	26 μl	86 μl	0,27	0,88	2,81	8,99
OECD (ml)	40	39,97	39,91	39,73	39,12	37,19	31,01

Table 21, Concentrations for Arquad 2C-75

Concentration (mg/l)	Control	0,0045	0,013	0,04	0,12	0,36
Add from stock (ml)	0	17 μl	49 μl	0,15	0,450	1,35
OECD (ml)	40	39,98	39,95	39,85	39,55	38,65

Determination of algae cell concentrations

To be able to ensure equally growth in all test vessels the same amount of algae cells have to be added. 10^4 algae cells are required for each test vessel and to know the amount of inoculum that needs to be added measurements were carried out photo metrically, using a UV/VIS Spectrophotometer at 436 nm in a 4 cm cuvette. To establish the relation between extinction and number of cells in a certain inoculum volume, a calibration curve was made. From the relation between extinction (E) and counted cell number (N) the following calibration curve was determined using linear regression:

$$N = (2.5 \cdot 10^6 \times E) - 1.812 \cdot 10^5$$

The calibration curve was used to determine the cell density of the inoculum and therefore the volume required to inoculate the test vessels. The extinction was measured to 0,844 and since it is desirable that each 100 ml Erlenmeyer flask with a test volume of 40 ml contains 10^4 cells the following calculations were done:

$$(2.5 \cdot 10^6 \times 0,844) - 1.812 \cdot 10^5 = 1,93 \times 10^6$$

$$1 \times 10^4 \times 40 / 1,93 \times 10^6 = 0,21 \text{ ml}$$

0,21 ml of the inoculum was added to each test concentration for Ethylan 1005, Ethomeen T/15 and Ethomeen T/25. The Erlenmeyer flasks were then incubated for 72 h and the extinction was measured to investigate the algae growth. After 72 h a small amount of growth was observed and to establish some more growth the Erlenmeyer flasks were incubated for additionally 24 h and the extinction was measured.

For the Arquad 2C-75 test another inoculum was used. The extinction was measured to 0,563.

$$(2.5 \cdot 10^6 \times 0,563) - 1.812 \cdot 10^5 = 1,226 \times 10^6$$

$$1 \times 10^4 \times 40 / 1,93 \times 10^6 = 0,33 \text{ ml}$$

The Arquad 2C-75 test was carried out a week later and because little growth was observed in the previous test a larger amount algae cells was added, 0,5 ml instead of 0,33 ml was added to Erlenmeyer flask and incubated for 72h.

Results

In table 7 below the algae results are presented.

Table 22, Toxicity results for Ethylan 1005, Ethomeen T/15, Ethomeen T/25 and Arquad 2C-75

Test substance	LOEC	NOEC	E _r C50
Ethylan 1005	N/A	N/A	8,37
Ethomeen T/15	0,07	<0,07	0,24
Ethomeen T/25	0,33	0,26	1,26
Arquad 2C-75	0,04	0,013	0,0383

Discussion and conclusion

The growth of the algae was less than expected both for the first test with Ethylan 1005, Ethomeen T/15 and Ethomeen T/25 as well as for the second test with Arquad 2C-75. Both tests were because of this observation prolonged to 96 h.

In the Ethylan 1005 test the substance was performed in to high concentration interval and no LOEC and NOEC could be calculated. The EC50 is in this case based on estimated lower test concentrations than what was preformed. It is recommended that the test is redone at a lower concentration interval to get an accurate result but it is possible to see that the substance tested is more toxic than the hypothesis. From previous result the EC50 was 49 mg/l which not is the case for the substance tested. In the statics for Ethylan 1005 it is shown that the curve is not perfect but it is possible to see that EC50 is around the lowest concentration tested.

In the case for Ethomeen T/25 it was possible to get a LOEC and NOEC (see table 9) and the concentration interval tested was much better than in the Ethylan 1005 test. As seen in the statistics a more appropriate curve was preformed and even tough a couple of outliers and that too few replicates

For the Ethomeen T/15 the test was performed at slightly too high concentration so no accurate NOEC could be calculated, the only available result is that the NOEC is lower than the LOEC (see table 9). As seen in the statistics the curve is even better in this case than for Ethomeen T/25. This result will probably also be good enough for screening even though the same problems with confidence limit was found , as in the Ethomeen T/25 case.

In the Arquad 2C-75 test the result a NOEC and LOEC could be calculated (see table 9) and because the EC50 is around the predicted value the result is considered quite good. If the test would be redone the concentration interval would be slightly adjusted and the test would have been done with more replicates to get the confidence limit right, as in all tests performed. The pH measurement showed that the algae had a grown very slowly, the measured value was much lower than expected. The expected value for 72 h and 96 h was around 8,9 – 9,0.

Appendix 3

In this appendix the statistics from the algae test 201 is presented.

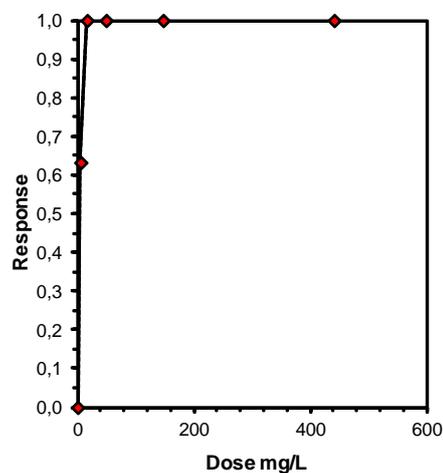
Algal Inhibition Test-Area (EbC50)					
Start Date:	Test ID: NG11043		Sample ID: Ethylan 1005		
End Date:	Lab ID:		Sample Type:		
Sample Date:	Protocol: OECD 201		Test Species: P.Subcapitata		
Comments:					
Conc-mg/L	1	2	3	4	
0	7,7760	4,7520	4,6080	6,1920	
5,4	2,1960	2,0880			
16,3	0,0000	0,0000			
49	0,0000	0,0000			
147	0,0000	0,0000			
441	0,0000	0,0000			

Conc-mg/L	Mean	N-Mean	Transform: Untransformed					N	Isotonic	
			Mean	Min	Max	CV%	Mean		N-Mean	
0	5,8320	1,0000	5,8320	4,6080	7,7760	25,381	4	5,8320	1,0000	
5,4	2,1420	0,3673	2,1420	2,0880	2,1960	3,565	2	2,1420	0,3673	
16,3	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	
49	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	
147	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	
441	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution ($p > 1$)	0,91299	0,788	0,85462	0,98839
F-Test indicates unequal variances ($p = 0,08$)	375,704	1,70923		

Linear Interpolation (200 Resamples)					
Point	mg/L	SD	95% CL(Exp)		Skew
IC05*	0,4267	0,0295	0,2411	0,7247	0,4887
IC10*	0,8535	0,0589	0,4821	1,4495	0,4887
IC15*	1,2802	0,0884	0,7232	2,1742	0,4887
IC20*	1,7069	0,1178	0,9643	2,8990	0,4887
IC25*	2,1337	0,1473	1,2053	3,6237	0,4887
IC40*	3,4139	0,2356	1,9285	5,7980	0,4887
IC50*	4,2673	0,2946	2,4107	7,2475	0,4887

* indicates IC estimate less than the lowest concentration



Algal Inhibition Test-Growth rate (ErC50)				
Start Date:	Test ID:	NG11043	Sample ID:	Ethlyan 1005
End Date:	Lab ID:		Sample Type:	
Sample Date:	Protocol:	OECD 201	Test Species:	P.Subcapitata
Comments:				

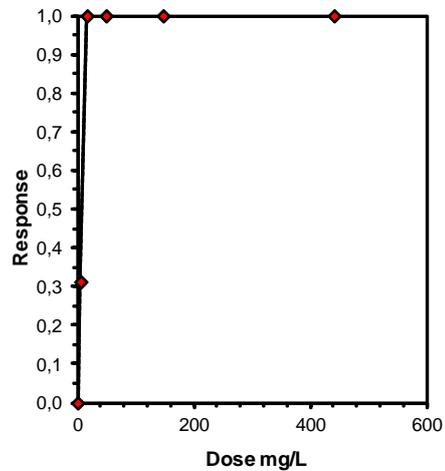
Conc-mg/L	1	2	3	4
0	0,0433	0,0369	0,0365	0,0403
5,4	0,0278	0,0260		
16,3	0,0000	0,0000		
49	0,0000	0,0000		
147	0,0000	0,0000		
441	0,0000	0,0000		

Conc-mg/L	Mean	N-Mean	Transform: Untransformed					N	Isotonic	
			Mean	Min	Max	CV%	Mean		N-Mean	
0	0,0392	1,0000	0,0392	0,0365	0,0433	8,210	4	0,0392	1,0000	
5,4	0,0269	0,6869	0,0269	0,0260	0,0278	4,697	2	0,0269	0,6869	
16,3	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	
49	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	
147	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	
441	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	0,0000	0,0000	

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution ($p > 1$)	0,93469	0,788	0,61515	-0,1967
F-Test indicates unequal variances ($p = 0,56$)	6,47542	1,70923		

Linear Interpolation (200 Resamples)					
Point	mg/L	SD	95%CL(Exp)		Skew
IC05*	0,8622	0,0865	0,3825	1,5697	0,5052
IC10*	1,7244	0,1731	0,7650	3,1395	0,5052
IC15*	2,5866	0,2596	1,1475	4,7092	0,5052
IC20*	3,4488	0,3461	1,5300	6,2790	0,5052
IC25*	4,3111	0,4310	1,9125	7,8487	0,4833
IC40	6,7783	0,4236	3,7497	9,5213	-0,1306
IC50	8,3652	0,3530	5,8414	10,6511	-0,1306

* indicates IC estimate less than the lowest concentration



Algal Inhibition Test-Area (EbC50)			
Start Date:	Test ID: NG11044	Sample ID:	Ethomeen T/25
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: EEC-C3-Algal Inhibition Test	Test Species:	SC-Selenastrum capricornutum
Comments:			

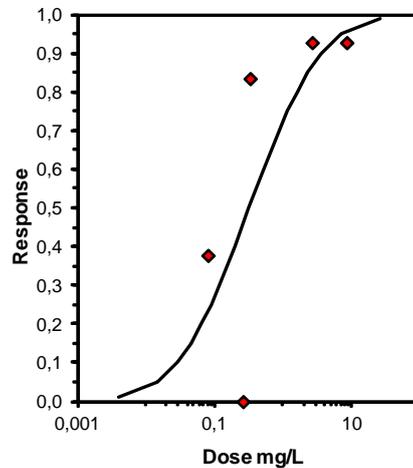
Conc-mg/L	1	2	3	4
0	7,7040	5,0400	4,8600	6,4080
0,08	3,3840	4,0896		
0,26	8,2080	4,0320		
0,33	1,0800	0,9000		
2,66	0,4320	0,4320		
8,5	0,4320	0,4320		

Conc-mg/L	Transform: Untransformed							1-Tailed				
	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
0	6,0030	1,0000	6,0030	4,8600	7,7040	22,123	4				6,0030	0,0000
0,08	3,7368	0,6225	3,7368	3,3840	4,0896	13,352	2	1,959	2,660	3,0773	3,7368	0,3775
0,26	6,1200	1,0195	6,1200	4,0320	8,2080	48,250	2	-0,101	2,660	3,0773	6,1200	-0,0195
*0,33	0,9900	0,1649	0,9900	0,9000	1,0800	12,856	2	4,333	2,660	3,0773	0,9900	0,8351
*2,66	0,4320	0,0720	0,4320	0,4320	0,4320	0,000	2	4,816	2,660	3,0773	0,4320	0,9280
*8,5	0,4320	0,0720	0,4320	0,4320	0,4320	0,000	2	4,816	2,660	3,0773	0,4320	0,9280

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Shapiro-Wilk's Test indicates normal distribution (p > 1)	0,91919	0,874	0,2029	1,1328						
Equality of variance cannot be confirmed										
Hypothesis Test (1-tail, 5)	NOEC	LOEC	Chv	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	0,26	0,33	0,29292		3,07729	0,51263	17,7953	1,78448	0,00277	5, 8

Parameter	Value	SE	95% Fiducial Limits		Maximum Likelihood-Probit						
			Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter		
Slope	1,21506	1,14998	-2,4447	4,87481	0	9,31323	7,81473	0,03	-0,4894	0,823	4
Intercept	5,59465	0,78814	3,08644	8,10285							

Point	Probits	mg/L	95% Fiducial Limits
EC01	2,674	0,00394	
EC05	3,355	0,01435	
EC10	3,718	0,02857	
EC15	3,964	0,04546	
EC20	4,158	0,06576	
EC25	4,326	0,09026	
EC40	4,747	0,20049	
EC50	5,000	0,32404	
EC60	5,253	0,52373	
EC75	5,674	1,16335	
EC80	5,842	1,59683	
EC85	6,036	2,30989	
EC90	6,282	3,67557	
EC95	6,645	7,31684	
EC99	7,326	26,6193	



Significant heterogeneity detected (p = 3,00E-02)

Algal Inhibition Test-Growth rate (ErC50)			
Start Date:	Test ID: NG11043	Sample ID:	Ethomeen T25
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: OECD 201	Test Species:	P.Subcapitata
Comments:			

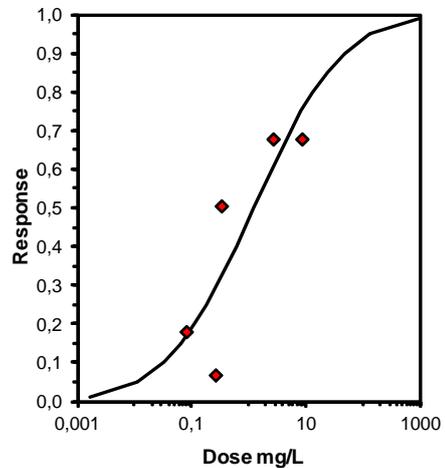
Conc-mg/L	1	2	3	4
0	0,0408	0,0592	0,0532	0,0530
0,08	0,0427	0,0417		
0,26	0,0541	0,0419		
0,33	0,0276	0,0233		
2,66	0,0165	0,0165		
8,5	0,0165	0,0165		

Conc-mg/L	Transform: Untransformed							1-Tailed				
	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
0	0,0515	1,0000	0,0515	0,0408	0,0592	15,009	4				0,0515	0,0000
0,08	0,0422	0,8186	0,0422	0,0417	0,0427	1,748	2	1,883	2,660	0,0132	0,0422	0,1814
0,26	0,0480	0,9311	0,0480	0,0419	0,0541	17,853	2	0,715	2,660	0,0132	0,0480	0,0689
*0,33	0,0255	0,4940	0,0255	0,0233	0,0276	12,085	2	5,253	2,660	0,0132	0,0255	0,5060
*2,66	0,0165	0,3205	0,0165	0,0165	0,0165	0,000	2	7,053	2,660	0,0132	0,0165	0,6795
*8,5	0,0165	0,3205	0,0165	0,0165	0,0165	0,000	2	7,053	2,660	0,0132	0,0165	0,6795

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Shapiro-Wilk's Test indicates normal distribution (p > 1)	0,89934	0,874	-0,7819	2,03716						
Equality of variance cannot be confirmed										
Hypothesis Test (1-tail, 5)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	0,26	0,33	0,29292		0,01321	0,25624	0,00061	3,3E-05	3,2E-04	5, 8

Parameter	Value	SE	95% Fiducial Limits		Maximum Likelihood-Probit						
					Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	0,80909	0,42586	-0,0256	1,64377	0	2,03445	7,81473	0,57	0,09545	1,23595	3
Intercept	4,92277	0,29048	4,35343	5,49211							

Point	Probits	mg/L	95% Fiducial Limits
EC01	2,674	0,00166	
EC05	3,355	0,01155	
EC10	3,718	0,03247	
EC15	3,964	0,06523	
EC20	4,158	0,11357	
EC25	4,326	0,18273	
EC40	4,747	0,6058	
EC50	5,000	1,24581	
EC60	5,253	2,56199	
EC75	5,674	8,49352	
EC80	5,842	13,6664	
EC85	6,036	23,792	
EC90	6,282	47,7953	
EC95	6,645	134,403	
EC99	7,326	934,76	



Significant heterogeneity detected (p = 5,70E-01)

Algal Inhibition Test-Area (EbC50)			
Start Date:	Test ID: NG11043	Sample ID:	Ethomeen T15
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: EEC-C3-Algal Inhibition Test	Test Species:	SC-Selenastrum capricornutum
Comments:			

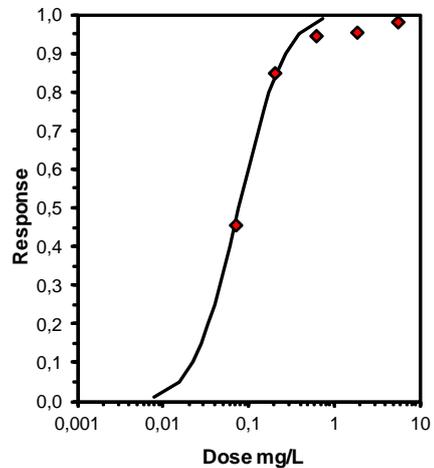
Conc-mg/L	1	2	3	4
0	7,7040	5,0400	4,8600	6,4080
0,07	3,2040	3,3120		
0,2	0,7560	1,0440		
0,61	0,3240	0,3240		
1,83	0,3240	0,2160		
5,49	0,1080	0,1080		

Conc-mg/L	Transform: Untransformed							1-Tailed				
	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
0	6,0030	1,0000	6,0030	4,8600	7,7040	22,123	4				6,0030	0,0000
*0,07	3,2580	0,5427	3,2580	3,2040	3,3120	2,344	2	3,878	2,660	1,8829	3,2580	0,4573
*0,2	0,9000	0,1499	0,9000	0,7560	1,0440	22,627	2	7,209	2,660	1,8829	0,9000	0,8501
*0,61	0,3240	0,0540	0,3240	0,3240	0,3240	0,000	2	8,023	2,660	1,8829	0,3240	0,9460
*1,83	0,2700	0,0450	0,2700	0,2160	0,3240	28,284	2	8,099	2,660	1,8829	0,2700	0,9550
*5,49	0,1080	0,0180	0,1080	0,1080	0,1080	0,000	2	8,328	2,660	1,8829	0,1080	0,9820

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Shapiro-Wilk's Test indicates non-normal distribution (p <= 1)	0,7871	0,874	0,88581	4,09977						
Equality of variance cannot be confirmed										
Hypothesis Test (1-tail, 5)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	<0,07	0,07			1,88285	0,31365	17,2195	0,66805	9,8E-05	5, 8

Parameter	Value	SE	95% Fiducial Limits		Maximum Likelihood-Probit						
			Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter		
Slope	2,35353	1,33265	-0,2585	4,96552	0	0,21068	7,81473	0,98	-1,1143	0,42489	7
Intercept	7,62259	1,39054	4,89714	10,348							

Point	Probits	mg/L	95% Fiducial Limits
EC01	2,674	0,00789	
EC05	3,355	0,01537	
EC10	3,718	0,02194	
EC15	3,964	0,02788	
EC20	4,158	0,03373	
EC25	4,326	0,03973	
EC40	4,747	0,05998	
EC50	5,000	0,07686	
EC60	5,253	0,09847	
EC75	5,674	0,14868	
EC80	5,842	0,1751	
EC85	6,036	0,21186	
EC90	6,282	0,26928	
EC95	6,645	0,38421	
EC99	7,326	0,74839	



Significant heterogeneity detected (p = 9,80E-01)

Algal Inhibition Test-Growth rate (ErC50)			
Start Date:	Test ID: NG11045	Sample ID:	Ethomeen T15
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: EEC-C3-Algal Inhibition Test	Test Species:	SC-Selenastrum capricornutum
Comments:			

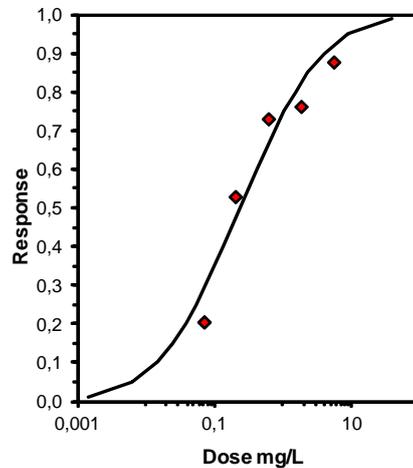
Conc-mg/L	1	2	3	4
0	0,0408	0,0592	0,0532	0,0530
0,07	0,0446	0,0373		
0,2	0,0224	0,0260		
0,61	0,0139	0,0139		
1,83	0,0139	0,0106		
5,49	0,0063	0,0063		

Conc-mg/L	Transform: Untransformed							1-Tailed				
	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
0	0,0515	1,0000	0,0515	0,0408	0,0592	15,009	4				0,0515	0,0000
0,07	0,0409	0,7937	0,0409	0,0373	0,0446	12,605	2	2,350	2,660	0,0120	0,0409	0,2063
*0,2	0,0242	0,4695	0,0242	0,0224	0,0260	10,795	2	6,043	2,660	0,0120	0,0242	0,5305
*0,61	0,0139	0,2691	0,0139	0,0139	0,0139	0,000	2	8,327	2,660	0,0120	0,0139	0,7309
*1,83	0,0122	0,2372	0,0122	0,0106	0,0139	18,987	2	8,690	2,660	0,0120	0,0122	0,7628
*5,49	0,0063	0,1218	0,0063	0,0063	0,0063	0,000	2	10,005	2,660	0,0120	0,0063	0,8782

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Shapiro-Wilk's Test indicates normal distribution (p > 1)	0,88592	0,874	-1,0334	3,66623						
Equality of variance cannot be confirmed										
Hypothesis Test (1-tail, 5)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	0,07	0,2	0,11832		0,01204	0,23349	0,00088	2,7E-05	4,2E-05	5, 8

Parameter	Value	SE	95% Fiducial Limits		Maximum Likelihood-Probit						
					Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	1,0488	0,5459	-0,0212	2,11876	0	0,49784	7,81473	0,92	-0,6193	0,95347	3
Intercept	5,64954	0,39086	4,88345	6,41563							

Point	Probits	mg/L	95% Fiducial Limits
EC01	2,674	0,00145	
EC05	3,355	0,00649	
EC10	3,718	0,01441	
EC15	3,964	0,02469	
EC20	4,158	0,03786	
EC25	4,326	0,05465	
EC40	4,747	0,13776	
EC50	5,000	0,24026	
EC60	5,253	0,41903	
EC75	5,674	1,0563	
EC80	5,842	1,52456	
EC85	6,036	2,33826	
EC90	6,282	4,00502	
EC95	6,645	8,89208	
EC99	7,326	39,6999	



Significant heterogeneity detected (p = 9,20E-01)

Algal Inhibition Test-Area (EbC50)			
Start Date:	Test ID: Arquad Lin	Sample ID:	
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: OECD 201	Test Species:	P.Subcapitata
Comments:	Screening of Arquad 2c-75		

Conc-mg/L	1	2	3	4
0	1,8360	2,0880	1,8720	2,0160
0,0045	2,2320	2,6280		
0,013	2,1600	2,2320		
0,04	0,6840	0,7560		
0,12	0,0000	0,0000		
0,36	0,0000	0,0000		

Conc-mg/L	Transform: Untransformed							1-Tailed				
	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD		
0	1,9530	1,0000	1,9530	1,8360	2,0880	6,090	4				1,953	0
0,0045	2,4300	1,2442	2,4300	2,2320	2,6280	11,523	2	-4,388	2,660	0,2891	2,43	-0,2442
0,013	2,1960	1,1244	2,1960	2,1600	2,2320	2,318	2	-2,235	2,660	0,2891	2,196	-0,1244
*0,04	0,7200	0,3687	0,7200	0,6840	0,7560	7,071	2	11,343	2,660	0,2891	0,72	0,63134
*0,12	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	17,967	2,660	0,2891	0	1
*0,36	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2	17,967	2,660	0,2891	0	1

Auxiliary Tests		Statistic	Critical	Skew	Kurt						
Shapiro-Wilk's Test indicates normal distribution (p > 1)		0,96573	0,874	0,05428	0,92294						
Equality of variance cannot be confirmed											
Hypothesis Test (1-tail, 5)		NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test		0,013	0,04	0,0228		0,28914	0,14805	2,65825	0,01575	6,9E-08	5, 8

Algal Inhibition Test-Growth rate (ErC50)				
Start Date:	Test ID:	Arquad Lin	Sample ID:	
End Date:	Lab ID:		Sample Type:	
Sample Date:	Protocol:	OECD 201	Test Species:	P.Subcapitata
Comments:	Screening of Arquad 2c-75			

Conc-mg/L	1	2	3	4
0	0,0144	0,0159	0,0149	0,0156
0,0045	0,0150	0,0200		
0,013	0,0162	0,0165		
0,04	0,0075	0,0078		
0,12	0,0000	0,0000		
0,36	0,0000	0,0000		

Conc-mg/L	Mean	N-Mean	Transform: Untransformed					N	t-Stat	1-Tailed Critical	MSD	Isotonic	
			Mean	Min	Max	CV%	Mean					N-Mean	
0	0,0152	1,0000	0,0152	0,0144	0,0159	4,493	4				0,0164	1,0000	
0,0045	0,0175	1,1505	0,0175	0,0150	0,0200	19,892	2	-1,753	2,560	0,0033	0,0164	1,0000	
0,013	0,0163	1,0729	0,0163	0,0162	0,0165	1,364	2	-0,850	2,560	0,0033	0,0163	0,9979	
*0,04	0,0076	0,5023	0,0076	0,0075	0,0078	3,447	2	5,799	2,560	0,0033	0,0076	0,4671	
0,12	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2				0,0000	0,0000	
0,36	0,0000	0,0000	0,0000	0,0000	0,0000	0,000	2				0,0000	0,0000	

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Shapiro-Wilk's Test indicates normal distribution (p > 1)	0,91432	0,842	-0,009	2,70777						
Bartlett's Test indicates unequal variances (p = 0,05)	7,68544	2,4E-06								
Hypothesis Test (1-tail, 5)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	0,013	0,04	0,0228		0,00334	0,21973	4E-05	2,3E-06	0,0022	3, 6

Linear Interpolation (200 Resamples)					
Point	mg/L	SD	95%CL(Exp)	Skew	
IC05	0,0154	0,0023	0,0000	0,0166	-1,0754
IC10	0,0180	0,0016	0,0006	0,0199	-0,9678
IC15	0,0205	0,0016	0,0041	0,0232	-0,9192
IC20	0,0231	0,0015	0,0077	0,0264	-0,8557
IC25	0,0256	0,0015	0,0112	0,0297	-0,7759
IC40	0,0332	0,0013	0,0219	0,0396	-0,4338
IC50	0,0383	0,0014	0,0287	0,0482	0,1036

