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The effects of two crude oil solutions to phytoplankton species

Masters Thesis

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1. Introduction

Crude oil is a widely used fossil fuel, which is transported mostly by tankers via waterways. The energy conspumtion and the need for crude oil (hereafter „oil“) and its derivatives continues to grow. The consumption of oil has risen from 1512,8 million tonnes in 1965 to 4059,1 million tonnes in 2011 – that is approximately 63% in 46 years or 1,36% per year (BP 2012). With the expansion of oil consumption and transportation, the hazard of accidental or illegal pollution also increases. History has seen many major oil and fuel spill accidents, for example Ixtoc 1 oil well accident in 1979, the Exxon Valdez oil spill in 1989 and of more recent accidents – the sinking of the Prestige oil tanker in 2002 and the Deepwater Horizon blowout in 2010. The Baltic Sea is one of the most heavily trafficked seas in the world and oil tankers make up about 20% of the ships. According to a Helsinki Commision report, the number of large tankers and the amounts of oil being shipped are on the rise and should increase greatly in the next few years (HELCOM 2010a). With the growing oil transportation, the probability of accidents that result in pollution also increases. Besides accidents, illegal oil discharges also occur. Even though the frequency and amount of oil spilled during illegal discharges have shown a slight decline in the last few years, the hazard of oil pollution still remains at a high level (HELCOM 2010b).

Oil and its derivates have been found to be toxic to a wide array of marine organisms from bacteria and archea to large seabirds and –mammals. The effects of oil on single organisms may add up to affect populations and communities, as well as whole ecosystems. Phytoplankton, the major primary producer in the marine environment, is also sensitive to environmental stressors such as pollution. Phytoplankton plays a crucial role in the inflow of primary energy to coastal food webs, thereby changes in phytoplankton may cause significant damages to the functioning of marine ecosystems (Hallare et al. 2011).

There have been many studies of oil spills regarding their toxicity to different marine organisms both in laboratories and with natural phytoplankton communities (Gordon 1973; Harwell 2006; Mohammady et al. 2005; Taş et al. 2010; Varela et al. 2006). However, there have not been many studies conducted with brackish water species.

This study focuses on examining the effects of oil on some common phytoplankton species of the Baltic Sea to see wheather the responses of the algae may be species-specific.

2. The effects of crude oil pollution on phytoplankton.

2.1 Crude oil composition, toxicity and spill dynamics.

Crude oil is a dark oily liquid formed from ancient plant and animal remains (mostly zoo-and phytoplankton) in conditions of high temperature and pressure (Hsu and Robinson 2006a). Crude oil or petroleum is a myriad of different chemical components, which is why crudes from different locations, depths and of different age have different compositions (Hsu and Robinson 2006b).

Crude oil is distilled to make fuel and extract more volatile fractions of higher economic value (Perez et al. 2010). Some crude oil componenets, such as naphthalene are also toxic individually. The toxic effect of polycyclic aromatic hydrocarbon mixtures may be antagonistic or synergetic in their cumulative effects on phytoplankton (Gilde and Pinckney 2012), meaning that they may act individually or their toxicity may increase with other components.

The chemicals in oil can be arranged into groups in accordance with their solubility in organic solvents, molecular weigh and/or boiling point.

The saturates

Alkanes and cycloparaffins have a low boiling point and have been shown to act as an anesthetic, producing a narcotic state in lower animals at low concentrations and causing cell damage or even death at higher concentrations in a wide variety of lower animals (Blumer 1972). The toxicity of alkanes is related to their chain lenght, which correlates with their solubility in water and also hydrophobicity/ lipophilicity. Cycloalkanes have the ability to inhibit oxygen uptake and disrupt the permeability

barrier of the inner membrane in mitochondria and thus slow down respiration, while impairing ATP synthesis (Sikkema et al. 1995).

The aromatics

The aromatics (mono-, di-, and polynuclear aromatic compounds of the arene class) fraction is divided into low boiling aromatics such as BTEX (benzene, toluene, etylbenzene, xylene), which are acute poisons (Blumer 1972), and high boiling aromatic hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs), that are considered to be the most toxic fraction of petroleum due to their high adsorption affinity for particulate surfaces (Blumer 1972; Jiang et al. 2010; Perez et al. 2010) . Polycyclic aromatic hydrocarbons have a number of aromatic rings ranging from 2 to 10. They are often described as mutagenic, teratogenic and carcinogenic to different organisms (Blumer 1972; Saco-Alvarez et al. 2008). The most abundant PAHs in oil are naphthalene, phenanthrene, fluoranthene and acenaphene (Gilde and Pinckney 2012)**.** PAHs have a high solubility in water and a high bioavailability to phytoplankton (Pelletier et al. 2006). But aromatics also volatilize quite rapidly, which is why the depressive effect of PAHs on phytoplankton growth is concidered to be limited to a few hours (O'Brien and Dixon 1976). When exposed to ultraviolet radiation, PAHs photodegrade to form more reactive compounds with an increased toxicity in comparison with parental compounds. The increased toxicity of photomodified PAHs may be contributed to their higher solubility and thus higher bioavailability (Pelletier et al. 2006). Due to a high molecular weigh and hydrophobicity, PAHs adsorb particularly well onto surfaces, including cell walls. The aromatic fraction has been shown to impair energy transduction, damage the cytoplasmic membrane and cause the leakage of macromolecules from the cell (Sikkema et al. 1995).

The resins and the asphalthenes

The resins (aggregates with a multitude of building blocks such as pyridines, quinolines, carbazoles, thiophenes, sulfoxides, and amide) and the asphalthenes (aggregates of extended polyaromatics, naphthenic acids, sulfides, polyhydric phenols,

fatty acids, and metalloporphyrins) have high molecular weigh and form tar balls, which may persist in the environment for many years, because they are not easily (bio)degraded due to their small surface area (Tkalich et al. 1999). These residues stick easily to surfaces and may coat different organisms.

In addition, heterocyclic compounds containing N, S, O, metals and other elements are also present in crude oils, which are relatively highly soluble and may therefore accumulate in cells (Barron 1999).

In general, smaller hydrocarbon molecules within a class are more toxic than larger ones due to their higher penetration ability through cell walls (O'Brien and Dixon 1976). Important parameters affecting a compound's ability to penetrate cells are the boiling point and viscosity of the compounds, which are related to their molecular size. The toxicity of the components also grows with the increase of carbon chain and/or benzene rings in a particular compound. The overall toxicity is a function of individual compound toxicity, their joint toxicity and the existance of other environmental stressors such as ultraviolet radiation and other pollutants (Jiang et al. 2010). Soluble components of oil bioaccumulate and may also contribute to toxicity (Barron 1999).

Many of the compounds in oil have a high lipophilicity, which depends on various chemical and physical characteristics (e.g molecular surface area, molecular volume, polarity). The lipophilic compounds dissolved in water act as non-specific metabolic inhibitors (Perez et al. 2010) and are taken up passively by cells and are accumulated mostly in the cytoplasmic membrane (Sikkema et al. 1995).

As mentioned, the smaller the molecular weight and/or lower degree of saturation, the higher the solubility. Since higher solubility means higher bioavailability, then the more soluble compounds have a potentially higher toxicity. Solubility and dissolution is also dependent upon temperature, pH, salt concentration and other parameters (Sikkema et al. 1995). Solubility is also important in dispersing toxic components through the water column (O'Brien and Dixon 1976).

Factors affecting the oil spill

Once spilled, oil forms a thin film over the surface of the water called and oil slick, that travels downwind 3-4% of the wind speed (Tkalich et al. 1999). The spreading of the oil and the thickness of the slick depend on interfacial tensions between the oil and the water surface, the nature of the oil and the temperature of the water (Tkalich et al. 1999). Atmospheric conditions affect the physical parameters that are involved in the spreading of the oil, e.g surface tension, specific gravity, viscosity etc. Spreading is increased if the surface tension is low. The latter is reduced with increasing temperatures. Therefore oil is more likely to spread in warmer waters and will remain in place at lower water temperatures in winter. Most crudes are lighter than water and thus will accumulate on the surface of the water. However, the specific gravity of an oil spill may increase as the volatile fraction of the oil evaporates. Another factor influencing the spreading of the oil is viscosity. The smaller the viscosity of the oil, the greater the tendency for it to spread. Viscosity is also reduced with rising temperatures, therefore the spreading of a spill is faster in warmer waters and in summertime (Riley and Skirrow 1975). The bigger the area of the spill, the more phytoplankton communities it can affect, therefore the effects of crude oil may be more pronounced during the summer.

The spilled oil is subject to weathering – volatilization, hydrolysis, photolysis, oxidation, and biodegradation processes, that ultimately remove most of the oil. But since those processe take time, oil may be washed up on shores or into bilogically sensitive tidal areas or estuaries, it may accumulate in different organism or sink to the seafloor and affect the bentos communities (Tkalich et al. 1999). Spilled oil is also a subject to photodegradation via ultraviolet radiation (280-320 nm). Ultraviolet radiation may also be reffered to as an additional stressor to phytoplankton, which makes them more susceptible to the water soluble fraction of the oil (Pelletier et al. 2006). Concurently with the weathering and photolysis, the spilled oil is constantly diluted and the concentration of the toxic components decreases over time.

2.2 The effects of oil on phytoplankton

Oil can affect phytoplankton in both direct and indirect. The effects of crude oil on specific phytoplankton groups or species depend on the type and the amount of oil spilled (Taş et al. 2010), the time of the year and the prevailing weather conditions of the the spill occurence, and also the environmental and physical conditions of the spill location, phytoplankton density and composition at the time of the spill (O'Brien and Dixon 1976), and the size of the cells (Echveste). Additionally the presence of other pollutants and prior exposure of the area to oil plays also an important role in the overall toxic effect (O'Brien and Dixon 1976).

The type and the amount of the spilled oil

As mentioned, crudes obtained from different locations have different compositions and proportions of the ingridient chemicals. The toxicity depends on the toxicity and concentration of individual chemicals. Crude oils have been found to be less toxic to phytoplankton than refined fuel oils (Ansari 1997; El-Sheekh et al. 2000; Sikkema et al. 1995). Refined oils as a rule are enriched with different aromatic compounds (e.g biphenyl, naptalene, toluene, benzene) in order to enchance the octane rating of the fuel (the higher the octane rating the better the fuel) (Sweeting 1993) an thus the increased concentration of aromatics makes refined oils more toxic to phytoplankton (and various other organisms).

In the study of oil toxicity to phytoplankton, the water soluble fraction (WSF) of the oil is often used, which is concidered to be saturated with oil compounds (Saco-Alvarez et al. 2008). The WSF is the part of the oil that remains in the water after the weathering processes and is simpler in composition than the original oil (Ansari 1997). High concentrations of WSF have been shown to inhibit greatly phytoplankton growth and decrease cell density (Huang et al. 2011). Low concentrations of WSF have been shown to restrain significantly phytoplankton growth. On one hand the toxicity of oil acts through the decrease of CO_2 and nutrients absorption. On the other hand, it inhibits

both respiration and photosynthesis, damages membranes and the antioxydation defence system and blocks nuclear acid on protein synthesis (Tkalich et al. 1999)

The more tolerant species of algae may use the oil hydrocarbons as a additional carbon source (Tkalich et al. 1999). Low concentrations of WSF have a less pronounced toxic effect and may even promote growth (Jiang et al. 2010). This would explain the bloom of certain kinds of algae at low concentrations of oil under suitable conditions (Tkalich et al. 1999). For example, the elevated growth level of *Chlorella vulgaris* under heavy oil pollution is due to its supposed ability to make use of certain oil hydrocarbons (O'Brien and Dixon 1976).

Furthermore, it is also possible that at low concentrations of oil contamination, oxygen free radicals present in the weathering and photo-activated oil may promote the production of superoxide dismutase (SOD), which may enchance the defensive reactions in the cells (Huang et al. 2011). This may be one of the reasons for the higher resistance to oil pollution in the tolerant species. Contrarily, some reasearches have found that at very low concentrations the growth of phytoplankton is severely inhibited by petroleum hydrocarbons (Ansari 1997) and even sub-lethal levels of oil can induce stress in phytoplankton communities (Gilde and Pinckney 2012).

The occurence time and location of the spill

The season during which the spills occurs is very important because of the environmental conditions (atmospheric conditions and water temperature), nutrient availability and phytoplankton composition. The negative effects of a spill decrease if it occurs during winter (Taş et al. 2010) since the oil spread slower in lower temperatures. Furthermore, since the intensity of ultraviolet radiation is lower during wintertime, there are less photoderivatives of oil produced. The amount of solar irradiance and hence UV radiation arriving at the surface of the water varies with the time of the day, season, latitude and also cloudiness (Saco-Alvarez et al. 2008).

The location of the spill is important in the spill dynamics and thus the extent and toxic effects of the pollution. Additionally, there may be other pollutants in the oil spill area, which may have a joint toxic effect to phytoplankton. An additive effect of a wide

array of pollutants at low concentrations may induce a joint toxic effect equivalent to a single chemical at a high concentration (Echeveste et al. 2010b).

Density and composition of the phytoplankton at the time of the spill

It has been noted that if an oil spill occurs during an algal bloom, the inhibition of phytoplankton growth is less apparent compared to a „normal“ state. This is possibly due to the decreased amount of oil each cell receives when the gross cell count is high. Hence the sensitivity of cells to oil contamination decreases when the cell concentration increases (Hing et al. 2011). On the other hand, the decreased cell abundance in polluted area cause more toxicants available per cell and therefore the toxic effect is enhanced (Perez et al. 2010)

There is a natural shift in phytoplankton species composition depending on the season. During an algal bloom, usually one-two species are dominant, while the number of other species remain relatively low. After the bloom, the dominant species change and a new bloom may occur. In case of a spill, algal biomass decreases overall, but some groups may actually increase in relative abundance, resulting in a change in community structure. (Gilde and Pinckney 2012).

Microalgal species show a variability in their sensitivity to different petroleum hydrocarbons (Ansari 1997; Jiang et al. 2010) in reference to growth rate and photosynthetic assimilation (O'Brien and Dixon 1976). The stress of pollution and the natural variability in the tolerance of different phytoplankton groups results in an abnormal species succession (Huang et al. 2011; Jiang et al. 2010) – the less tolerant species, which may be the dominant species in the area, decrease or even vanish, while the more resistant ones may gradually become dominant (Jiang et al. 2010). The tolerant groups may maximize their reproductive potential (and thus show an increase in their biomass) because the competition for nutrients and solar radiation decreases after the elimination of the more susceptible species (O'Brien and Dixon 1976). Overall, it has been shown that the diversity of phytolankton in polluted areas is greatly reduced, species number and cell density may decrease by up to 50% of the pre-accident conditions (Jiang et al. 2010). This is because growth under petroleum contamination could only be possible with some kind of adaptation. It is assumed that this can be

achieved as a result of physiological acclimatization supported by modifications of gene expression (Romero-Lopez et al. 2012). The gross effect of the spill on phytoplankton populations will thus be influenced by the original species composition at the time of the oil discharge and may consequently display a clearly defined seasonal variability in the outcome of the spillage (O'Brien and Dixon 1976).

Cell size

The toxicity tresholds of individual hydrocarbons are dependent upon the size of the cells (Echeveste et al. 2010a; Urakawa et al. 2012). Picophytoplankton, that often dominates the phytoplankon communities, is strongly affected by PAHs and the decline is more pronounced with the growing toxicants concentration. The smaller cells have a higher surface/volume ratio and hence a bigger specific surface to which oil compounds may adsorb (Echeveste et al. 2010a).

2.2.1 Inhibition of growth, and biomass.

Individual cells are most sensitive to oil contamination when steady state growth is achieved. If the oil concentration does not surpass the tolerance limit of specific species, the growth rate will be in equilibrium with the dilution of the oil in the environment and the steady state will be maintained (Hing et al. 2011). This is confirmed by the recovery of phytoplankton exposed to low concentrations of oil (Perez et al. 2010). If, however, the amount of the oil exceeds the cell tolerance, it may prevent the cells from growing.

Growth may be inhibited due to the prolongation of the cell cycle (Perez et al. 2010) rather than inducing mortality, although some studies have found that cell density decreases and the percentage of the dead cells increases after the addition of petroleum hydrocarbons (Echeveste et al. 2010a; Gilde and Pinckney 2012). The reduction of growth may also be contributed to the decrease in cellular ATP content due to the impairment of the photosynthetic apparatus (Perez et al. 2010). Additionally, a reproductive inhibition occurs (O'Brien and Dixon 1976).

Although the overall cell density of phytoplankton decreases, there may be an apparent increase in phytoplankton biomass due to reduced grazing. Zooplankton is shown to be more sensitive to oil pollution than phytoplankton and hence the top-down effect weakens (Hallare et al. 2011; Jiang et al. 2010). The seeming increase in one group of phytoplankton may also be due to a decrease in other groups (Gilde and Pinckney 2012).

Still, these inhibitory effects are considered to be more or less temporary, because the oil in the environment is constantly diluted, weathered and (bio)degraded (Hing et al. 2011), while the phytoplankton has a quite short generation time. It has been shown that the subsiding of PAHs due to volatilization results in an increase in the chlorophyll concentration (Hallare et al. 2011).

There is also evidence that some cyanobacteria and microalgae can adapt to oil pollution rather quickly by single gene mutations and are also able to proliferate under low oil concentration as a result of physiological acclimatization (Romero-Lopez et al. 2012).

2.2.2 Damages to the cell membranes

The cytoplasmic membrane constists of phospfolipid bilayer and plays a crucial role in maintaining the homeostasis of the cell. Because of the high lipid content, highly lipophilic compounds penetrate relatevly easily through the outer membrane. The cytoplasmic membrane has a low permeability for polar and charged molecules, but PAHs are apolar and can thus easily penetrate the lipid bilayer. The accumulation of lipophilic compounds and interaction with the phospholipid layer of the membranes results in the loss of membrane integrity and modification of membrane fluidity. This may cause the swelling of the bilayer and affects the functioning of the membrane (Sikkema et al. 1995). The membranes of the chloroplasts and mitochondria are also affected, resulting in the hindering of the metabolic pathways (O'Brien and Dixon 1976).

In addition to hydrocarbons, membranes may also be damaged by the oxygen free radicals (see 2.2.4) through the peroxidation of the bilayer lipids (Saco-Alvarez et al. 2008).

2.2.3 Inhibition of photosynthesis

Photosynthesis is a chain of reactions to make organic matter from inorganic carbon with the energy from the sun. Photosynthesis is the main process in phytoplankton that provides energy needed for the functioning of the cells (Young and Beardall 2003).

Different crude oil types inhibit photosynthesis of natural phytoplankton communities in proportion to their concentrations (O'Brien and Dixon 1976). Firstly, the oil acts as a physical barrier for the diffusion of CO_2 across cell walls and absorption of nutrients, resulting in the decrease of chlorophyll *a* and reduction of primary productivity (Jiang et al. 2010), whereby the inhibition of carbon uptake is increased with increasing oil concentration (Gordon 1973). An oil slick forms a dark coating on the surface of the water that may additionally hinder or completely block the penetration of sunlight into the water column. The decrease of available sunlight may significantly inhibit photosynthesis. The slick also limits the gas exchange between the water and the atmosphere. Because CO_2 is used as a carbon source for photosynthesis, the decrease of CO2 diffusion into the water also limits the photosynthesis.

The lipophilic hydrocarbons of the water soluble fraction bioaccumulate in phytoplankton and can have a direct toxic effect or suppress their photosynthesis by reducing the primary photochemical yield (Jiang et al. 2010; Pelletier et al. 2006). The degradation products of WSF such as naphthoquinones and hydroxylated compounds can also potentially block photosynthesis (Pelletier et al. 2006). Naphthoquinones have a structural similarity with plastoquinones, which serve as electron carriers between photosytem II (PS II) and cytochrome complexes (Richmond 2007) and thus compete with each other.

Oil acts primarily on the PSII (Singh and Gaur 1990) and blemishes photosynthesis probably through a direct interaction with the electron transport system. Petroleum

damages membranes and proteins associated with the photosynthetic electron transport, resulting in excitation pressure on PSII (Perez et al. 2010).

Chloroplasts have a lipid membrane and a high lipid content, which functions as a carbohydrate reservoir (Benson et al. 1959), therefore compounds with the highest lipid solubility exert the most damage to the cells (O'Brien and Dixon 1976) and tend to accumulate in the chloroplasts (Perez et al. 2010). The light reactions of the photosynthetic cycle are carried out in the thylakoid membranes, which are composed of a lipid bilayer with embedded proteins (Richmond 2007), that are stacked on top of each other forming grana (Barsanti and Gualtieri 2006). The thylakoid membranes enclose a narrow space called the lumen, which is the site where the key steps in photosynthetic electron transport take place and is as a reservoir of protons for ATP synthesis (Kana et al. 2009). It has been suggested that hydrocarbons dissolve in the lipoid phase of the grana of chloroplasts. Additionally, membrane distortion causes an increase in distance between individual chlorophyll molecules and may also impair the ability to photosynthesize (O'Brien and Dixon 1976). Further, petroleum has the ability to destroy chlorophylls and also precursor pigments, that may cause severe assimilatory problems even if the chlorophyll molecules remain unharmed. The chlorophyll *a* amount is often used as an indirect measure of the phytoplankton productivity (Echeveste et al. 2010b), therefore the reduction of chlorophyll is also a decrease in productivity. The loss of photosynthetic pigments, however, is evidently a reversable process in sublethal environments (O'Brien and Dixon 1976).

In addition to the reduction of the electron transport capacity, the WSF also decreases the activity of the release of oxygen centers. In these conditions, where the production of organic molecules (sugars etc.) is inhibited, respiration echances to satisfy the energy demand of the cells (Jiang et al. 2010). However, petroleum also penetrates mitochondria and damages their membranes, resulting in the loss of the ability to respirate aerobically (O'Brien and Dixon 1976). The impairment of both photosynthesis and respiration ability may be fatal to the cells.

2.2.4 Influence on genes and DNA

On the genetic level, the chemicals in the oil have been shown to diminish the synthesis of nucleic acids and reduce DNA, RNA and the protein content in phytoplankton, damage the DNA and prevent the replication of DNA (Jiang et al. 2010). Exposure to PAHs in particular alters and reduces expression of genes in charge of photosynthetic pigment production (chlorophylls and carotenoids) (Romero-Lopez et al. 2012) and silica-associated proteins necessary for cell divisions in diatoms (Gilde and Pinckney 2012) and prevents the replication of DNA (Jiang et al. 2010).

Upon receiving UV irradiation, extremely reactive free radicals are formed, when the absorbed radiation from PAH molecules is transfered to oxygen molecules (Saco-Alvarez et al. 2008). Photoproducts and -derivates are also formed and they may have an increased toxicity compared with the original chemicals found in oil, for example carbonyl compounds, phenolic acids, peroxides, fluorenone) (Larson et al. 1977). These chemicals and the free radicals damage DNA and RNA (El-Sheekh et al. 2000) and induce gene mutations and may cause cell abnormalities (Jiang et al. 2010).

3. Materials and methods

3.1 The algae species and culturing conditions

To assess the inhibitory effects of oil on phytoplankton, seven algae species were used. The algal strains used in the current study were *Rhodomonas salina* (Tvärminne culture collection), *Brachiomonas submarina* (Tvärminne culture collection), *Prymnesium parvum* (Kalmar University Algal Collection, KAC 39), *Thalassiosira pseudonana* (Tvärminne culture collection, TV5), *Pavlova lutheri* (Tvärminne culture collection), *Isochrysis galbana.* (Tvärminne culture collection, CCAP 927/12), and *Monoraphidium contortum*. The *Monoraphidium contortum* was isolated from the Gulf of Finland in 2012. Single cells were collected with a micropipette and transferred to multidish wells each containing filtered sea water (salinity 6 ppt) and after a week cells were transferred to new multidish wells containing f/2 medium.

Cryptophyte *Rhodomonas salina* is an unicellular brownish-green microalga with two hairy flagella (Barsanti and Gualtieri 2006). *R. salina* has a single large two-lobed chloroplast, that contains chlorophylls *a* and c_2 (Kana et al. 2009; Skovgaard 1998). Additional light-harvesting pigments found in Cryptophyta are α-carotene, diatoxanthin, phycoerythrin, and phycocyanin. The cell of *R. salina* is encircled by a periplast consisting of a plasma membrane and plates underneath it (Lee 2008).

Brachiomonas submarina is a single celled green algae with two flagella from the phylum Chlorophyta. Like *R. salina, Brachiomonas submarina* also has only one large chloroplast, but the chlorophylls found in *B.submarina* are chlorophylls *a* and *b*. The major carotenoids are lutein and β-carotene. Unlike in other microalgae, the storage product (strach) is stored in the chloroplast as opposed to the cytoplasm. The main ingridients in the cell walls of *B. submarina* are cellulose and glycoproteins (Lee 2008). Another species of the same phylum is *Monoraphidium contortum*. It is a unicellular alga with a S-like shape.

Prymnesium parvum, Pavlova lutheri and *Isochrysis galbana* represent the phylum Prymnesiophyta (also called Haptophyta). All of these species are motile unicellular microalgae and possess a unique organelle called the haptonema, which is

used to induce rapid backward swimming in case of obstacles or to capture food (Edvardsen et al. 2000). Prymnesiophytes usually have two chloroplasts per cell, which contain chlorophylls *a* and c_1 or c_2. The main carotenoids are β-carotene, diadinoxanthin and diatoxanthin. P.lutheri has single two-lobed chloroplast with an eyespot (Lee 2008). *P. parvum*, a common species in the Batic Sea, may form toxic blooms in low-salinity and nutrient-rich conditions (Fistarol, 2003). *P. lutheri* and *I. galbana* on the other hand do not produce toxins. Prymnesiophyta cells are commonly covered with elliptical organic scales embedded in a mucilaginous substance (Lee 2008).

Centric diatom (Heterocontophyta) *Thalassiosira pseudonana* possesses chlorophylls *a,* c_1 and c_2 and the main carotenoid is fucoxanthin, which is part of the PS II (Lee 2008). The cells of diatoms are enclosed in a frustule – a special box-like structure consisting of two halves fitting into each other, and consists mainly of silica (Lee 2008).

The phytoplankton cultures were grown in Schott Duran 100ml flasks as non-axenic batch cultures in f/2 medium (Andersen 2005; Guillard and Ryther 1962) and kept in a Termaks algae incubator (Termaks AS) at 20^{o}C with a 16h light:8h dark cycle, under an irradiace of 200μmol $m^{-2}s^{-1}$. The f/2 culture medium was prepared with filtered (2μl Whatman® filter papers) and autoclaved Baltic Sea water (with a salinity of 6 ppt), at 120^{o}C. The sea water was collected off the coast of North-Estonia in the Lahemaa national park region near Viinistu. The cultures were diluted with fresh medium every 3-4 days to avoid nutrient limitation and to assure cultures were in exponential growth phase. The cultures used in the experiments were in good physiological condition with fluorescence yield (Fv/Fm) of at least 0,50 (Lippemeier et al. 2001).

3.2 Measurements of the phytoplankton species photosynthetic efficiency.

Because oil affects the electron transport in photosystem II, fluorescence yield measurments were chosen to study the inhibitory effects of oil solutions to phytoplankton. The fluorescence yield is used to assess the photosynthetic efficiency of the reaction centres of PSII. In the current study (negative) changes in the fluorescence yield were used as an indicator of physiological stress caused by the oil.

The fluorescence yield (F_V/F_M) (here also reffered to as photosythetic efficiency) is a ratio between variable fluorescence (F_V) and maximum fluorescence (F_M) and has a value between 0 and 1. The variable flourescence F_V is the difference between maximum flourescence F_M, where the reaction centres are closed (electron acceptors are reduced) and minimum flourescence F_0, in which case the reaction centres are open (electron acceptors are oxidised). The fluorescence yield of PS II is calculated from the following equation (1):

$$F_V/F_M = (F_M - F_0)/ F_M) \quad (1)$$

The fluorescence yield of the algae cultures was measured at the beginning of the experiments (day 1) to be used as a reference point for both the control and treatment replicates. The measurments of the fluorescence yield were done on the second, third and fourth day or approximately 24h, 48h and 72h after the start of the experiments using the Phyto-PAM Phytoplankton Analyzer (Heinz Walz GmbH, Effeltrich, Germany) equipped with the Optical Unit ED101-US. A a 10 × 10 mm quartz cuvette was used for measuring the cultures and the controls and a disposable 12,5 x 12,5 mm cuvette for the treatment measurments.

3.3 Experimental setup

Each phytoplankton culture was divided into 6 batches , 3 of which were used as control replicates and 3 as treatment replicates. The density of the cultures was measured with CASY TT Particle Counter and Analyser (Schrfe System, Germany) to make sure the cultures had approximately equivalent densities. The mean density of the cultures was approximately 300 000 cells per milliliter.

For the controls, 3 batches of 5 ml of the cultures were added to 25 ml glass tubes. The treatments were prepared with a crude oil type, which is commonly transported in the Baltic Sea. The crude oil solutions were prepared with prefiltered

autoclaved Baltic Sea water with a salinity of 6 ppt. The oil – water emulsion was mixed using the Branson Digital Sonifier equipped with a microtip, to achieve a more homogenous blend. The treatment solutions were added to the cultures in a certain proportion, to achieve approximately 1000-fold and 250-fold dilutions (hereafter treatments). Exact concentrations could not be attained, because there were certain losses of the oil due to it sticking to the surfaces of the equipment used.

200 µl of medium was added to both control and treatment batches to prevent nutrient limitation, because the latter may cause additional changes in the fluorescence yield (Kolber et al. 1988; Lippemeier et al. 2001). All of the glass tubes were covered with plastic caps to minimize evaporation and the volatilization of the volatile fractions (mainly PAHs) of the treatments. The controls and the treatments were placed on a mixer (Heidolph Instruments Unimax 2010) at a medium speed of about 140 rpm. Experimental temperatures and irradiances were the same as as described for the maintanance of the cultures.

3.4 Statistical Analyses

The statistical analyses were carried out using the statistical computing software R (Crawley 2007). Two-way interaction ANOVA was used to reveal a potential interaction between the different oil concentrations (the treatment effect) and different species.

4. Results

Measurments of the fluorescence yield were done approximately 24, 48 and 72 hours after the addition of oil-water solutions. During this time, the fluorescence yield of the controls remained relatively stable, while a significant decrease in the yield of the treatments was observed. Both treatments reached their lowest fluorescence yield value by day 2 of the experiment or appoximately 24h after the addition of oil (Figure 1). For this reason the fluorescence yield of the second day was compared to the fluorescence yied of the first day of the experiment before the addition of oil. The latter is referred to as the reference yield.

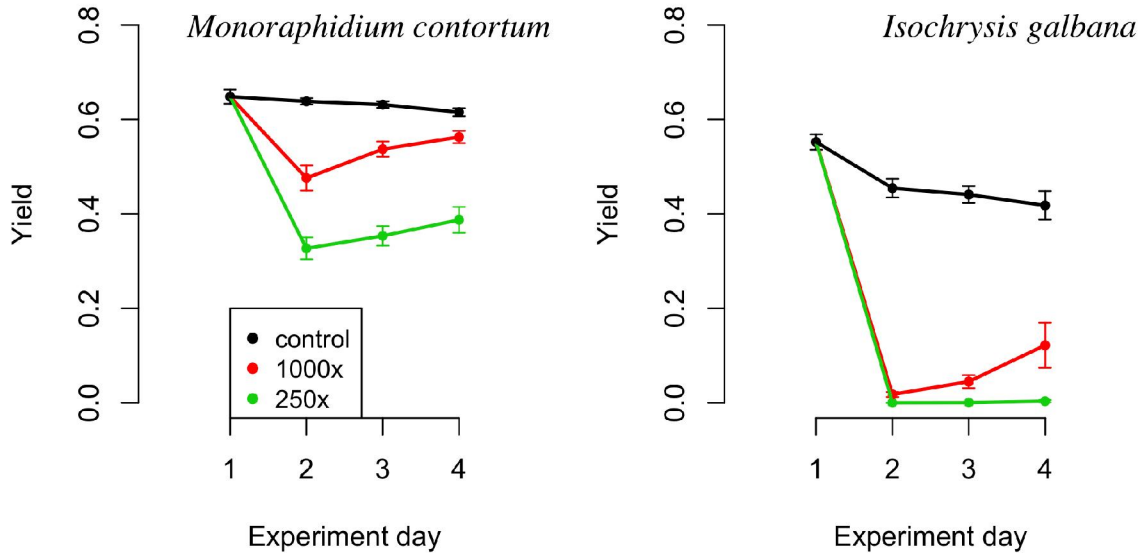

Figure 1. Examples of the varying response levels across species to the different treatments. Error bars indicate 95% confidence intervals (1.92* SE.).

Because the reference points of different cultures being slightly different, the decline of the fluorescence yield was not calculated as an absolute value, but rather as a percentage of the remaing yield. This way the comparison of the species would be coequal.

Table 1. Analysis of Variance of the changes in the fluorescence yield.

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Treatment	2	1427291	713646	4396.954	$< 2.2^{e-16}$
Species	6	169559	28260	174.116	$< 2.2^{e-16}$
Treatment+species	12	112229	9352	57.623	$< 2.2^{e-16}$
Residuals	1203	195252	162		

The response of different species to the tretments was variable, therefore the interaction between the treatment effects and species response was calculated. It is a mean response not attributable to the additive effect of the treatments and species. The effects of the separate factors as well as the interaction between the two factors on the treatment response (change in the yield) were statistically significant (Pr (>F) < $2{,}2^{e-16}$) (Table 1).

The controls (calculated after *B.submarina* as the intercept) showed a mean decrease of approximately 9% compared to the reference yield. There were significant differences in the effects of the two treatments – the 1000-fold oil dilution accounted for an additional drop of approximately 29% by day 2 of the experiment compared to the controls and the 250-fold dilution for an 79% decrease (Tabel 2).

Table 2. Changes in the fluorescence yield on day 2 of the experiment. The Estimate is the percentage of the mean yield remaining by day 2 of the experiment compared to the reference yield of day 1.

Factor	Estimate	Std Error	T value	Pr(>|t|)
Intercept (*Brachiomonas salina* control)	90.961	1.280	71.040	$< 2^{e-16}$
Treatment1000	-28.894	2.290	-12.615	$< 2^{e-16}$
Treatment250	-79.134	2.155	-36.717	$< 2^{e-16}$
Isochrysis galbana	-10.743	1.973	-5.444	6.30^{e-08}
Monoraphidium contortum	7.800	1.811	4.308	1.79^{e-05}
Pavlova lutheri	6.174	1.973	3.129	0.001796
Prymnesium parvum	9.243	1.973	4.684	3.13e-06
Rhodomonas salina	13.150	1.773	7.418	2.24e-13
Thalassiosira pseudonana	2.931	1.855	1.580	0.114447
Treatment1000 + *Isochrysis galbana*	-47.782	3.465	-13.788	$< 2^{e-16}$
Treatment250 + *Isochrysis galbana*	-1.029	3.378	-0.305	0.760582
Treatment1000 + *Monoraphidium contortum*	6.269	3.239	1.935	0.053164 .
Treatment250 + *Monoraphidium contortum*	29.293	3.048	9.611	$< 2^{e-16}$
Treatment1000 + *Pavlova lutheri*	-14.546	3.465	-4.197	2.90^{e-05}
Treatment250 + *Pavlova lutheri*	-13.144	3.378	-3.892	0.000105
Treatment1000 + *Prymnesium parvum*	-13.655	3.333	-4.097	4.46^{e-05}
Treatment250 + *Prymnesium parvum*	-1.026	3.593	-0.285	0.775337
Treatment1000 + *Rhodomonas salina*	-27.635	3.123	-8.848	$< 2^{e-16}$
Treatment250 + *Rhodomonas salina*	-7.697	3.025	-2.544	0.011082
Treatment1000 + *Thalassiosira pseudonana*	-52.431	3.400	-15.422	$< 2^{e-16}$
Treatment250 + *Thalassiosira pseudonana*	-13.935	3.075	-4.532	6.42e-06

Multiple R-squared: 0.8975, Adjusted R-squared: 0.8958
F-statistic: 526.5 on 20 and 1203 DF, p-value: $< 2.2^{e-16}$

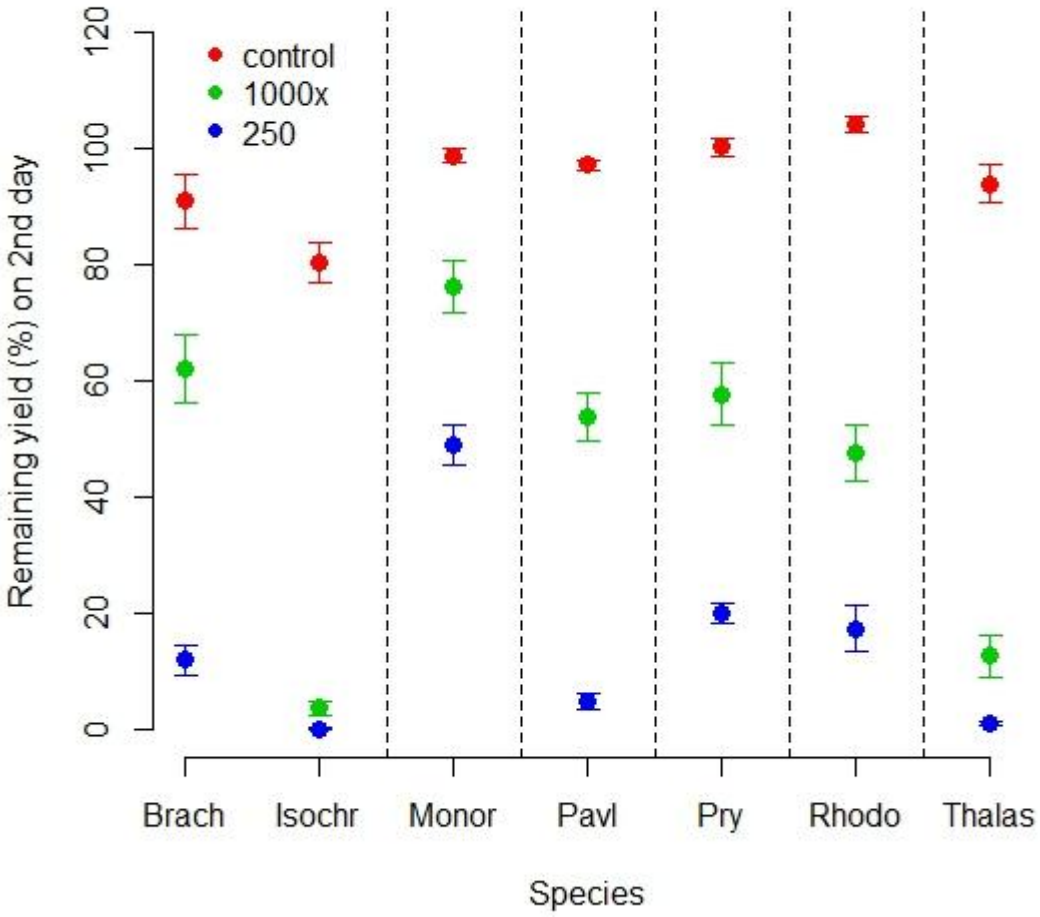

Figure 2. The mean response on species to oil treatments. Error bars indicate the confidence intervals of the mean (1.96*standard error).

In general, the 1000-fold oil dilution had a weaker toxic effect than the 250-fold dilution. However, there was a notable difference in the response of the phytoplankton species (Table 2). *T. pseudonana* and *I. galbana* were greatly affected by both treatments, while the other species showed a higher discrepancy in the response to the different treatments (Figure 2). The drop in the fluorescence yield below the value 0,20 suggests a severe impairment of the the electron transport system of PSII and thus a significant inhibition of photosynthesis. The fluorescence yield value of zero may be considered lethal. Therefore it can be assumed that both *T. pseudonana* and *I. galbana* may have suffered near-lethal impact of the oil.

5. Discussion

The aim of the study at hand was to investigate crude oil influence on phytoplankton species and observe possible differences in species tolerance to oil toxicity. The level of crude oil toxicity on the plankton organisms depends on various factors including crude oil concentration in the environment, the physiological state of the organism, cell membrane composition, the availability of nutrients, community diversity and density (O'Brien and Dixon 1976; Taş et al. 2010). A target culture with better tolerance levels will have a higher chance of survival in crude oil experiments.

In this study, microalgae was harmed in the crude oil treatments. Direct contact with crude oil influenced the photosynthetic efficiency of the phytoplankton in a negative way. The decline in the fluorescence yield of the treatments may be attributed to a possible decrease in the chlorophyll concentration, which is mainly affected by the PAHs present in the treatments (Hallare et al. 2011). The chlorophyll concentration may decline due to increased cell mortality or the damages to the chloroplasts and destruction of the pigments.

Results indicate that there is a variability in the susceptibility of distinct phytoplankton species to oil. In general, exposure to crude oil inhibits phytoplankton photosynthetic efficiency, but some species can be tolerant or slightly influenced or even growth simulated by crude oil toxicity (Hallare et al. 2011). In the conducted experiment, phytoplankton cells were affected markedly at 250-fold crude oil dilution. The photosynthetic efficiency of the untreated phytoplankton species was similar in all cultures, but after the oil was added, microalgae photosynthetic efficiency decreased in crude oil treatments.

In most algal cells the maximum value of photosynthetic efficiency is ca 0.65 ~0.70, but can be significantly lower if the cells are nutrient starved or otherwise stressed (Falkowski and Kolber 1995). This means photosynthetic efficiency is a good proxy to estimate the physiological stress of phytoplankton cells.

Almost all species in this study showed a considerable inhibition of the photosynthetic efficiency by the oil; some species, namely *T. psuedonana* and *I. galbana* seem to be more susceptible to oil (Figure 2). The best example of toxicity

tolerance on the other hand was probably the experiment with *Monoraphidium contortum*.

Although without further study of phytoplankton cell structures and membrane functioning, it is difficult to argue about the reasons underlying the variability in the tolerance of the distinct species, it can be theorised that the differences may be due to the differences in the cell wall compositions of distinct species or a presence of a native physiological and chemical defence system.

Both Chlorophytes *M. contortum* and *B. submarina* were relatively less affected by the oil treatments compared to the other species. Results indicate that the crude oil tolerance levels of different species of Chlorophytes varies. The variation can occur due to the observed species having different cell membrane compositions. Chlorophytes with flagellae (including *B. submarina*) have a glycoprotein based cell membrane, whereas the cell membrane of Chlorophytes without flagellae consists of sturdy polysaccharides. Our results show that out of the two Chlorophytes *M. contortiom* tolerated the effects of crude oil better than *B. submarina*.

Of the Prymnesiophytes, *P.parvum* showed the highest tolerance tresholds. Because *P. parvum* is a toxin-producing species with a native defence mechanism against self-toxicity (Olli and Trunov 2007), it is possible that this mechanism is also responsible for its higher resistance to oil contamination.

Diatoms have been reported to be one of the most sensitive groups to oil and oil derivatives (Perez et al. 2010). The response of *T. pseudonana* to the oil treatments in this study seem to confirm this statement. Diatoms are abundant in both marine and freshwater environments and produce up to 45% of the yearly marine organic carbon (Armbrust et al. 2004; Lee 2008). For this reason, diatoms play an important role in the marine silicon cycle; they both control and are limited by the available silicon in the water (Yool and Tyrrell 2003). A widespread oil pollution may therefore have a detrimental effect on the total phytoplanktonic biomass (Pelletier et al. 2006) and alter the marine silicon cycle. In the Baltic Sea, diatoms are one of the most important bloom-forming species. In case of an oil pollution, diatoms may lose their dominance due to a low resistance to oil, making it possible for the more toletant groups of algae to proliferate and form a bloom. The more tolerate groups may be toxin-producing and

therefore harmful blooms may become more frequent. This scenario will have a notable negative effect on the Baltic Sea ecosystem.

The results of the current study support the view that the variability in the resistance to oil pollution of distinct phytoplankton species may lead to a non-normal species succession, with the more tolerant species becoming the dominant group (Huang et al. 2011; Jiang et al. 2010). In this case, transformations of phytoplankton communities may induce changes in the functioning of the planktonic ecosystem by means of bottom-up effects (Jiang et al. 2010). Zooplankton communities may be altered due to selective feeding on phytoplankton (Perez et al. 2010) and thus the biomass of other marine animals may be also affected. Thus, shifts in phytoplankton communities can alter the structure, stability and function of marine ecological systems (Jiang et al. 2010). Furter, a contaminant-induced phytoplankton crash may release important carbon and nutrient sources for bacteria and may enchance their growth. Bacteria show a lesser sensitivity to oil and PAHs and may use the petroleum hydrocarbons as a energy source (Lekunberri et al. 2010). Hence a transition from an phytoplankton-based food web to a microbial one may occur. There is a very little downward transport of carbon in a microbial loop, since all of the carbon is recycled and utulized in the upper layers of the water (Pelletier et al. 2006). The export of carbon to the sediments is crucial to the functioning of the biological carbon pump. When the the cell density and biomass decreases, the flux of the carbon incorporated in the photosynthesis to the seafloor is also reduced and the capacity of the marine biological pump is diminished (Echeveste et al. 2010b). This represents a significant and a potentially far-reaching ecological risk.

In the 1000-fold dilution experiments, all of the species except *I. galbana* showed a slight recovery on the third and fourth day of the experiment. However, the recovery in the 250-fold dilution experiments was only observed in *P. parvum, R. salina, B. submarina* and *M. contortum.* Yet none of the species returned to the controls level during the continuation of the experiments. The recovery may occur due to the increase of the chlorophyll content, caused by the weathering and removal of the oil from the treatments. The recovery of the phytoplankton was not examined here, however it would be interesting to investigate in a subsequent study the (lethal) concentrations of crude oil in which recovery would not occur.

6. Conclusion

Different phytoplankton species show a variability in their tolerance to oil. All of the phytoplankton species used in this study were inhibited by the additions of oil-water solutions. In general the 1000-fold dilution had a lower toxic effect than the 250-fold dilution. The most sensitive species were *Thalassiosira pseudonana* and *Isochrysis galbana* showing an acute response to the both treatments, while *Monoraphidium contortum* seemed to be the least affected of the all species.

The reasons underlying the differences in the tolerance to oil are remain unclear, but it is possile that the variability is caused by the differences in the cell membranes or the presence of a native physiological and chemical defence system. The variability in the tolerance to oil pollution may cause shifts in the phytoplankton community resulting in the altering on food webs via the bottom-up control mechanism.

7. Kahe naftalahuse mõju fütoplanktoni liikidele

Naftareosuse oht on aktuaalne küsimus Läänemere ökoloogias. Saastumine nafta ja selle produktidega (näiteks mootorikütus) mõjutab paljusid organisme, sealhulgas fütoplanktonit. Fütoplanktonil kui primaarprodutsendil põhineb suur osa toiduahelaist, seetõttu võiks muutused fütoplanktoni koosluses mõjutada kogu ökosüsteemi funktsioneerimist.

Antud töös uurisin, kas erinevate mikrovetikaliikide vahel võiks esineda teatud erinevust resistentsuses naftareostusele, mis võiksid esile kutsuda muutusi fütoplanktonikooslustes. Selleks kasutasin seitset harilikku laboris kasvatatud Läänemere fütoplanktoniliiki, mille peal testisin kahe naftalahuse inhibeerivat mõju. Leidsin, et nii 1000-kordne naftalahjendus kui ka 250-kordne lahjendus inhibeerisid oluliselt mikrovetikate fotosünteesi efektiivsust, kusjuures 250-kordse lahjenduse toksiline mõju oli markantsem kui 1000-kordse lahjenduse mõju. Liikide vahel eksisteeris selgeltmärgatav erinevus nende vastupidavuses naftale. Enim mõjutatud liikideks osutusid ränivetikas *T. pseudonana* ja prümnesiofüüt *I. galbana*. Suhteliselt resistentemad olid aga rohevetikad *M. contortum* ja *B. Submarina*, prümnesiofüütidest

aga *P. parvum*. Võib oletada, et erinevused nafta taluvusvõimes võivad tuleneda rakkude erinevast rakumembraani koostisest või teatud keemilise või füsioloogilise kaitsekohastumuse olemasolust. Nii või teisiti võib erinev vastupanutase põhjustada muutusi vetikakooslustes, mispuhul resistentsematel liikidel oleks naftareostuse puhul teatud eelis vähemresistentsemate ees.

Eksperimentide lõpupoole ilmnes osade liikide puhul vähene tõusutrend fotosünteesi efektiivsuses, mis võiks olla märgiks populatsiooni taastumisest. Tulevased uuringud võiksid selgitada, kas piisava aja möödudes võiks toimuda täielik taastumine ning milliste naftakontsentratsioonide puhul taastumist ei toimuks.

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Lihtlitsents lõputöö reprodutseerimiseks ja lõputöö üldsusele kättesaadavaks tegemiseks

Mina, Lara Podkuiko

(sünnikuupäev: 18.05.1988)

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„The effects of two crude oil solutions to phytoplankton species“

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