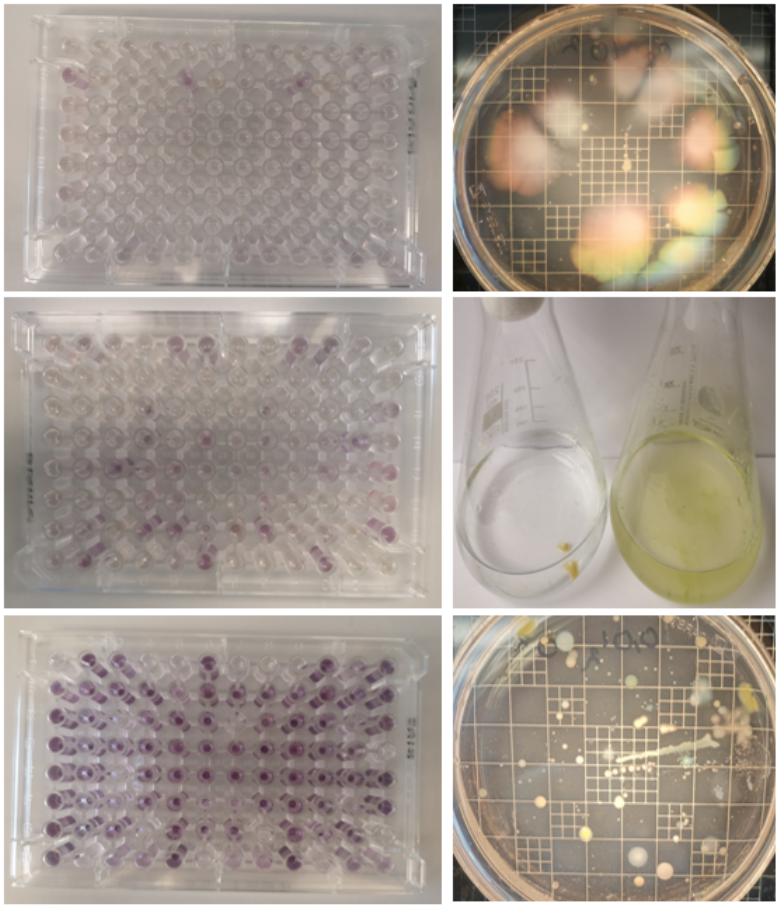
Biolog Ecoplate® as a supplementary tool for evaluating ecotoxicity in a freshwater microplanton community



Emilie May Battersby

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Faculty of Engineering and Natural Sciences
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Abstract

Due to the production and release of chemicals into the environment, is happening at a pace that is outrunning the capacity of assessment and monitoring, it is important to consider alternative methods for evaluating the ecotoxicity of chemicals. The aim of this project is to evaluate Biolog Ecoplates® as a community level method for assessment of toxicity and VUV-facilitated toxicity mitigation. Laboratory scale microcosms with a freshwater microplankton community were contaminated with one of the three selected biocides (benzalkonium chloride, chlorhexidine, and didecyldimethylammonium chloride) or 1 herbicide (glyphosate), as well as a set of microcosms each contaminated with one of the selected chemicals, that had undergone VUV-treatment. After 9 days microcosm incubation, a sample from each microcosm was then incubated in a Biolog Ecoplate. To accommodate the evaluation of Biolog Ecoplates® as a method for toxicity assessment, measurements of ATP concentration and colony-forming-units in the microcosms were also included. Biolog Ecoplates did not succeed in identifying a toxic effect of the examined biocides on the activity in the Biolog Ecoplates, as was otherwise expected. The Biolog Ecoplates were however to some degree able to differentiate the substrate utilisation patterns of the contaminated microplankton communities, from the communities of the control microcosms. These results indicate a change in functional diversity and therefore a possible shift in community structure, which was especially found in the microcosms contaminated with either didecyldimethylammonium chloride or glyphosate as well as the microcosms contaminated with either VUV-treated didecyldimethylammonium chloride or glyphosate. The Biolog Ecoplates identified the toxicity mitigation of VUV-treatment by preventing a decrease in functional diversity, that was otherwise observed for the microcosms contaminated with the examined biocides. The overall findings in this project therefore indicate that Biolog Ecoplates® have potential as a community level method for assessment of chemical toxicity on a freshwater microplankton community.

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Introduction 1

1.1 Current status of chemical use and toxicity testing

Chemicals play a key role in maintaining the lifestyle of many people in 2024. Chemical industry is an integrated part of the upstream supply chain for manufacturing, agriculture, energy and healthcare sectors [European Environment Agency, 2023]. Globally over $3.5 * 10^5$ chemicals and mixtures of chemicals have been registered for production and use [Wang et al., 2020], and in 2022, EU consumed 274 million tonnes of chemicals [Eurostat, 2023].

The production and widespread use of different chemicals does however also have a negative side. The prevention of persistent chemicals from harming Europe's freshwater and marine ecosystems is proving to be a challenge, and cases are emerging of hazardous chemicals found in humans, adversely effecting their health [European Environment Agency, 2022].

European Environment Agency [2023] has reported that we have now passed the planetary boundary for chemical pollution. This statement has come after the research conducted by Persson et al. [2022], who examined the safe operating boundary for chemical use and release in regards to maintaining the ecosystems in which modern societies have evolved. Steffen et al. [2015] introduced chemical pollution (under the category novel entities) as part of the 9 categories regarding the planetary boundary concept, and states that the introduction of chemicals to the environment is of global concern, if the chemicals exhibit persistence, mobility leading to widespread distribution and potential impact on vital ecosystems. Persson et al. [2022] concluded that we have passed the planetary boundary in regards to chemicals, due to the production and release of chemicals happening in a pace that is outrunning the capacity of assessment and monitoring. Furthermore, Steffen et al. [2015] appeals to the research community to develop a knowledge base that allows

the screening of chemicals for properties that may predispose them towards becoming global problems, before they are released into the environment.

Today, risk assessment for contaminants are, among other things, based on the results from a set of short term toxicity tests conducted on aquatic organisms - alga, *Daphnia* and fish. For the risk assessment of contaminants on microbial activity in waste water treatment plants, bacteria respiration inhibition tests are also included [Bureau, 2003]. There is several standardised methods for toxicity testing on aquatic species. For alga, the acute toxicity test is the measure of inhibition of growth rate or biomass yield after 72h of exposure and the no-observed-effect-concentration (NOEC) is considered as the chronic endpoint. *Daphnia magna* is the most popular invertebrate for ecotoxicity testing and the acute toxicity is evaluated on the effect of immobilisation after 24h of exposure and the chronic toxicity is then based on the NOEC on reproductive success after 21 days of exposure. The acute toxicity testing of fish is based on the lethal endpoint after 96h of exposure. However due to ethical issues, methods evaluating fish embryo on mortality, growth and deformation are replacing the traditional in vivo fish testing. To examine if the chemical will have an adverse effect on the aquatic life, the species examined are of different trophic levels, representing different positions in the food chain. [Escher et al., 2021].

The adequacy and quality of basing a toxicity evaluation of contaminants purely on single-species short-term laboratory tests is however debatable, as the toxicity of contaminants towards a species can be highly dependent on the community structure in the environment and interactions between species, as well as environmental factors such as temperature [Bérard et al., 1999]. Furthermore, in regards to ecotoxicology, the ability to evaluate an environmental risk based on these single species toxicity test is questionable, as the ecotoxicity of a contaminant can depend widely on the season and the composition of species in the community [Pesce et al., 2009].

The toxicity of the degradation products from the parent chemical is another important aspect when conducting an ecotoxicology evaluation. A review conducted by Boxall et al. [2004], revealed that 80% of degradation products of synthetic chemicals were less toxic or of similar toxicity to their parent molecule. However the analysis also showed that 20 % were $>$ 3 times more toxic, of which 9% were $>$ 10 times more toxic than the parent

molecule. In a few of the examined cases, the degradation product turned out to be $>$ 100 times more toxic than the parent molecule. The ecotoxicity of the intermediate degradation products is both relevant for chemicals that are rapidly degraded in the environment, but also in regards to chemicals treated with the increasing field of water processes like advanced oxidation processes (AOP). AOP have received a great deal of attention as attractive and effective methods for removal of a wide range of pollutants in water and waste water [Lucas et al., 2021]. When evaluating the toxicity of a chemical, it is therefore also relevant to examine the ecotoxicity of the intermediate degradation products due to natural degradation or AOP facilitated degradation.

The need for a higher degree of chemical ecotoxicity assessment, is clear and the method has to be quick and effective to keep up with the speed of chemical development, production and release to the environment. To address this concern, alternative methods for examining the effect of a compound and it's degradation products on a community scale should be evaluated.

Biolog Ecoplates® are a method that has been used to identify spatial and temporal changes in microbial communities in e.g. soil, water and wastewater. At the request of a group of microbial ecologists, Biolog Ecoplates® were created for community analysis and microbial ecological studies [Biolog, Inc., 2023]. Microplates from Biolog was first described in 1991 by Garland and Mills [1991] as a rapid community-level method to characterise and classify heterotrophic microbial communities in soil, water and the rhizosphere. Today, Biolog Ecoplates® are widely used in studies examining the effect on microbial communities from various human activities and waste products, such as different agricultural practices [Gao et al., 2024], industrial waste contaminated soil and water [Lukhele and Msagati, 2024] and antibiotic traces in the environment [Pino-Otín et al., 2023, 2024]. Based on the utilisation pattern of ecological relevant carbon substrates, a community-level physiological profile (CLPP) can be drawn of the examined microbial community [Christian and Lind, 2006]. A community level analysis using Biolog Ecoplates® could therefore be a promising supplementary tool for the environmental risk assessment of chemicals, leading to an improved ecotoxicology assessment.

1.2 Project objective

The aim of this project is to evaluate Biolog Ecoplates® as a community level method for assessment of toxicity and VUV-facilitated toxicity mitigation of 3 selected biocides (benzalkonium chloride, chlorhexidine, and didecyldimethylammonium chloride) and 1 herbicide (glyphosate) on a freshwater microplankton community kept in a laboratory microcosm setup. To accommodate the evaluation, measurements of ATP concentration and colony-forming-units will also be included.

Theory 2

2.1 Organisms in a freshwater ecosystem

An overall classification of the freshwater organisms is presented in figure 2.2 and the approximate size of the different groups of organisms in freshwater ecosystems are presented in figure 2.1. The freshwater plankton community examined in this project is in the size range of 45 μm and under (orange square in figure 2.1), including bacteria, and some phytoplankton and microzooplankton (20-200 μm) [Calbet, 2022].

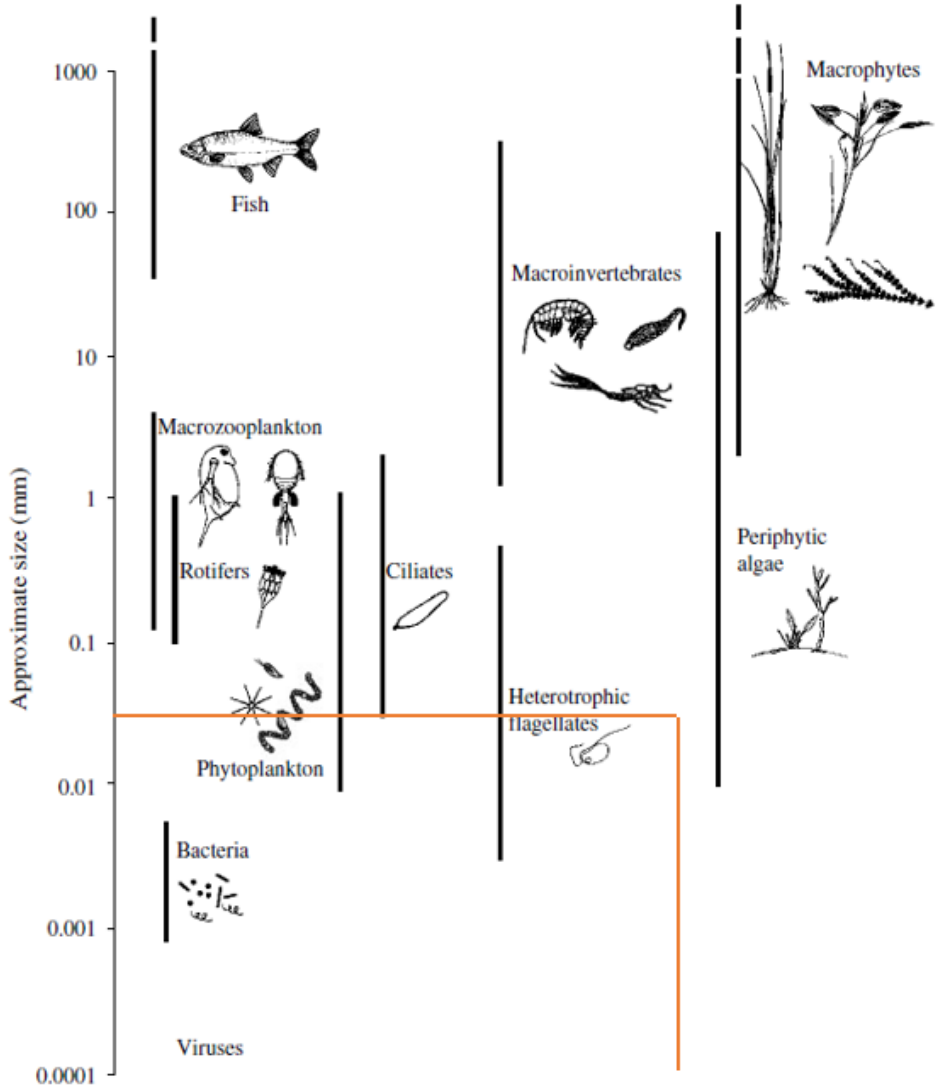

Figure 2.1. The size spectrum of groups of organisms found in the freshwater ecosystem. The figure is from Brönmark and Hansson [2005] and the orange square indicates the size and groups of organisms examined in this project.

Bacteria are typically found within the size range 0.2-5 μm and are very important in the lake metabolism as they are major actors in the degradation process of dead material [Brönmark and Hansson, 2005]. Furthermore, unlike most eukaryotes, bacteria can uptake dissolved organic carbon that has been excreted from phytoplankton or as a result of autolysis. The dissolved organic material is converted into CO_2 and bacterial biomass, ensuring the reentry of carbon into to the food chain again, providing a food source for the zooplankton [Reineke and Schönmann, 2023]. This part of the aquatic food chain is called the microbial loop and is an important part of the carbon cycle [Reineke and Schönmann, 2023]. Bacteria are an important food source for various grazers such as

rotifers, crustacea and protozoa, and may be found in concentrations of 10^6 per ml water. When examining the community in the size range presented in figure 2.1, phytoplankton and blue-green alga are responsible for the primary production [Brönmark and Hansson, 2005]. The particulate carbon in the form of phytoplankton or detritus is then a carbon source for herbivorous zooplankton, which in turn serves as a food source for carnivorous zooplankton [Reineke and Schönmann, 2023]. Most phytoplankton rely on photosynthesis for their production of energy, however euglenoids is a group of phytoplankton, where many are facultative heterotrophs and can therefore also consume bacteria and detritus as their energy source.

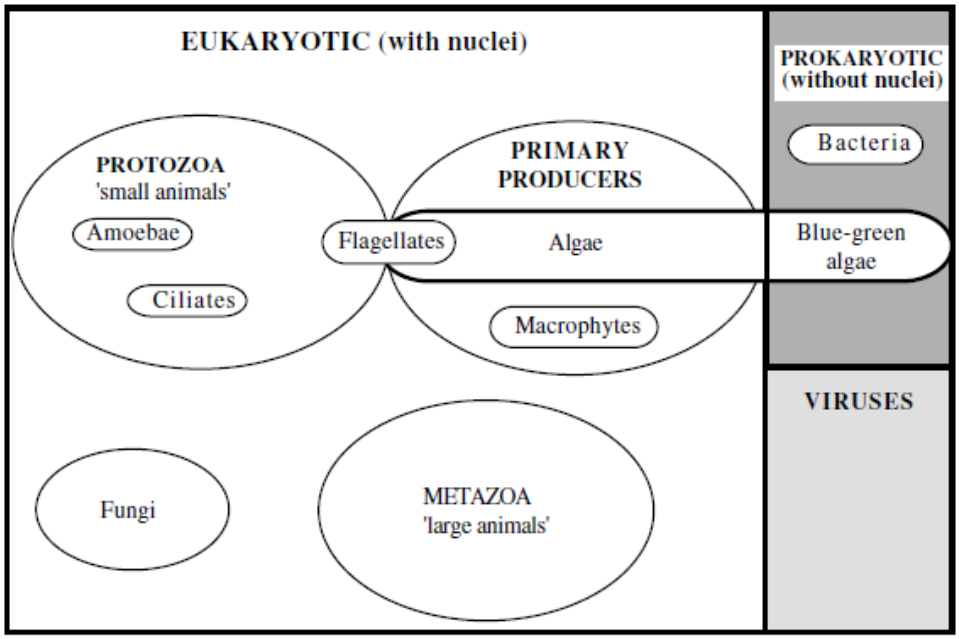

Figure 2.2. An illustration of commonly used classifications and groups of freshwater organisms [Brönmark and Hansson, 2005].

Some flagellate are also autotrophs able to ingest particulate carbon in addition to photosynthesis. Others are exclusively heterotrophs and mainly feed on bacteria, playing an important roll in reducing bacterial abundance. Together with amboebae and ciliates, flagelatte are under the protozoa category (heterotrophic unicellular organisms) that consume complex organic molecules or particles, including bacteria [Brönmark and Hansson, 2005].

2.2 Ecotoxicology

If a contaminant is introduced to an ecosystem, each population within the community may be affected in one of the ways illustrated in figure 2.3. Some species decrease in abundance, with some becoming locally extinct. Other populations remain stable, while some species increase in abundance [Walker et al., 2012]. The change in population size can both be due to the direct effect of the toxicant, or indirectly via changes in inter-specific competition or predator-prey ratio. In general, the size of an isolated population over time follows an exponential growth curve slowly transferring to a logistic growth pattern as the population reaches the carrying capacity of the system. At this point the resources of the system is fully utilized and the intra-specific competition has reduced the growth rate to zero, stabilising the population size around the carrying capacity of the system [Townsend et al., 2008]. If abiotic factors are improved in the ecosystem, by for exsample introducing a contaminant that works as a nutrient, the carrying capacity of different species in the system may increase, leading to a higher abundance and possible species richness in the community [Brönmark and Hansson, 2005]. Some populations in the community might be competing for the same resources (exploitation competition) adding an inter-specific competition as a regulating element of the size of the populations, also known as the Lotka-Volterra model. By lowering or increasing the amount of inter-specific competition, the carrying capacity of the population in question is either lowered or increased leading to a different ratio of this population in the community, resulting in a shift in community composition. The predator-prey relationship can also affect the population size in question, illustrated in the Lotka-Volterra predator-prey model, which shows the behavior of joint populations that undergo coupled cycles in abundance [Townsend et al., 2008]. If the abundance of a predator species is reduced due to the introduction of a toxicant, the abundance of the prey might increase, and opposite, if it is the abundance of the prey that is reduced, the abundance in predator might also be reduced. The community structure and/or the total functional state might therefore be affected by the contamination of the toxicant.

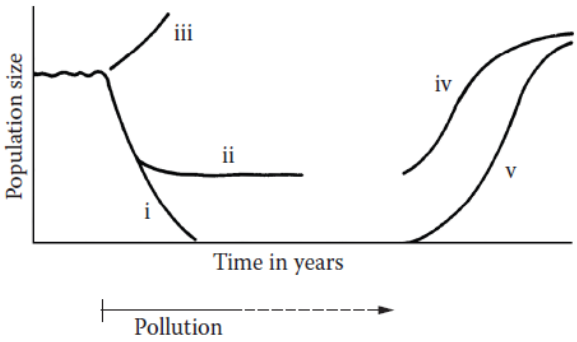

Figure 2.3. A figure from Walker et al. [2012] illustrating the possible effects a pollutant can have on population size (i-iii). The first possibility is that the population will become locally extinct. The second option is a decline in abundance, when the population endures the effect of the pollutant. The third possibility is an increase in population size to levels higher than pre-pollution. Possibility (iv-v) are possible pathways for the population size once the pollution is over (iv-v) due to removal of the suppressing factor (iv) or due to immigration (v).

2.2.1 Community level methods for evaluation of ecotoxicity

When studying the changes in communities or ecosystems due to pollution, two distinctive approaches are used namely a community structural approach and the community functional approach [Walker et al., 2012]. Both approaches help identify the overall effect on the examined community due to the possible abundance pathways each species may follow upon introduction of a pollutant, which was explained above.

The structural approach is examining the absence or presence of species and the overall balance of species abundance in the examined community. A very common structural approach for determining the state of a freshwater river ecosystem is by using the River invertebrate prediction and classification (RIVPACS) method. Based on environmental variables of the site, the fauna of the location in it's natural state is predicted and compared to the actual structure of the community, thereby calculating a degree of shift in community composition from it's natural state. More generally, to evaluate the structural composition of a community, diversity indices are used, combining species richness and the abundance of each species. Shannon's diversity index is the most commonly used diversity index however there are some issues to remember when including a diversity index when evaluating the effect of a pollutant on a community or ecosystem. The diversity indexes do not take into account which species are present, if these species are beneficial or not for the ecosystem. Furthermore, the diversity of different communities respond differently

to different pollutants, e.g. a negatively correlated or bell-shaped diversity response to increasing pollution [Walker et al., 2012].

The functional approach gives an indication of the functional state of the ecosystem, and often involves measuring the effect of pollution on the carbon and nitrogen cycle. A widely used method for determining the toxicity of chemicals to soil microorganisms, is by measuring the formation of CO_2. Another method can be to measure adensine triphosphate (ATP), which is known as a universal indicator of biomass [Bochdansky et al., 2021], and ATP levels in cultured alga and lake plankton have been found to correlate well with other standard biomass parameters such as chlorophyll a, and dry weight [Brezonik et al., 1975]. The measurement of ATP has been found to be a sensitive bioassay for toxicity [Brezonik et al., 1975; Seyfried and Desjardins, 1987], outcompeting the agar plate test and the resazurin reduction test in determining the bacterial viable cell count following metal contamination [Seyfried and Desjardins, 1987].

It is clear that combining the two approaches (structural and functional), increases the overall evaluation of the pollution effect on the ecosystem. Biolog Ecoplates® can be thought of a method that could combine the structural and functional approach of microbial ecotoxicity evaluation. The Biolog Ecoplate® has 96 wells containing 31 carbon substrates and 1 control well, all repeated three times for data replica. Included with the carbon source is a tetrazolium dye that produces a purple colour when reduced. If the incubated microorganisms can utilize the carbon source, their respiration will reduce the dye to the violet formation, and a colour pattern produced, rate of colour formation and colour intensity will give a metabolic fingerprint that can be used to characterise the microbial community [Biolog, Inc., 2023]. The 31 different carbon substrates in Biolog Ecoplates® have been divided into 6 substrate groups as done by Christian and Lind [2006], which can be seen in figure 2.4. The analysis of which substrates have been utilised and the evenness of utilisation among the different substrates can give an indication of functional diversity in the examined microbial community [Garland, 1997]. Together with an analysis of substrate types that are highly utilised, the identification of a possible shift in community structure might be identified.

Amino Acids	Carbo-hydrates	Carboxylic Acids	Amines	Phenols	Polymers
L-arginine	D-cellobiose	γ-hydroxy-butyric acid	phenylethyl-amine	2-hydroxy-benzoic acid*	α-cyclo-dextrin
L-asparagine	i-erythritol	α-ketobutyric acid	putrescine	4-hydroxy-benzoic acid*	glycogen
glycyl-L-gluta-mic acid	D-galactonic acid-γ-lactone	D-galacturonic acid			Tween 40
L-phenylalanine	N-acetyl-D-glu-cosamine	D-glucosaminic acid			Tween 80
L-serine	glucose-l-phos-phate	itaconic acid			
L-threonine	β-methyl-D-glucoside	D-malic acid			
	D,L-α-glycerol phosphate	pyruvic acid methyl ester			
	α-D-lactose				
	D-mannitol				
	D-xylose				

* Commonly grouped with the carboxylic acids

Figure 2.4. The distribution of the 31 carbon sources in the Biolog Ecoplates into 6 substrate groups. Figure and classification taken from Christian and Lind [2006]

Base on the overall rate of colour development, the carbon substrate utilisation pattern from the Biolog Ecoplates® may also provide an estimation of overall potential activity or density of the examined microbial community [Garland, 1997], which can give an indication of the functional state of the ecosystem. The reduction of tetrazolium dye by mammalian cells has been found to be mainly due to the oxidation of NADH [Berridge et al., 2005]. The color respons in the Biolog Ecoplates® could therefore been seen as a result of total activity in each well. In the case of a freshwater plankton community, the Ecoplates may therefore produce a response both due to the activity of organisms able to use the carbon as an energy source, but also as a response due to the activity of the carnivorous plankton.

2.3 The chemicals used in this ecotoxicity experiment

Benzalkonium chloride (BAC), chlorhexidine (CHX), didecyldimethylammonium chloride (DDAC) and glyphosate (GLY) are examples of important chemicals, regarding food safety and human health. These chemcials are found both in waste water treatment plants and in the environment far from the site of application [Martínez-Carballo et al., 2007; Östman et al., 2007; Pérez et al., 2011; DeLeo et al., 2020].

CHX, DDAC and BAC are three widely used biocidal chemicals that act as the active ingredient in a wide range of antiseptic and disinfectant products as well as preservatives. They all target the cytoplasmic membrane, however with different mechanisms of action [McDonnell and Russell, 1999]. BAC and DDAC are quartinary amonium compounds (QAC) [Flanjak et al., 2024], which is a group of chemicals known for their antimicrobial properties and is often found in cleaning and personal care products as well as durable consumer goods [Arnold et al., 2023]. Because of the SARS-CoV-2 pandemic, the use of QAC's has likely increased given the common use of QAC's in disinfectant wipes and surface spray cleaners [Hora et al., 2020]. Furthermore, BAC and DDAC are approved in some countries for use in outdoor cleaning and biocidal products, resulting in the possible leaching and run-off to the environment [Flanjak et al., 2024]. CHX is likewise also found in a variety of products such as detergents, cleaning and personal care products and perfumes [ECHA, NAc], in particular hand washing and oral products [McDonnell and Russell, 1999]. The three biocides have been found to be toxic to a range of test organisms including bacteria and alga, with EC50 values in the range of $10^{-}3 - 2 * 10^{0}$ mg/L [Flanjak et al., 2024; Jesus et al., 2013] and CHX and BAC have been classified as very toxic to aquatic life with long lasting effects [ECHA, NAc,N].

GLY is the most frequently used herbicide, both worldwide and in the EU, as the active ingredient in a range of chemical mixtures for weed control, used in agriculture, public areas such as railway tracks and for private use [European Commission, 2023; Annett et al., 2014]. The mode of action for GLY is the disruption of the Shikimate pathway, which is important for the biosynthesis of vital amino acids. The pathway is most commonly found in plants and the disruption ultimately leads to cell disarray and death [Annett et al., 2014; Gandhi et al., 2021]. GLY has played a prominent roll in agriculture in the last 50 years [Gandhi et al., 2021]. It's use has however been heavily debated up to the renewal of approval in 2023 [European Commission, 2023], due to increasing concern of negative effect on non-target organisms, including humans [Gandhi et al., 2021]. The effect on target and non target organisms has therefore been extensively studied and GLY is considered a good test candidate for evaluating the results of the community analysis obtained via Biolog Ecoplates on chemical toxicity evaluation.

2.4 VUV-treatment of water contaminated with organic pollutants

The field of water treatment and processes to remove resistant contaminants is receiving a growing focus. In recent years, advanced oxidation processes (AOP) have received a great deal of attention as attractive and effective methods for removal of a wide range of pollutants from water and waste water [Lucas et al., 2021]. The principle behind AOPs is in site production of a strong oxidative agent (hydroxyl radicals (·OH)), resulting in non-selective oxidation of organic pollutants [Lucas et al., 2021; Ghime and Ghosh, 2020]. Vacuum-UV is an AOP [O.Legrini et al., 1993] that effectively generates ·OH, and has been found to successfully increase the degradation of organic trace compounds and dissolved substrates [Zoschke et al., 2014; O.Legrini et al., 1993]. The technique has the advantage of being a relative simple process where no addition of chemicals is needed [O.Legrini et al., 1993].

Materials and Methods 3

3.1 Chemicals

The chemicals used are Alkyldimethylbenzylammonium chloride (Benzalkonium chloride, BAC)(CAS nr. 8001-54-5), Chlorhexidine digluconat (CHX-Dig)(CAS nr. 18472-51-0), Didecyldimethylammonium chloride (DDAC)(CAS nr. 7173-51-5) and Glyphosate (GLY)(CAS nr. 38641-94-0).

3.2 Freshwater sample

A freshwater sample was collected in March 2024, from the lake: "Nols Sø", located in the north of Jutland, Denmark. The lake water sample was kept in an aquarium, under light at 20 °C with an air-stone until the microcosm setup (1 week).

3.3 Chemical solutions and UV-treatment

Four chemical solutions of each 20 mg/L of the examined chemicals (BAC, CHX, DDAC and GLY) were prepared in tap water and stored at 5 °C for 1 day until the microcosm setup. The UV-treatment in this project was a combination of VUV and UVC, as low-pressure mercury vapor lamps emit both wave lengths [Zoschke et al., 2014]. 4 liters of each chemical solution with the concentration 20 mg/L were VUV irradiated in a continuous-flow VUV photoreactor (ULTRAAQUA A/S, Aalborg, Denmark). The photoreactor consists of a tubular stainless-steel reactor, a low pressure high output amalgam VUV mercury lamp (UltraTherm 200 W LPHO TOC UV, ULTRAAQUA A/S, Denmark) and a cooling spiral operating at 10 °C to prevent heating. The maximum UVC radiance of the tap water was 156 W/m^2. The solutions were circulated in the reactor at a speed of just over 3 L/min. (3.060 L/min) for 30 min.

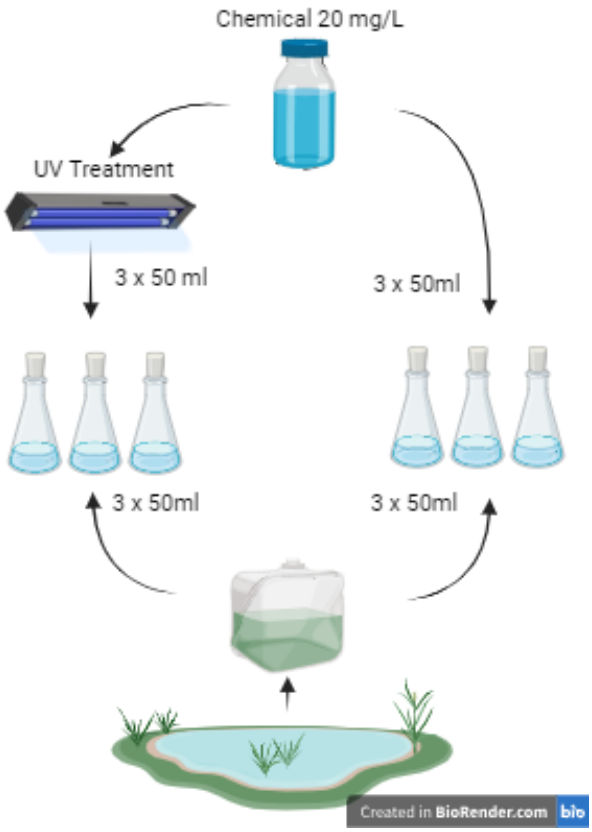

Figure 3.1. Principle behind the microcosm setup. For each chemical (BAC, CHX, DDAC and GLY) the following microcosms were prepared: three microcosm containing lake water and chemical contaminated tap water, and three microcosms containing lake water and chemical contaminated tap water that has been VUV-treated.

3.4 Microcosm setup

Each microcosm was constructed in 250 ml erlenmeyer flasks and consisted of 100ml 1:1 mixture of lake water and the chemical solution that was either UV or non-UV treated. A diagram of the experiment setup can be seen in figure 3.1. The lake water was filtered (45 μm) before incubation in the microcosms to remove larger organisms (macro-, meso- and some microplankton). Each flask was closed with a cotton stopper and incubated on an orbital shaker (100 rpm), at 20 °C, under constant 24h light (7000 - 10000 lux)(fluorescent tube, Philips Master TL-D 36W/840)(see figure 3.2). For each chemical (BAC, CHX, DDAC and GLY) the following microcosms were prepared: three replica microcosm containing lake water and chemical contaminated tap water (BAC, CHX, DDAC and GLY), and three replica microcosms containing lake water and chemical contaminated tap water that has been VUV-treated (BAC-VUV, CHX-VUV, DDAC-VUV and GLY-

VUV). As a control, 3 microcosms containing lake water and tap water were prepared (Control), as well as 3 microcosms containing lake water and VUV-treated tap water (Control-VUV). The microcosms were incubated for 9 days, and samples for analysis of bacteria abundance and ATP concentration were collected on day 0, 3, 6 and 9.

Figure 3.2. The setup for the incubation period of the microcosms.

Some microcosms developed large alga aggregates during the 9 days of incubation. To decrease the variation in samples from the microcosms due to the heterogeneity of the solution, larger alga aggregates in the sample were left to sediment for 15 minutes, before a smaller sample of the supernatant was collected. This smaller sample was then vortexed to break up any remaining aggregates and to remix the solution. From here a sample for cell count and ATP analysis was collected. On the 9th day of incubation of the microcosms, in addition to the cell count and ATP analysis, a part of the sample from each microcosm after pre-treatment was also incubated in Biolog Ecoplates.

3.5 Viable heterotrophic bacteria cell count

The viable microorganism cell count was estimated using the Plate count technique with an R2A Agar medium (BD DIFCO(TM) R2A Agar). The pre-treated sample as explained in the section above was then serial diluted and 100 μl of each dilution was spread on R2A Agar plates and incubated at 22 °C for three days after which the CFU/ml was determined. The estimated CFU/ml was then calculated using the following equation:

$$\frac{CFU}{ml} = \frac{\text{Number of colonies counted on plate} * \text{Dilution factor}}{0.1ml} \tag{3.1}$$

3.6 ATP concentration

From the prepared sample as described in section 3.4, 100 μL was sampled and 100 μL of the reagent from the Microbial Cell Viability Assay kit (BacTiter-Glo(TM)) was added. The added reagent supports cell lysis and provides the reactants for the oxygenation of luciferin if ATP is present in the sample, which in turn leads to the production of a luminescence signal [Promega, 2019]. After 1 min. the luminescence signal was read on a Glomax 20/20 luminometer (Promega). The luminescence from blank values were then subtracted from the sample measurements and devided by the blank values to obtain the relative luminescence unit (RLU), before the RLU of the samples were converted to an ATP concentration using the constructed standard curve presented in figure 3.3.

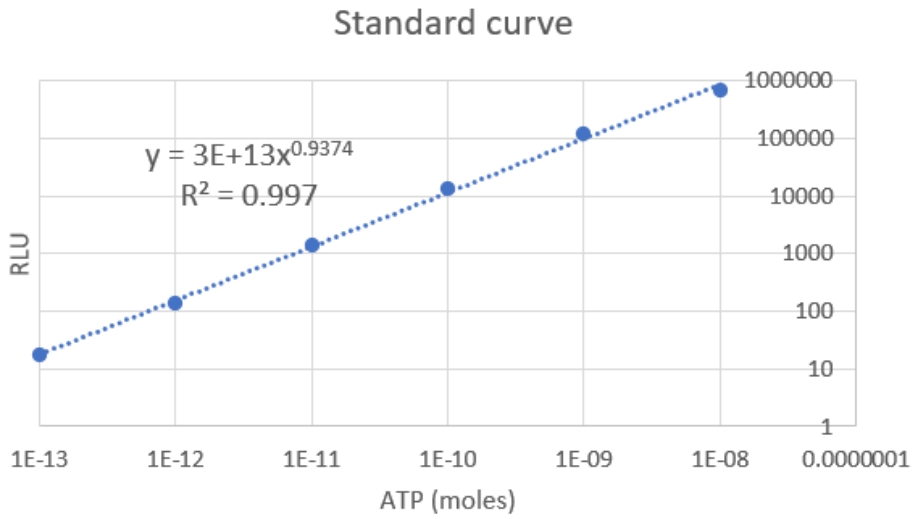

Figure 3.3. Standard curve for determining the ATP concentration (moles) based on measured RLU.

3.7 Community analysis using Biolog Ecoplates

The sample used for ATP and CFU/ml was also incubated in Biolog Ecoplates. An ecoplate of 96 wells containing 3 replications of 31 substrates and 1 blank (water) was used for each microcosm and 150 μl from the prepared sample was transferred to each well. The plates were kept in the dark, at 20 °C on an orbital shaker at 100 rpm. The absorbance was measured at 550nm on a microplate photometer (Multiskan(TM) FC, Thermo Scientific) and was measured at T0 and every 24h for 12 days.

For some substrates an immediate increase in absorbance was measured at time 0, which could be due to the dissolution of the carbon substrate in the water sample. The absorbance at time 0 for each well was therefore subtracted from the following measurements as done in Pino-Otín et al. [2023]; Németh et al. [2021]. For each time step, an average of the absorbance in the control wells (no-carbon substrate)(n=3) was subtracted from the absorbance of each well as done in Sala et al. [2010]; Németh et al. [2021].

As done by Németh et al. [2021], the average well colour development (AWCD) for each sample at each time point was then calculated by the equation:

$$\text{AWCD} = \frac{\sum_{i=1}^{N} OD_i}{N} \tag{3.2}$$

where OD_i is the corrected optical density of each substrate-containing well (i) and N is the number of substrates (N=31). Similarly for every substrate category (see figure 2.4), the substrate group average well colour development (SGAWCD) was calculated for each sample at each time point as presented by Németh et al. [2021] using the equation:

$$\text{SGAWCD} = \frac{\sum_{i=1}^{N} OD_i}{N} \tag{3.3}$$

where OD_i is the corrected optical density of each substrate-containing well (i) in the substrate category, and N is the number of substrates in the category. For each treatment, the carbon utilisation pattern for each treatment has been analysed by calculating the area under curve (AUC) both for each SGAWCD curve and for each individual substrate. The method is presented by Guckerta et al. [1996] and the equation used is the following:

$$\text{AUC} = \sum_{i=1}^{T} \frac{(OD_i + OD_{i-1})}{2} * (t_i + t_{i-1}) \tag{3.4}$$

where OD_i is the corrected optical density at reading i (either for each individual substrate or for the SGAWCD), $OD_i{-}1$ is the corrected optical density at reading i-1 (either for each individual substrate or for the SGAWCD). t_i is the time at reading i and t_{i-1} is the time at reading i-1. T is the total number of readings (in this case 12).

For each sample the percentage of each substrate group of the total carbon utilised is calculated using the following equation:

$$\text{Substrate group } \% = \frac{AUC_{\text{Substrate group}}}{\sum_{i=1}^{N} AUC_i} * 100 \tag{3.5}$$

where $\text{AUC}_{\text{Substrategroup}}$ is the area under the curve of the SAWCD for the substrate group, which is divided by the sum of all of the areas under the curve for each substrate group (N=6.

Shannon's diversity index (SDI) was calculated as presented by [Németh et al., 2021] using the following equation:

$$SDI = -\sum_{i=1}^{N} P_i * ln(P_i) \tag{3.6}$$

where N is the number of substrates (N=31). In this project P_i is the ratio of AUC for each substrate out of the total area for the sample, and is calculated for each substrate i using the following equation:

$$P_i = \frac{AUC_i}{\sum_{i=1}^{N} AUC_i} \tag{3.7}$$

For the principle component analysis (PCA), the P_i for each substrate is then converted to a percentage, and the PCA is then conducted on all 31 substrates as 31 individual variable. The test is done in R using the function prcomp(). For some of the PCAs, the data was also scaled using the Z score as seen in the equation below:

$$x_{\text{i scaled}} = \frac{x_i - mean(x)}{sd(x)} \tag{3.8}$$

3.8 Statistical analyses

All data treatment has been conducted in R [R Core Team, 2023]. The data for the substrate class percentage of the total carbon utilised for each treatment (figure 4.8) were found to be normally distributed using the Shapiro-Wilk test. However an equal variance was not found using the Bartlett's test, and therefore to determine differences in substrate group utilisation between treatments, the non-parametric Kruskal-Wallis test and the post hoc Dunn's Test have been conducted. To examine the difference between the Shannon's diversity index of the different treatments in figure 4.9,A,B and C, one-way ANOVA and the post hoc Tukey test have been used. To identify a difference in Shannon's diversity index between the samples collected from the microcosms contaminated with GLY and VUV-treated GLY as well as the control microcosms, the non-parametric Kruskal-Wallis

test and the post hoc Dunn's Test was used, as the samples from the VUV-treated GLY contaminated microcosms were not normally distributed. For all tests a significance level of 0.05 was used.

Results 4

4.1 ATP concentration, CFU/ml and alga growth in microcosms

On day 9 of the microcosm incubation, alga growth was clear in the microcosms contaminated with GLY and to a higher degree in the microcosms contaminated with VUV-treated GLY (figure 4.2). The microcosms contaminated with CHX did not show visible alga growth on day 9, however an alga aggregate was visible in the microcosms contaminated with VUV-treated CHX of similar size to the alga aggregate visible in the control microcosms (see the red circles in figure 4.1). For the microcosms contaminated with BAC or DDAC as well as the microcosms contaminated with VUV-treated BAC and DDAC, no visible alga growth was observed on day 9 of the microcosm incubation.

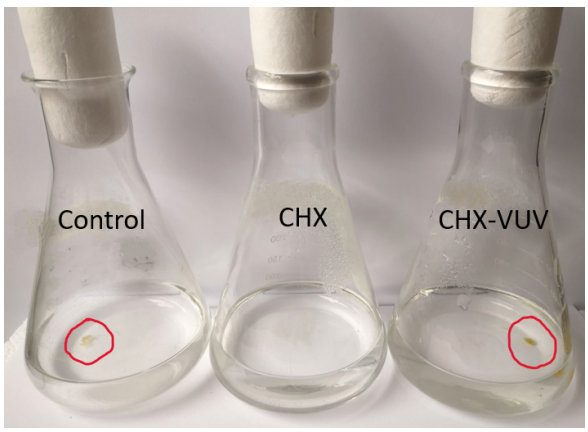

Figure 4.1. Three microcosms on day 9 of incubation. From the left: A control microcosm, a microcosm contaminated with chlorhexidine and a microcosm contaminated with chlorhexidine that has been VUV-treated. The red circles indicate alga aggregates.

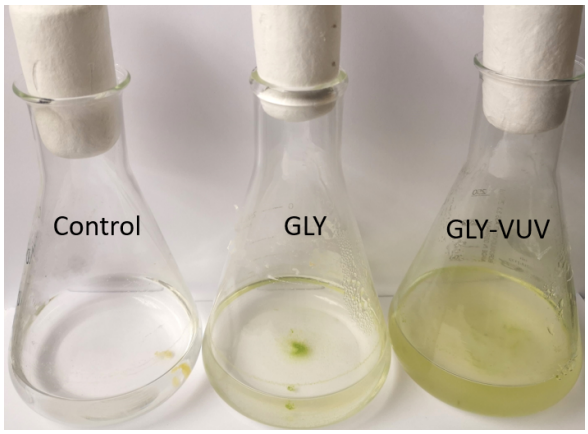

Figure 4.2. Three microcosms on day 9 of incubation. From the left: A control microcosm, a microcosm contaminated with gluphosate and a microcosm contaminated with glyphosate that has been VUV-treated.

Figure 4.3 presents the ATP concentrations measured in the microcosms at each time point: day 0, 3, 6 and 9 of incubation. The ATP concentrations in both the control and control microcosm contaminated with VUV-treated tap water (Control-VUV) vary between $7.2 * 10^{-}5 - 1.6 * 10^{-}4$ micro molar. In the microcosms contaminated with BAC, the ATP concentrations decreased from day 0 to 3 and stayed low for the rest of the incubation period. The microcosms that are contaminated with VUV-treated BAC (BAC-VUV) are less affected by the contamination, with only a slight decrease in ATP concentration over the incubation period. In the microcosms contaminated with CHX, the decrease in ATP concentration is less rapid, however still leading to an ATP concentration well below the concentration measured in the control microcosms. In the microcosms contaminated with VUV-treated CHX (CHX-VUV), a clear increase in ATP concentration is seen from day 0 to day 3, and the ATP concentrations throughout the incubation period are in the same range as the ATP concentration measured in the control microcosms contaminated with VUV-treated tap water. In the microcosms contaminated with DDAC and VUV-treated DDAC (DDAC-VUV), the ATP concentrations measured are similar throughout the incubation period and are below the ATP concentrations measured in the control microcosms and the control microcosms contaminated with VUV-treated tap water. In the microcosms contaminated with GLY, the ATP concentration has a small decrease from day 0 to 3 and then the ATP concentration increases to concentrations higher than the control on day 6 and 9. For the microcosms contaminated with VUV-treated GLY, the ATP concentration increased from day 0 to day 3 to an ATP concentration higher than in the control and Control-VUV and it continued the increase throughout the incubation period.

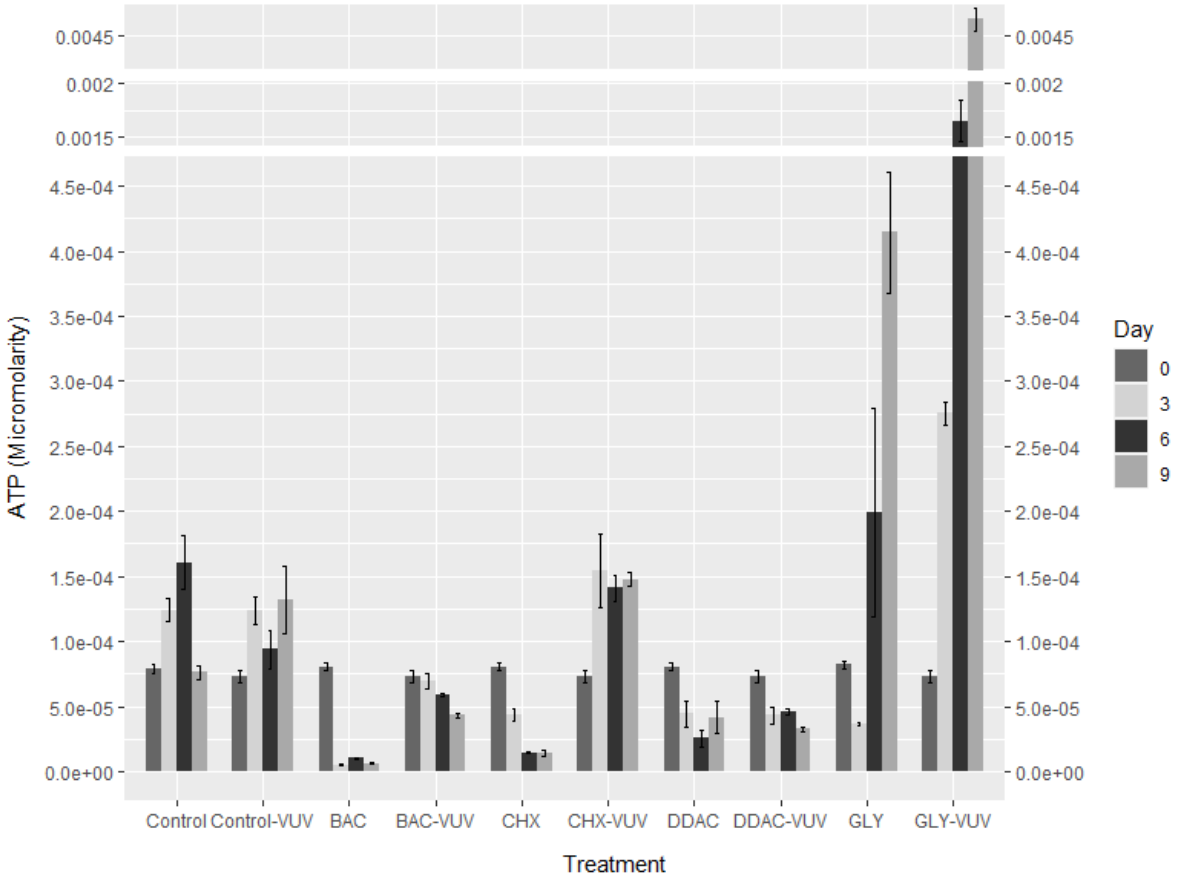

Figure 4.3. The average measured ATP (micro molar) and standard error (n=9) on day 0, 3, 6 and 9 of the incubation for the following microcosms. The microcosms contaminated with benzalkonium chloride (BAC), chlorhexidine (CHX), didecyldimethylammonium chloride (DDAC) and glyphosate (GLY) and the microcosms contaminated with VUV-treated benzalkonium chloride (BAC-VUV), chlorhexidine (CHX-VUV), didecyldimethylammonium chloride (DDAC-VUV) and glyphosate (GLY-VUV). The figure also includes the ATP concentrations measured in the control microcosm (Control) and the control microcosm contaminated with VUV-treated tap water (Control-VUV). Note the sliced y-axis. Note also that day 0 is also time 0, and there is therefore no effect from the different treatments yet.

The average concentration of bacteria (CFU/ml) in the different microcosms after 9 days of incubation are presented in figure 4.1. For the microcosms contaminated with BAC, CHX, DDAC and GLY the CFU/ml values are higher than the values in the control microcosm. Likewise for the microcosms contaminated with VUV-treated BAC (BAC-VUV), DDAC (DDAC-VUV) and GLY (GLY-VUv), the CFU/ml on day 9 is higher than the CFU/ml for the control microcosm contaminated with VUV-treated tap water (Control-VUV). The CFU/ml for the microcosm contaminated with VUV-treated CHX (CHX-VUV) is lower than the CFU/ml in the control microcosm contaminated with VUV-treated tap water (Control-VUV). However the standard error for the VUV-treated control is quite high ($\pm 9.4 * 10^3$ CFU/ml), and a difference in CFU/ml between the microcosms contaminated with VUV-treated CHX and the control microcosms contaminated with VUV-treated tap water is therefore not clear.

Table 4.1. Average Colony-forming-units /ml (CFU/ml) ± standard error (n=3) from day 9 of the microcosm incubation. Top row contains the CFU/ml in the control microcosm and the contaminated microcosms with benzalkonium chloride (BAC), chlorhexidine (CHX), didecyldimethylammonium chloride (DDAC) and glyphosate (GLY) respectively. The second row contains the CFU/ml in the control microcosm contaminated with VUV-treated tap water (Control-VUV) and the microcosms contaminated with VUV-treated benzalkonium chloride (BAC-VUV), chlorhexidine (CHX-VUV), didecyldimethylammonium chloride (DDAC-VUV) and glyphosate (GLY-VUV) respectively.

Control	**BAC**	**CHX**	**DDAC**	**GLY**
$3.1 * 10^4$	$1.5 * 10^5$	$1.6 * 10^5$	$5.7 * 10^5$	$1.6 * 10^6$
$\pm 7.6 * 10^3$	$\pm 2 * 10^4$	$\pm 2.3 * 10^4$	$\pm 4.7 * 10^4$	$\pm 5.2 * 10^5$
Control-VUV	**BAC-VUV**	**CHX-VUV**	**DDAC-VUV**	**GLY-VUV**
$1.5 * 10^4$	$7.7 * 10^5$	$1.3 * 10^4$	$3.9 * 10^5$	$2.4 * 10^6$
$\pm 9.4 * 10^3$	$\pm 2.6 * 10^5$	$\pm 1.3 * 10^3$	$\pm 5.8 * 10^4$	$\pm 7.0 * 10^5$

4.2 Activity and community structure in Biolog Ecoplates

On day 9 of the microcosms incubation, samples from each microcosm were collected for incubation in the Biolog Ecoplates. From the absorbance values measured every 24 hours, the AWCD for each treatment has been calculated (equation 3.2) and presented in figure 4.4 and 4.5. A clear trend in AWCD for all three examined biocides and VUV-treated biocide is found (figure 4.4 A, B and C). The AWCD for the samples collected from biocide contaminated microcosms (BAC, CHX and DDAC) follow the same trend as the AWCD for both the samples collected from the control microcosms and the control microcosm contaminated with VUV-treated tap water (Control-VUV). The AWCD for the samples collected from the DDAC contaminated microcosms (DDAC in figure 4.4,C), have a slight different development pattern. From 48-144 hours of Ecoplate incubation, the AWCD for the DDAC contaminated microcosms was slightly lower than the AWCD for the control, and then from 168-288 hours of incubation the AWCD of the samples from the DDAC contaminated microcosms were slightly lower than the the AWCD of the control samples. The AWCD for the samples collected from the microcosms contaminated with a VUV-treated biocide are for all three examined biocides (BAC-VUV, CHX-VUV and DDAC-VUV), higher than the AWCD for the samples collected from the control microcosm contaminated with VUV-treated tap water (Control-VUV) from 48-288 hours. The biggest difference is observed for the sample collected from the microcosm contaminated with VUV-treated DDAC (DDAC-VUV in figure 4.4,C), followed by VUV-treated CHX (CHX-VUV in figure 4.4,B) and then VUV-treated BAC (BAC-VUV in figure 4.4,A). The AWCD for the samples collected from the microcosm contaminated with GLY (GLY in figure 4.5) have a higher AWCD from 48-288 hours, than the samples collected from the control microcosms. The AWCD for the samples collected from the microcosms contaminated with VUV-treated GLY (GLY-VUV) are even higher throughout the Ecoplate incubation period.

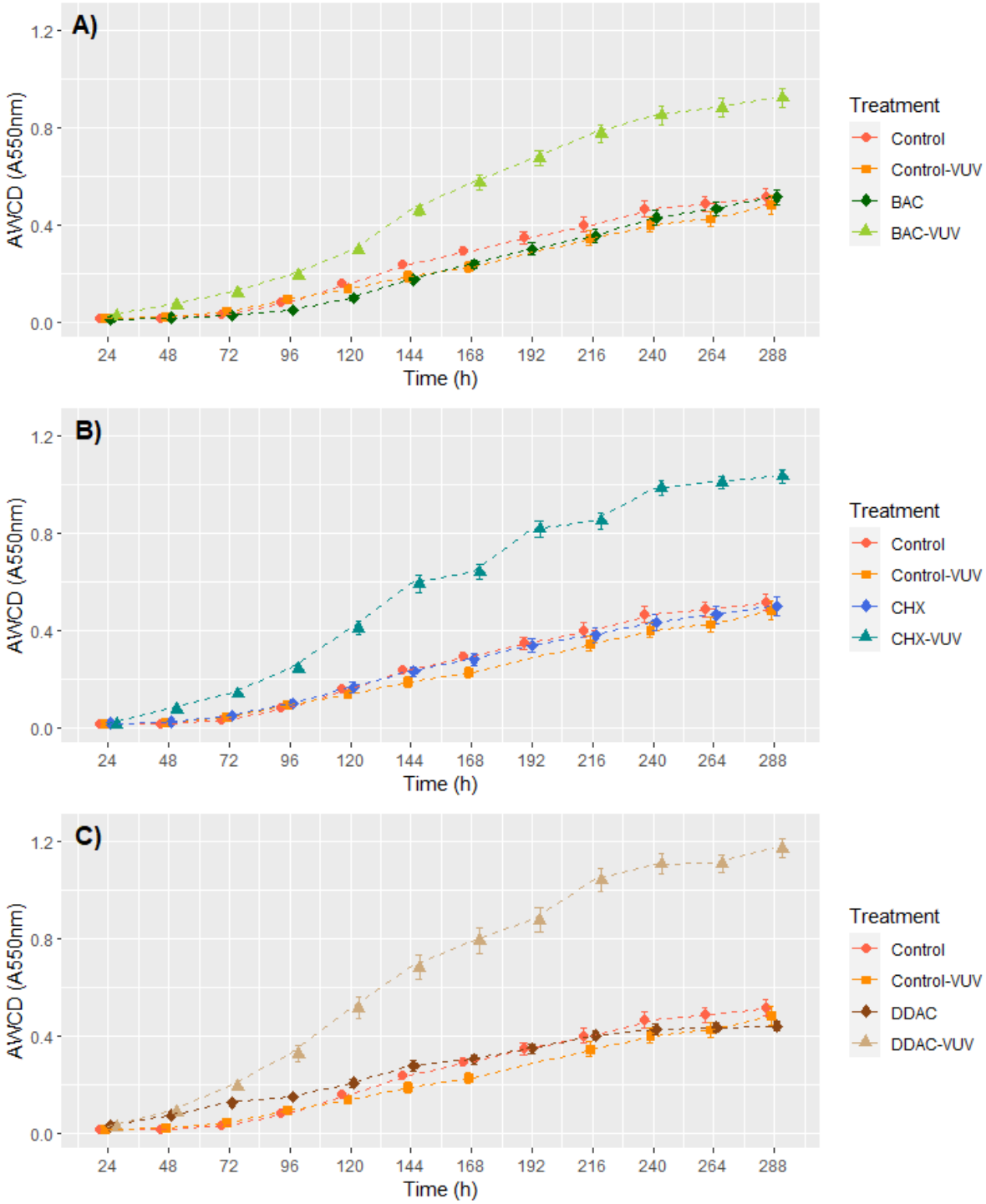

Figure 4.4. The average of the AWCD and standard error (n=9) for each treatment throughout the Ecoplate incubation period. The treatments are samples collected on day 9 of the microcosm incubation from the following microcosms: Control microcosm, Control microcosm contaminated with VUV-treated tap water (Control-vUV) and A) microcosm contaminated with benzalkonium chloride (BAC) and microcosm contaminated with VUV-treated and benzalkonium chloride (BAC-VUV), B) microcosm contaminated with chlorhexidine (CHX) and micrososm contaminated with VUV-treated chlorhexidine (CHX-VUV) and C) microcosm contaminated with didecyldimethylammonium chloride (DDAC) and micrososm contaminated with VUV-treated didecyldimethylammonium chloride (DDAC-VUV).

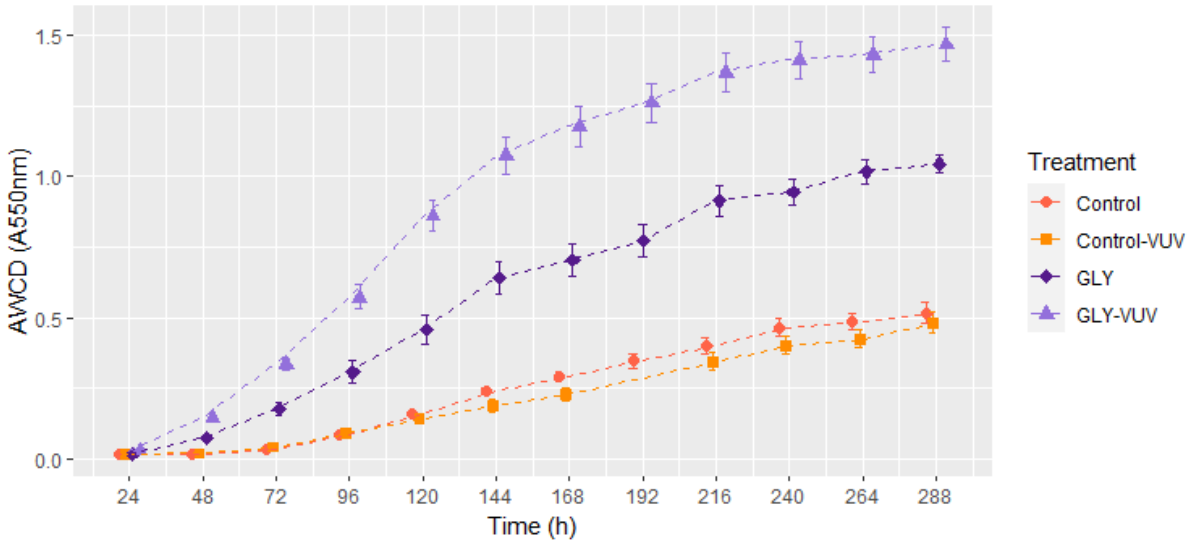

Figure 4.5. The average of the AWCD and standard error (n=9) for each treatment throughout the Ecoplate incubation period. The treatments are samples collected on day 9 of incubation from the following microcosms: Control microcosm, Control microcosm contaminated with VUV-treated tap water (Control-VUV), microcosm contaminated with glyphosate (GLY) and micrososm contaminated with VUV-treated glyphosate (GLY-VUV).

For each substrate group, the SGAWCD (equation 3.3) was calculated. An example of the average SGAWCD for the samples collected from the DDAC contaminated microcosm and the control microcosm is shown in figure 4.7 and 4.6. The rest of the calculated SGAWCD for each substrate group can be seen in appendix A. Some of the treatments have big standard deviations resulting in an uneven variance between groups and therefore the non-parametric methods have been used to identify a difference in substrate group utilisation between treatments. The area under the curve was then calculated 3.4, and the percentage of each substrate group out of the total carbon utilised was then calculated (equation 3.5).

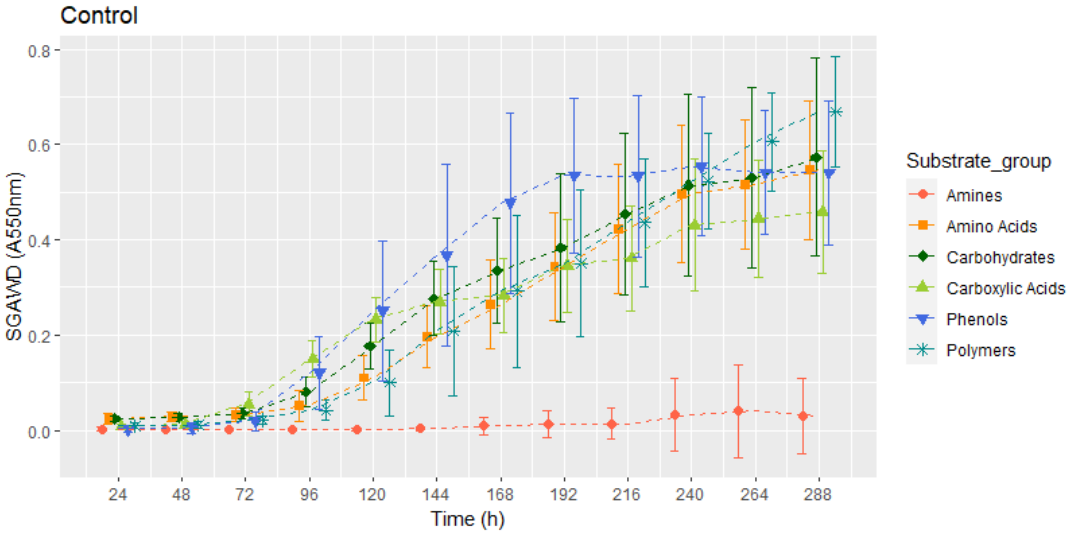

Figure 4.6. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the control microcosms.

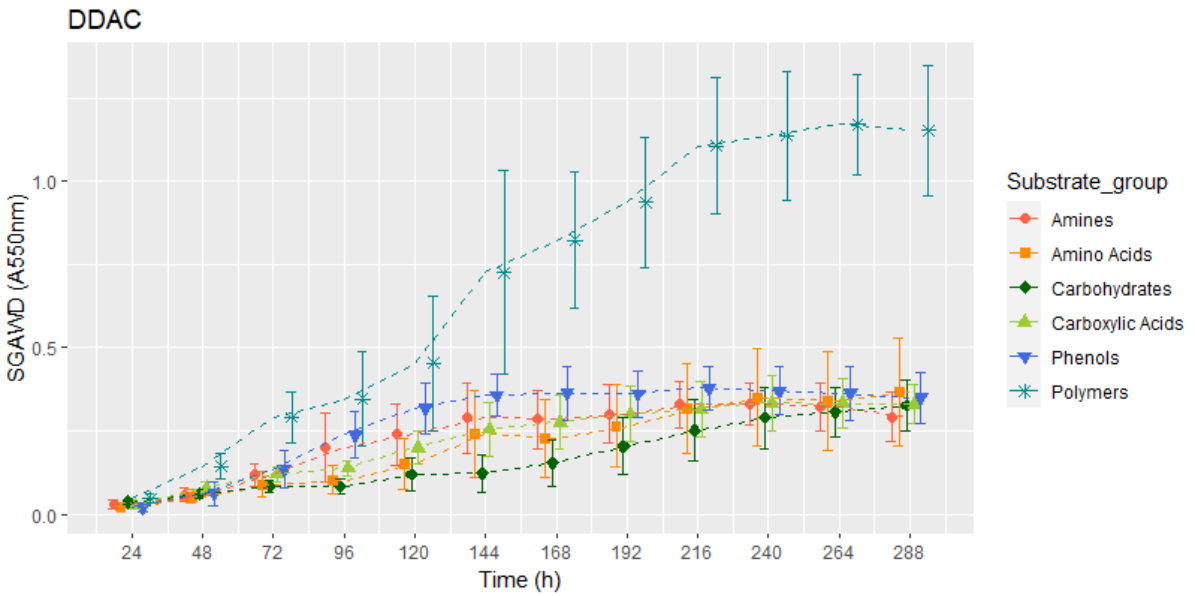

Figure 4.7. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days from the microcosms contaminated with DDAC

The samples collected from the microcosms contaminated with BAC show a significant lower percentage utilised phenol compounds compared to the samples collected from the control microcosms (figure 4.8,A)(Dunn's Test, Bonferroni corrected $P < 0.025$). For the samples collected from the microcosms contaminated with CHX, a significant lower percentage of utilised amino acids was found compared to the samples collected from the control microcosms (figure 4.8,B)(Dunn's Test, Bonferroni corrected $P < 0.025$). For the

rest of the substrate groups for each of the two treatments (BAC and CHX), no significant difference in percentage substrate utilisation compared to the control microcosms was found. For the samples collected from the microcosms contaminated with DDAC (figure 4.8,C), the utilisation amount of amines and polymers is found to be significantly higher than what is found in the samples collected from the control microcosm. For carbohydrates, amino acids and carboxylic acid, the percentage utilisation is significantly lower (Dunn's Test, Bonferroni corrected $P < 0.025$). For the samples collected from the microcosms contaminated with GLY (figure 4.8,D), the utilisation percentage of amino acids is found to big significantly lower than what is found in the samples collected from the control microcosm. For polymers the utilisation amount percentage is significantly lower (Dunn's Test, Bonferroni corrected $P < 0.025$).

For all examined VUV-treated chemicals (figure 4.8, A, B, C and D) (BAC-VUV, CHX-VUV, DDAC-VUV and GLY-VUV), a significant increase in the percentage of amines utilised and a significant decrease in percentage phenols utilised was found in comparison to the samples collected from the control microcosms contaminated with VUV-treated tap water (Control-VUV)(Dunn's Test, corrected $P < 0.025$). For all three examined VUV-treated biocides (BAC-VUV, CHX-VUV, DDAC-VUV) the percentage of total polymer utilisation is significantly higher than the percentage polymers utilised in the samples collected from the control microcosms contaminated with VUV-treated tap water (Control-VUV)(Dunn's Test, Bonferroni corrected $P < 0.025$).

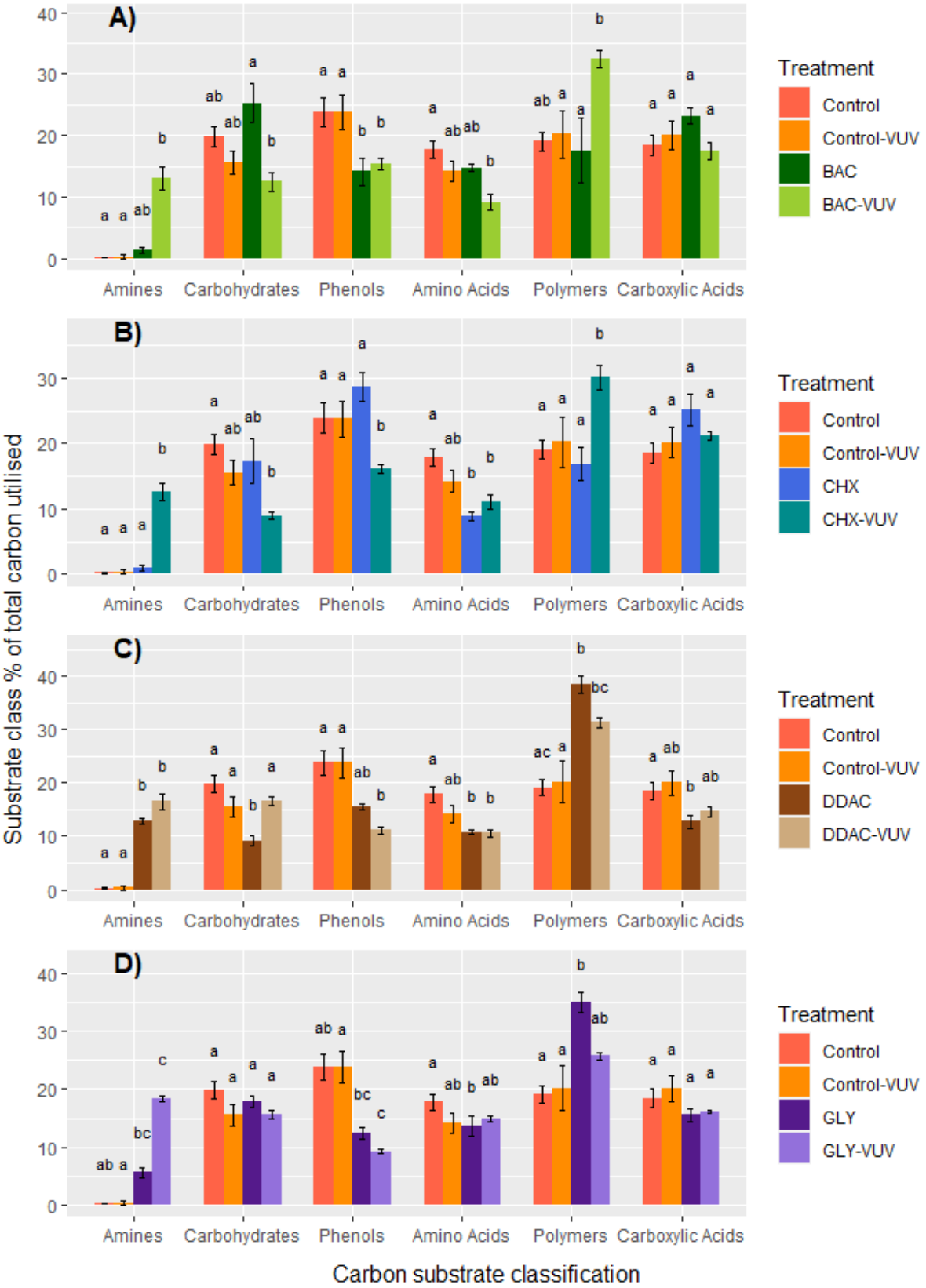
A)
B)
C)
D)
Substrate class % of total carbon utilised
Carbon substrate classification
Amines
Carbohydrates
Phenols
Amino Acids
Polymers
Carboxylic Acids
Treatment
Control
Control-VUV
BAC
BAC-VUV
CHX
CHX-VUV
DDAC
DDAC-VUV
GLY
GLY-VUV

Figure 4.8. The average percentage of each substrate group out of the total carbon utilised and standard error (n=9) for each treatment. For each graph the treatments consist of samples collected from the control microcosm and the control microcosm contaminated with VUV-treated tap water (Control-VUV). The graphs also include samples from the microcosms contaminated with A) benzalkonium chloride (BAC) and VUV-treated benzalkonium chloride (BAC-VUV), B) chlorhexidine (CHX) and VUV-treated chlorhexidine (CHX-VUV), C) didecyldimethylammonium chloride (DDAC) and VUV-treated didecyldimethylammonium chloride (DDAC-VUV) and D) glyphosate (GLY) and VUV-treated glyphosate (GLY-VUV). The difference in letter between treatments for each substrate group indicates a significant difference in the percentage of the substrate group utilised out of the total substrate utilised (Dunn's Test, Bonferroni corrected $P < 0.025$).

Shannon's diversity index (equation 3.6), which is based on the area under the curve for each substrate, was found to show similar tendencies for all three biocide treatments (figure 4.9,A,B,C), where the samples collected from the microcosms contaminated with the biocides (BAC, CHX or DDAC) have a significant lower Shannon's diversity than the samples collected from the control microcosms (around a 5% decrease)(Tukey test, adjusted $p < 0.05$). For each of the samples collected from the microcosms contaminated with the VUV-treated biocides (BAC-VUV, CHX-VUV and DDAC-VUV), the Shannon's diversity is found to be significantly higher than the samples collected from the control microcosms contaminated with VUV-treated tap water (control-VUV) (8, 10 and 17% for BAC-VUV, CHX-VUV and DDAC-VUV respectively)(Tukey test, adjusted $p < 0.05$). The samples collected from the GLY contaminated microcosms did not show a significant difference in the calculated Shannon's diversity index compared to the value obtained for samples from the control microcosm. The samples collected from the microcosms contaminated with VUV-treated GLY (GLY-VUV) did however show a significant increase in Shannon's diversity compared to the samples collected from the control microcosm contaminated with VUV-treated tap water (Control-VUV)(figure 4.9,D)(Dunn's Test, Bonferroni corrected $P < 0.025$).

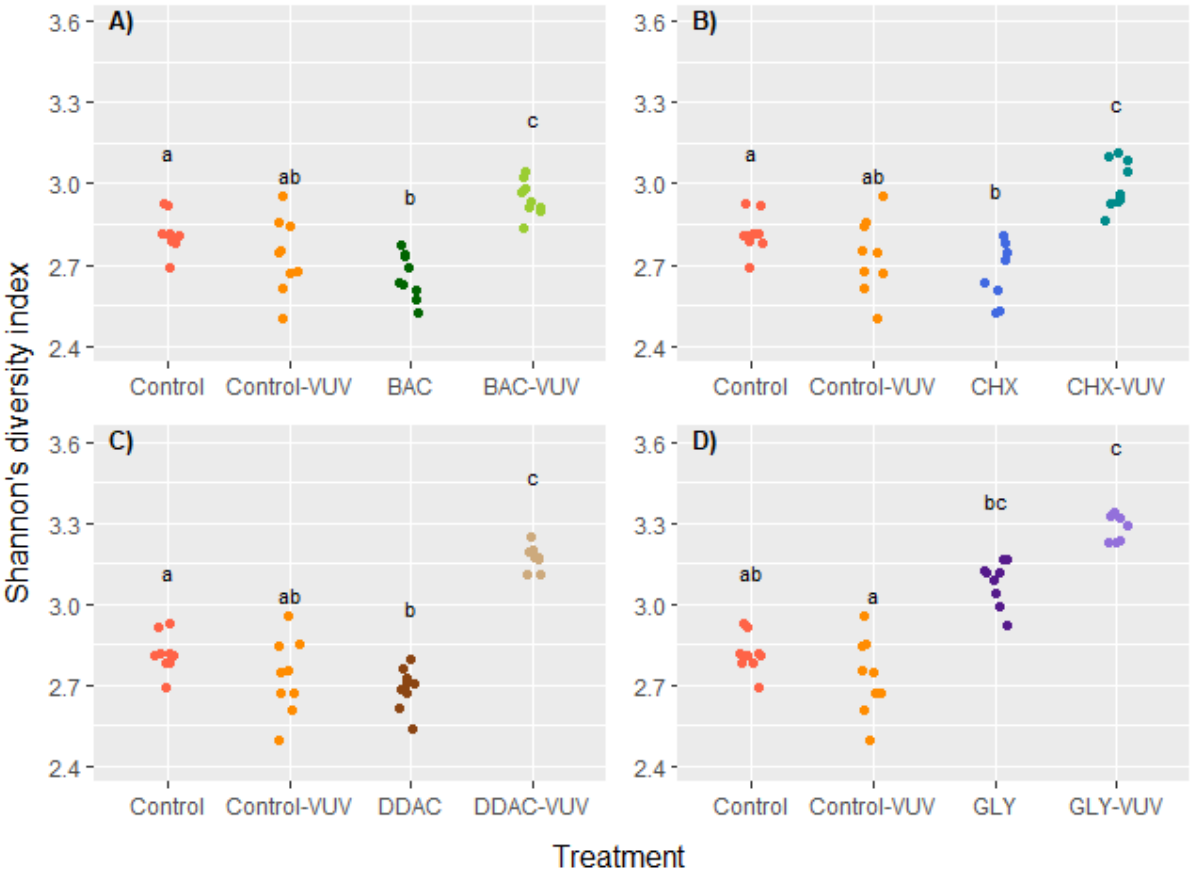

Figure 4.9. Shannon's diversity index for each treatment (n=9). Each graph presents the Shannon's diversity index calculated for the control and control microcosm contaminated with VUV-treated tap water (Control-VUV). The graphs then also include the Shannon's diversity index for the microcosms contaminated with A) benzalkonium chloride (BAC) and VUV-treated benzalkonium chloride (BAC-VUV), B) chlorhexidine (CHX) and VUV-treated chlorhexidine (CHX-VUV), C) didecyldimethylammonium chloride (DDAC) and VUV-treated didecyldimethylammonium chloride (DDAC-VUV) and D) glyphosate (GLY) and VUV-treated glyphosate (GLY-VUV). The difference in letter between treatments indicates a significant difference in the Shannon's diversity index which is based on the ratio of each substrate out of the total substrate utilised. For A, B and C the Tukey test, adjusted $p < 0.05$ has been used, for D the Dunn's Test, Bonferroni corrected $P < 0.025$ has been used).

4.3 Principle component analysis

For each chemical tested in this project, a PCA was conducted to compare the carbon utilisation pattern obtained from the contaminated microcosms and the control microcosms. When the data was scaled (equation 3.8), the PCA was able to differentiate between samples collected from some of the contaminated microcosms from samples collected form the control microcosms (C and CUV). An example is presented in figure 4.10, where the PCA is able to differentiate between the carbon utilisation pattern from

the microplankton communities exposed to DDAC and the carbon utilisation pattern of microplankton communities exposed to VUV-treated DDAC (DDACUV). The carbon utilisation pattern from both of these treatments were differentiated from the pattern obtained from the control microcosms (C) and the control microcosms contaminated with VUV-treated tap water (CUV), which were not differentiated in the PCA. The rest of the PCAs for the remaining examined chemicals can be found in appendix A.

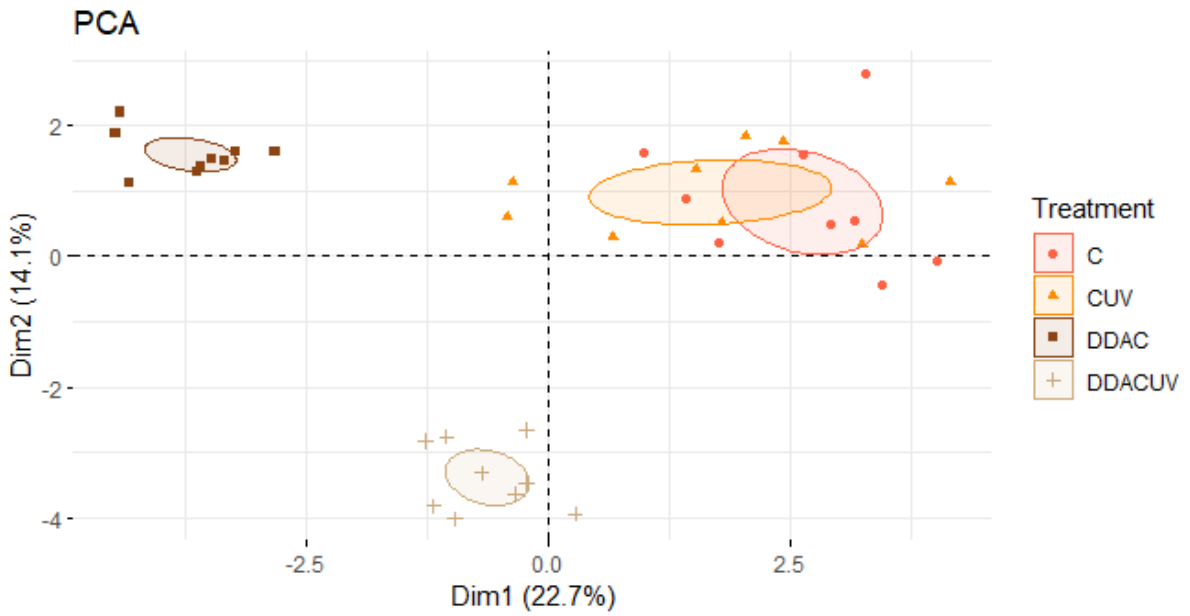

Figure 4.10. PCA of samples collected from the following microcosms: The control microcosm (C), the control microcosm contaminated with VUV-treated tap water (CUV), the microcosm contaminated with didecyldimethylammonium chloride(DDAC) and the microcosm contaminated with VUV-treated didecyldimethylammonium chloride (DDACUV). The PCA is based on 31 substrates as 31 variables, and the value is the percentage of the area under the substrate curve out of the total area under the substrate curves. Each value was then scaled as a measure of relative standard deviation of the variable.

Figure 4.11 presents the loading values for the first two principle components (Dim 1 and Dim 2) from the scaled PCA presented in figure 4.10. Figure 4.12 presents the Cos2 values for the first two principle components (Dim 1 and Dim 2). Both figures show a range of substrates that are of importance when differentiating the samples from the microcosms contaminated with DDAC and VUV-treated DDAC from the control microcosms. For example the substrate glycogen has a high Cos2 value in dimension 2. As dimension 2 is especially important when differentiating between the samples collected from the microcosms contaminated with VUV-treated DDAC (DDACUV in figure 4.10) from the samples collected in the control microcosms (C and CUV), the high Cos2 value of glycogen

indicates that the variance of this substrate was captured well in the second principle component. The loading value of glycogen in dimension 2 is close to -1, indicating that the samples collected from the microcosms contaminated with VUV-treated DDAC have a high substrate utilisation of glycogen. Dimension 1 is the principle component responsible for differentiating samples collected from the DDAC contaminated microcosms, from the samples collected from the control microcosms.

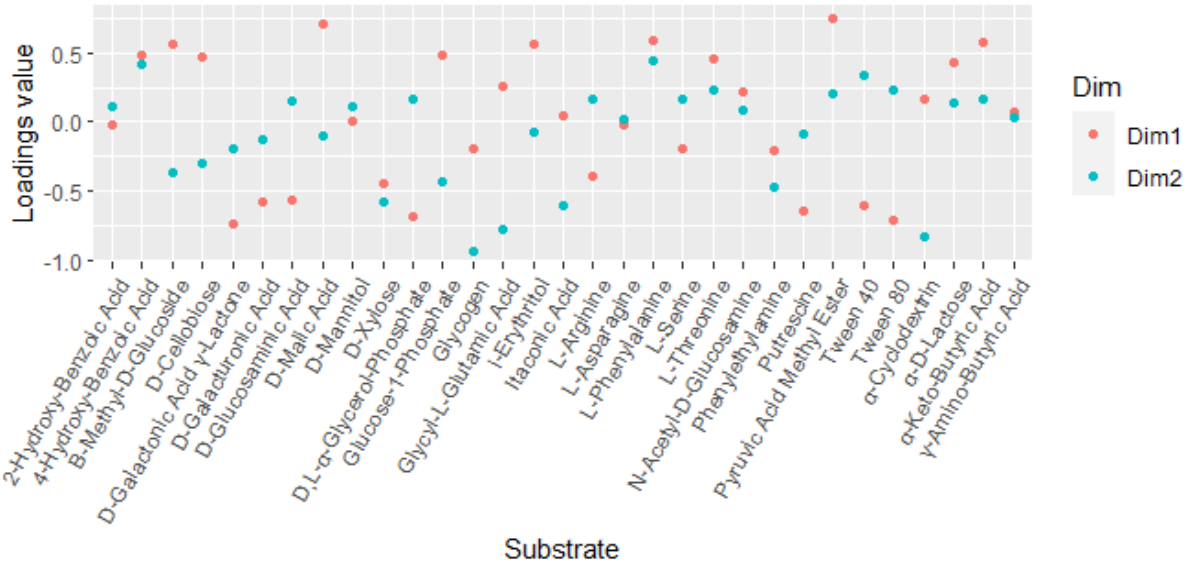

Figure 4.11. The loadings for each variable in the first and second principle component (Dim 1 and Dim 2) for the PCA done on scaled data from samples collected from the control microcosm (c), the VUV-treated microcosm (CUV), the didecyldimethylammonium chloride contaminated microcosm (DDAC) and the microcosms contaminated with VUV-treated didecyldimethylammonium chloride (DDACUV).

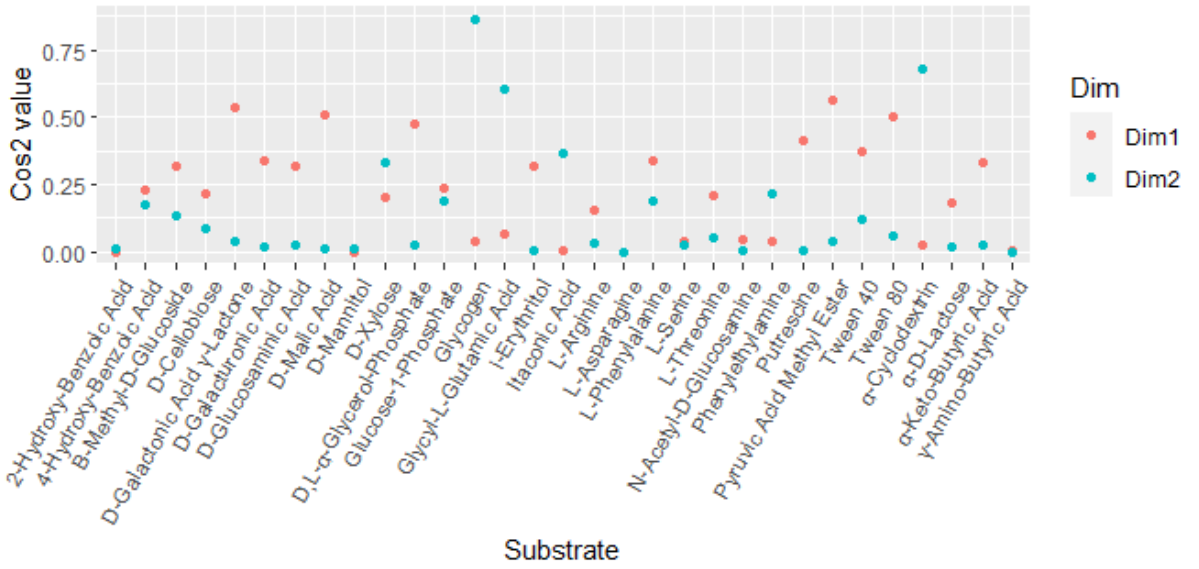

Figure 4.12. The Cos2 value for each variable in the first and second principle component (Dim 1 and Dim 2) for the PCA done on scaled data from samples collected from the control microcosm (c), the VUV-treated microcosm (CUV), the didecyldimethylammonium chloride contaminated microcosm (DDAC) and the microcosms contaminated with VUV-treated didecyldimethylammonium chloride (DDACUV).

As the PCA analysis is conducted on a dataset where all variables are already using the same unit (percentage), scaling each variable as described in equation 3.8, can be argued to be unnecessary. As all data points after scaling are presented as a measure of standard deviation, scaling the data before PCA could result in an amplification of differences between samples that are otherwise not of importance and making an otherwise large difference between sample of less importance.

For the same dataset, a new PCA was conducted without scaling the data (see figure 4.13), however this PCA was only able to differentiate between the different groups of samples (DDAC and VUV-treated DDAC) from the samples collected from the control microcosms (C and CUV) along the first principle component (Dim 1). When analysing the loadings and the Cos2 values for this principle component (figure 4.14 and 4.15) a few important substrates are identified. Especially the polymer Tween-80 has a high Cos2 value of 45 for dimension 1, indicating that the variance of this substrate has a high representation in the first principle component (Dim 1). As it is along this principle component, that the samples from the microcosms contaminated with DDAC and VUV-treated DDAC are separated from the samples collected from the control microcosms, the high Cos2 value of

the substrate Tween-80 indicates that utilisation of this substrate has been important in differentiating the samples from the different treatments (DDAC and DDACUV from the control samples). The loading value of Tween-80 in the first principle component (Dim 1) is around 7,5, and as the samples collected from the DDAC contaminated microcosms are situated on the far positive side of the first principle component, this would indicate that the samples collected from the microcosms contaminated with DDAC have a high substrate utilisation of the polymer Tween-80.

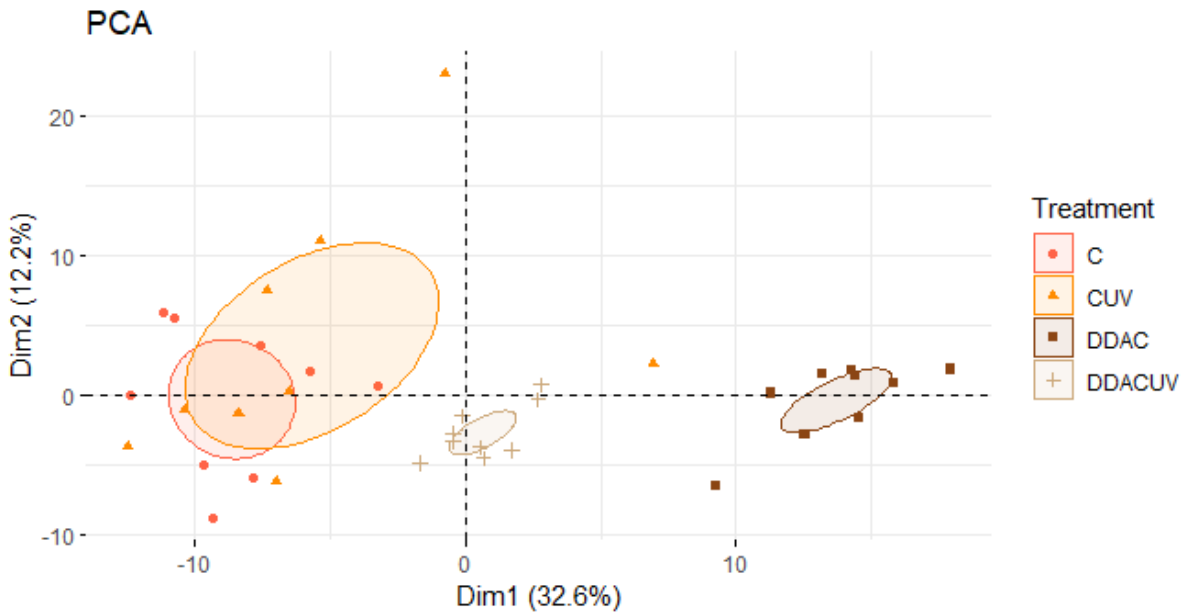

Figure 4.13. PCA of samples collected from the following microcosms: The control microcosm (C), the control microcosm contaminated with VUV-treated tap water (CUV), the microcosm contaminated with didecyldimethylammonium chloride(DDAC) and the microcosm contaminated with VUV-treated didecyldimethylammonium chloride (DDACUV). The PCA is based on 31 substrates as 31 variables, and the value is the percentage of the area under the substrate curve out of the total area under the substrate curves.

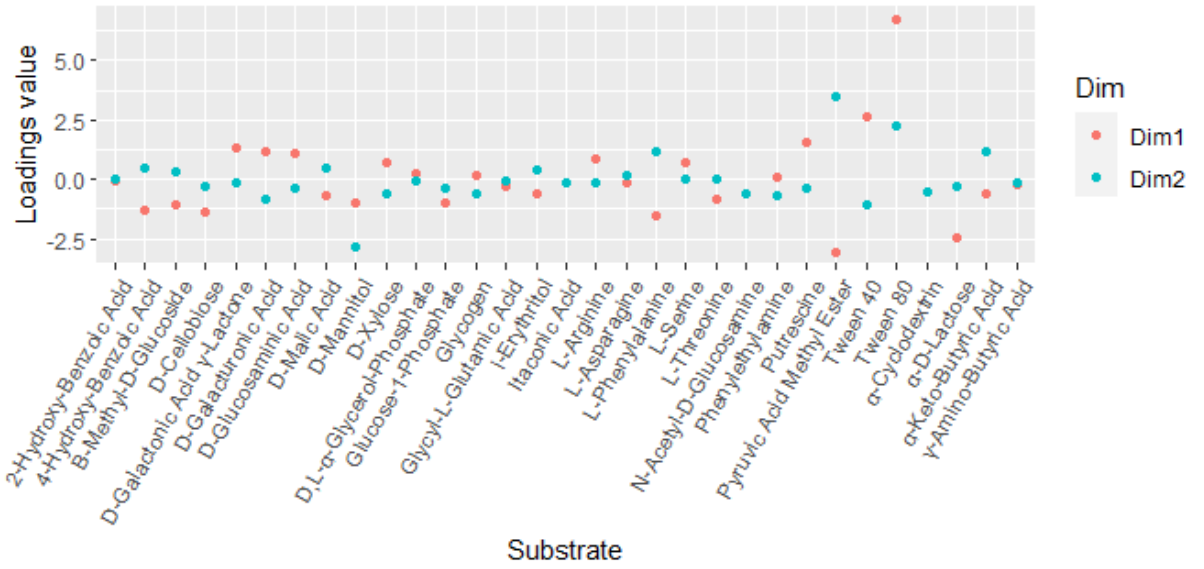

Figure 4.14. The loadings for each variable in the first and second principle component (Dim 1 and Dim 2) for the PCA done on scaled data from samples collected from the control microcosm (c), the VUV-treated microcosm (CUV), the didecyldimethylammonium chloride contaminated microcosm (DDAC) and the microcosms contaminated with VUV-treated didecyldimethylammonium chloride (DDACUV).

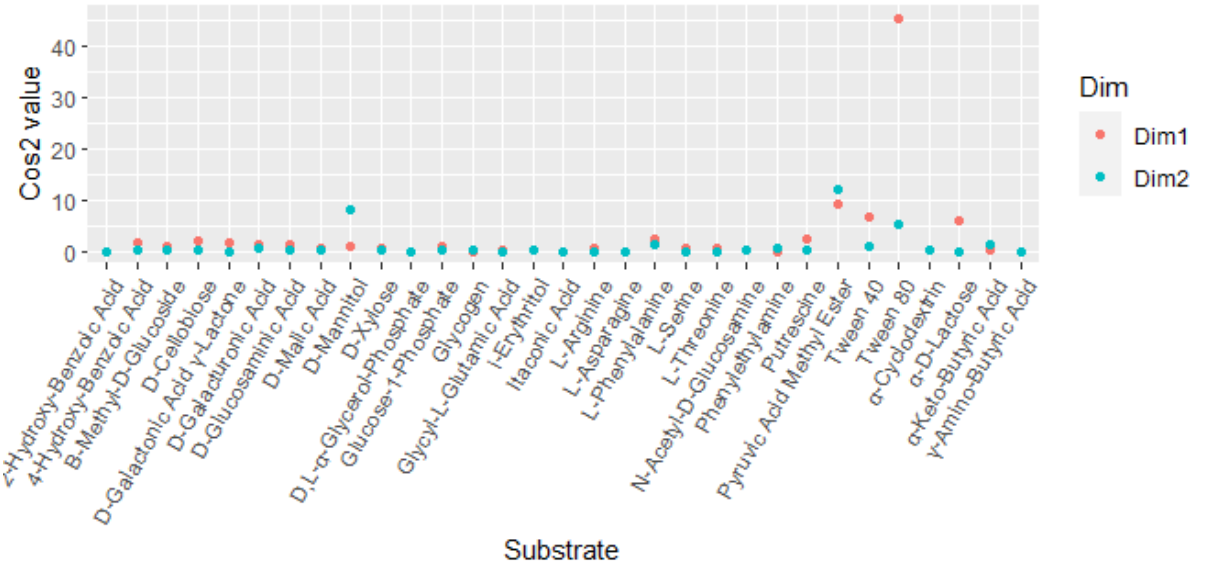

Figure 4.15. The Cos2 value for each variable in the first and second principle component (Dim 1 and Dim 2) for the PCA done on scaled data from samples collected from the control microcosm (c), the VUV-treated microcosm (CUV), the didecyldimethylammonium chloride contaminated microcosm (DDAC) and the microcosms contaminated with VUV-treated didecyldimethylammonium chloride (DDACUV).

For both of the PCAs, the total variability explained by the 1. and 2. dimensions is low.

36.8% for the scaled PCA in figure 4.10 and 44.8% for the non scaled PCA in figure 4.13. Furthermore, a great part of the variables are not found to be strongly correlated (see figure A.14 in appendix), indicating that a PCA is not a suitable statistical method for analysing the data in this project.

Discussion 5

The design of the Ecoplate allows for two different evaluations of the microbial community's response to a pollutant. The AWCD can provide information about the overall change in abundance and activity of the microbial community and the substrate utilisation pattern can give an indication of a change in functional diversity [Stefanowicz, 2006].

5.1 Community activity

5.1.1 The effect of GLY and VUV-treated GLY on community activity

GLY has proven to have an effect on a range of other organisms [Pérez et al., 2011], including test organisms such as the bacteria *Aliivibrio fischeri*, the microalga *Raphidocelis subcapitata* and the crustacean *Daphnia magna* with EC_{50} values around 0.99-3.67 mg/L [Papagiannaki et al., 2020]. The decrease in ATP concentration from day 0 to day 3 in the GLY contaminated microcosms indicates that GLY has had an overall negative effect on the total activity of the freshwater microplankton community from day 0 to day 3 after contamination. As the samples analysed in the Biolog Ecoplates were only collected on day 9 of incubation, this decrease in total activity for the GLY contaminated microcosms in the beginning of incubation was not detected. The results from the biolog Ecoplates do however indicate an increase in activity in the samples collected from the GLY contaminated microcosms on day 9 of incubation compared to the samples from the control microcosms. The increase in activity is also identified in the increase in ATP concentration and CFU/ml in the glyphosat contaminated microcosms from day 3 to 9 and in the increase of visible alga in the microcosms.

Several reports have found that the degradation of GLY results in the release of macro-

nutrients that in turn results in increased alga growth. [An et al., 2023] also found a significant photodegradation of GLY in pond, ditch and lake water samples, up to 86% after 96h, using a 500 W-xenon lamp to simulate the sunlight. The degradation was greatly affected by the humus concentration in the water and GLY was found to be oxidised into glyoxylic acid and AMPA that was further oxidised into orthophosphate and other small molecules, including nitrate and nitrit. [An et al., 2023] furthermore found that the photodegredation products of glyhosate could stimulate the growth of the alga *Microcystis aeruginosa*. A large scale microcosm experiment (1.2m x 25 m2) examining the effect of Roundup on freshwater ecosystems found similar results after 42 days. With a single dose of Roundup, equal to a concentration of 8 mg/L of the active ingredient GLY, Vera et al. [2010] found a significant increase in total phosphorus in the treated microcosms, which favored an eutrophication process resulting in a shift from clear to turbid water. Vera et al. [2010] explains the eutrophication process being due to the degradation of the applied Roundup with a GLY half life of 4.2 days similar to the half life values of 5.77 and 7.37 days for 6 and 12 mg/L glphosat found by [Pérez et al., 2007]. Under similar microcosm experiment setup as in this project, Pesce et al. [2009] found that GLY concentrations were decreased by 30% at 9 °C and at 14 °C GLY was degraded to under the detection limit of (0.5 ug/L) from a starting value of 10 ug/L in 14 days. Pesce et al. [2009] explained the degradation of GLY as possibly due to microbial degradation in the microcosms that was increased with increasing temperature. It is therefore also possible that GLY has also been degraded in this experiment, leading to the release of macronutrients that can support an increase in alga growth. A higher alga growth could support a bigger heterotrophofic plankton community. This is confirmed by an increase in CFU/ml which is also observed in the increased AWCD in the Biolog Ecoplates from the samples collected from GLY contaminated microcosms compared to the control microcosms.

The AWCD in the Biolog Ecoplates indicated a higher total activity in the samples collected in the microcosms contaminated with VUV-treated GLY compared to the samples collected from GLY contaminated microcosms and the control microcosms. This trend is also found in both the visual alga growth in the microcosms, in the ATP concentrations and CFU/ml measured in the microcosms. GLY has been found

to be degraded by VUV radiation [Yang et al., 2021], and in combination with the above described release of nutrients due to GLY degradation, the contamination of the microcosms with VUV-treated GLY was expected to increase the activity of the freshwater microplankton community which is visible in both the ATP concentrations and the AWCD in the Biolog Ecoplates.

5.1.2 The effect of the BAC, CHX and DDAC on community activity

As the examined biocides have been found to be toxic to a range of test organisms in the range of μg to mg/L, the contamination of each of the three tested biocides was expected to have an effect on the microplankton community [Flanjak et al., 2024; Jesus et al., 2013]. The ATP concentrations in the microcosms contaminated with BAC, CHX and DDAC respectively also all show a decrease in total microplankton activity. On the contrary, the AWCD in the Biolog Ecoplates does not show a difference in activity between the samples collected from the control microcosms and the biocide contaminated microcosms. The measurement of ATP concentration has been found to be a sensitive method for toxicity testing, capturing not only a decrease in viable cell numbers, but also a decrease in cellular ATP as a physiological stress response to the toxicity of the examined contaminant [Brezonik et al., 1975]. The transfer of the samples from the contaminated microcosms to the substrate rich environment of the Biolog Ecoplates, could have increased the activity in some of these otherwise lethargy organisms, due to more favorable living conditions, resulting in a similar AWCD as the samples collected from the control microcosms.

As the ATP concentration can be used as a measure of total biomass [Bochdansky et al., 2021], the larger phyotplankton in the microcosms can be suspected to have a large effect on the total ATP concentration measured. The visible alga aggregates in the microcosms contaminated with VUV-treated CHX supports this hypothesis as these microcosms also have an ATP concentration similar to the control microcosms. In comparison, the microcosms contaminated with VUV-treated DDAC and BAC have ATP-concentrations lower than the control microcosms and also do not show visible growth of alga. The AWCD in the Biolog Ecoplates show opposite results to the ATP concentrations in regards to the microcosms contaminated with VUV-treated biocides. The higher AWCD for all three

samples from VUV-treated biocides compared to the AWCD of the controls, indicate that these microcosms have a larger amount of (obligate and facultative) heterotrophic microorganisms able to thrive in substrate rich environments compared to the control microcosms. The degradation of the organic contaminants due to the VUV-treatment can have lead to smaller substrates, increasing the whole heterotrophic community which can be confirmed by the increase in CFU/ml. These results show how Biolog Ecoplates and the measurement of ATP concentration give an analysis focused on different groups of the examined microplankton community, and how these tools can supplement each other when evaluating the overall effect of a contaminant on a plankton community.

5.2 Carbon substrate utilisation pattern

The second element of Biolog Ecoplates is the possibility to compare the carbon substrate utilisation pattern between different treatments to identify a possible shift in the community structure.

5.2.1 The effect of GLY and VUV-treated GLY on the carbon substrate utilisation pattern

A change in the carbon substrate utilisation pattern of the microcosms treated with GLY and VUV-treated GLY was expected, both due to the change in possible carbon sources and nutrient availability from the degradation of GLY. But also due to the varying toxic effect that GLY and GLY-based herbicide solutions have been found to have on single aquatic organisms [Pérez et al., 2011] and the microbial community composition of freshwater ecosystems Pesce et al. [2009]; Vera et al. [2012]; Pérez et al. [2007]; Vera et al. [2010]. The changes examined are however mainly focused on the change in phytoplankton abundance and the increase in picocyanobacteria and bacterioplankton, with Vera et al. [2012] also describing an indirect effect of the herbicide on the zooplankton by the increase in the abundance of the rotifer *Lecane* spp. as a consequence of the improved food availability given by picocyanobacteria and bacteria. With a significant increase in percentage of carbon polymers utilised, and a decrease in percentage of amino acids utilised, the carbon substrate utilisation pattern obtained from the Biolog

Ecoplates indicate that the functional diversity in the GLY contaminated microcosms is slightly different from the functional diversity found in the control microcosms. A shift in functional diversity is also indicated for the microcosms contaminated with VUV-treated, as the samples collected from these microcosms show a significant increase in percentage amines utilised and a significant decrease in percentage phenols utilised. The carbon substrate utilisation pattern from the Biolog Ecoplate has identified a possible shift in community structure of the freshwater microplankton community after the contamination of glyphosat and VUV-treated GLY.

A reduction in Shannon's diversity index of the samples collected from the glyphosat contaminated microcosms was expected compared to the samples from the control microcosms, as a decrease in sensitive species upon the introduction of the toxicant can reduce the biodiversity of the sample [Stefanowicz, 2006]. If the samples for Biolog Ecoplate analysis had been collected on day 3 of microcosm incubation, a decrease in Shannon's diversity for the GLY contaminated microcosms might have been observed as the ATP concentrations in these microcosms had decreased from day 0, indicating a decrease in viable cells. However, after applying a single dose of 10 ug/L GLY in a 14 day microcosms experiment, Pesce et al. [2009] did not find a difference in bacterial community diversity of a riverine microbial community using the 16S PCR-TTGE detection method.

For the samples collected from the microcosms contaminated with VUV-treated GLY, the significant increase in functional diversity indicates an increase in species diversity of the heterotrophic microplankton community. These results could have been expected as an visual increase in alga growth was observed and an increase in species richness is often found with increasing primary productivity to a certain degree, due to the increased available energy in the system supporting additional species and trophic levels [Brönmark and Hansson, 2005]. As the Biolog Ecoplates in this experiment primarily examines the heterotrophic microplanktonic community that is normally a part of a freshwater ecosystem, the indication of an increase in biodiversity does not mean that an increase in biodiversity will be found in the rest of the ecosystem, in particular the benthic and periphytic communities, as an increase in the primary production in the planktonic community can lead to a decrease in favorable abiotic living circumstances for a large amount of species that normally inhabit parts of these ecosystems [Brönmark and

Hansson, 2005]. These results indicate that the results of the community structure from Biolog Ecoplates should be collected from several different communities in the ecosystem, to capture a more broad view of the contamination effect on the examined ecosystem.

5.2.2 The effect of the BAC, CHX and DDAC on the carbon substrate utilisation pattern

Only a slight difference in community structure is indicated by the carbon substrate utilisation pattern of the Biolog Ecoplates for BAC and CHX, with only one substrate group utilisation percentage being significantly different to the percentage found in the samples collected from the control microcosms. For the samples collected from the DDAC contaminated microcosms, the carbon substrate utilisation pattern on the other hand identifies a significant difference in functional diversity (5 out of 6 substrate groups), from the samples collected in the control microcosms. An explanation could be that CHX is found to be poorly biodegradable [ECHA, NAa] and BAC was found to be biodegraded in activated sludge only after complete utilisation of the available glycose in the experiment setup [Zhang et al., 2011]. On the other hand, DDAC is found to be readily biodegradable in an activated sludge systems [DeLeo et al., 2020], and the clear difference in substrate utilisation pattern compared to the samples from the control microcosm could be due to the commence of DDAC degradation. This argument is supported by the similarity in substrate utilisation pattern of the samples collected from the microcosms contaminated with VUV-treated DDAC, where degradation products of DDAC after VUV-treatment are assumed to be present and can function as carbon substrates for the heterotrophic microplankton community. The substrate utilisation pattern between the samples collected from the GLY contaminated microcosms and the VUV-treated GLY contaminated microcosms has not been found to be significant different in any of the 6 substrate groups examined, and these findings support the sugestion, that Biolog Ecoplates are a useful method to examine if a contaminant can be biodegraded in an environmental setting.

The PCA is able to differentiate between the carbon substrate utilisation pattern of the samples collected from the DDAC contaminated microcosms and the microcosms contaminated with VUV-treated DDAC, indicating that there is a difference in the

functional community structure between the two communities. Further analysis of the loadings and Cos2 value for the first principle component (Dim 1) of the non scaled PCA, indicates that it is an increased utilisation of the substrate Tween 80 that is responsible for differentiation the microplankton community affected by DDAC from the microplankton community affected by the VUV-treated DDAC.

For all of the samples collected from the microcosms contaminated with VUV-treated biocides and to a certain degree also the VUV-treated GLY, a similar substrate utilisation pattern is observed, with an increase in percentage amines and polymers utilised compared to the samples collected from the controls. These results indicate that the VUV-treatment of the chemicals examined in this project did not reduce the effect of the contamination on the heterotrophic community. However when including the calculation of the functional diversity index for the three biocides, by VUV-treating the biocids, a decrease in biodiversity was avoided which was also what was expected when considering the toxicity of the biocides and the toxicity mitigation via VUV-treatment found for DDAC and BAC [Flanjak et al., 2024].

5.3 Areas to consider when using Biolog Ecoplates as a community level tool for ecotoxicology evaluation

A big benefit of Biolog Ecoplates is that they provide an easy way to obtain a carbon substrate utilisation profile that can help identify changes in microbial community structure. In general, there is an agreement that it is important to adjust the result obtained from the Biolog microtiter plates or Ecoplate for difference in incubation numbers before analysing the CLPP [Garland, 1997], and to do so there are several different methods. Dividing the absorbance value with the mean AWCD is a common approach, however the difference in AWCD due to a difference in the number of positive wells should be accounted for [Garland, 1997]. Garland [1997] suggests choosing samples of equivalent AWCD, but not necessarily equivalent incubation times, as a better method for limiting the effects of difference in the overall rate of color development on the CLPP analysis. This method was also evaluated in this project, however the substrate responsible for

differentiation between contaminated and control samples varied depending on the chosen reference AWCD, as also reported by Garland [1997]. No clear trend was also not found in the calculated diversity index. Garland [1997]; Stefanowicz [2006] also proposes to use a kinetic analysis of the well colour development as a more effective way of capturing differences in sample composition, and could increase the amount of information that can be obtained from the Biolog Ecoplate results. By fitting a model based on density-dependent logistic growth to the often observed sigmoidal curve, provides parameters describing the curve of optical density development such as the lag phase, rate of color development or the asymptote value [Garland, 1997; Stefanowicz, 2006; LINDSTROM et al., 1998]. For the optical density measurements collected in this report a kinetic analysis approach fitting the Gompertz equation to the measurements of optical density, as suggested by Stefanowicz [2006] was examined. The Gompertz equation did however not fit several of the optical density developments during the incubation period, resulting in 18% of the collected optical density time curves not presenting a valid estimation of the growth rate or asymptote due to standard errors bigger than the estimated parameter. Several of the optical development curves obtained in this report were more closely fitted to a straight line. Garland [1997]; Stefanowicz [2006] also propose determining the integral of the color development of each well and this report found that the area under the curve was a method that could successfully capture the total difference in, abundance (lag fase), growth rate and maximum activity between each substrate.

Some of the developments of the absorbance over time for each substrate found in this paper had double exponential "growth" periods throughout the incubation period of the Ecoplates. This could be an indication of a change in the dominating species in the isolated community of the single substrate well of the ecoplate. This argument is based on the findings of Christian and Lind [2006], who found a change in morphological ratios (measured as the ratio of cocci to bacilli) over the incubation time (4 days) for all substrates, and Christian and Lind [2006] conclude that the Biolog Ecoplate is a selective culturing technique. They further argue that their findings may suggest that the CLPP drawn from Biolog Ecoplates are a measure of functional potential and not in situ ecological potential, which is also stated by [Stefanowicz, 2006], who argues that the design of the Ecoplate favors the microorganisms that grow well in high nutrient

environments and is dependent on the organisms that are able to reduce the tetrazolium dye. Hence, Stefanowicz [2006] states that Biolog Ecoplates should only be used in comparison research, for example comparing contaminated and non-contaminated sites, and care should be taken when drawing conclusions on functional structure. This further raises the issue of having a control sample to compare and determine the degree of effect that the pollution has on the community. If a control sample is already slightly affect by contaminants, the overall evaluation of the effect the toxicant has on the community, might be falsely determined. Furthermore, as the degree of toxicity can depend widely on the season and the composition of species in the community [Pesce et al., 2009], the time of sampling a control from the environment can also affect the results from the Biolog Ecoplate's ecotoxicity evaluation. Overall it is clear that in order to use Biolog Ecoplates as a supplementary method for ecotoxicity evaluation, there is a need for a more standardised method for both the experimental setup including the choice of control sample, and for the following data treatment.

6 Conclusion

The Biolog Ecoplates did not show a toxic effect of each of the three examined biocides on total activity, which in comparison was identified in the measurements of ATP concentration of the whole microplanktonic community. The results from the Biolog Ecoplates agreed with the ATP concentration and CFU/ml measurements on the effect of glyphosat contamination on total activity. The Biolog Ecoplate was to some degree able to differentiate the substrate utilisation patterns of the contaminated microplankton communities from the communities of the control microcosms, indicating a change in functional diversity and therefore a possible shift in community structure, especially in the microcosms contaminated with DDAC and GLY.

The results from the Biolog Ecoplate also showed that the VUV-treatment of the examined chemicals, was able to increase the functional diversity in comparison with the decrease in functional diversity observed in the microcosms contaminated with chemicals that were not VUV-treated. The ability of Biolog Ecoplates to evaluate the toxicity mitigation of VUV-treatment is however not clear, as the results of the total activity in the Biolog Ecoplates were quite different to the results of the ATP concentrations for the microcosms contaminated with the examined VUV-treated chemicals.

Similar substrate group utilisation patterns for the VUV-treated and non-VUV-treated contaminated microcosms for both DDAC and GLY suggest that Biolog Ecoplates might be a tool for identifying the biodegradability of a pollutant.

Biolog Ecoplates are found to be a useful community level method for assessment of chemical toxicity on a freshwater microplankton community. The results of this project indicate the importance of including other toxicity methods in addition to Biolog Ecoplates, when evaluating the degree of toxicity mitigation and chemical toxicity on an ecosystem.

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Data A

A.1 Substrate group timelines for each treatment

The 31 substrates on the Biolog Ecoplate are divided into 6 substrate groups as can be seen in figure 2.4. For each substrate group the average well colour development is calculated by equation 3.3 and presented in the following graphs together with the standard deviation.

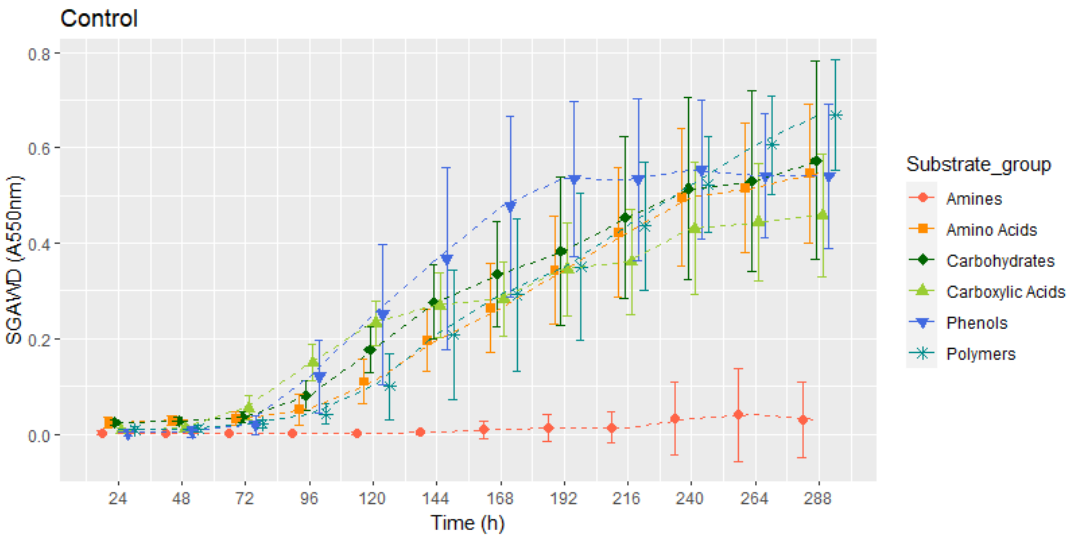

Figure A.1. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the control microcosms.

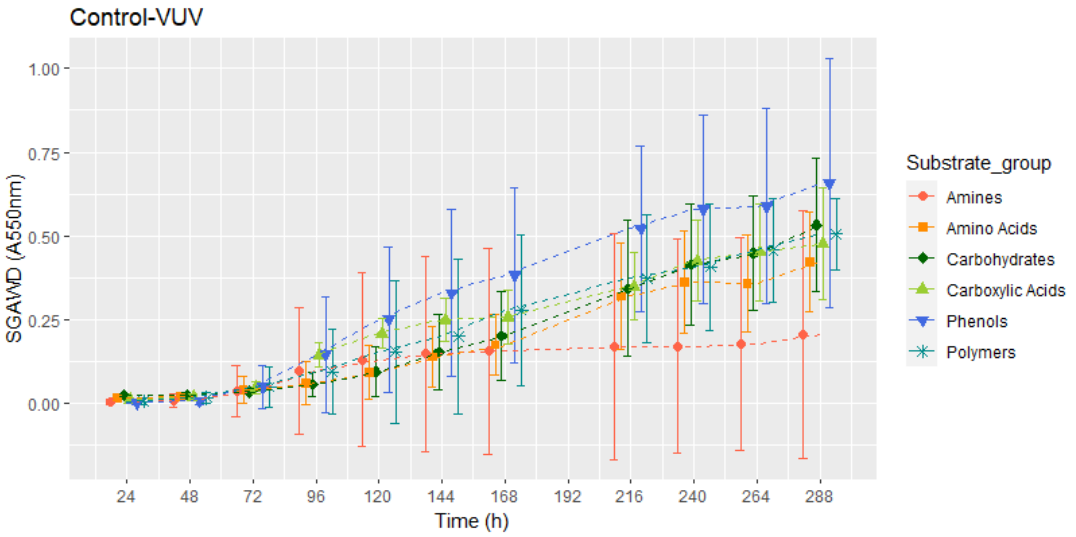

Figure A.2. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the control-VUV microcosm contaminated with VUV-treated tap water. The measurements at 192h have been removed due to error in absorbance reading.

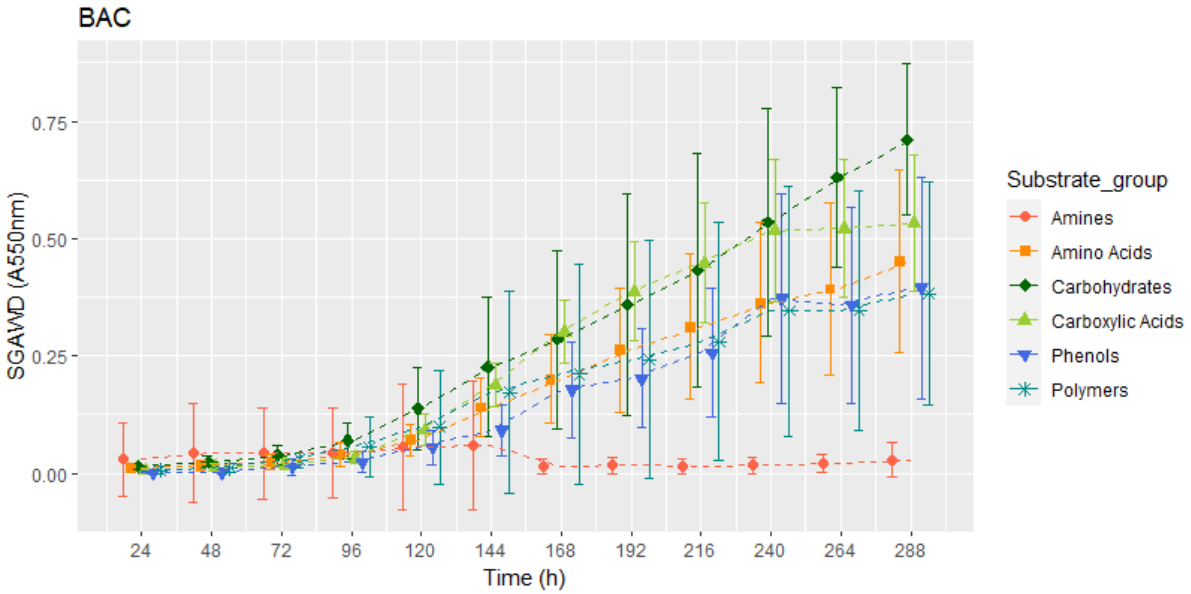

Figure A.3. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the microcosms contaminated with BAC.

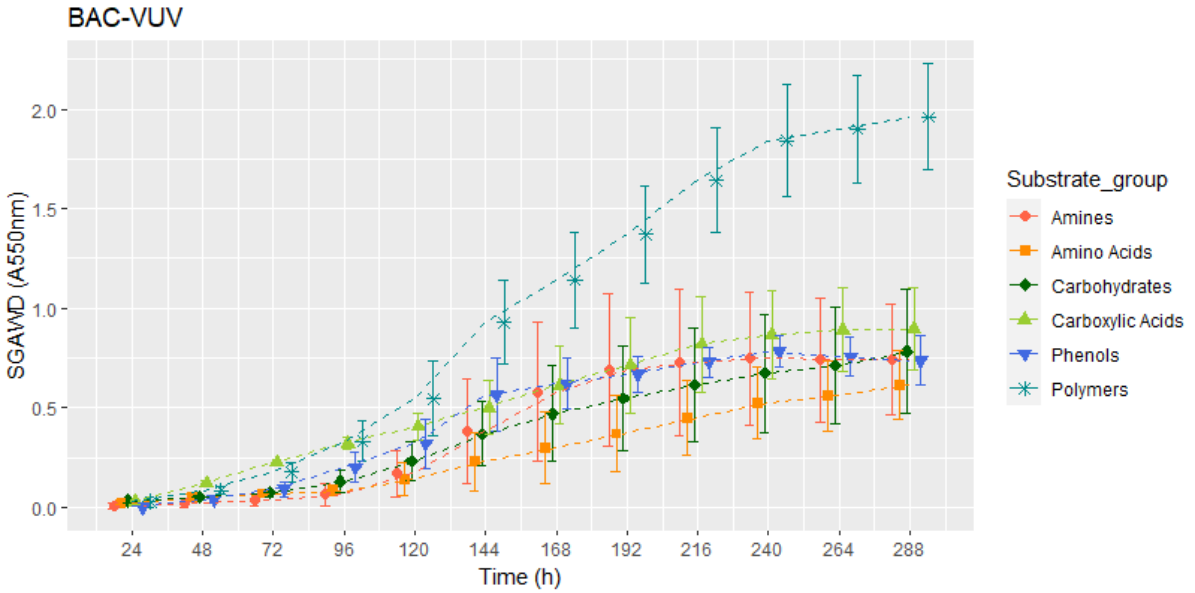

Figure A.4. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the microcosms contaminated with VUV-treated BAC.

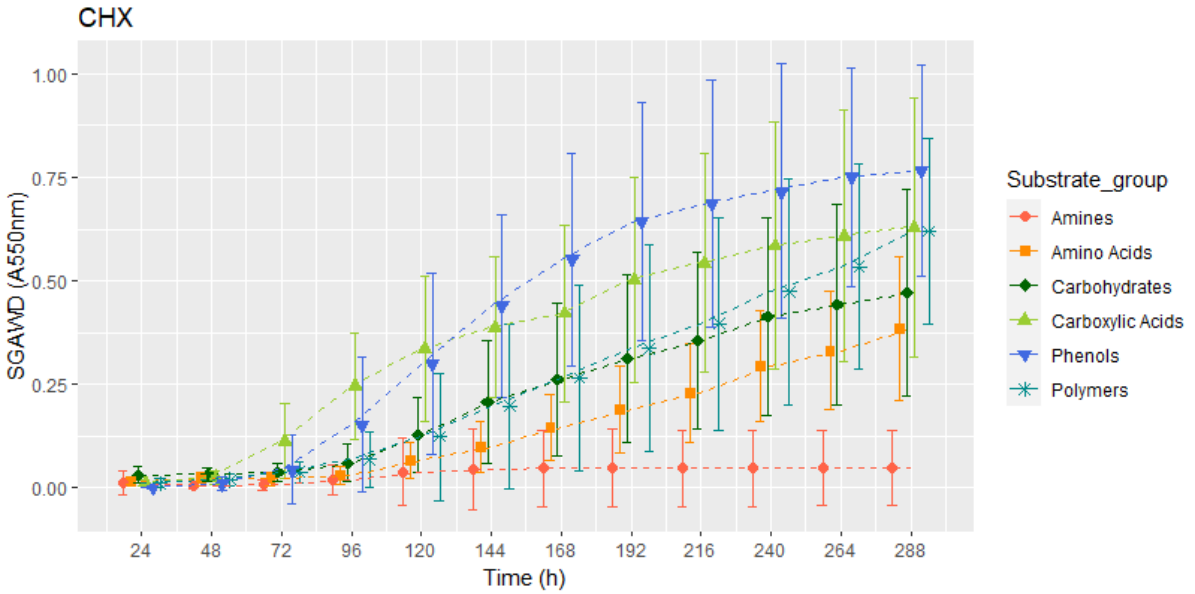

Figure A.5. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days from the microcosms contaminated with CHX.

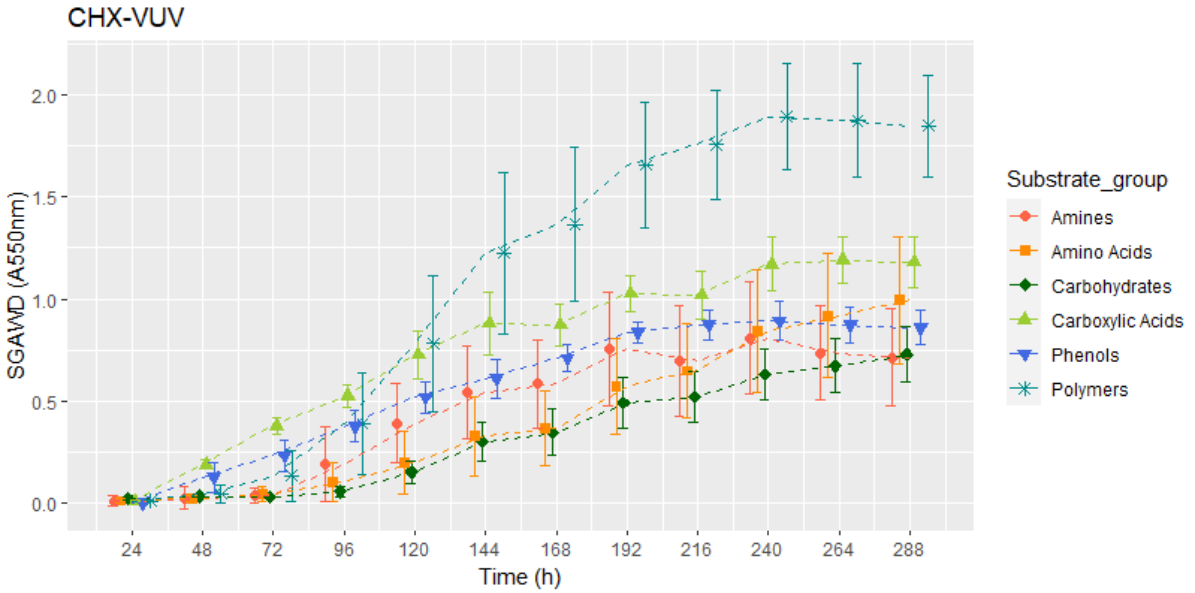

Figure A.6. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the microcosms contaminated with VUV-treated CHX.

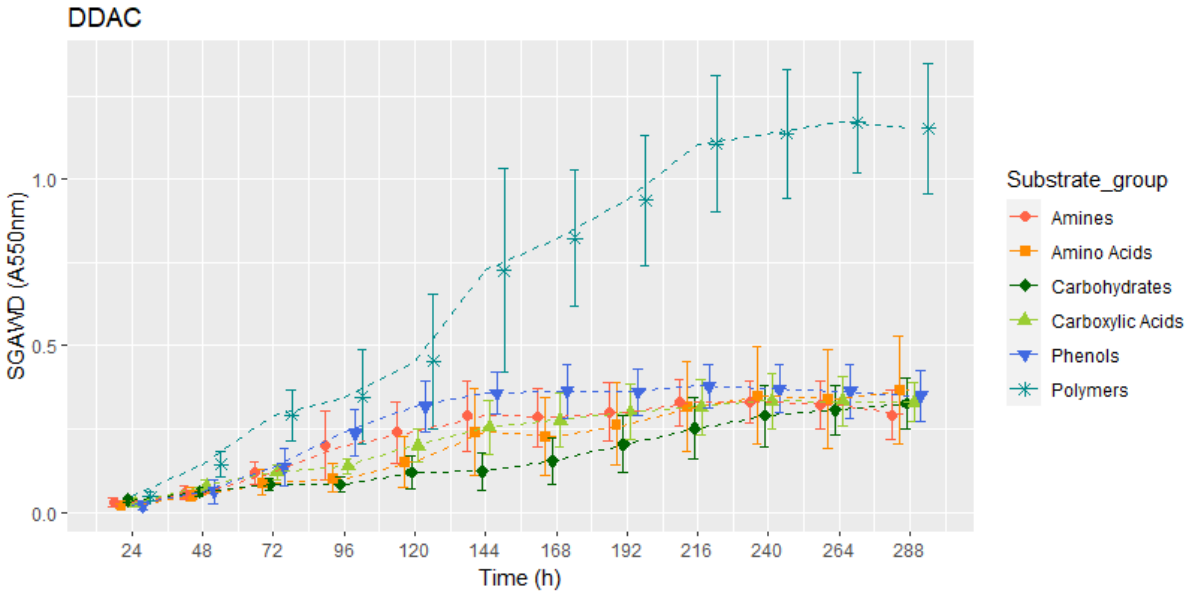

Figure A.7. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days from the microcosms contaminated with DDAC

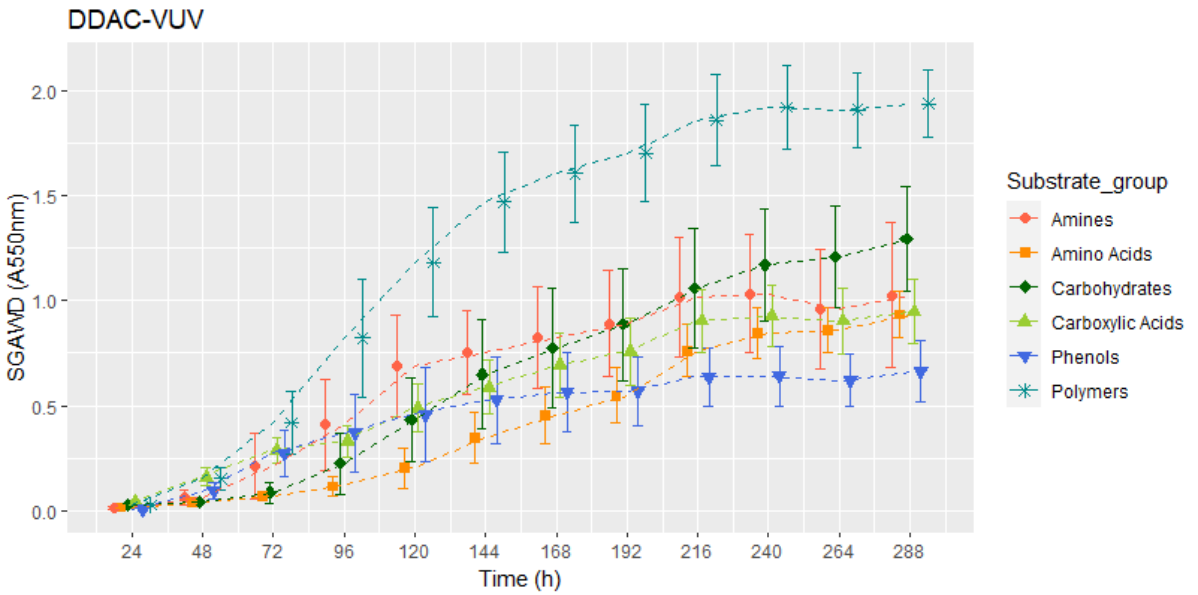

Figure A.8. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the microcosms contaminated with VUV-treated DDAC.

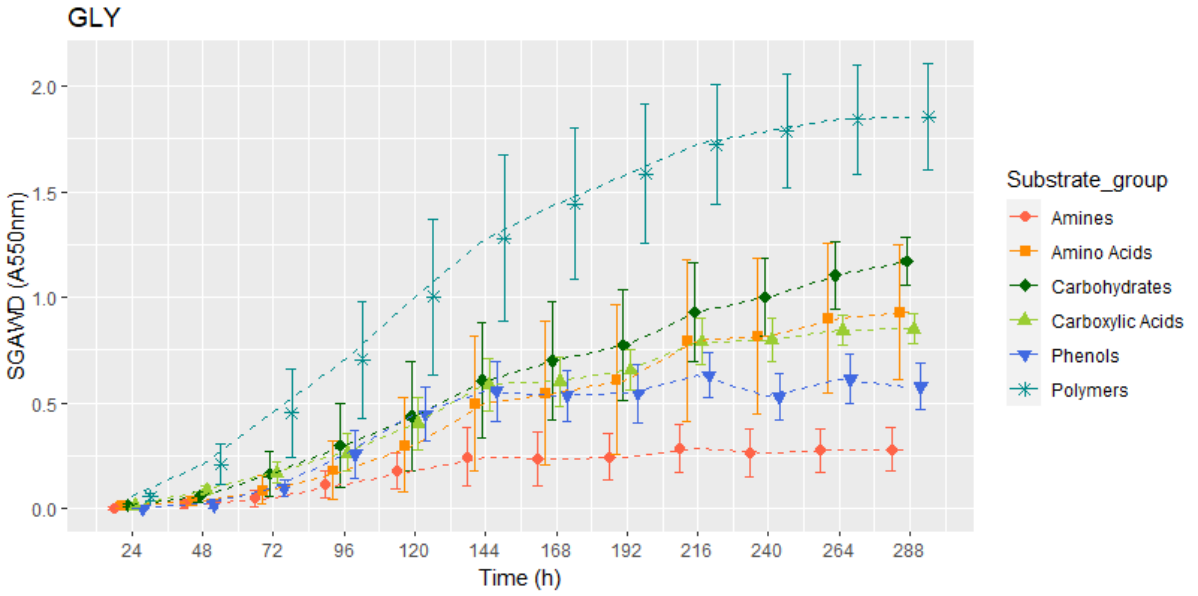

Figure A.9. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days from the microcosms contaminated with GLY.

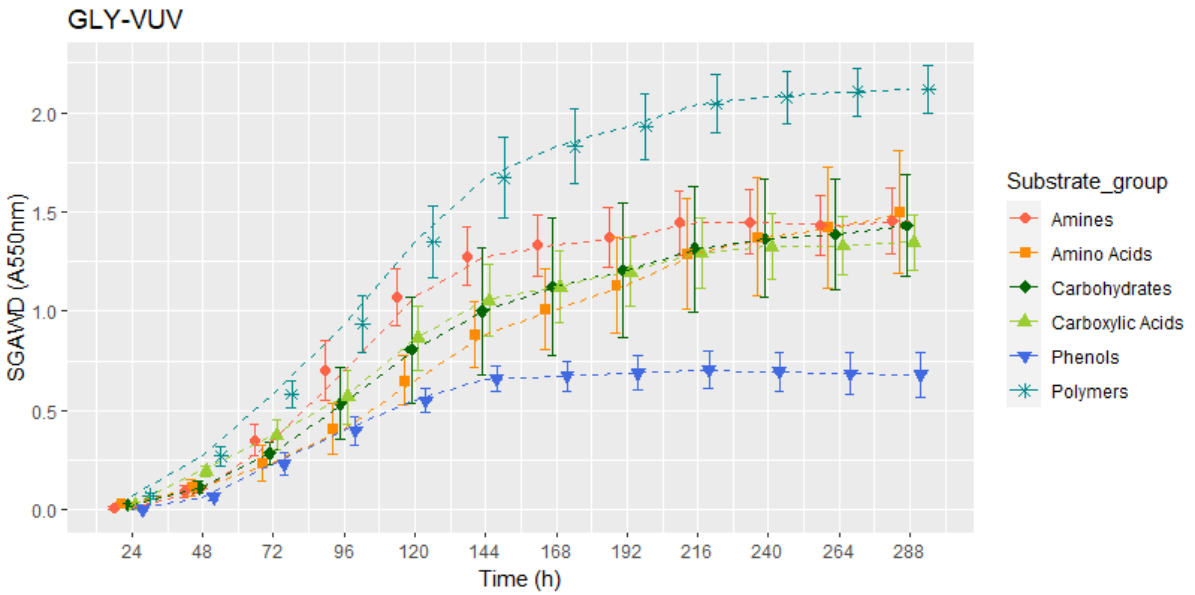

Figure A.10. The average absorbance of each substrate group and standard deviation (n=9) during the incubation period (1-12) days, from the microcosms contaminated with VUV-treated GLY.

A.2 Scaled PCA

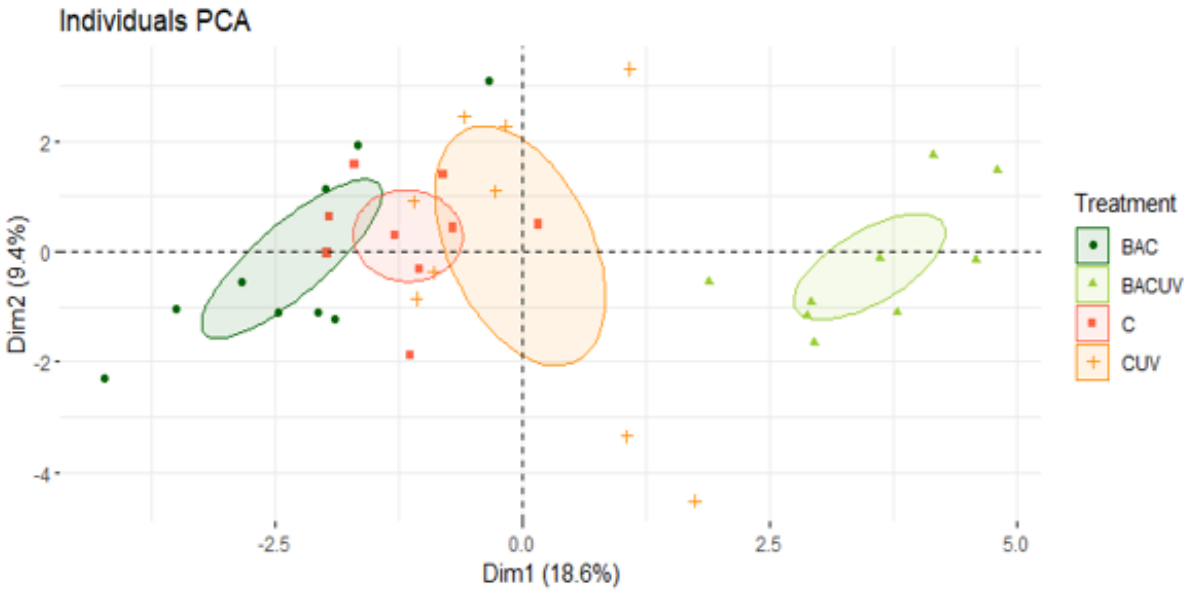

Figure A.11. PCA analysis of the samples collected from the following microcosms: The control microcosm, the control microcosm contaminated with VUV-treated tap water, the microcosm contaminated with BAC and the microcosm contaminated with VUV-treated BAC. For each variable, the data points are scaled and centered.

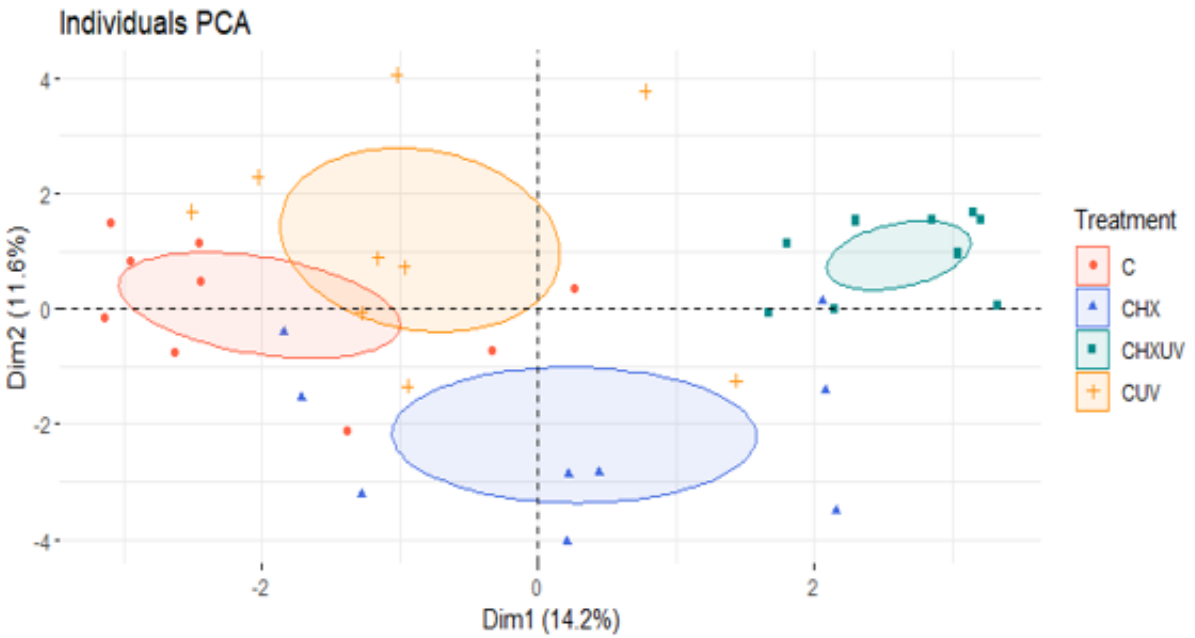

Figure A.12. PCA analysis of the samples collected from the following microcosms: The control microcosm, the control microcosm contaminated with VUV-treated tap water, the microcosm contaminated with CHX and the microcosm contaminated with VUV-treated CHX. For each variable, the data points are scaled and centered.

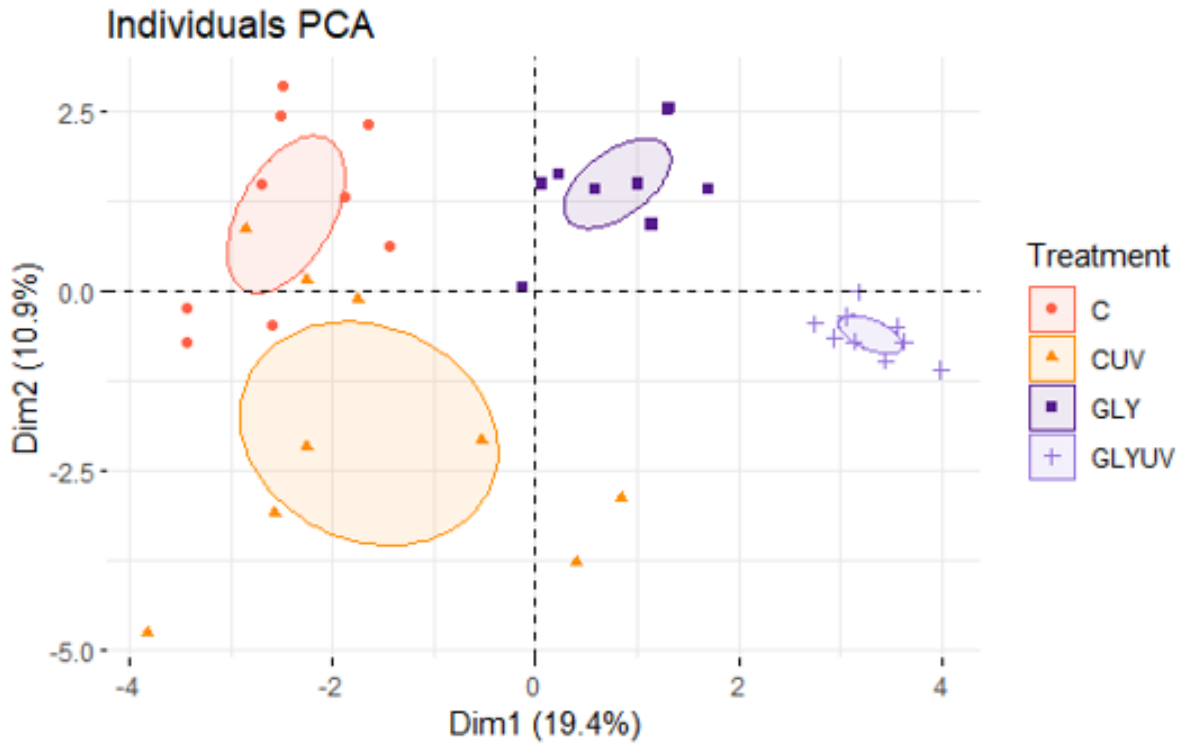

Figure A.13. PCA analysis of the samples collected from the following microcosms: The control microcosm, the control microcosm contaminated with VUV-treated tap water, the microcosm contaminated with GLY and the microcosm contaminated with VUV-treated GLY. For each variable, the data points are scaled and centered.

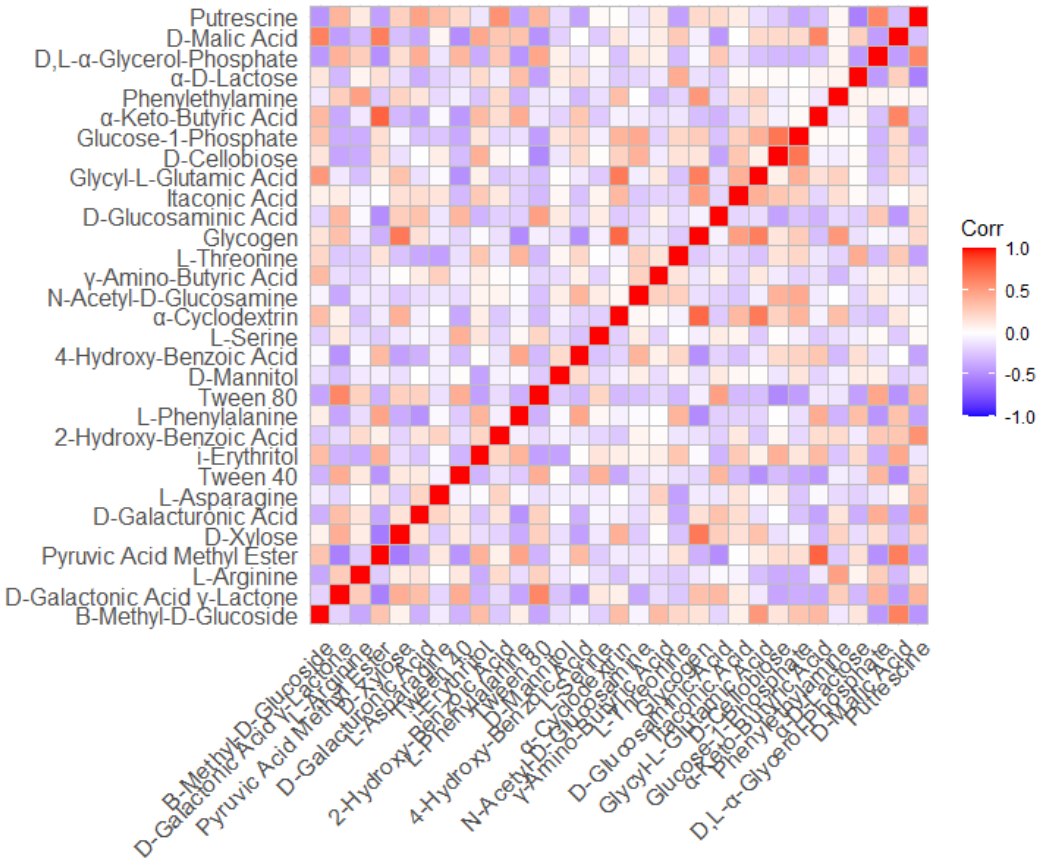

Figure A.14. The correlation matrix between each of the substrates that are a variable in the non-sacled PCA for the samples collected from the control microcosm, the control microcosm contaminated with VUV-treated tap water, the microcosm contaminated with DDAC and the microcosm contaminated with VUV-treated DDAC.