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Capacity of a flow-through biofilter system to secure K-selection and microbial stability in the out-flowing water

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Biotechnology (5 year)
Submission date: August 2018
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Abstract

Normally water treatment and design of aquaculture systems focuses on optimizing physiochemical water quality and tries to have low bacteria numbers. High bacteria numbers, within reasonable limits, are not always a problem as long as the system is bio-stable, and the bacterial community is suitable for the living organisms in the water. There is a need to develop methods and technology targeting a beneficial microbial community composition and bio-stability. Previous work serving as a basis for the present project indicates that communities established under K-selection is suited to achieve this goal.

The underlying hypothesis for the present project was that it is possible to get more competition and K-selection in the water of bioreactors by introducing carriers for biofilm growth. The biofilm can have a low maximum growth speed without being removed from the reactor. The bacteria in the biofilm will consume the substrate resulting in reduced carrying capacity in the water. This results in K-selection.

In this project the impact of the presence of biofilm-carriers in relation to the microbial water quality was investigated. The experiment was performed with a starting point of 250 ml seawater and 50ml medium in lab-scale continuous reactors. Three levels of biofilm carriers was applied by supplying the reactors with either zero (Treatment Z, zero), 343 cm^2 (Treatment L, low) or 666.4 cm^2 (Treatment H, high) carrier surface per reactor. Three replicate reactors were operated for each treatment, for comparisons and validating of results. The effect on cell number, amount of K-selection and microbial community composition were examined by the use of, among other things, flow cytometry and Illumina 16S rRNA amplicon sequencing.

The cell numbers of the planktonic communities in the L reactors were higher than in the H reactors and Z reactors. The latter was an unexpected result. There was no tendency of the number of cells in the biofilm on the carriers to increase over time, suggesting maturity with respect to cell numbers after only one week.

A higher number of K-strategists were found in the H-reactors than in the other treatments during the whole experiment. The biofilm communities could have higher competition due to the surface area and got less substrate per cell. This led to K-selection. In the L and H reactors there were a mix of planktonic communities with moderate competition and biofilm

communities which were strongly limited. Due to the high number of bacteria in the biofilm in the H reactor, which used more of the substrate, resulted in the planktonic community to also be more K-selected in this reactor.

The planktonic bacterial communities were not influenced by the co-existing biofilm bacterial communities. There was little difference in the bacterial communities between the Z, L and H reactors. In the later weeks of the experiment, the planktonic bacterial communities in all reactors kept changing and there was no obvious stabilizing trend.

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1. Introduction

1.1 Microbial population and growth strategies

Normally water treatment and design of aquaculture systems focuses on optimizing physiochemical water quality and tries to have low bacteria numbers. High bacteria numbers, within reasonable limits, are not always a problem as long as the system is bio-stable, and the bacterial community is suitable for the living organisms in the water.

A microbial population is the sum of all cells of microorganisms of the same species that inhabit an ecosystem at a given time. They try to survive and reproduce, and to achieve this they use different strategies of growth. This can be used to differentiate between the populations. Under suitable resources and growth conditions the microorganisms will grow to form populations. Similar microbial populations which use the same resources in a similar way is called guilds. A living area, habitat, shared by a guild and which have the necessary conditions and resources for growth in a cell is called a niche (Madigan, 2015).

The diversity in a community can be expressed by the richness (number of different species) and abundance (proportion) and can change quickly in a short time period.

1.2 Nutrient levels and growth rates

In an ecosystem resources typically enter sporadic with a pulse of nutrients which can be followed by nutrient deprivation. Due to this microorganisms in nature have variable growth conditions. Long periods of exponential growth are rare in nature and the growth rates are therefore normally lower than the maximum growth rates seen in laboratories. Estimates based on research shows that soil bacteria grow in nature at less than 1% of the maximum growth rate. The slower growth rates in nature compared to laboratory culture shows the differences in resources and growth conditions (Madigan, 2015).

Competition for resources between microorganisms in a habitat can be strong and the end result is depending on several factors. These include growth rates, nutrient uptake and metabolic rates (Madigan, 2015). A habitat contains different optimized microorganisms and their relation to each other is based on how each niche resembles its realized niche. The theory of r- and K- selection can contribute to an explanation of this relationship.

1.3 Theory on r- and K-selection

The theory on r- and K- selection is based on how organisms adapt within two different selection regimes and the selection is based on adaption to one out of two strategies and no option for a middle ground. Opportunistic and non-opportunistic (competition strategic) bacteria species is another description for the r and K strategists, respectively. The basis of the theory is the logistic equation of growth where the terms r and K is taken from: dN/dt = rN ((K-N/K), where r is the rate a population increase, N is the population size and K is the carrying capacity, i.e. the maximum biomass that the environment can sustain (McArthur, 2006)

Table 1.1. Characteristics of opportunists (r-strategists) and non-opportunists (K-strategists) species and pioneer and matured microbial communities (Adapted from Vadstein et al., 1993)

Species level	**r-strategist**	**K-strategist**
Maximum growth rate	High	Low
Effect of enrichment	Rapid growth	Slow growth
Affinity to substrate	Low	High
Competitive ability, low nutrient per capita	Poor	Good
Community level	**Pioneer community**	**Mature community**
Biological control	Low	High
Stability to perturbation	Poor	Good
Diversity	Low	High
Specialization	Low	High

The opportunistic bacteria, r-strategists, grows at high rates in uncrowded environments with high amounts of substrate. Selection for non-opportunistic bacteria, K-strategists, occur when the environment is crowded and there is a low supply of substrate (Table 1.1 and Vadstein et al., 1993). This is due to the fact that the maximum growth rate of the r-strategists is high, but their competitive ability is low at low substrate supply. The last is where the K-strategists thrive. R-strategists are often the first colonizers in an environment and will dominate due to

low competition for resources. When the environment stabilizes, and competition becomes stronger, the K-strategists take over the community due to competitive superiority.

A community established under r-selection is referred to as a pioneer community, whereas a community established under K-selection is referred to as a mature community. A pioneer community is normally an unstable system with low diversity and low biological control. A mature community on the other hand is a stable system with high diversity and high biological control (Table 1.1 and Vadstein et al., 1993).

In aquaculture facilities selection for K-strategists creates a stable and mature environment, but the selection regime may vary. Disinfection is a standard water treatment step in aquaculture as a biosecurity barrier toward the external environment and due to the perception that the rearing water needs to have a low microbial level. The microbial community is therefore perturbed. The environment after disinfection has a low biomass level in comparison to the carrying capacity due to the disinfection resulting in dead biomass in addition to feces and uneaten feed. This community selects for microorganisms able to exploit high supply of nutrients. Opportunistic r-strategists therefore thrive here. Selection for K-strategists by creating a stable environment with high competition would lead to less pathogens that attack individual creatures in the environment (deSchryver and Vadstein, 2014).

A microbially matured flow through system (MMS) and a recirculating aquaculture system (RAS) can be used to select for K-strategists. A K-selective water treatment gives a more stable and mature composition of the microbial community of the water going into the tanks, resulting in a higher survival rate. A mature, stable microbial community is established in MMS by the use of reservoirs with biofilter media with a minimum of 12h HRT after the water passes the UV-filter. In this way competition for resources is secured by a high biomass of bacteria as biofilm on the biofilter. In a normal flow through system (FTS) the water goes to a reservoir without a biofilter and has therefore fewer bacteria to compete for available resources (Attramadal et al., 2014). The capacity in MMS and RAS to create a K-selected community is not known and leads to the problem of how to efficiently select for K-strategists.

1.4 Biofilm and surfaces

An important microbial habitat is surfaces. These can offer better access to nutrients and give defense against predators and physicochemical disturbances. It also functions as a habitat and prevents microbes to be washed away. Flow over a colonized surface increases the nutrient transport to the surface and results in more resources provided than to planktonic cells. The biofilms are often K-selected with a mature and stable community composition. This results in slower growth than in the planktonic environment (Madigan, 2015).

During bacterial growth on surfaces they form biofilms. Biofilms is bacterial communities attached to a surface enclosed in an adhesive matrix. The matrix is the product of cell death and excretion products by cells. The biofilm traps the nutrients and prevents the detachments of cells, which can happen in flowing systems, but the diffusion of nutrients and oxygen into the biofilm is not easy. Biofilms is different from planktonic environments by supporting critical transport and transfer processes. “Species growing in biofilm can be up to 1000 times more tolerant to an antimicrobial substance than planktonic cells of the same species.” (Madigan, 2015).

In a biofilter system planktonic and surface-attached communities experience different selection regimes. The difference in selection is mainly due to the fact that the loss rate is equal to the dilution rate for the planktonic community, and much slower for the attached community. It is important to realize that the two communities interact by attachment and detachment. Thus, the effect of water treatment on the composition and the dynamic of the microbial community composition is an important part of selection.

1.5 Aim of the study

The overall goal of this master thesis was to identify factors that are crucial for creating a K-selected, stable microbial community in flow-through bioreactors, and thereby increase the understanding of how to obtain K-selection with the use of biofilters. This master thesis was part of the EU ERANET project MicStaTech.

In the MicStaTech project, the aim was to evaluate the capacity of a flow-through biofilter system to secure K-selection and microbial stability in the out-flowing water.

Key experimental variables are dilution rate of the system and biofilter surface area per volume unit of bioreactor. The underlying hypothesis is that at one level the supply of substrate (proportional to flow rate) is so high that the system will swap to r-selection, and that this point is dependent on the surface area of the reactor. This last point is related to the fact that biofilter systems have both a planktonic and a surface-attached community that experience different selection regimes, including both competition and loss.

Some detailed questions that will be addressed are:

- How does planktonic and biofilm bacterial communities develop over time in continuously operated bioreactors started with natural seawater bacterial communities and clean biofilm carriers?
- How will the planktonic bacterial communities be influenced by the co-existing biofilm bacterial communities?
- How long does it take the bacterial communities of the reactors to stabilize?
- How does the three different area per reactor volume affect the answers to the questions above?

2. Materials and methods

2.1 Experimental design and setup

The experiment was performed in seawater in lab-scale continuous reactors. A hydraulic retention time of 0.5 d was used throughout the experiment. Each of the 9 reactors were filled with 300 mL medium in addition to biofilm carriers, as described below. Three levels of biofilm growth area were applied by supplying the reactors with either zero (Treatment Z, zero), 70 (Treatment L, low) or 136 (Treatment H, high) biofilm carriers per reactor. Three replicate reactors were operated for each treatment, for comparisons and validating of results. The area per carrier was 4.9 cm^2.

Two custom-made plexiglass PMMA (poly methyl methacrylate) water baths were placed on top of two MIXdrive6 magnetic stirrers and used for keeping a stable temperature of 18 °C in the reactors. Two A MX07-20V12V cooling generators were connected to the water baths by the use of silicone tubes, inlet and outlet in each bath. The generators were pumping water through the baths by having one slot receiving water from the baths and one pumping the water back in the baths.

One of the water baths contained 6 bioreactors (Treatment L and H) and the other contained 3 (Treatment Z). In order to make the 0.5 L bioreactors airtight, silicon corks were put on the outlet and inlet of the lids, and the caps on the lids were in addition sealed with a multi-purpose sealant (silicone). Air and medium were supplied to the reactors through the same inlet tube, and culture and air was discharged through the outlet. When medium and air was pumped into the reactors, the pressure would pump the water out through the outlet, thereby maintaining a constant water volume in the reactors. After assembly and autoclavation the reactors were placed in the water baths described above.

A tube network from the medium reservoir to the reactors was connected via a peristaltic pump by the use of Tubing Tygon SI 3350-2stop ID 1.52 mm silicon tubes. The pump was calibrated according to the flow rate in the experiment, 0.937 mL/min resulting in a HRT of 0.5 Day^{-1}. The tubes were replaced if they deviated with more than 10% from the target flow rate.

The reactors were filled with 250 mL filtrated seawater and 50 mL medium (Table 2.1) in addition to carriers corresponding to treatment Z, L and H. The reactors were maintained stagnant for 2 days. Three replicate reactors were used for each treatment resulting in a total of 9 reactors. The duration of the experiment was 12 weeks, and samples were taken once a week.

Table 2.1. Components for the medium (Seawater M65) preparation

Component	**Concentration (g/L)**	**Addition (µl/L)**	**Final concentration (mg/L)**
M65 stock	150 g/L	33.3 ul/L	5 mg/L
f/2 medium			
NaNO3	75 g/L	20 ul/L*	1,5 mg/L
NaH2PO4 *H20	5 g/L	20 ul/L*	0,1 mg/L
Na2SiO3 *9 H20	30 g/L	20 ul/L*	0,6 mg/L
Trace metal solution		20 ul/L*	-
Vitamin solution		10 ul/L*	-
0.2 um filtrated seawater/Spring water		* 1:50 from the original f/2 medium	

2.1.1 Medium preparation

The medium used in the experiment was the f/2 medium with the addition of M65 stock to provide organic matter for the heterotrophic bacteria (Table 2.1). The water used in the medium was collected from 90 meters depth in the Trondheimsfjord and delivered to NTNU Gløshaugen.

For medium preparations, the nutrients were added to 18 L of seawater. Before addition of nutrients, the seawater was filtrated through a 3.0 µm followed by a 0.2 µm filter by using a vacuum pump. The 0.2 µm filter was washed with 100 mL MQ water to avoid contamination.

Nutrients were added to 18 L filtrated seawater (Table 2.1) in a sterile bench by using a burner to avoid contamination of the nutrients and the medium.

The medium was autoclaved for 40 min at 121 °C, the lid was half open. A probe was put into a 20 L Nalgene PP carboy with the same amount of water as the medium for temperature measurements during autoclaving.

2.2 Sampling

The following samples were taken weekly for 12 weeks.

2.2.1. Sampling for flow-cytometry based analyses

Samples were taken for flow-cytometry based methods from the reactors and medium. 495 µl water sample were mixed with 5 µl glutaraldehyde (50%) followed by snap freezing in liquid nitrogen after 15 minutes fixation in room temperature. To check for contamination in the system introduced by the medium, samples were taken from each medium tank, to assure that it was not contaminated. All the samples were kept in a freezer at – 20 °C.

2.2.2. Sampling for DNA-based analysis

Biofilm carriers: These samples were taken for later analysis of bacterial community composition, and quantitative DNA extraction from carrier was also used to estimate the number of bacteria per carrier. Two carriers were taken from each reactor at each sampling time and put in the freezer after snap freezing in liquid nitrogen

Water: For later illumina amplicon sequencing of bacterial community composition, planktonic bacteria in a 50 mL water sample from the reactor was collected by filtering the water through a filter by using a pipette.

2.3 Analytical methods

2.3.1 Quantification of r-strategists

The fraction of r-strategists in the community were estimated by nutrient pulsing and quantification of the frequency distribution of the RNA content of cells. To achieve the maximum specific growth rate (μ_{max}) of water bacteria, nutrients were added to water samples (Table 2.2). The samples were incubated at 20°C for 3 hours for the cells to achieve the maximum RNA-content. The samples that had not been pulsed by flow cytometry analysis were used as a control for estimation of cell counts. The nutrient-pulsed samples were used to determine r-strategists by the use of high max RNA content (Brandsegg, H. Protocol).

Table 2.2. Composition of nutrients added to samples to induce maximum specific growth rate (μ_{max}) (Brandsegg, H. Protocol.)

Nutrient solution	**Concentration stock**	**Amount added**	**Final concentration**
M65 stock medium (Appendix A)	150 g/L	166.7 µl/L	25 µg/ml of the three components in total
f/2 Trace metal solution	Appendix A	100 µl/L	Appendix A
NaH2PO4 • H2O	5.0 g/L	100 µl/L	0.5 µg/ml
NH4Cl	0.9 M	100 µl/L	90 µM

Flow cytometry analysis of RNA content of single cells is described below, the method used is further described in Brandsegg, H. Protocol.

2.3.2. Flow cytometer analysis

The samples were thawed at room temperature and mixed well. Mixing was done to achieve a homogeneous sample. For cell counting a dilution of 1:12 was used for the normal samples and 1:10 for samples pulsed with nutrients to achieve cell counts less than 1000 events/µl. The samples were diluted with 0.2 µm filtrated water 1/10 TE-buffer.

Staining of the RNA was done with SYBR Green II RNA Gel Stain, 10.000X in DMSO (Life Technologies, Thermo Fisher Scientific Inc., Waltham). On the same day as analysis, a working solution of 1:50 was prepared from the stock solution. It was diluted with 1/10 filtrated TE-buffer. 10 µl of this working solution was added to 1 ml diluted sample, mixed and incubated in the dark for 15 minutes. During analysis and incubation, the samples were covered in aluminum foil and they were all analyzed within the stability period of 27 minutes after staining (Brandsegg, H. Protocol).

The samples were analysed on a BD Accuri C6 Flow Cytometer (BD Bioscience, San Jose) with medium flow (34.5 µl/min) and 3 minutes of counting. A medium flow was generally recommended due to improved accuracy for small cells, instead of higher and lower flow rates (Brandsegg, H. Protocol

Data was processed with BD Accuri C6 Software (Bd Biosciences, San Jose). To define the cells of interest of unpulsed and pulsed sample, the cell density and the frequency distribution of the maximum RNA per cell were used to determine the ratio of r-strategists. RNA-content was analysed for single cells and clotted cells were excluded from the analysis by the use of thresholding based on the SYBR Green II/RNA complex. In forward scatter (FSC) a threshold of 10^5 was set and all the values over this were excluded from the analysis. Lower limit and cut off in the FL1-A (RNA content) was set to 10^4. Everything below this value was considered noise.

2.3.3 Isolation of DNA

A QIAamp DNA mini kit was used for DNA extraction from biofilm carriers and water samples. The concentration of the DNA-extracts was measured by the use of qubit.

The results from the qubit analysis was calculated into DNA/mL to make the results readable. This was done by multiplying the qubit result with the dilution factor and the amount of eluate used followed by a division of the amount of sample filtrated. The results were changed by a factor of 1000 from ng/µl into ng/ml.

2.3.4 Illumina sequencing of 16S rDNA amplicons

To study microbial diversity in the samples bacterial 16S rDNA amplicons were sequenced by illumina.

2.3.4.1. 1.st stage PCR: Amplification of the v3-v4 region of bacterial 16S rDNA amplicons

The v3-v4 region of the bacterial 16S rRNA gene was amplified using Phusion Hot Start Polymerase (molecular Biology, Thermo Fisher) and the primers (used in the PCR was) ill338F and ill805R and the reaction mixture contained the components given in Table 2.3. The PCR was carried out with the cycling conditions given in Table 2.4.

Table 2.3. Components in PCR (total reaction volume of 20 µL).

Component	**Supplier**	**Amount x1**
dH20	-	11.95 µl
5x Phusion buffer HF (7,5 mM MgCl2)	Phusion Kit Illumina*	4.0 µl
ill338F (10um)	Eurofins	0.6 µl
Ill805R (10um)	Eurofins	0.6 µl
BSAx100	Biolabs	0.8 µl
dNTP (10mM each)	VWR	0.5 µl
MgCl2 (50mM)	Phusion Kit Illumina*	0.4 µl
Phusion Hot Start DNA polymerase	Phusion Kit Illumina*	0.15 µl
Template		1.0 µl

Table 2.4. PCR program setup.

Temperature (°C)	**Time**	**Cycles (number)**
98ºC	1 min	
98ºC	15 sec	|
50ºC	20 sec	| 35
72ºC	20 sec	|
72ºC	5 min	
4ºC	1 min	
10ºC	∞	

To check the quantity and quality of the PCR products, they were analyzed by agarose gel electrophoresis. The gel was made with 4 g agarose in 400 mL 1X Tris-acetate (TAE) buffer (Appendix B) followed by boiling in the microwave. When the gel was casted, 50 mL was mixed with 2.5 µL gel red. In each well, 5 µL PCR product mixed with 1 µL loading buffer,

was applied. As a size reference, a Gene Ruler 1kb Plus DNA ladder was used in one of the wells.

The gel was run in 1x TAE at 140 volts for 45minutes, and visualized under UV light and photographed in a G:box (Syngene, Cambridge) by using the program GeneSnap (Syngene, Cambridge). If the gel showed lack of PCR products, the PCR was repeated with the relevant samples with increased cycle number (38 cycles).

Normalization and purification of the PCR products was done with a sequal prep normalization plate kit (Invitrogen). The kit gave an output of 1-2 ng for each amplicon.

2.3.4.2. Indexing- 2. stage PCR

Each amplicon was marked with a unique set of 10 nt index-sequences in a second round of PCR. The indexing kit (Illumina*:Nextera XT Index Kit, Ref: 15055294) contained 8x12 unique indexing sequences which can give an unique marking of 96 samples. 17.5 µl mastermix was added to each well followed by index 1, index 2 and template. In total a reaction volume of 25 µl (Table 2.5). The PCR was carried out with the cycling conditions given in Table 2.6.

Table 2.5. Mastermix for indexing PCR with a total reaction volume of 20 µL.

Component	**Supplier**	**Amount x1**
dH20		11.687 µl
5x Phusion buffer HF (7,5 mM MgCl2)	Phusion Kit Illumina*	5.0 µl
dNTP(10mM each)	VWR	0.625 µl
Phusion Hot Start DNA polymerase	Phusion Kit Illumina*	0.188 µl
Index 1 (orange cork, N-serie)	Illumina*	2.5 µl
Index 2 (white cork, S-serie)	Illumina*	2.5 µl
Template (normalized from 1. stage)		2.5 µl
$MgCl_2$		0.25 µl

Table 2.6. PCR program setup.

Temperature (°C)	Time	Cycles (number)
98ºC	1 min	
98ºC	15 sec	|
50ºC	20 sec	| 8
72ºC	20 sec	|
72ºC	5 min	
4ºC	1 min	
10ºC	∞	

An agarose gel electrophoresis was run after the PCR to check the amount and sizes of the PCR-products. Some samples had to be run again with 10 cycles due to weak bonds.

Normalization of the PCR products was done with a Sequal Prep Normalization plate kit, Invitrogen. The kit contained cleaning and normalization and gave an output of 1-2 ng.

2.3.4.3. Preparation of the amplicon library and Illumina sequencing

After the normalization all the samples was pooled into one tube and the concentration was measured on NanoDrop. A gel was run of the pooled amplicon sample to check the quality/quantity of the library.

The amplicon library was sequenced at the Norwegian Sequencing Centre at UiO.

2.3.5 Processing of sequencing data

The datafiles which was sent to NTNU, the Illumina sequencing data, were processed with the USEARCH pipeline (version 9.2; https://www.drive5.com/usearch/). At the step of merging paired reads, primer sequences were trimmed, and reads shorter than 400 base pairs were filtered out. The processing further included demultiplexing, quality trimming by the Fastq filter command (with an expected error threshold of 1). Chimera removal and clustering at the 97% similarity level was performed using the UPARSE-OTU algorithm (Edgar, 2013). Taxonomy assignment was based on the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 15).

Statistical analysis was performed using the program package PAST v. 3 (Hammer et al., 2001) followed by the use of excel and past3 to analyse the data.

2.4 Calculations and statistical analysis

2.4.1 Diversity analyses

The concept of diversity can be divided into two. The first is richness, the number of different species within a community. The concept is easy to define but can be hard to quantify with certainty due to the difficulty of sampling exhaustively. Evenness is the second factor, how evenly species are distributed within a sample. An even community are a community with species present in similar amounts. A single value is often given for the concepts of richness and evenness: diversity index. Diversity indices are normally used to describe alpha-diversity and beta-diversity. Alpha diversity is the diversity of a defined unit, sample or habitat and can be described with species or OTU-richness and Shannon's index. Beta diversity is the degree of similarity between to communities and can be described by Bray-Curtis similarity which is an extension of the Sørensen index (Pepper et al., 2015).

2.4.1.1 The Shannon index

The Shannon index (H´) is based on that the number of species in a community and their abundances affects the complexity of a community. Both the richness and evenness are a factor when calculating the community diversity and increases with increasing richness and evenness (Equation 2.4.1). Lower Shannon values reflect communities with fewer species and/or an uneven distribution among the species (Pepper et al., 2015).

$$H' = -\sum_{i=1}^{K}(p_i \ln p_i) \tag{2.4.1}$$

Where p_i = proportion of the i^{th} species in the community and K= richness. The given value for the proportion of the individual species in the community multiplied by the log is calculated for every species and summed. This gives the Shannon index score (Pepper et al., 2015).

2.4.1.2 Chao 1

Chao 1 looks at the singletons and doubletons and estimates an amount of species that would theoretically be captured if the whole community was captured exhaustively (Pepper et al., 2015).

2.4.1.3 Bray-Curtis similarity

Bray-Curtis similarity gives the degree of similarity between two communities and is used for quantitative data (abundances). The values are given from 0 to 1. A value of 1 indicates that

the communities share all their species and have the same abundance (=100% similarity). A value of 0 shows that they share no species (Pepper et al., 2015).

3. Results

To increase the understanding of how to obtain K-selection, the influence of biofilm carriers on r-/K-selection was tested.

The experiment was performed in seawater in lab-scale continuous reactors. A hydraulic retention time of 0.5 d was used throughout the experiment. Each of the 9 reactors were filled with 300 mL medium in addition to biofilm carriers. Three levels of biofilm growth area were applied by supplying the reactors with either zero (Treatment Z, zero), 70 (Treatment L, low) or 136 (Treatment H, high) biofilm carriers per reactor. Three replicate reactors were operated for each treatment, for comparisons and validating of results.

3.1. Estimation of bacterial density in water and biofilm carrier samples

Planktonic cell density were determined by the use of flow cytometry and biofilm determined with DNA per carrier. The DNA content in each cell was determined for the planktonic samples. The QIAamp DNA Mini Kit was used for quantitative DNA extraction and the Qubit was used for the quantitation of DNA in the samples by using fluorescent analysis.

3.1.1. Determination of DNA concentrations

The DNA concentrations of water samples varied throughout the experiment. The H reactors had lower average values than the Z and L reactors. The average values in week 2 for all the reactors are lower than the other weeks (Table 3.1).

Table 3.1. DNA concentrations of water samples by the use of QIAamp DNA Mini Kit and Qubit in ng DNA/mL. Calculated from a DNA dilution of 1:10 in addition to 450 µl eluate. 25 mL sample was taken from each reactor for each sample time.

	Reactor								
Week	Z1	Z2	Z3	L1	L2	L3	H1	H2	H3
1	7.362	3.402	2.772	5.598	12.492	586.8	58.32	44.82	19.44
2	80.28	41.04	89.28	20.52	49.32	22.86	14.4	24.48	49.14
3	43.38	17.064	34.2	8.604	11.988	20.34	19.08	10.386	6.048
4	46.26	11.898	14.022	20.34	8.208	61.02	26.64	22.32	8.01
5	68.04	128.52	144	39.6	36	32.94	14.04	73.44	50.04
6	33.48	39.6	68.04	24.66	51.12	15.012	6.12	6.552	61.56
7	116.28	71.28	56.88	126	87.84	57.96	50.4	31.86	79.56
8	150.84	105.84	149.76	165.6	99.72	46.44	30.78	141.84	40.68
9	221.4	140.04	207	75.96	56.16	47.16	196.2	54.72	66.24
10	80.46	18.36	28.08	12.834	8.514	3.06	16.506	25.92	19.98
11	179.82	23.04	25.56	31.86	7.992	18.36	8.928	51.12	19.44
12	32.22	61.2	32.04	86.4	74.88	43.2	15.948	39.6	29.52

To estimate the cell count on the biofilm carriers the DNA concentration was calculated for extracts based on 1 carrier. The same method was used for the results from the water samples, with some modifications. 1 carrier was used per extraction and replaced the amount of filtrated water used in the water samples in the calculation. The results are presented in Table 3.2.

Table 3.2. DNA concentrations of carrier samples by the use of QIAamp DNA Mini Kit and Qubit in ng DNA/mL. Calculated from a DNA dilution of 1:10 in addition to 450 µl eluate.

	Reactor							
Week	L1	L2	L3	H1	H2	H3	Average L	Average H
1	43.2	68.0	115.2	97.2	100.8	64.8	75.5	87.6
2			52.7	61.7	161.1		52.7	111.4
3	44.1	41.0	59.4	58.5	38.3	89.6	48.2	62.1
4	75.6	71.1	112.1	88.7	251.1		86.3	169.9
5	145.8	32.9	72.9	54.5	91.8	38.7	83.9	61.7
6	132.3	32.9	56.3	123.8	90.9	1215.0	73.8	476.6
7	215.1	82.8	266.9	123.8	108.9	33.8	188.3	88.8
8	52.2	165.2	239.4	29.7	49.5	193.1	152.3	90.8
9	35.6	123.8	37.8	359.1	134.6	111.6	65.7	201.8
10		86.0	117.9	236.3	44.6	104.9	101.9	128.6
11	68.4	93.2	540.0	317.3	81.5	189.0	233.9	195.9
12	47.3	68.0	119.7	57.2	27.9		78.3	42.5

3.1.2. Estimation of amounts of DNA per cell

To estimate the DNA per cell flow cytometry data was analysed by calculating fg (10^{-15}) DNA/cell based on cells/mL from each reactor in addition to ng DNA/mL from the qubit results.

3.1.2.1. Cellular DNA content in water samples

The average cellular DNA content for the reactors water samples (Table 3.3) showed that of the samples from the bacterial cells in the Z reactors had higher DNA content than those of the other reactors.

Table 3.3. Cellular DNA content for water samples given as fg DNA/cell. Aberrant results are removed from the table.

Week	Z1	Z2	Z3	L1	L2	L3	H1	H2	H3
1	21.0	10.6	21.5	3.6			10.8		8.1
2	13.7	5.7	13.4	4.5	10.0	3.8	2.2	4.9	10.3
3	8.6	5.9	13.5	3.6	1.9	7.4	6.7	4.3	2.5
4	5.0	2.0	2.2	5.6	1.0	6.6	6.3	3.6	0.7
5	14.1	31.6	35.1	7.0	3.7	4.6	3.3		4.5
6	22.8	5.5	7.1	1.9	3.0	0.8	2.6	1.7	3.7
7	31.5	22.5	7.8		11.3	9.1		12.6	9.2
8	61.5	42.1	17.5			8.7	13.9		16.4
9	25.8	21.3	13.7	5.9	3.9	3.3		10.9	11.9
10	16.3	2.8	5.0	1.9	0.7	0.4	3.6	10.5	6.1
11	30.2	2.9	3.6	4.8	0.7	2.0	3.8		6.6
12	6.3	20.5	7.1	13.3	9.8	6.7	6.8		7.9
Avg	21.4	14.4	12.3	5.2	4.6	4.8	6.0	6.9	7.3
SD	15.4	13.1	9.2	3.3	4.2	3.1	3.8	4.3	4.3

3.1.2.2. Quantification of bacterial cells on biofilm carriers

By using the surface of each carrier samples and the quantitative DNA data. The number of bacterial cells per cm^2 was calculated for the biofilm samples. This was needed to further calculate the percentage of planktonic or biofilm bacterial cells in each reactor.

In the biofilm samples from reactors with low number of carriers. The values were more stable over time than in the reactor with high number of carriers (Figure 3.1). The number of cells per carrier did not increase at a steady rate and had variations from $87*10^6$ to $27*10^6$.

The L reactors had lower average cells per cm^2 than in the H reactors with both having a growing trend from the beginning, but with cell numbers in week 12 similar to those in the first week.

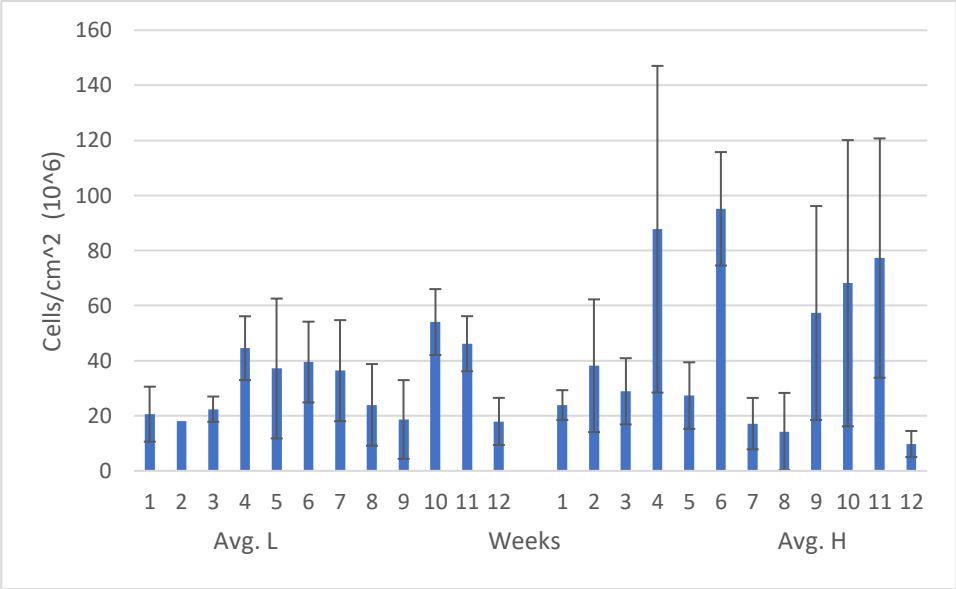

Figure 3.1. Bacterial cell density for biofilm carriers from reactors with high (H) and low (L) amounts of carriers. There were 3 replicates for each treatment and the average value is shown. Error bars represent standard error of the mean.

3.1.3. Distribution of bacterial cells in water and on biofilm carriers in the reactors

The distribution of bacterial cells between water and biofilm carriers was based on the amount of water cells counted in cells/mL (Table 3.4) and the biofilm samples in cells/cm^2. Total cell number in water and biofilm in each reactor was determined by the use of the total water volume of 300 mL and the total biofilm carrier surface area in the reactors. The total cells/ml in the L reactors was higher than the cells/ml in the H reactors.

Table 3.4 Cells/ml from the unpulsed water samples in each reactor for all weeks. The results are presented as $*10^6$.

	Reactor								
Week	Z1	Z2	Z3	L1	L2	L3	H1	H2	H3
1	0.35	0.32	0.13	1.56	0.44	30.57	5.42	2.27	2.40
2	5.86	7.26	6.65	4.58	4.91	5.95	6.58	5.03	4.77
3	5.06	2.91	2.53	2.40	6.41	2.76	2.84	2.44	2.39
4	9.26	6.02	6.27	3.64	8.61	9.26	4.26	6.12	12.17
5	4.84	4.07	4.10	5.70	9.77	7.15	4.27	4.19	11.17
6	1.47	7.26	9.57	12.83	16.99	17.92	2.39	3.76	16.46
7	3.69	3.17	7.28	4.68	7.77	6.39	1.86	2.52	8.62
8	2.45	2.51	8.54	6.03	0.14	5.36	2.22	0.09	2.48
9	8.57	6.58	15.14	12.81	14.25	14.41	7.93	5.01	5.58
10	4.95	6.47	5.56	6.86	12.70	8.39	4.57	2.48	3.26
11	5.95	7.93	7.08	6.61	11.41	9.24	2.37	2.01	2.95
12	5.10	2.98	4.54	6.48	7.60	6.49	2.34	2.18	3.76

The total surface in the low number carrier treatment was 343 cm^2 for the L reactors and 666 cm^2 in the H reactors. The average amount of water sample cells in each treatment was $16.0*10^8$ +/- $2.9*10^8$ in the Z reactors, $25.0*10^8$ +/- $6.2*10^8$ in the L reactors and $13.4*10^8$ +/- $4.9*10^8$.

Table 3.5. The percentage of biofilm cells of the total cells (biofilm and water) in each reactor.

Week	Z1	Z2	Z3	L1	L2	L3	H1	H2	H3	Avg L & H	Avg L	Avg H
1	0	0	0	46.4	82.9	10.5	52.1	72.9	62.0	54.5	46.6	62.3
2	0	0	0			25.7	41.7	70.9		46.1	25.7	56.3
3	0	0	0	49.4	25.4	53.4	68.0	61.8	79.5	56.2	42.7	69.8
4	0	0	0	55.1	32.8	41.7	70.5	82.5		56.5	43.2	76.5
5	0	0	0	56.5	14.6	34.1	55.7	68.3	25.4	42.4	35.0	49.8
6	0	0	0		16.4	24.1	91.1	82.6		42.8	13.5	86.9
7	0	0	0	50.4	19.1	48.0	74.0	65.0	14.4	45.1	39.2	51.1
8	0	0	0	13.5	95.4	44.6	31.8	94.9	73.1	58.9	51.1	66.6
9	0	0	0	8.3	22.0	7.9	74.1	62.9	55.8	38.5	12.7	64.2
10	0	0	0		29.1	46.0	85.9	67.9	79.1	61.6	37.5	77.6
11	0	0	0	40.3	34.8			83.8	89.0	49.6	37.6	57.6
12	0	0	0	16.0	19.0	32.6	55.4	39.4		32.5	22.5	47.4
Avg												
Week 1-12	0	0	0	33.6	35.6	33.5	58.4	71.1	59.8	48.7	34.0	63.8
+/-SEM				6.5	7.9	4.3	7.0	3.9	8.9	2.5	3.5	3.4
Avg week 9-12	0	0	0	21.5	26.2	28.8	53.9	63.5	74.6	45.5	27.6	61.7
+/-SEM				7.9	3.1	9.1	16.5	7.9	8.0	5.6	5.3	5.5

In the H reactors the biofilm constituted a 30% larger part of the bacterial cells than in the L reactors. There are 0 percent of cells in biofilm in the Z reactors due to the fact that there are no carriers in this treatment. By looking at the average values for all the weeks in Table 3.5 the values are consistent at 33% of the total and 66% of the total cell numbers for L and H respectively. There are no tendency of the amount of cells on the carriers to increase over time.

3.2 r/K strategists in the pulsed sample from flow cytometry

A flow cytometry-based method by Hege Brandsegg was used to estimate the fraction of K strategists in planktonic communities in the reactors.

The vast majority of the bacteria in the reactors were found to be r-strategists (Figure 2). This indicated an uncrowded environment with enough substrate. The percentage of K-strategists

in the Z-reactors was under 10% during the whole experiment. The average fraction of K-strategists in L-treatments were under 5% (Figure 3.2). The H-reactors had higher fractions of K-strategists than the other treatments during the whole experiment. A larger number of cells in the biofilm gave a higher number of K-strategists in the planktonic community.

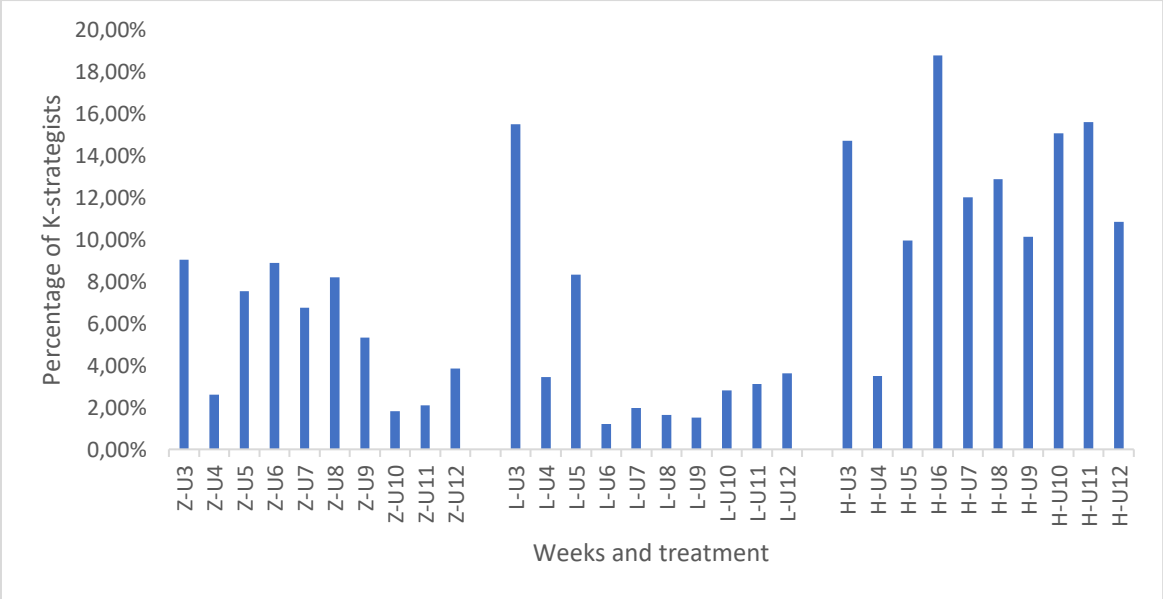

Figure 3.2. The percentage of K strategists in the planktonic bacterial communities for each reactor throughout the experimental period. Z. L and H is the treatments and the week number are given after the "U".

3.3 Microbial community composition and diversity

The microbial community associated with water and biofilm samples from the Z, L and H reactors was analysed by Illumina sequencing of 16S rDNA amplicons.

3.3.1 Alpha diversity of the microbial communities associated with water and biofilm samples

The average number of reads for each sample taken for the biofilm and planktonic communities were 47609 +/- 595 and 65478 +/- 419, respectively.

The observed OTU richness, Shannon diversity index (H) and the estimated Chao-1 richness were calculated (by using PAST).

There was a higher OTU richness in the planktonic communities in the L and H treatment than in the Z treatment without carriers (Figure 3.3). The richness was in addition higher in the L than H treatment for most weeks other than week 2, but neither treatment kept a stable richness. The water samples had a lower overall richness for the Z, L and H treatments than the biofilm samples.

Chao-1 was compared with the observed OTU richness to estimate the coverage of the sequencing analysis (Figure 3.3). The coverage was generally high, over 80%, for the water and biofilm samples (Table 3.6) and is a good representation of the theoretical numbers of OTUs in each sample. The sequencing coverage was at average 3.3% higher for the biofilm samples than in the planktonic samples (Table 3.6).

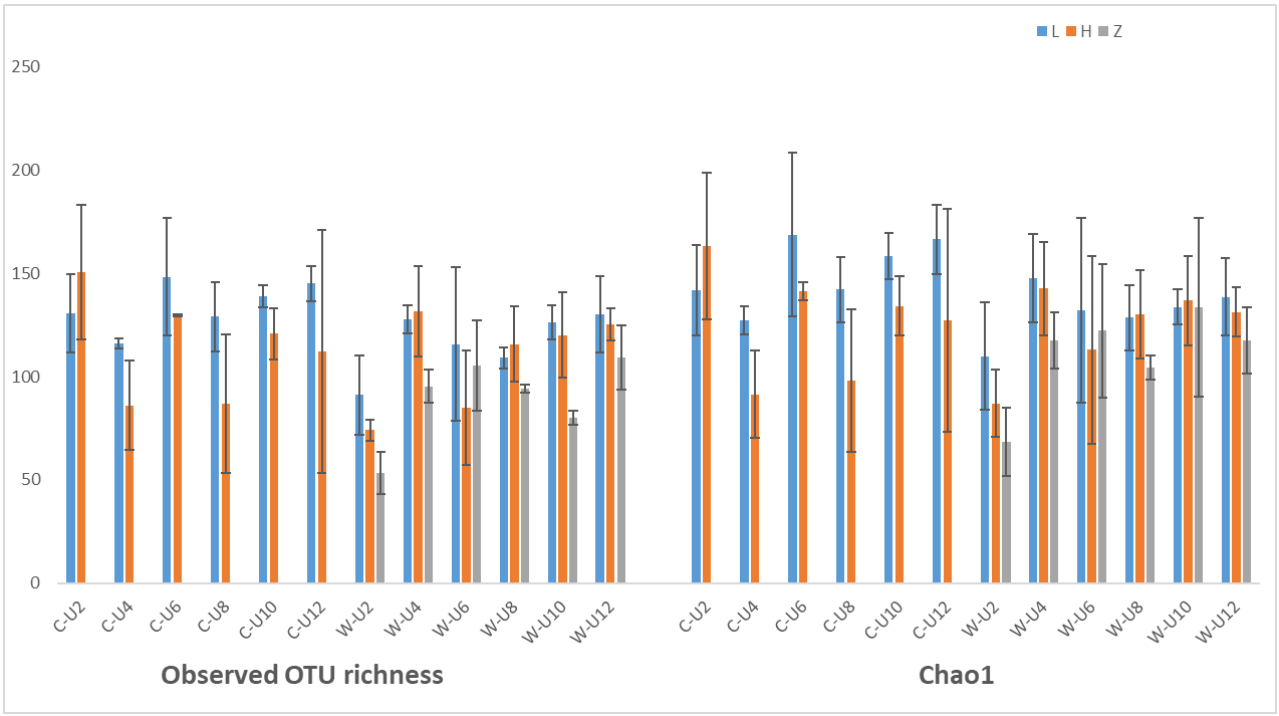

Figure 3.3. Observed OTU richness and Chao-1 for carrier and water samples from the three types of treatment during the 12 weeks of the experiment. The identity of the samples/communities are given as biofilm (C) or planktonic (W), followed by sample week (U) and the different treatments (L (blue), H (orange), or Z (grey).

Table 3.6. Average sequencing coverage for biofilm (C)- and planktonic (W) communities determined as the percentage of observed OTU richness of the estimated Chao1 richness. The average coverage for treatment C and W based on weeks is shown (U) in addition to standard errors of the mean.

Treatment	U2	U4	U6	U8	U10	U12	Avg. Coverage
Coverage W	83.2	87.0	84.2	88.3	81.9	94.2	86.5
Coverage C	92.3	92.6	90.1	89.0	89.0	85.7	89.8

Standard error

Treatment	U2	U4	U6	U8	U10	U12	Avg. Coverage
Coverage W	10,4	6,8	10,3	7,6	16,5	3,1	9,1
Coverage C	3,5	3,5	3,4	3,7	2,6	9,9	4,4

The Shannon's index seemed to be lower in the planktonic community in week 2 in comparison to the other sample weeks (Fig. 3.4). The Shannon index increased for all the treatments in the first six weeks. The diversity for the following weeks varied, but they all seemed to stabilize during the last weeks. (Figure 3.4).

The carrier samples generally had an (overall) higher Shannon diversity than the water samples and the highest diversity was seen in the carrier samples from the H-reactors.

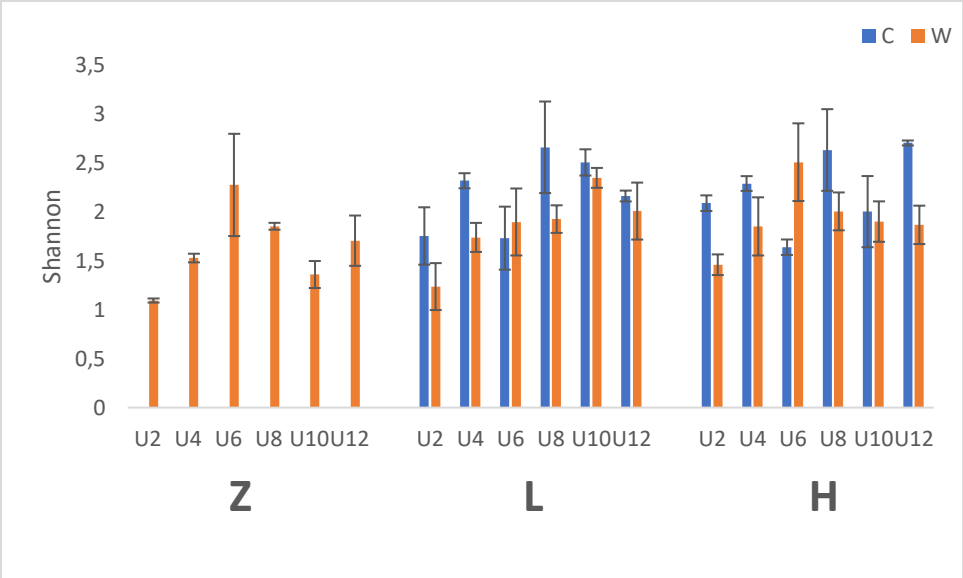

Figure 3.4. Shannon diversity index (H). The identity of the samples/communities are given as biofilm (C) and planktonic (W) followed by sample week (U) and the different treatments (Z, L and H). The average value of the treatments is shown.

The community composition at the class level is shown for each sample in Figure 3.5. Alpha-proteobacteria was the most abundant class both in water and carrier samples. Bacilli was mostly found in the water communities and was less abundant in the biofilm communities. The opposite was the case for the Flavobacteria and Gamma-proteobacteria, which were more common in the biofilm than in the water, but they were decreasing as the experiment progressed. In the biofilm treatment L, there are less Flavobacteria than in treatment H in the first weeks of the experiment. The fraction of Flavobacteria stays stable the following weeks until the percentage drops the three last weeks.

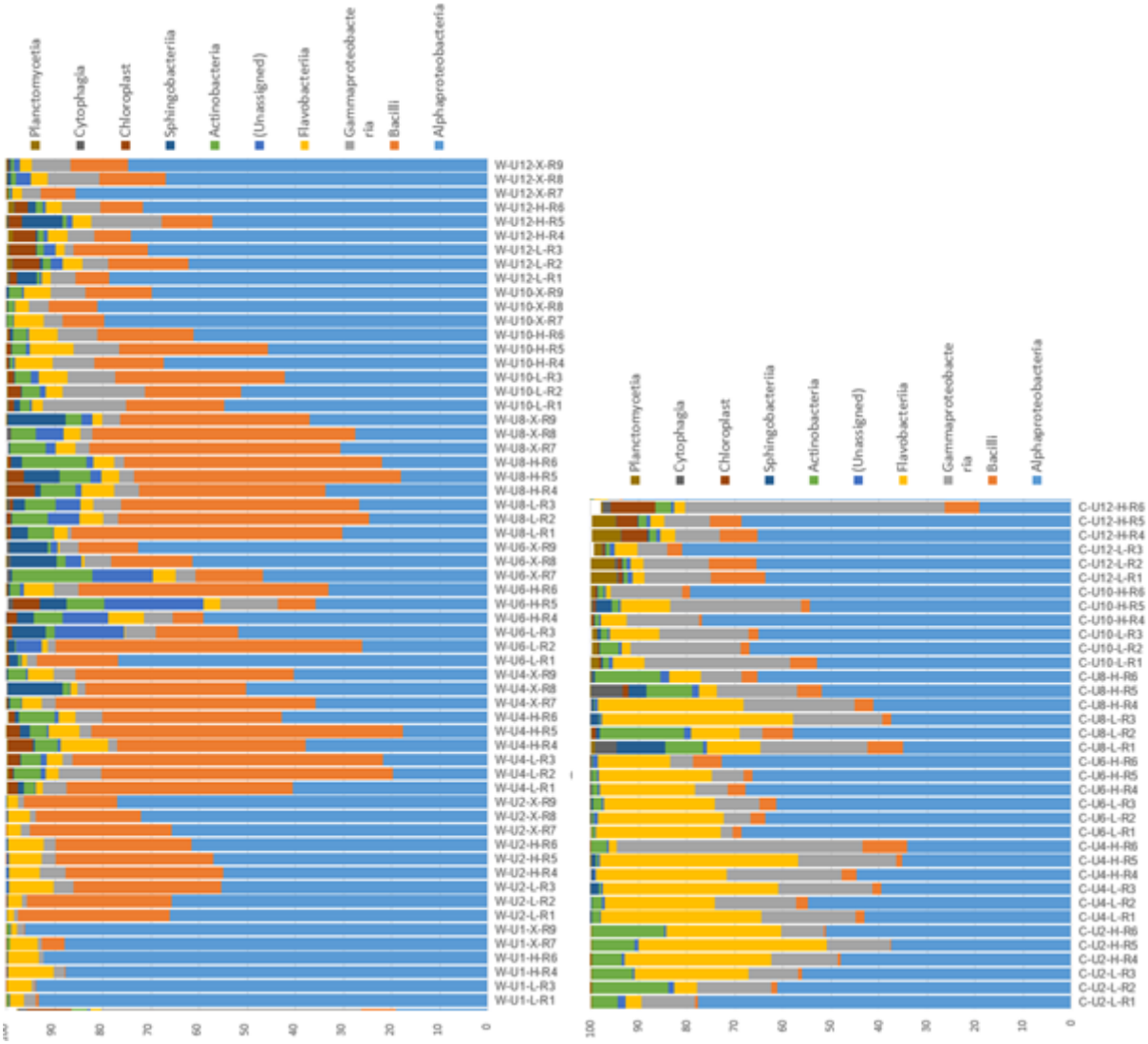

Figure 3.5. Percentages of different bacterial classes in water and carrier samples. Only classes represented by a proportion of ≥1% in at least one of the samples are shown. The samples are identified by biofilm (C) and planktonic (W) followed by sample week (U) and the different treatments (L, H and X(Z)). For a larger figure, see Appendix C.

3.3.2. Beta diversity of microbial community structure: biofilm and water samples for the whole experimental period

PcoA analysis was used for visual comparison of microbial communities associated with water and biofilm samples (Figure 3.6). There was a clear overall difference in the microbiota between the water and biofilm communities. Both the biofilm and the planktonic communities appeared to be relatively similar between treatments.

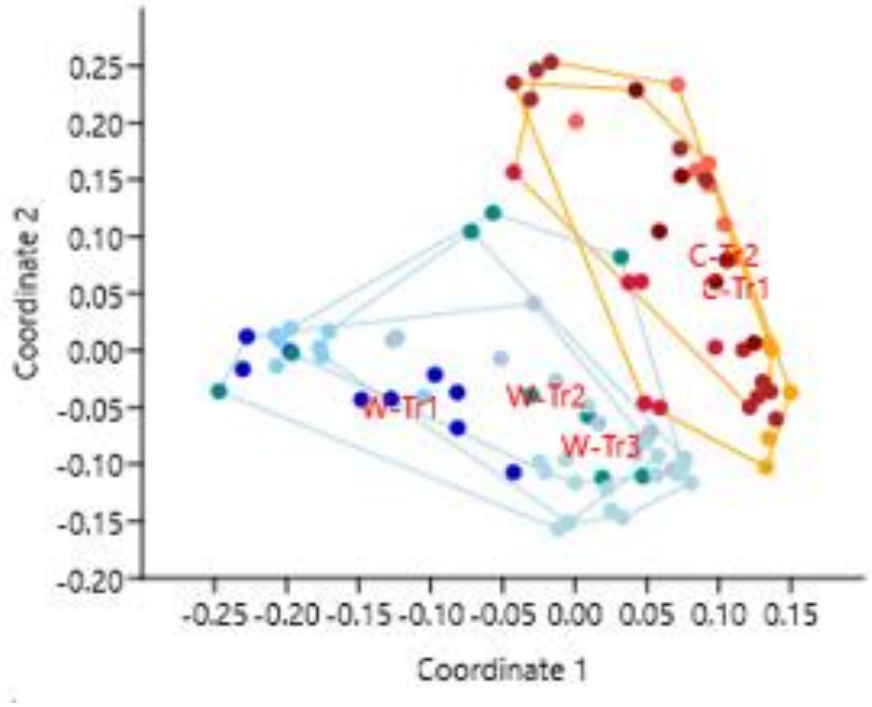

Figure 3.6. PcoA plots based on Bray-Curtis similarities for comparison of community profiles for water- and biofilm samples. The labelling indicates the differences between water (W) and biofilm (C) samples in addition to treatment (Tr). The blue colours indicate water samples and red colours indicate the carrier samples.

A permanova test confirmed that the differences in the microbial community composition was significant between the water and biofilm communities (p=0.0001).

To see if the communities developed similarly between each system, the microbial communities of water and biofilm was compared between the Z, L and H reactors (Figure 3.7). The PcoA plots indicated no clear overall difference in the microbiota for the water (a) and biofilm (b) between the treatments. For the water samples the largest differences between the treatments are seen in week 6 between the L and H reactors (Figure 3.7.a). The water communities in week 8 and 12 are similar to each other between treatments (Figure 3.7.a). The water communities are very similar between treatments in week 12.

In the biofilm samples, there are differences between the treatments in week 12, where the H treatment stands out from treatment L. It appeared to be no visible differences between the biofilm communities in week 6 and 8 (Figure 3.7.b)

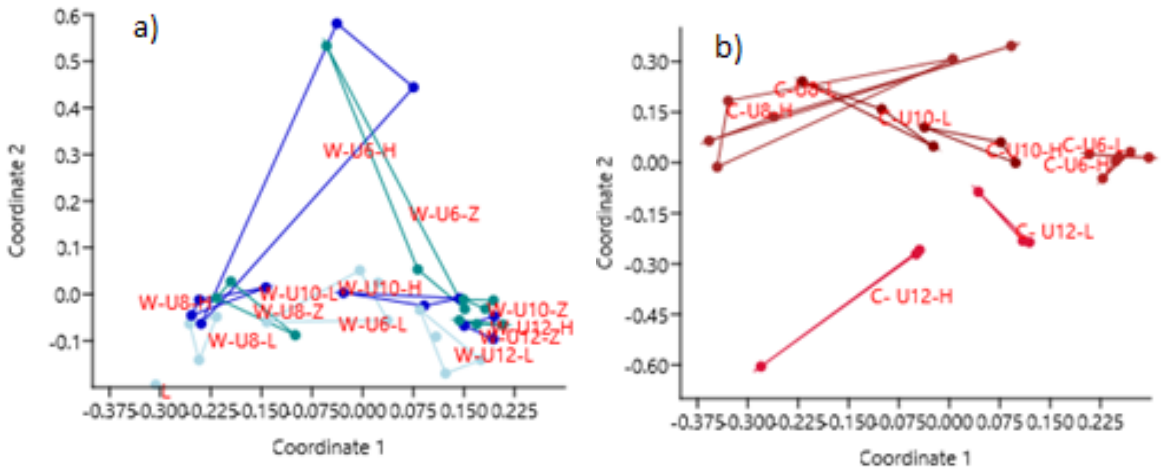

Figure 3.7 PcoA plots based on Bray-Curtis similarities for comparison of treatments for water- and biofilm samples. The labelling indicates the differences between water (W) and biofilm (C) samples in addition to treatment Z, L and H. The blue colours indicate water samples (a)and red colours indicate the biofilm samples (b).

To find out which OTUs that caused most of the differences between the planktonic- and biofilm communities, a SIMPER (similarity percentages) analysis was used. The top 10 most significant OTUs are presented in Table 3.7. OTU_2 was the main OTU responsible for the differences, contributed with 21.14% of the dissimilarity, and constituted 27.6% of all the reads in the water samples, but only 3.6% in the biofilm samples. This OTU was assigned to *Bacillus*. This results in the high number of Bacillus in the water samples (Fig 3.5) OTU_1, the second most contributing OTU, was only 10% more common in water samples (42% of the reads in water samples) and was classified as the family Rhodobacteraceae (Alphaproteobacteria).The third most contributing OTU to the differences was OTU_5, Croceibacter, which was 10 times more common in the biofilm than the water samples. The four most contributing OTUs to the difference in community composition between water and carrier communities, added cumulatively up to 55.8% of the dissimilarity.

Table 3.7. Results for Simper analysis showing the 10 OTUs contributing the most to the Bray Curtis dissimilarity between planktonic (W) and biofilm (C) communities. The amount of dissimilarity they contributed with is shown, in addition to the average abundance of the total of W and C samples.

Taxon	Contribution %	Average abundance C	Average abundance W	OTU_ID
OTU_2	21.1	0.036	0.276	Bacillus
OTU_1	19.6	0.384	0.424	Rhodobacteraceae
OTU_5	9.2	0.113	0.014	Croceibacter
OTU_3	5.9	0.077	0.017	Marinobacter
OTU_10	4.6	0.055	0.002	Reichenbachiella
OTU_13	4.1	0.055	0.010	Gaetbulibacter
OTU_4	3.2	0.009	0.041	Erythrobacter
OTU_6	2.0	0.026	0.020	Muricauda
OTU_17	1.5	0.019	0.007	Methylophaga
OTU_12	1.5	0.008	0.016	Micrococcus

Average Bray-Curtis similarities were calculated for both water and carrier samples, and for comparisons between water and biofilm samples for each week (2, 4, 6, 8, 10 and 12) (Figure 3.8). The differences between the planktonic- and biofilm communities appeared to increase during the experiment, but with a tendency to become more similar in the last two weeks of sampling (Figure 3.8). There were no specific temporal tendencies for either the planktonic or biofilm communities (Figure 3.8). A value of 0.3-0.5 for the comparison between water and carrier samples showed that the communities are clearly different from each other (Figure 3.8), which was confirmed by the permanova test for the comparison between water and carrier (L+H) samples at each timepoint ($p=0.0001$)

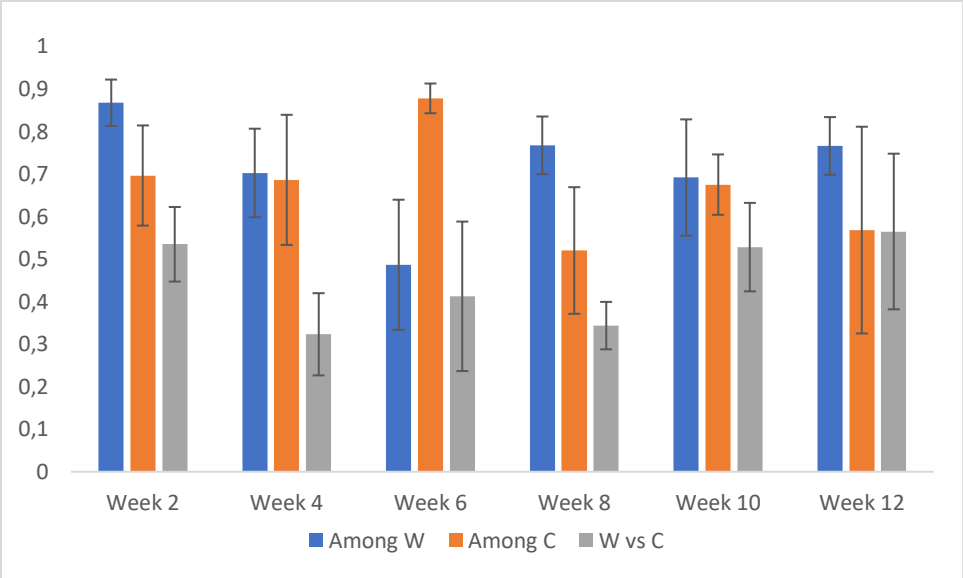

Figure 3.8. Average Bray-Curtis similarities for comparisons of community profiles within and between water and carrier samples for each sampling time. Among W: comparisons among water samples; Among C: comparisons among carrier samples (L+H). W vs C: comparisons between water and carrier samples. The error bars indicate standard deviation.

3.3.3. Comparison of microbial succession: time development within each treatment

To find out how the microbial communities developed over time in each treatment a new PcoA analysis was performed independently for each treatment. The planktonic- and biofilm microbiota appeared to change over time in all treatments. (Figure 3.9).

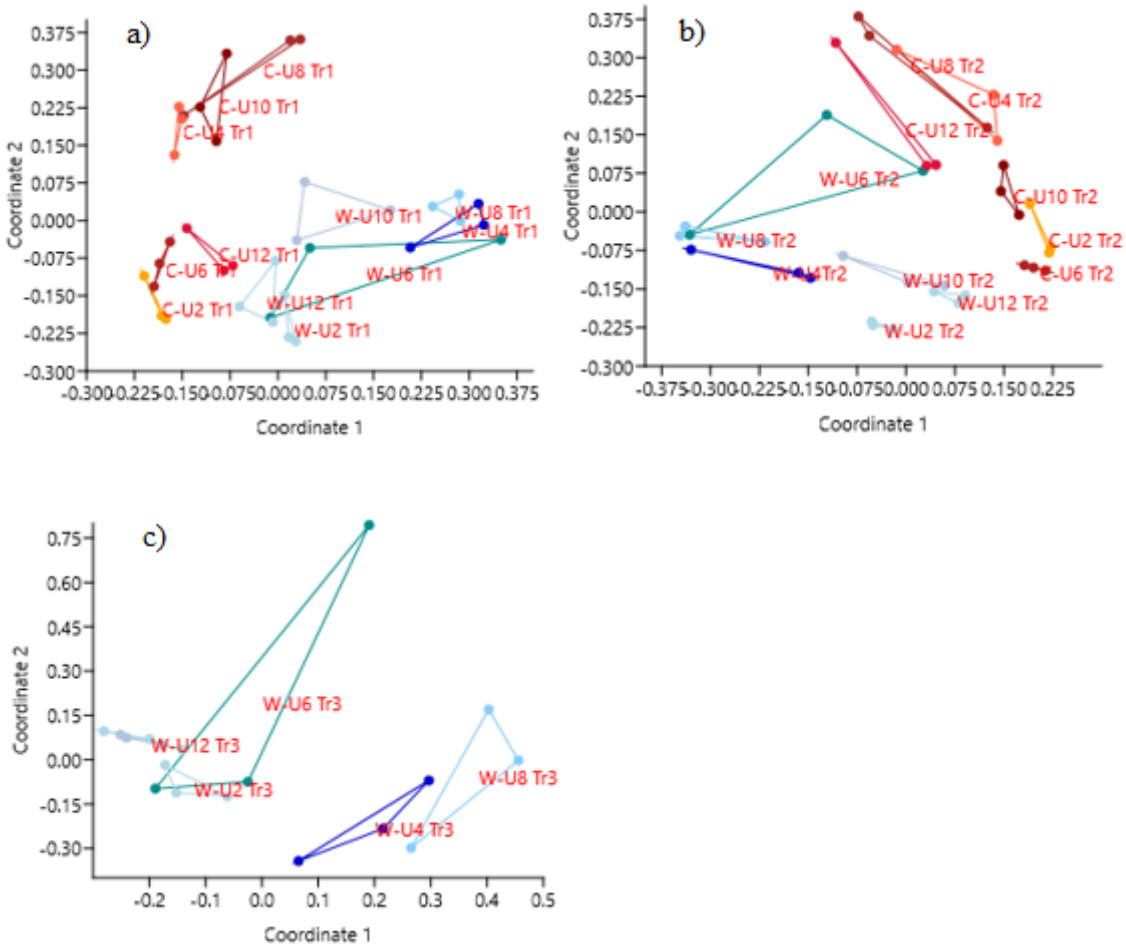

Figure 3.9. PcoA-plot based on Bray-Curtis similarities for comparison of community profiles for biofilm (C)- and planktonic (W) samples in treatment system L (a), treatment system H (b) and treatment system Z (c). A blue colour variant for the water samples and a red colour variant for the carrier samples are utilised.

Average Bray-Curtis similarities were calculated for both water and carrier samples, and for comparisons between water and biofilm samples for each week within treatment L (Figure 3.9.a) (week 2, 4, 6, 8, 10 and 12). The communities were different from each other between

sampling times, but there were less variations between the samples in the first and last weeks of the experiment (Figure 3.10). By looking at Figure 3.5 the same tendency was seen where Flavobacteria was more common in biofilm communities and Bacilli was more common in the planktonic communities. (Figure 3.5). The similarity in between the water and carrier communities in the last week of the experiment was due to a reduced amount of Flavobacteria and Bacilli.

Flavobacteria in the biofilm had a decreasing fraction during the weeks. The same tendency is seen in Actinobacteria where it was most abundant during the first four weeks of the experiment. In the water samples Bacilli had a growing tendency the first four weeks, before stabilizing until week 10, where it decreased in fraction until the end of the experiment.

Both Flavobacteria in the biofilm and Bacilli in the water samples follow the same tendency within their own treatment (Figure 3.5).

The similarities between water and biofilm communities in week 12 are approximately same as the similarities within each sample group (Fig. 3.10). A similar tendency is seen in week 10 and 2.

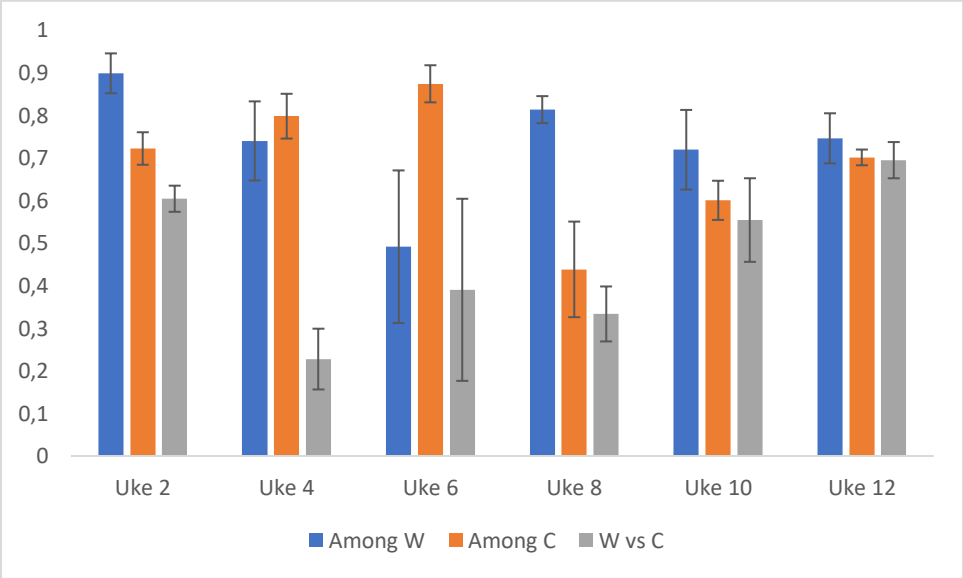

Figure 3.10. Average Bray-Curtis similarities for comparisons of community profiles within treatment L. Among W: comparisons among water samples; Among C: comparisons among carrier samples; W vs C: comparisons between water and carrier samples. The error bars indicate standard deviation.

The same trends are not seen in the community profiles within treatment H (Figure 3.11). The Bray-Curtis similarities are lower for comparisons between the water and biofilm communities. Again, this was shown by Flavobacteria in the biofilm communities and Bacilli in the planktonic communities (Figure 3.5). The L treatment had similar water and biofilm community profiles in the last weeks, this was not the case in the H treatment. Figure 3.5 showed the varying percentages of Gammaproteobacteria resulting in the differences in the H treatment.

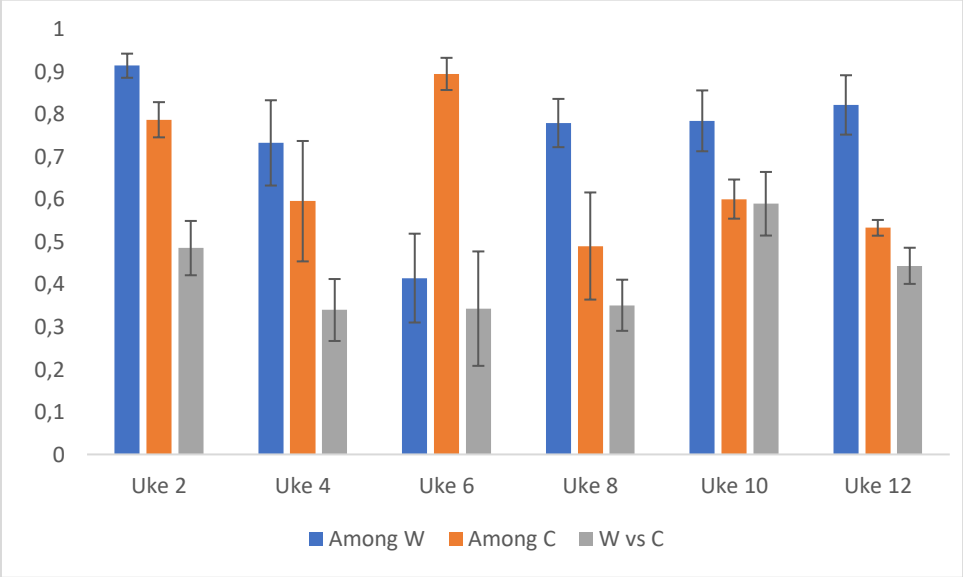

Figure 3.11. Average Bray-Curtis similarities for comparisons of community profiles within treatment H. Among W: comparisons among water samples; Among C: comparisons among carrier samples; W vs C: comparisons between water and carrier samples. The error bars indicate standard deviation.

By calculating average Bray-Curtis similarities for comparisons of community profiles between two successive sampling weeks the speed of succession was shown (Figure 3.12 and 3.13). During week 6-8 the differences in the community profiles are small, the communities does not change much over time during these weeks. This tendency is seen in week 4-6 in the H treatment too, but not in the L treatment. The water communities between week 8-10 and 10-12 are different and changed during this period.

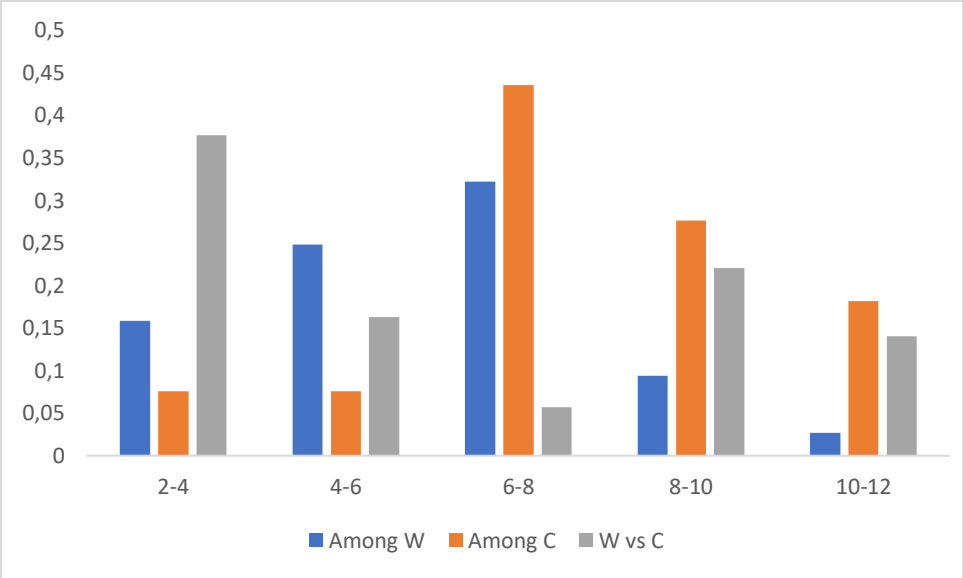

Figure 3.12. Average Bray-Curtis similarities for comparisons of community profiles within treatment L between two weeks. Among W: comparisons of water samples between two weeks; Among C: comparisons of carrier samples between two weeks; W vs C: comparisons between water and carrier samples between two weeks.

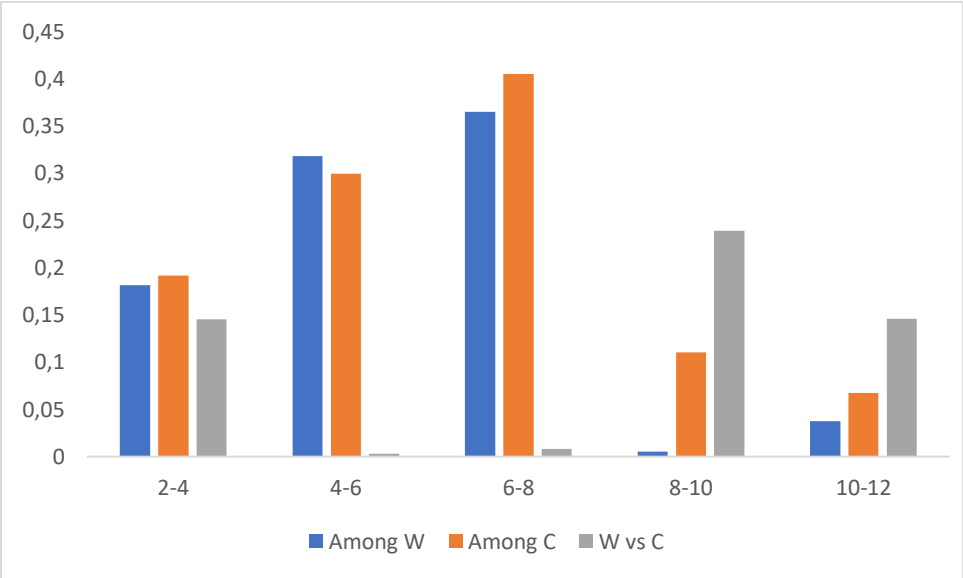

Figure 3.13. Average Bray-Curtis similarities for comparisons of community profiles within treatment H between two weeks. Among W: comparisons of water samples between two weeks; Among C: comparisons of carrier samples between two weeks; W vs C: comparisons between water and carrier samples between two weeks.

4. Discussion

The project aimed to investigate how planktonic and biofilm bacterial communities developed over time in continuously operated bioreactors started with natural seawater bacterial communities and clean biofilm carriers. A second objective was to determine how the planktonic bacterial communities would be influenced by the co-existing biofilm bacterial communities and how long it took the bacterial communities of the reactors to stabilize. The effect of the three-different area per reactor volume was also applied to the objectives above.

4. 1 Evaluation of methods

4.1.1 Nutrient pulsing, sampling and analysis.

To quantify the fraction of r- and K-strategists in natural communities a protocol by Hege Brandsegg was used (Brandsegg, H. Protocol).

The maximum RNA content of bacterial cells correlates with the maximum specific growth rate (Morse and Carter 1949). In growing cells rRNA is the dominating RNA in cells that are growing. R-strategists are fast growing in comparison to K-strategists and have a higher maximum RNA content per cell which can be used to determine different selection regimes based on flow cytometry. By the addition of nutrients to shift the cells into having maximum RNA content and maximum specific growth rate (μ_{max}). If cell division happened the composition of the community would change due to the fast numeric response. The shift up in RNA content is dependent on an incubation period at a given temperature. The length of incubation depends on the temperature used. The number of r-strategists in the system determines the optimal incubation time and. This could lead to a high cell density after the incubation time, (where anything under an 20% increase was acceptable).

The pulsed samples should have been incubated at in situ temperature, which was 18 °C, but was incubated at 20 °C due to space problems. This could have led to a non-optimal incubation time. The incubation time should be so short that the cell number does not increase by more than 20%.

A plot of FL1 versus FSC (forward scatter) was created for each sample. Only single cell formations were of interest in the data analysis of the samples. Aggregated cells were not counted in the analysis and were excluded by the use of gating. The gating was considered for each sample individually, which could lead to variable results.

The thresholds used for determining fractions of r- or K-strategists was taken from the protocol. They were based on assumptions in r-and K-selected communities and by evaluating a threshold that gave the highest dynamic variability of the estimate. The limits were created in experiments done at 15 °C and the threshold might be different for higher or lower temperatures. The thresholds used may have wrongly impacted the amount of r- and K-strategists in the reactors.

The goal of the evaluation was to analyze microbial communities in the reactors that had been under r- or K-selection over time. As seen in the results, a larger number of cells grew in biofilm due to a larger surface area, which gave a higher fraction of K-strategists in the planktonic community. This made sense due to less substrate available in the water resulting in increased competition (Vadstein et al., 1993).

Before maximum RNA content was achieved the cells went through pulsing with nutrients and incubation. Optimal growth conditions were not certain and was an important source of potential error in this project.

4.2 Development of planktonic and biofilm bacterial communities over time

Each of the three treatments had three replicas to secure statistical relevance. Each of these had to be analyzed independently before they were used together as a unit. The PcoA-plot based on Bray-Curtis similarities showed how the microbial communities developed during the weeks in each treatment. The planktonic- and biofilm microbiota changed over time but appeared to be similar between treatments (Figure 3.6). This was further confirmed by comparing the microbial communities of water and biofilm in the Z, L and H reactors (Figure 3.7).

There was no tendency of the number of cells on the carriers to increase over time, suggesting maturity with respect to cell numbers after one week. The time until the biofilm was considered mature was low compared with a volatile solid-based evaluation of the different stages of growth in biofilm done by Zhu et al (Zhu at al., 2014). By the use of biomass three stages in growth was determined. Biofilm accumulation (0-16 days), sloughing and updating (16-28 days) and maturation (28-45 days).

The Shannon's index increased for all treatments the first six weeks but stabilized during the last weeks of the experiment. The bacterial diversity in Zhu et al fluctuated in the first weeks

of biofilm formation (Zhu et al., 2014), but stabilized during the last weeks as seen in this project.

4.3 Planktonic bacterial communities and co-existing biofilm bacterial communities

The bacterial cells in the water samples in the Z treatment had higher DNA content than those of the other treatments. The Z and H treatments consisted of carriers which showed that the biofilm growth on carriers had an impact on the water samples.

The H-reactors had higher fractions of K-strategists than the other treatments during the whole experiment. This corresponded to the expectations. The cells in the biofilm consumed the substrate which led to a reduced substrate concentration in the water. This led to increased competition for the planktonic community resulting in K-selection.

The maximum growth rate for the planktonic communities in the Z reactors was determined by the dilution rate. With a dilution rate of 0.5/day the limitations in growth was low. This also applied for the planktonic communities in the L and H reactors. The biofilm communities could have higher competition due to the surface area and got less substrate per cell. This led to K-selection. In the L and H reactors there were a mix of planktonic communities with moderate competition and biofilm communities which were strongly limited. Due to the high number of bacteria in the biofilm in the H reactor, which used more of the substrate, resulted in the planktonic community to also be more K-selected in this reactor.

Average Bray-Curtis similarities for comparisons of community profiles within treatment L and H gave interesting results when compared between water and carrier samples. In the last week of the experiment the similarities between water and carrier samples were as high as the similarities within each treatment. This gave the indication of a stable microbial community with little differences between the community compositions in the end of the experiment.

The planktonic- and biofilm microbiota changed over time. Average Bray-Curtis similarities for comparisons of (between) water and biofilm samples showed that they were different from each other. The differences were the smallest in the first and last weeks of the experiment. The main contributors for this were Flavobacteria, which were abundant in the biofilm communities, and Bacilli, which were abundant in the planktonic communities.

4.4 Stabilization of the bacterial communities in the reactors

In the H reactors the biofilm constituted a 30% larger part of the total bacterial cells than in the L reactors. By looking at the average values for all the weeks in Table 3.5, the values were consistent at 33% of the total and 66% of the total cell numbers for L and H respectively.

There was no tendency of the number of cells on the carriers to increase over time, suggesting maturity with respect to cell numbers after one week. A certain amount of biofilm must be released from the carriers corresponding to the growth in biofilm to cause this result. The thickness of the biofilm is also important. Normally substrate penetration is less than 100 μm and evenly distributed on the surface (Ødegaard, 2006)

Turbulence in the reactor was needed to achieve a thin biofilm and to transport nutrients. An additional factor causing the cells on the carriers to not increase overtime was collisions between the carriers due to the movement causing abrasion.
Mass transfer of substrate and usage of the substrate is the basis for growth of biofilm. The growth is dependent on the rate of which the limiting substrate is acquired, the biomass yield and density. The detachment of biomass from a biofilm is impacted by abrasion, but the detachment is not the same for growing and non-growing biofilm. In steady state the detachment is balancing the growth (Loosdrecht et al,. 1995).

4.5 Beta diversity of microbial community structure: biofilm and water samples for the whole experimental period

Alphaproteobacteria was the most abundant class in both water and carrier samples. Flavobacteria and Gamma-proteobacteria were more common in the biofilm- than in the planktonic communities and Bacilli was more common in the planktonic communities.

The most important difference between biofilm communities and planktonic communities was Bacillus, which was more common in the planktonic communities. The second most contributing OTU was Rhodobacteraceae (Alphaproteobacteria) which was 10% more common in the water samples.

Rhodobacteraceae is a key element of the initial formation of biofilms formed in Eastern Mediterranean coastal seawater. By the use of culture dependent techniques, the most dominant bacteria on biofilm in the coastal Atlantic and Pacific Oceans were the Alphaproteobacteria class, especially the Rhodobacteraceae family (Elifantz et al., 2013).

These were followed by Gammaproteobacteria and Bacteroidetes phylum (Dang & Lovell, 2000, 2002; Jones et al., 2006). In the results Rhodobacteraceae was more common in the water samples, than in the biofilm samples, but by looking at the average abundance for biofilm (0.348) it is clear that it played an important role in the biofilm.

Flavobacteria is common in the oceans but can also dominate in mature freshwater biofilms (Manz et al., 1999). The system in the reactors are based on seawater, but the same tendency is seen where Flavobacteria is common in biofilm communities.

4.6 Further work and theories

By extending the experimental period further changes between the microbial conditions could have been spotted, especially by turning up the dilution rate (connected with supply of substrate) in the end of the experiment to see how the different systems reacted. With an increase in substrate concentration at one level the supply of substrate could have been so high that the microbiota in the H reactors swapped to r-selection and this would be dependent on the surface area of the reactor. This is due to biofilter systems having a planktonic and surface-attached community that experience different selection regimes with competition and loss.

Additional reactors which was not washed and autoclaved on a regular basis might have led to a different outcome in the Z and L reactors due to biofilm growth on the reactors and could have given interesting results in comparison to the washed reactors. Based on the theory of r- and K-selection these unwashed reactors would have favoured a K-selected community (Schryver and Vadstein, 2014).

A lower substrate concentration in the reactors in the beginning of the experiment could have shown the turning point between r- and K-selection for each of the Z and L reactors. This was not seen due to the substrate concentration in the reactors being higher than this point. The difference in the number of carriers in the L and H reactors caused a swap to K-selection in the H reactors showing the importance of more surface area with increased competition. If another triplet was created with a carrier number between the L and H reactors the development could have been shown better.

The flow cytometry-based method by Hege Brandsegg (Brandsegg, H. Protocol) could be further optimized for different conditions and temperatures in microbial communities in the nutrient pulsing.

5. Conclusion

The overall goal of this master thesis was to identify factors that are crucial for creating a K-selected, stable microbial community in flow-through bioreactors, and thereby increase the understanding of how to obtain K-selection with the use of biofilters.

The planktonic- and biofilm microbiota changed over time. Average Bray-Curtis similarities for comparisons of (between) water and biofilm samples showed that they were different from each other. The differences were the smallest in the first and last weeks of the experiment. The main contributors for this were Flavobacteria, which were abundant in the biofilm communities, and Bacilli, which were abundant in the planktonic communities.

A higher number of K-strategists were found in the H-reactors than in the other treatments during the whole experiment. This corresponded to the expectations, a larger number of cells in the biofilm gave a higher number of K-strategists in the planktonic community due to competition.

The maximum growth rate for the planktonic communities in the Z reactors was determined by the dilution rate. With a dilution rate of 0.5/day the limitations in growth was low. This also applied for the planktonic communities in the L and H reactors. The biofilm communities could have higher competition due to the surface area and got less substrate per cell. This led to K-selection. In the L and H reactors there were a mix of planktonic communities with moderate competition and biofilm communities which were strongly limited. Due to the high number of bacteria in the biofilm in the H reactor, which used more of the substrate, resulted in the planktonic community to also be more K-selected in this reactor.

Biofilm communities impacted the planktonic communities. The differences in the community composition (Figure 3.7) were not large, but there was a larger percentage of K-strategists in the planktonic communities in the H reactors. This indicated that there was a different community composition in the H reactors. The percentage of biofilm cells of the total cells (biofilm and water) in each reactor (Table 3.5) showed that the biofilm constituted a 30% larger part of the bacterial cells in the H reactors than in the L reactors. Planktonic cell numbers were twice as high in the L reactors than in the H reactors.

The bacterial cells in the water samples in the Z treatment had higher DNA content than those of the other treatments. The Z and H treatments consisted of carriers which showed that the biofilm growth on carriers had an impact on the water samples.

There was no tendency of the number of cells on the carriers to increase over time, suggesting maturity with respect to cell numbers after one week. An amount of biofilm bacteria must therefore be released from the carriers in amounts corresponding to the growth in the biofilm.

Planktonic bacterial communities were not influenced by the co-existing biofilm bacterial communities (Figure 3.7). There were no overall differences in the microbiota for the water and biofilm between the treatments.

By the use of Bray-Curtis similarities for comparisons of community profiles between two successive sampling weeks the speed of succession was shown. The bacterial communities of the reactors were stable in week 6-8 which showed that they did not change much during these weeks. In the later weeks the bacterial communities kept changing resulting in no stabilizing trend overall (Figure 3.12 and 3.13)

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Appendix A. Cultivation medium

Table A.1. M65 concentrated stock medium. Concentration of 50 g/l of each component resulting in 150 g/l in total.

Component	Amount	Supplier	Kat.nr/LOT
Yeast extract	5 g	Oxoid LTD.	LP0021/1214988-02
Bacteriological Peptone	5 g	Oxoid LTD.	LP0037/1432643
Tryptone	5 g	Oxoid LTD.	LP0042/450463
Distilled water	100 ml	Oxoid LTD.	

Table A.2. The f/2 trace metal solution components with concentrations for each and final concentration in the water.

Component	Stick solution (g/L)	Quantity used	Concentration in final medium (µg/ml)	Final concentration in sample (µg/ml)
$FeCl3 \cdot 6H2O$	-	3.15 g	3150.0	0.315
$Na2EDTA \cdot 2H2O$	-	4.36 g	4360.0	0.436
$MnCl2 \cdot 4H2O$	180.0	1 ml	180.0	0.018
$ZnSO4 \cdot 7H2O$	22.0	1 ml	22.0	0.002
$CoCl2 \cdot 6H2O$	10.0	1 ml	10.0	0.001
$CuSO4 \cdot 5H2O$	9.8	1 ml	9.8	0.001
$Na2MoO4 \cdot 2H2O$	6.3	1 ml	6.3	0.0006

Appendix B: Protocol TAE-buffer

50 x TAE-buffer

Per litre

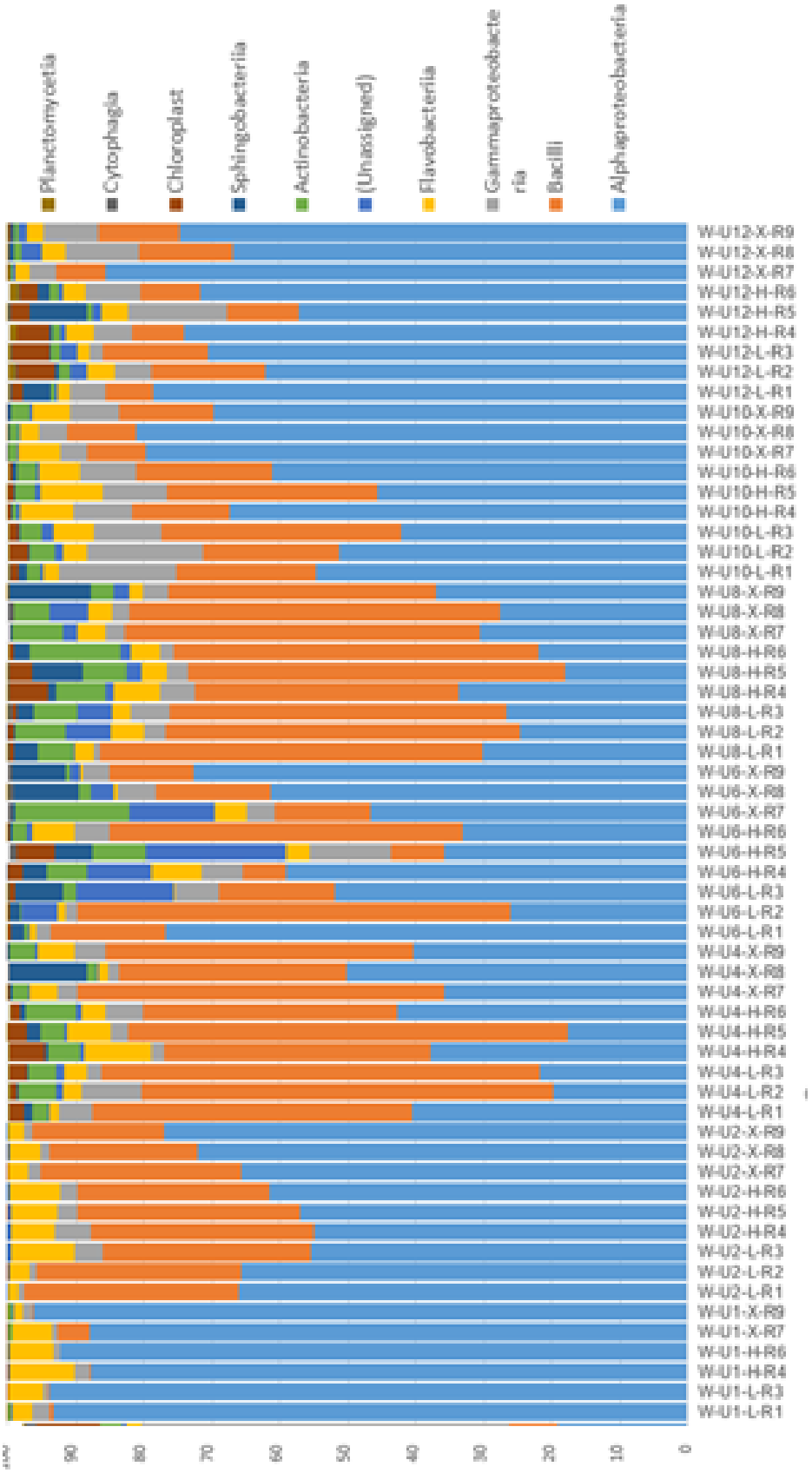
Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

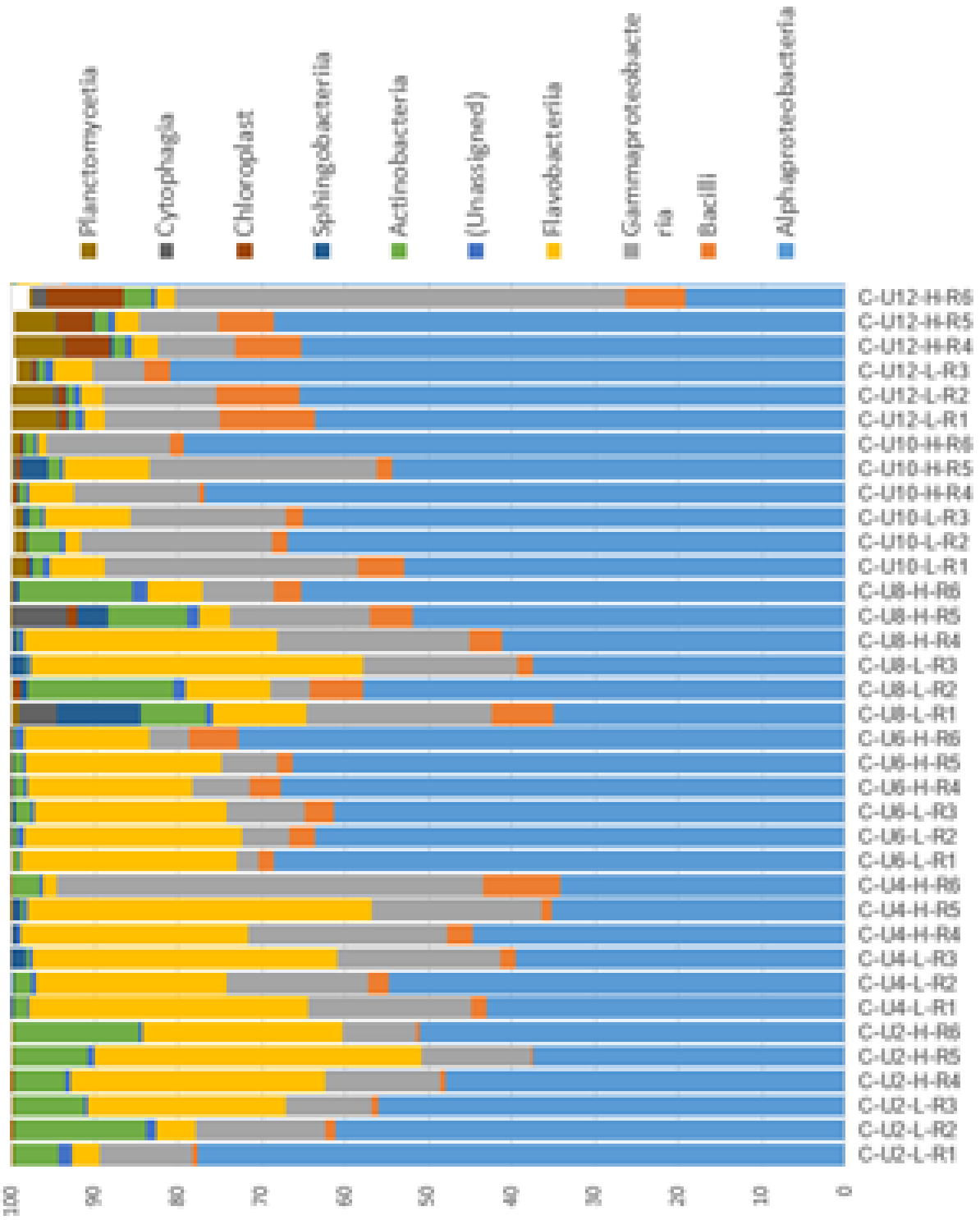
Add distilled water until the total volume is 1000 ml.

1X TAE-buffer

40 ml 50X TAE-buffer + 1960 ml MQ-water

Appendix C: Relative abundance of bacterial classes in water and carrier samples




Planctomycetia
Cytophagia
Chloroplast
Sphingobacteriia
Actinobacteria
(Unassigned)
Flavobacteriia
Gammaproteobacteria
Bacilli
Alphaproteobacteria
C-U12-H-R6
C-U12-H-R5
C-U12-H-R4
C-U12-L-R3
C-U12-L-R2
C-U12-L-R1
C-U10-H-R6
C-U10-H-R5
C-U10-H-R4
C-U10-L-R3
C-U10-L-R2
C-U10-L-R1
C-U8-H-R6
C-U8-H-R5
C-U8-H-R4
C-U8-L-R3
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C-U4-H-R5
C-U4-H-R4
C-U4-L-R3
C-U4-L-R2
C-U4-L-R1
C-U2-H-R6
C-U2-H-R5
C-U2-H-R4
C-U2-L-R3
C-U2-L-R2
C-U2-L-R1
100
90
80
70
60
50
40
30
20
10
0