

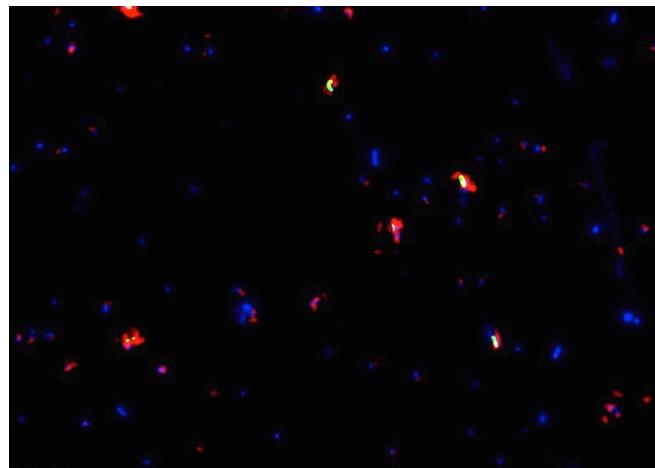


Master Thesis in Microbiology

Response of free-living chemotactic bacteria to a variable substrate field in Lake Zurich

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What I cannot create, I do not understand.

Richard Feynman

Abstract

Nutrients are heterogeneously distributed throughout the water column and they frequently emerge as microscale hotspots and gradients. Various processes contribute to the production of gradients in the lake and they can lead to behavioural and metabolic responses of the bacteria. Thus, two strategies have been evolved: Non-motile bacteria, which optimize nutrient uptake by diffusion and motile bacteria, which exploit these microscale gradients. Along with the ability to move, microbes have evolved a vast range of taxes. Chemotaxis is one of the most studied microbial taxes and it is defined as the directed movement towards a chemoattractant as well as the directed movement away from a chemorepellent. There have been many applications of chemotaxis assays to measure this behaviour in the past few years but there is still not a fully developed assay for freshwater systems. With the microfluidic-based *in situ* chemotaxis assay (ISCA) we tried to measure chemotaxis in Lake Zurich by using different substrates as chemoattractants. We could demonstrate that the ISCA was not applicable to Lake Zurich due to different reasons such as causing turbulence during deployment, which led to variability in the data, dilution of the motile, chemotactic fraction of bacterial cells present in the lake as well as some technical issues. An extensive knowledge of motility patterns of bacteria present in Lake Zurich is necessary to shed more light into this subject.

Microautoradiography and fluorescence *in situ* hybridization were conducted to investigate what taxa of free-living bacteria respond to a variable substrate field in Lake Zurich. Radiolabelled glutamine was used as tracer to assess microbial incorporation after two time points. We could demonstrate that the LD12 lineage of the *Alphaproteobacteria*, the acl cluster of the *Actinobacteria* and the *Betaproteobacteria* in general were quite abundant during August 2017 and revealed a moderate uptake activity for radiolabelled glutamine. Total uptake activity never reached 100 % due to oligotrophic adaptations of certain taxa, rapid tracer recycling and bacterial population demography.

More probes have to be tested in the future to investigate other bacterial groups active in glutamine uptake. Future experiments need to be conducted to reproduce these results, link them to their genetic background of chemotaxis and place it into a broader context of variability of the substrate field in Lake Zurich.

List of abbreviations

DOM	Dissolved organic matter
ISCA	In situ chemotaxis assay
MAR	Microautoradiography
FISH	Fluorescence in situ hybridization
CARD-FISH	FISH combined with catalysed reporter deposition
PDMS	Polydimethylsiloxane
Gln	Glutamine
Glc	Glucose
NAG	N-Acetylglucosamine
Leu	Leucine
Arg	Arginine
Put	Putrescine
P	Phosphate
FLW	Filtered lake water
FA	Formaldehyde
LB-medium	Luria-Bertani medium
NSY	Nutrient broth, soytone, yeast extract
IBM	Inorganic basal medium
RT	Room temperature
PES	Polyethersulfone
DAPI	4',6-Diamidin-2-phenylindol

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1 Introduction

1.1 Motility and chemotaxis in aquatic environments

Motility is one of the most studied types of microbial behaviour (Berg 1975; Mitchell et al. 1995; Grossart et al. 2001; Son et al. 2015). It promotes many processes such as interactions between bacterial cells and surfaces, responses of cells to stimuli and also cell-cell interactions (Son et al. 2015). Many bacterial isolates from different environments show motility (Mitchell et al. 1995). For example, previous studies investigating coastal marine bacteria could show that less than 10 % of the bacteria were actually motile. During fall and winter the fractions of motile bacterial cells were low (< 5 to 25 %) and during spring and summer the abundance of motility raised to up to 70 % (Grossart et al. 2001). The higher motility rates may be due to dynamic patterns between bacteria and the dissolved organic matter (DOM) (Stocker and Seymour 2012). DOM gradients are mainly produced by decomposition processes of dead organisms and can lead to behavioural and metabolic responses of bacteria (Azam 1998). Therefore, motility can be an important adaptation affecting biogeochemistry of freshwaters (Grossart et al. 2001).

Along with the ability to move, microbes are capable of perceiving a broad set of environmental stimuli like concentrations of nutrients and/or toxins as well as pH, oxygen, light and magnetic fields (Armitage 2001). In response to these external signals, bacteria have evolved a vast range of *taxis* (*taxis*: directed movement) (Krell et al. 2011). For instance these two well studies behaviour: phototaxis, bacterial movement towards a light source (Gest 1995) and chemotaxis, motion directed by chemical cues. Chemotaxis enables bacteria to navigate through the water column in response to chemical gradients (Stocker and Seymour 2012). Transmembrane chemoreceptors are activated to estimate chemical concentrations and sensory signals are transduced to process this information (Wadhams and Armitage 2004; Stocker and Seymour 2012). Chemotaxis allows motile bacteria to accumulate towards higher concentrations of attractants and to avoid repellents (Yang et al. 2015).

In pelagic habitats, microscale nutrient patches exist throughout the water column. Patches are available within limited time and space and represent resources like phytoplankton exudation, viral lysis of bacteria and allochthonous processes (Blackburn et al. 1998). This creates conditions that support bacterial motility to acquire nutrients from the environment, resulting in an accumulation of opportunistic bacteria. The opportunistic bacteria, also known as copiotrophs (Salcher 2014), are generally specialized on short-living DOM sources (Šimek et al. 2005) and might be found attached to particles (Grossart 2010).

Aquatic bacterial chemotaxis is mainly stimulated by a wide range of organic compounds (Dennis et al. 2013). Amino acids are one of the most valuable nutrient source types for many bacteria (Yang et al. 2015) since they can be used as sole nitrogen, carbon or energy sources (Halvorson 1972). Chemotaxis towards inorganic substrates like ammonium or phosphate might also have an impact on biogeochemical processes (Dennis et al. 2013). However, there is an indication of a high degree of substrate specialization among freshwater microbes which can lead to coexistence and diversity mediated by physiological niche separation (Salcher et al. 2013).

1.2 Chemotaxis assays

In 1888 Pfeffer performed one of the first chemotaxis assays by using a traditional capillary approach (Pfeffer 1888). Since then, several methods have been developed to assess chemotaxis. In 1966, a first quantitative method was designed for measuring this behaviour. The so-called “capillary assay” (Fig. 1) allows determining the distribution of bacteria throughout the capillary. The assay relies on the molecular diffusion of the attractant (Adler and Dahl 1967). The capillary tube containing the potential attractant is inserted into a bacterial suspension. The motile bacteria will accumulate around the tip of the capillary and later migrate inside (Fig. 1B). If the capillary contains a potential repellent, the bacteria do not migrate inside and instead swim away from it (Fig. 1C). After incubation for a certain amount of time, the capillary is removed and the contents can be transferred to liquid media for dilution and/or plating (Mazumder et al. 1999).

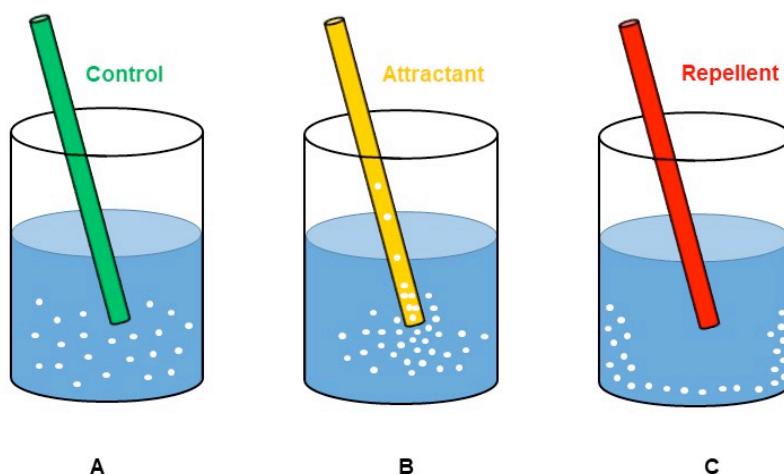


Figure 1: Schematic representation of a classical capillary assay as described by Alder and Dahl. A) Control-solution, for example water without nutrients. Random movement of the bacteria is expected and their motility occurs sporadically. B) Capillary contains an attractant. Directed movement towards the chemical attractant (chemotaxis) is expected. Bacteria start to migrate into the capillary. C) Capillary contains a repellent. Directed movement away from the repellent is assumed. Bacteria start to accumulate at the border of the beaker as the repellent diffuses into the adjacent water.

Other methods have been designed in the following years to asses chemotaxis (Mazumder et al. 1999; Seymour et al. 2008; DeLoney-Marino 2013). However, there is no specific method to study chemotaxis of freshwater bacteria close to in situ conditions. To quantify it in the lake, the microbial chemotactic behaviour must be measured in situ. For this purpose, the method of an in situ chemotaxis assay (ISCA) was developed by Lambert et al. 2017. This microfluidic-based assay allows simultaneous testing of various chemoattractants under identical conditions (Tout et al. 2015) (Fig. 2).

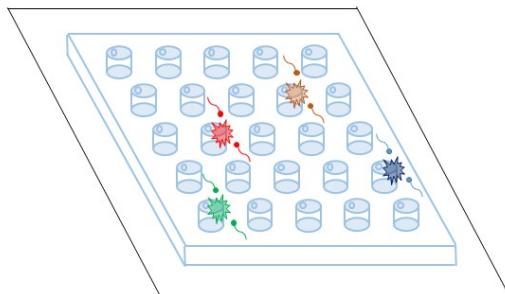


Figure 2: Design of the plate for the in situ chemotaxis assay (ISCA). The plate features 25 wells containing potential chemoattractants (different attractants represented in different colours), forming a gradient. Motile, chemotactic bacteria swim towards the attractant into the wells.

The ISCA was originally engineered using soft lithography, a technique for microfabrication of devices which features elastomeric materials such as polydimethylsiloxane (PDMS) (Whitesides et al. 2001). This idea has led to the fabrication of a microfluidic-based chemotaxis assay which allows the quantification of chemotaxis in real time, even monitoring several chemicals simultaneously (Seymour et al. 2008a). So far the ISCA was mainly applied in marine environments. It was used to study chemotactic responses in marine bacterial isolates, species of phagotrophic flagellates and phytoplankton (Seymour et al. 2008). Other applications of the ISCA could even show that chemotaxis is decisive for pathogenic bacteria to infect corals (Tout et al. 2015). Thus far there are no studies, that show an application of this assay in freshwater systems.

1.3 ISCA deployment in August 2016

In August 2016, Bennett Lambert from the Stocker Lab at ETH Zurich visited the Limnological Station to apply the ISCA on Lake Zurich for the very first time. Four different substrates were used: Glutamine, serine, glucose and aspartate (Gln, Ser, Glc and Asp) of which Gln and Glc showed a strong chemotaxis response (Gln: 7611 and Glc: 4854 cells / well) compared to the control (425 cells / well) (Fig. 3). To have a signal for chemotaxis the treatments must show a minimum of twice as much cells per well than the controls (Lambert

et al. 2017). Ser and Asp are very similar to the control (Ser: 448 and Asp: 338 cells / well) (Fig. 3).

The results were promising, therefore Prof. Jakob Pernthaler and his group decided to investigate this method further to develop an ISCA especially for freshwater bacteria.

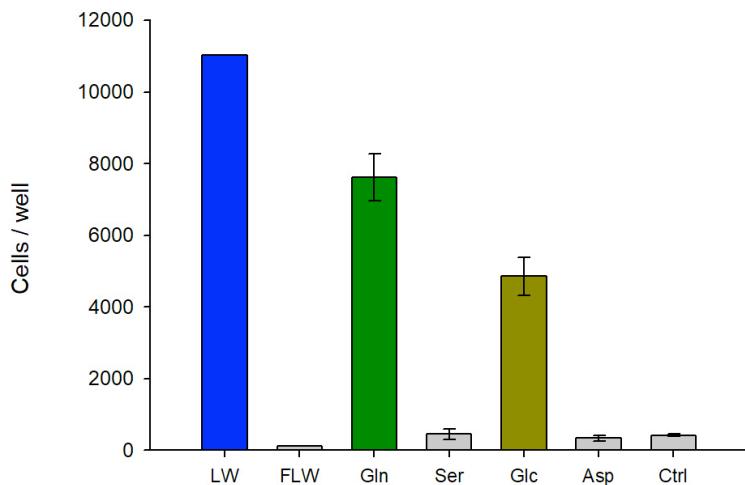


Figure 3: In situ chemotaxis assay (ISCA) from August 2016: Cell counts (cells / well) investigated from the wells with lake water (LW), filtrate lake water (FLW), glutamine (Gln), serine (Ser), glucose (Glc), aspartate (Asp) and control (Ctrl). Error bars show standard deviations.

1.4 Bacterial communities in freshwater systems

Bacteria play a prominent role in freshwater systems, as they have a great influence on water quality and on the carbon cycle of ecosystems (Arrigo 2005). Freshwater systems host diverse free-living bacteria such as the acI cluster of *Actinobacteria* (Warnecke et al. 2005; Salcher et al. 2013), the LD12 lineage of *Alphaproteobacteria* (Salcher et al. 2011), *Betaproteobacteria* (Glöckner et al. 1999) and also various *Flavobacteria* (Eiler and Bertilsson 2007). *Actinobacteria* were estimated as being amongst the most abundant freshwater bacteria (Salcher et al. 2013) and they appear to dominate a range of limnic systems like lakes and rivers. Their morphology is highly diverse (Shivlata and Satyanarayana 2015) but their cell size in freshwaters mainly appears to be small, which protects them from grazing (Allgaier et al. 2006). *Alphaproteobacteria* exhibit a pronounced depth-distribution pattern and seasonal fluctuations and they are rare in the pelagic zones of many limnetic systems (Salcher et al. 2011). Shifts in their community composition are greatly enhanced by microeukaryote grazing. As a response *Alphaproteobacteria* form filaments or aggregates to gain more resistance from grazing (Langenheder and Jürgens 2001; Newton et al. 2011). *Betaproteobacteria* show high abundances in freshwater systems

as well, reaching average fractions of up to 16 % (Glöckner et al. 1999). Especially the R-BT lineage of the genus *Limnohabitans* seems to represent an abundant fraction among *Betaproteobacteria*. They are fast growing and capable of rapidly changing their community composition (Šimek et al. 2008; Kasalický et al. 2010). *Flavobacteria* are known to be variable in freshwater systems (mostly eutrophic lakes) and depend on the availability of resources (Eiler and Bertilsson 2007).

Microbial abundances are affected by bottom-up and top-down processes and they might be related to the general ecosystem productivity in marine and freshwater microbial food webs (Pernthaler 2005). Bacterial growth rates are limited by substrate competition (bottom-up control); grazing and viral lysis lead to mortality (top-down control) (Langenheder and Jürgens 2001; Pernthaler 2005).

DOM represents a key source of nutrients and energy for microbes (Brailsford et al. 2017). It involves a vast range of individual compounds, which can be biologically processed. High molecular weight (HMW) compounds (> 1000 daltons) might be recalcitrant to microbial breakdown whereas low-molecular-weight (LMW) such as amino acids, nucleic acids and sugars are labile and prone to degradation (Kujawinski 2011; Brailsford et al. 2017). The components of DOM that support microbial growths involve amino acids, carbohydrates and carboxylic acids (Salcher 2014). These substrates vary greatly in time and space and are usually present in low substrate concentrations of about 13 nM (Meon and Jüttner 1999). Previous studies could show a patchy distribution of dissolved free amino acids (DFAAs) and highly variable concentrations (Horňák et al. 2016).

Aquatic organic matter appears to be heterogeneously distributed throughout the water column and displays high spatial and temporal fluctuations (Simon et al. 2002; Stocker and Seymour 2012). This pool of physically and chemically complex organic matter promotes a vast range of bacterial adaptations to their environment (Azam 1998).

1.5 Identification of bacterial communities

The vast majority of abundant bacterial communities in freshwater systems are classified as uncultivable (Salcher and Šimek 2016). Methods, which rely on cultivation, cannot be used to gain information about the *in situ* ecophysiological roles of these microbes. Therefore, it is challenging to link specific metabolic processes to the bacterial identity, e.g. based on 16S rRNA phylogeny (Okabe et al. 2004). Microautoradiography (MAR) was first introduced to microbial ecology in 1960 by the work of T.D. Brock. This method allows the determination of *in situ* uptake of specific radiolabelled substrates by individual cells (Brock and Brock 1966). Fluorescence *in situ* hybridization (FISH) is a technique, that uses fluorescent oligonucleotide probes to phylogenetically identify microbial taxa (Pernthaler et al. 2002). The combination of

both methods (MAR-FISH) represents a powerful technique to visualize the incorporation of radiolabelled substrates by individual cells of a specific (uncultured) bacterial population. The appropriate isotopes for this technique are mostly β -emitters like ^3H or ^{14}C , their disintegration is due to the loss of electrons. ^3H -substrates are preferable for incorporations since they have a low radiation energy, which causes silver grain deposition sufficiently close to the cells incorporating the substrate (Brock and Brock 1966; Alonso 2012).

The incorporation of amino acids is crucial for bacterial communities since it can be directly used for protein synthesis or serve as carbon, energy and/or nitrogen source (Burkovski and Krämer 2002). Glutamine for example, is a nitrogen-rich compound, which is used for the biosynthesis of other amino acids. It is essential for the biosynthesis of various nitrogen-containing compounds and it is involved in the primary reaction of ammonium assimilation (Forchhammer 2007).

1.6 Aims of the thesis

The aim of using the ISCA was to sort motile bacteria from environmental water samples, isolate chemotactic bacteria and screen their 16s rRNA genes. Since the production of this device turned out to be quite time-consuming, several other chemotaxis assays were reviewed to find an alternative method to the ISCA. Assaying chemotaxis was part of a broader project by Natalia Krempaska, which connects variable substrate concentrations with chemotaxis of motile bacteria. The objective here was to compare specific gene regions, which are expressed or down-regulated in chemotactic bacteria upon reaching a substrate patch in a variable water column. Alongside, another aim of the master thesis was to estimate substrate incorporation rates in bacterial cells using radiolabelled substrates and identify specific bacterial groups, that incorporated the given substrates by MAR-FISH. The idea was to link metabolic processes to the genetic background of distinct bacterial groups. At the moment there is little known about chemotactic bacteria in freshwaters and with this project we are trying to gain information about the dimension of chemotaxis in Lake Zurich.

2 Material and methods

2.1 In situ chemotaxis assay (ISCA) – Production, design and assembly

The aim of applying ISCA is to understand bacterial chemotactic behaviour and quantify their reaction upon encountering chosen substrates. The principle works as follows: the wells of the ISCA plate are filled with defined chemoattractants, that diffuse through the port into the surrounding environment (e.g. lake water). This results into a chemical gradient, which is sensed by chemotactic bacteria and gives them the cue to find higher concentrations and swim into the wells (Lambert et al. 2017).

For the production of the ISCA plate a 3D printed mould was kindly provided by the Environmental Microfluidics Group of Roman Stocker (Stocker Lab, Institute of Environmental Engineering, Department of Civil, Environmental and Geomatic Engineering, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland). The mould was designed using SolidWorks 2015 (Waltham, Massachusetts, USA) and was made from the polymer VeroGrey on an Object30 3D printer (Lambert et al. 2017). The ISCA plate consists of an array of 5 x 5 wells (Fig. 4) and each well has a diameter of 6.8 mm and a depth of 3 mm. The volume of one well corresponds to 110 µl. This volume was determined based on the lower limit of input needed for DNA extraction and sequencing (Jiang et al. 2015). The wells were spaced 9 mm apart within a row and 17 mm apart between rows (Lambert et al. 2017). This specific spacing was chosen to ensure minimal well-to-well interaction of the chemical plume formed during deployment. The port diameter of each well is 800 µm with a depth of 1.6 mm. To produce the ISCA plate, a mixture of polydimethylsiloxane (PDMS) and curing agent was prepared (10:1 PDMS base to curing agent, Sylgard 184, Dow Corning, Salzburg, Austria). The mixture was put into a vacuum chamber for 30 minutes to get rid of any bubbles produced during the mixing process. As soon as the mixture was completely clear and no bubbles were visible, 26 g of the PDMS and curing agent mixture were filled into the mould (Fig. 4A). Curing was conducted overnight at 40°C. The cured PDMS slice was carefully cut from of the mould using a razor blade. The port obstructions were opened using a biopsy punch of 800 µm diameters (ProSciTech, Kirwan, QLD, Australia). After that, the PDMS slice was plasma-bonded to sterile microscope slides (100 mm x 76 mm x 1 mm, VWR, Radnor, PA, USA) by exposing both, the PDMS slice and the microscopic glass to oxygen plasma for 5 min using a plasma sterilizer (Harrick Scientific, New York, USA). After bonding, the slice was heated at 90°C for 10 minutes to ensure the formation of covalent bonds between the two surfaces (Fig. 4B) (Lambert et al. 2017).

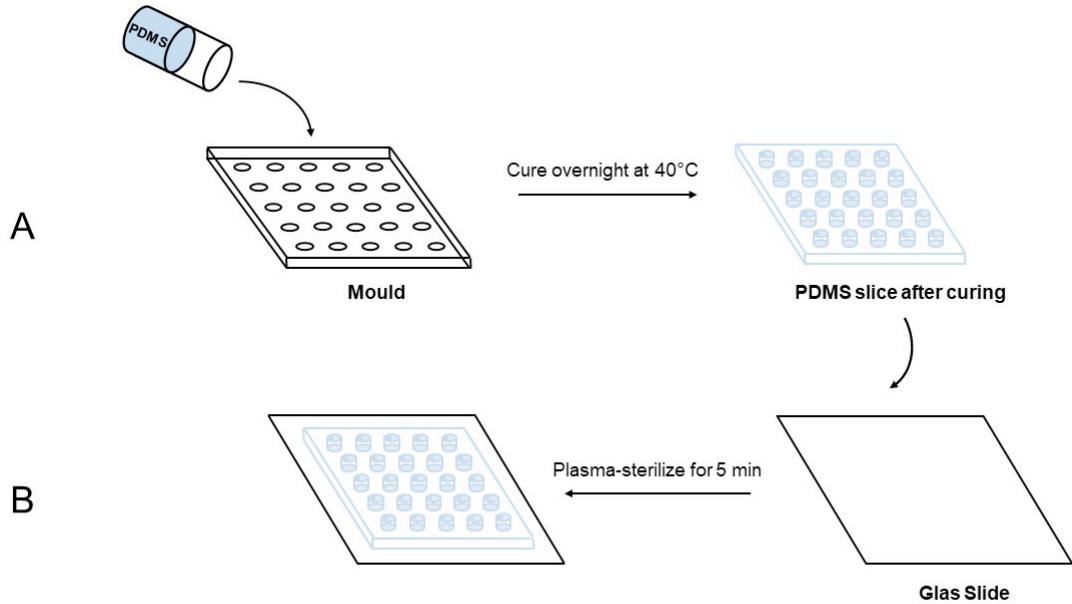


Figure 4: Production of in situ chemotaxis assay (ISCA) plates. A) A mixture of polydimethylsiloxane (PDMS) with curing agent is poured into a mould. The mixture is cured overnight. B) The resulting solid PDMS slice is plasma-bonded onto a microscopic glass slide, generating covalent bonding between the two surfaces.

2.2 Preparation of substrate stock solutions

Stock solutions of various substrates were prepared in different concentrations (10 µM, 100 µM, 1 mM) in order to use them as chemoattractants for ISCA. The stock solutions were prepared with sterile deionized water (Milli-Q, Sigma- Aldrich) and stored at -20°C until further use.

- L-Glutamine (Gln), BioUltra, Sigma-Aldrich, ≥ 99,5%
- D(+)-Glucose monohydrate (Glc)
- N-Acetylglucosamine (NAG), Cell Culture Tested
- L-Leucine (Leu), (S)-2-Amino-4-methylpentanoic acid, Sigma-Aldrich, ≥ 98%
- L-Arginine (Arg), (S)-2-Amino-5-guanidinopentanoic acid, Sigma-Aldrich, ≥ 98,5-101,0%
- Putrescine (Put), 1,4-Diaminobutane dihydrochloride, Sigma-Aldrich, ≥ 99.0% (AT)
- Potassium phosphate dibasic (inorganic P), Sigma-Aldrich

2.3 In situ chemotaxis assay (ISCA) – Experimental design

2.3.1 Sampling

For the chemotaxis experiment 5 L of lake water were taken from the regular sampling site of Lake Zurich ($47^{\circ}31' N$, $8^{\circ}58' E$) near the deepest point (Posch et al. 2012; Yankova et al. 2017). Lake Zurich is a large, prealpine, oligomesotrophic lake with a maximal depth of 136 m and a surface area of 66.8 km^2 . Due to climate change, the annual natural complete water turnover is hindered. Increasing temperatures have led to the accumulation of the cyanobacterium *Planktothrix rubescens*, which represents the dominant phototrophic organism in the lake (Posch et al. 2012).

2.3.2 Preparation and incubation of ISCA plates

1 L of lake water was filtered using a sterile filter tower with a polyethersulfone (PES) membrane (Sartolab RF/BT Filter System, 0.1 μm , Göttingen, Germany) under constant pressure at 100 mbar. Filtered lake water (FLW) was amended with according substrates to a final concentration of 10 μM and the mixture was filled into the wells of the ISCA plate with sterile syringes (0.01 ml – 1 ml, Injekt-F, Braun, Hessen, Germany) and a hypodermic needle (0.24 x 13 mm, TSK STERiJECT, Oisterwijk, The Netherlands). The wells were filled until a drop emerged through the port to ensure that no air was trapped in the wells. Five wells within the same row were filled with the same substrate. In a first trial, wells were filled horizontally with the same substrate. In subsequent experiments, substrates were filled vertically to prevent potential contamination by diffusion through the pore (Fig. 5). Unamended FLW was used as negative control (Fig. 5A). Subsequently, plates were incubated in triplicates individually in 2000 ml beakers (SIMAX, Prague, Czech Republic) in 1.4 L of fresh unfiltered lake water for 1 h (Fig. 5B). After the incubation, ISCA plates were carefully removed and the samples from the five wells within a column were collected and pooled together into a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany) by using 200 μl pipette tips. Half of the sample was used for cell count analysis by flow cytometry and the other half for sequencing and bacterial diversity analysis (Fig. 5B).

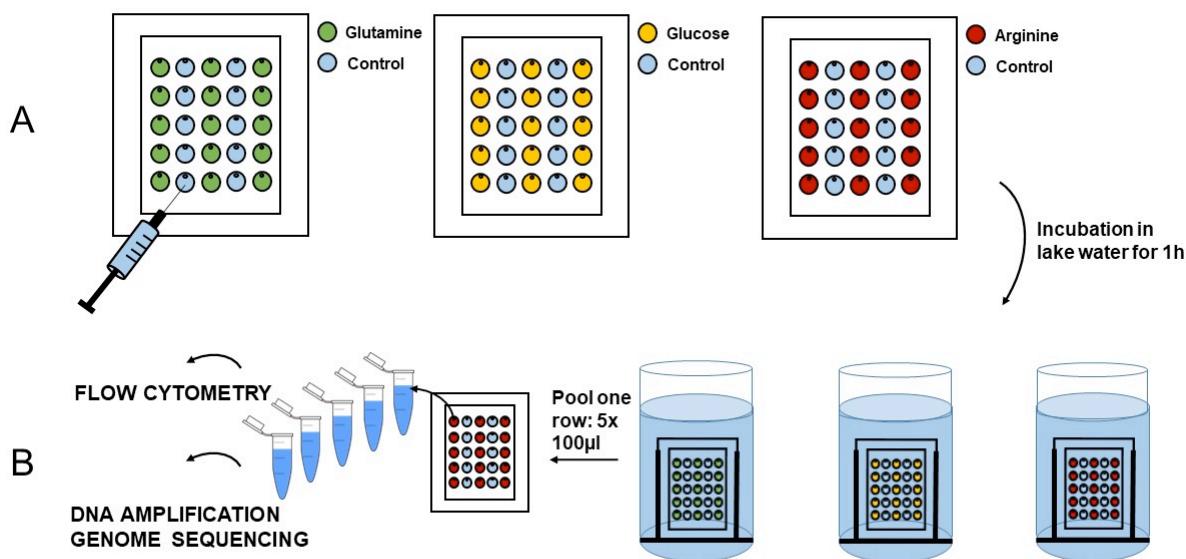


Figure 5: Scheme of the *in situ* chemotaxis assay (ISCA) workflow shown for three different substrates. A) Glutamine, glucose and arginine were filled each in three different ISCA plates. Filtered lake water (FLW) was used as negative control. B) The plates were incubated in lake water for 1 h. The samples of the five wells were collected and pooled for flow cytometry and DNA sequencing.

2.3.3 Flow cytometry

To quantify the strength of chemotactic accumulation within each well, cell counts were enumerated by flow cytometry (Lambert et al. 2017) (Fig. 6). By comparing cell counts between loaded wells and control, we could assess if motile bacteria were able to swim into the wells.

Samples of 100 µl were fixed with a formaldehyde solution (FA, ≥ 37 %, Carl Roth GmbH, Karlsruhe, Germany) to a final concentration of 2 %. Subsequently the samples were stained with 5 µl of 10x SYBR Green (5×10^{-5} dilution of commercial stock, Invitrogen, Oregon, USA). Cell counts were determined by a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA, USA) and analysed by the software CytExpert 2.0 (Beckman Coulter, Bream CA, USA). Bacteria normally consist of two distinct groups, the high nucleic acid (HNA) and the low nucleic acid (LNA) bacteria (Salcher et al. 2011). Figure 6 shows a cytogram where background noise was removed and gates were set for the HNA (green) and the LNA (dark green) bacteria of the total bacterial community (red). Given that SYBR Green intercalates with the DNA, both groups vary significantly in fluorescence, thus nucleic acid content (Lebaron et al. 2001). The gates were drawn around dense spots in the cytogram (Fig. 6). For the ISCA to be successful, the cytogram should show a distinct pattern of HNA. The

motile fraction of the bacteria are most likely amongst the HNA and therefore should display a chemotactic response towards a chemoattractant.

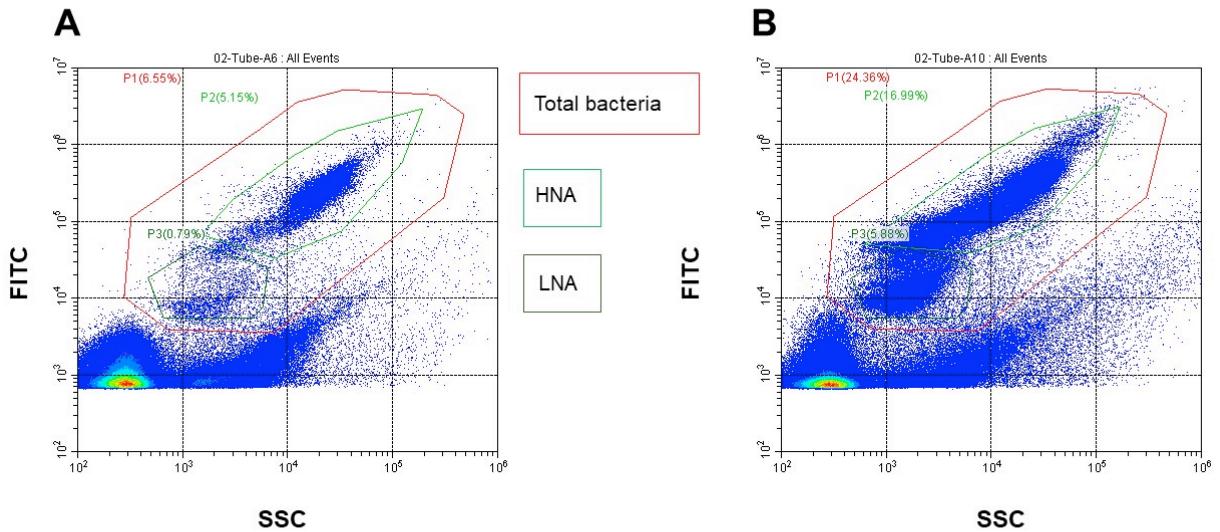


Figure 6: Example of two cytograms with gate settings for total bacteria (red), high nucleic acid (HNA) (green) and low nucleic acid (LNA) bacteria (dark green). X-axis: SSC=488/8 nm, y-axis: FITC=525/40 nm; A) Example of a cytogram showing the bacteria which were present in the well with no substrate (control). B) Example of a potential chemotactic response towards a substrate. The motile bacteria are most likely amongst the HNA (top right accumulation).

2.3.4 Enrichment experiment

The aim of this experiment was to test the efficiency of the ISCA. Since many of the performed ISCA experiments were unsuccessful, we wanted to assess the technical parameters of ISCAs under ideal conditions. To create ideal conditions, generally more cells had to be found in the wells in order to detect the differences in cell counts above the baseline. For this reason, we enriched lake water overnight to allow the cells to grow.

The overnight enrichment of 10 L of lake water was performed with Luria-Bertani (LB)-medium (0.1 % LB final concentration) (Mitchell et al. 1996). Three ISCA plates were deployed with substrate amendments of 100 µM Gln, 5%, 10% and 20% of LB-medium. As a control, FLW was used. The lake water and LB-medium was double-filtered, first with a 0.2 µm Sterivex filter then with a 0.2 µm Millex FG (Millipore) and each of the same substrates were spaced within a row (Fig. 7). The ISCA plates were incubated in enriched lake water for 1 h and then processed as mentioned above (see chapter 2.3.2)

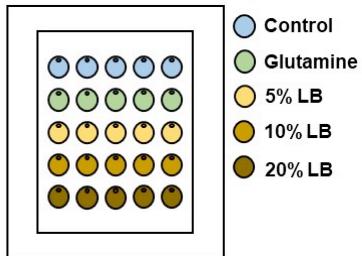


Figure 7: Plate set-up for the enrichment experiment. Rows are filled with control, 100 µM glutamine and 5 – 20% LB-medium

2.4 Chemotaxis assays

Different chemotaxis assays were developed and performed additionally to the ISCA. The objective of developing other assays was to see if previously obtained isolates from earlier ISCA experiments could be back-tested and identified as motile bacteria, which perform chemotaxis. The principal basis for most chemotaxis assays is the capillary assay. During the past years other assays followed with alterations like increasing the number of capillaries for each individual experiment (Malmcrona-Friberg et al. 1990) and newly developed assays use microfluidic-based platforms (Stocker et al. 2008). We followed the idea of the capillary assay and tried to test it for chosen motile freshwater isolates of Lake Zurich.

2.4.1 Cultivation of different bacterial strains for chemotaxis assays

For all the chemotaxis assays (except ISCA) the same culture medium was used, consisting of a mixture of 1 g nutrient broth (Merck, Darmstadt, Germany), 1 g soytone (Becton, Dickinson and Company, Sparks, USA) and 1 g yeast extract (Merck, Darmstadt, Germany) (NSY) dissolved in 1 L of inorganic basal medium (NSY in IBM medium) according to Hahn et. al. (Hahn et al. 2004). For more information see Table A11 and A12 in the Supplementary materials.

Bacterial candidates for the chemotaxis assays were *Paenibacillus* sp., *Rhodoferax* sp., *Modestobacter* sp., *Bradyrhizobium* sp. and *Flavobacterium* sp. They were isolated by previous ISCAs and exhibited motile behaviour during direct observation under the microscope.

For the re-inoculation of bacterial strains 24-well plates (VWR Internationals, Radnor, PA, USA) were prepared in triplicates for each bacterial strain by filling each well with 1 ml of the culture medium mentioned above (NSY in IBM medium). 10 µl of the bacterial liquid culture were used for inoculation. The re-inoculated cultures were incubated for two days at room temperature (RT) on a shaker (20 rpm) to let them grow. Turbid wells were chosen and the cultures were screened under the inverted microscope (10 – 80x magnifications, Zeiss 2 Axio

Vert. A1, Carl Zeiss AG, Germany) for motility and cell homogeneity, indicative for a potential pure culture. If a culture exhibited heterogeneous cell shapes and sizes, the liquid culture was used to inoculate agar plates with the same NSY in IBM medium concentration as described above. The culture was plated with inoculation loops by constantly diluting the bacterial inoculum within one plate in order to obtain single distinct colonies after incubation. Some of these distinct colonies were then picked and inoculated in new liquid NSY in IBM medium to assess fresh liquid cultures.

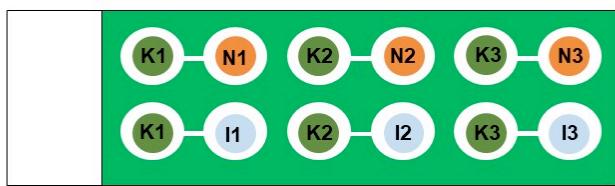
Modestobacter sp. was chosen as the best candidate for chemotaxis assays due to its unambiguous motility and growth. *Rhodoferax sp.* and *Bradyrhizobium sp.* seemed to have lower growth rates, reached overall lower cell abundances and only few cells were motile. *Paenibacillus sp.* and *Flavobacterium sp.* were also not as active as *Modestobacter sp.*

To assure a clear chemotactic reaction, the motile bacterial strain has to reside in a nutrient poor environment first and starve. As soon as a nutrient source of higher concentrations is added, bacteria sense the higher nutrient concentrations chemotactically and swim keenly to the nutrient source (Adler 2008). To achieve this starvation mode, *Modestobacter sp.* was washed by centrifugation or dilution and re-suspended in 1 % IBM medium (Kjelleberg and Hermansson 1984). The centrifugation step at 12'000 rpm for 10 minutes seemed to be too destructive because the strain did not show any motility afterwards. For this reason, the strain was diluted 10 times with 1 % IBM.

2.4.2 Multi-well assay

Multi-well microscopic slides (Immuno-Cell, Mechelen, Belgium) with 2 x 6 wells were used for this chemotaxis assay.

A channel of around 500 µm was scored by a razor through the coating of the glass slide to connect the two adjacent wells (Fig. 8). On the left well of the two connected wells, 2.5 µl of a fresh diluted *Modestobacter sp.* culture was applied (Fig. 8, green "K"). On the right well, 2.5 µl of NSY in IBM medium was applied to act as chemoattractant (Fig. 8, orange "N"). Subsequently, a cover slip was placed on top of the microscopic slide to generate a connection between the fluids.



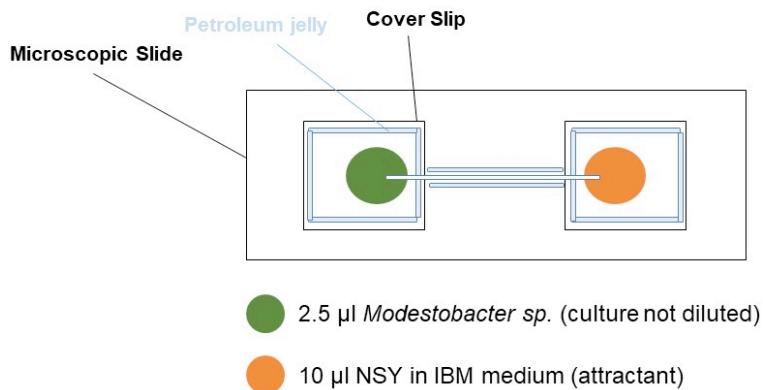
- 2.5 µl *Modestobacter* sp. (diluted 10x with IBM)
- 2.5 µl NSY in IBM medium (attractant)
- 2.5 µl 1 % IBM (control)

*Figure 8: Experimental set-up of the multi-well microscopic slide. K1-K3 represent the bacterial isolate *Modestobacter* sp., N1-N3 represent the attractant nutrient broth, soytone and yeast extract (NSY) in inorganic basal medium (IBM) and I1-I3 the control.*

The experiment was conducted in triplicates: K1-K3 represent the wells where *Modestobacter* sp. culture was placed and N1-N3 the wells where the NSY in IBM medium was set, which should act as an attractant (Fig. 8). In the second row instead of NSY in IBM only 1% IBM (sterile-filtered) was used as a control. After 1 h the slide was examined under the microscope for a development of a bacterial gradient towards the attractant. As a reference, a bacterial gradient towards the control should not develop. To quantify chemotaxis, photos were taken at the beginning (t_{0h}) and the end of the experiment (t_{1h}).

2.4.3 Capillary assay with petroleum jelly

The bacterial strain was initially not diluted to see if the microscope could actually resolve cells in the capillary. On a microscopic glass slide the attractant (NSY in IBM medium) and the bacterial culture were applied in a volume of 10 µl and 2.5 µl (Fig. 9). A thin capillary of about 100 – 500 µm diameters was created by holding a glass Pasteur pipette under the flame of a Bunsen burner for a few seconds and simultaneously pulling the capillary apart on both ends. A barrier was drawn using petroleum jelly and a cover slip was put each over the inoculum and the medium (Fig. 9). The slide was observed in real-time under the inverted microscope.



*Figure 9: Experimental set-up of the chemotaxis assay with a capillary and petroleum jelly. The drop with the *Modestobacter* sp. culture (green) was linked to the attractant nutrient broth, soytone and yeast extract (NSY) in inorganic basal medium (IBM) (orange).*

2.4.4 Vertical capillary assay

The capillary is filled with an attractant, which is inserted into a suspension of a bacterial culture. The attractant forms a spatial gradient, which is sensed by the bacteria. They start to migrate up and enter the tube (Bainer et al. 2003).

A Pasteur pipette made out of glass was applied as a capillary. 1 ml of NSY in IBM medium was used as attractant and 1 % IBM as control. A rack was required to stabilize the capillary, which was dipped into 2.5 – 5 µl of bacterial culture (Fig. 10). The opening of the glass pipette was carefully sealed with petroleum jelly to ensure capillary forces. After 30 – 60 minutes of incubation time the content of the glass pipette was emptied and observed under the inverted microscope.

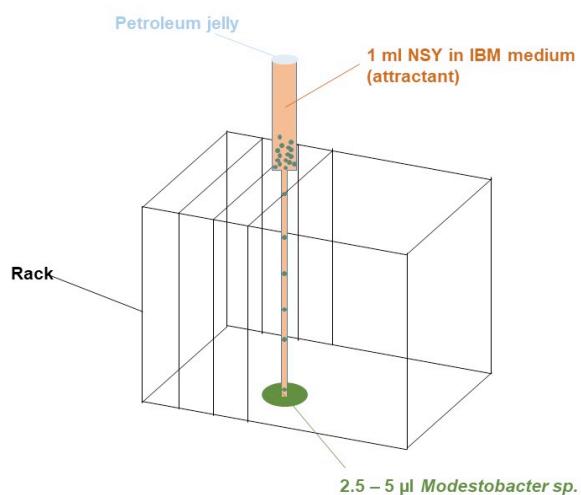


Figure 10: Experimental set-up of the vertical capillary assay. Glass capillary filled with attractant nutrient broth, soytone and yeast extract (NSY) in inorganic basal medium (IBM) (orange) is dipped into a bacterial culture (green). Bacteria should migrate upwards through the tube by chemotaxis.

2.5 Incubation experiment

The objective of the incubation experiment was to create a transcriptome and metagenome of two distinct time points (t_{1h} and t_{4h}) with addition of a given substrate. The aim was to find gene regions, which are expressed during Gln uptake and link the up-regulation of Gln transporters of according bacteria to their chemotactic ability or inability. The focus was to gain background information to complement the metagenome and transcriptome data. The samples obtained from the same incubation experiment were further processed for MAR-FISH analysis to find distinct bacterial populations, which are involved in Gln uptake.

2.5.1 Sampling and procedure of the incubation experiment

The incubation experiment was performed in August due to potential increased motility, which underlies seasonal variability. The fraction of motile cells are larger in summer than in winter (Stocker and Seymour 2012).

The set-up contained 24 2000 ml bottles (Schott glass bottles, Sigma-Aldrich), which were pre-washed with 2 M HCl solution and additionally autoclaved. Lake water (80 – 85 L) was taken with a Friedinger-sampler from 5 m depth at the regular sampling site of Lake Zurich (47°31' N, 8°58' E) (Posch et al. 2012; Yankova et al. 2017) (Fig. 11). The water was filtered through a 32 µm sieve (Haver & Boecker, Oelde, Germany) and distributed into four 25 L-containers. Pre-filtered lake water was distributed into 24 bottles (Fig. 11). Around 10 ml of water sample was taken at first, fixed with FA (37 %, final concentration of 2 %) and counted by flow cytometry to obtain background information about the bacterial abundance at this time point.

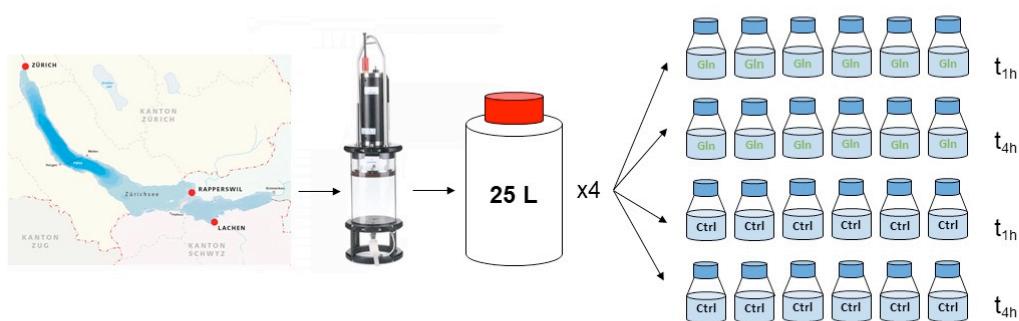


Figure 11: Schematic representation of the sampling for the incubation experiment. Lake water was taken with a Friedinger-sampler, filtered and distributed into four 25 L-containers. Pre-filtered lake water was distributed into 24 bottles, 12 of them were amended with glutamine (Gln), and the other 12 were used as negative controls (Ctrl).

Six bottles were prepared for the first (t_{1h}) and second time point (t_{4h}) with amended Gln as well as control (no added substrate) (Fig. 12). Gln was added to a final concentration of 10 μ M. After 1 and 4 h of incubation, six bottles of each substrate and control were filtered with a delivery pump (BVP Standard, ISMATEC, Wertheim, Germany) for 10 minutes at level 9 on 0.2 μ m polycarbonate-filters (GPWP, 45 mm diameter, Millipore, Merck, Darmstadt, Germany). During incubation, the bottles were kept in the dark at 18°C (Fig. 12). Afterwards, the filters were taken off very carefully with sterile forceps (they were held over a flame for a few seconds), put into 2 ml micro tubes (Biosphere, SARSTEDT, Nümbrecht, Germany) and temporarily stored in liquid nitrogen. At the end of the experiment, the filters were stored at -80°C and used later for RNA-seq (RNA extraction, RNA depletion, RNA sequencing) (Fig. 12). In addition, time point 0 (t_0) was also considered in the beginning of the experiment. Six RNA- and two DNA-filters were produced at t_0 meaning they were filtered immediately with the delivery pump without incubation and no substrates were added. This incubation experiment will be repeated around May 2018.

Water Sample	Incubation	Filtration	Processed Sample	Storage	Purpose
<i>Incubation Experiment</i>					
Ctrl Gln x6	1h 	Delivery pump 	RNA 6 x Gln / Ctrl		
Ctrl Gln x6	4h 		RNA 6 x Gln / Ctrl		
T ₀	x8	direct filtration	6 x RNA 2 x DNA	 →  → -80°C	Metagenomics Transcriptomics

Figure 12: Experimental set-up of the incubation experiment showing an overview of the water sample, incubation, filtration, processed sample, storage and purpose of each step. The bottles were incubated for either 1 or 4 h and filtrated with a delivery pump. In the end 6 x glutamine / control (Gln / Ctrl) RNA filters for each time point (t_0 , t_{1h} , t_{4h}) as well as two DNA filter for t_0 were processed, folded into micro tubes, kept temporarily in liquid nitrogen and stored permanently at -80°C.

2.6 Bacterial bulk activity

A scintillation counter (Tri-Carb 3170TR/SL, PerkinElmer, Germany) was used to measure the radioactivity in DNA in order to measure the extent of cell division (growth rate) that has occurred in response to radiolabelled Gln addition. The counter measures the radioactive 3 H-thymidine, which was incorporated into new strands of chromosomal DNA during mitotic cell division (Kirchman 2001). The idea was to have a first overview of the incubation rate of Gln and decide if MAR-FISH should be applied or not depending on the presence of an activation for the substrate after 4 h.

2.6.1 Samples for scintillation counter measurements

Water samples of 10 ml were taken in triplicates from the incubation experiment in August (see chapter 2.5). Triplicates of each treatment as well as two prefixed (37 % FA, final concentration 2 %) negative control samples for each time point were incubated in the dark with ^3H -glutamine (^3H -Gln; 10 nM; specific activity 60 Ci mmol $^{-1}$, ANAWA / Biotrend, Wangen, Switzerland), which was added in a concentration of 10 nM (Fig. 13A).

To measure bulk incorporation rates the samples were filtered at low pressure (< 200 MM Hg) onto 0.2 μm nitrocellulose filters (GSWP, Millipore, Merck, Darmstadt, Germany) (Fig. 13A). The filters were washed twice with 3 ml of ice-cold trichloroacetic acid (TCA 5%) and twice with 3 ml of ice-cold EtOH (80%). After filtration, the filters were air dried and put into 5 ml scintillation vials (VWR Internationals, Radnor, PA, USA). 0.5 ml ethyl acetate was added in a way that the filters were completely covered. They were dissolved overnight by the ethyl acetate. This extraction method was described in Kirchman (2001).

After one night of incubation, 4 ml of scintillation cocktail was added to the samples and measured with the scintillation counter (Fig. 13A).

Water Sample	Incubation	Filtration	Processed Sample	Storage	Purpose
<i>Scintillation Counter</i>					
A			3 x ^3H -Gln (1h) 3 x ^3H -Gln (4h) 2 x Ctrl		Measurement of the ^3H -Gln incorporation rate
<i>MAR-FISH</i>					
B			3 x ^3H -Gln (1h) 3 x ^3H -Gln (4h) 2 x Ctrl		Discovery of distinct bacterial communities involved in Gln uptake

Figure 13: Experimental set-up of the scintillation counter measurements and MAR-FISH experiment showing an overview of the water sample, incubation, filtration, processed sample, storage and purpose of each step. A) For scintillation counts water samples were amended with radiolabelled glutamine (^3H -Gln) in triplicates for each time point ($t_{1\text{h}}$ and $t_{4\text{h}}$). Duplicates for each time point were proceeded as well as negative controls (Ctrl). the filters were immediately processed with the scintillation counter. B) For MAR-FISH experiments, the same set up was used and the filters were air-dried and stored at -20°C.

2.7 Sample and filter preparation for MAR-FISH

Water samples of 10 ml were taken in triplicates from the incubation experiment in August (see chapter 2.5.1) and put into 15 ml falcon tubes (TPP, Trasadingen, Switzerland) for MAR-FISH filters and incorporation experiments. 6 µl of ^3H -Gln were added in the dark.

The treatments were incubated for 1, respectively 4 h in the dark and fixed afterwards with FA (37%, final concentration 2 %) (Fig. 13B). Controls were taken in duplicates from bottles, fixed immediately with FA and incubated for 1, respectively 4 h. 3 ml of each treatment was filtered onto polycarbonate-filters (GTTP, 0.2 µm pore size, 25 mm diameter, Millipore, Merck, Darmstadt, Germany), washed twice with 1 ml sterile pre-filtered deionized Milli-Q water (0.2 µm polycarbonate syringe attachment filter, Whatman, Maidstone, United Kingdom), air dried and stored at -20°C until further processing (Fig. 13B).

For CARD-FISH (FISH combined with catalysed reporter deposition) experiments, filters (5 m depth) from the regular sampling campaign of Prof. Thomas Posch (Posch et al. 2012; Yankova et al. 2017) were used since they were produced only two days after the incubation experiment (16/08/17).

2.7.1 Oligonucleotide probes used in (MAR-) FISH experiments

Different oligonucleotide probes were used for CARD-FISH to obtain a first broad impression about the communities present in Lake Zurich. Different oligonucleotide probes were used for MAR-FISH to identify general and specific bacterial groups, which took up ^3H -Gln (Table 1). For more information about each probe (specificity, % formamide in HB and reference) see Table B4 and B5 in the Supplementary materials.

Table 1: Listing of the different oligonucleotide probes used for CARD- and MAR-FISH.

Probes for CARD-FISH	Probes for MAR-FISH
GAM42a	
CF968	BET42a
LimA-23S-1435	
R-BT065	acl-853
Bet2-870	
Cyc715	R-BT065
ALF968	
LD12-115	ALF968
acl-853	
acl-B1-23S-2669	LD12-115
acl-A7-23S-1420	

2.8 CARD-FISH

Relative abundances of different bacterial subgroups were determined by CARD-FISH according to the protocol published by Sekar *et al.* (Pernthaler et al. 2002; Sekar et al. 2003). Depending on the probes used for CARD-FISH, several modifications were implemented in the protocol. To minimize background signals, the filters hybridized with acl and LD12 were washed for 60 instead of 30 minutes in the preheated washing buffer. To obtain stronger FISH-signals the formamide concentration of the hybridization buffer was decreased by 5 %.

2.9 MAR-FISH

2.9.1 Preparation of slides for autoradiograms

MAR-FISH was performed based on the protocol established by Alonso and Pernthaler (2005) with modifications by Salcher et al. (2008). After successfully completing the CARD-FISH protocol (Fig. 14A), the filters were embedded in 2 % agarose (Standard Agarose, Type LE, BioConcept). For this a clean microscopic cover slip (24 x 600 mm, Menzel-Gläser, Thermo Fisher Scientific, Waltham, United States) was dipped into molten 2 % agarose with the hybridized filter sections faced down. The cover slips were dried at 40°C. Accordingly, the outline of the filters were drawn and the filters were carefully peeled off from the cover

slip. The cover slip was then attached to a microscopic glass slide (76 x 26 mm, Thermo Fisher Scientific) with the bacteria faced up. Meanwhile an aliquot of 20 ml of the autoradiography emulsion (Type NTB, Carestream, NY, USA) was heated for 60 minutes in a water bath at 43°C.

The following steps were executed in the dark: The first slide was put into the autoradiography emulsion and gently agitated for several seconds in a way that the whole slide was covered. After that, the slide was taken out and put into a light-tight box. This procedure was conducted for all of the slides. The slides were exposed for only one day at 4°C in the dark for the formation of a latent image (Fig. 14B). This incubation time was modified due to high uptake activity of the bacteria.

2.9.2 Development of samples

The following steps were executed in the dark: The slides were put in the developer (Kodak Professional Dektol Developer, Eastman Kodak Company, NY, United States) for 2 minutes, in Milli-Q for 10 seconds, in fixer (Kodak Professional Fixer, Eastman Kodak Company, NY, United States) for 5 minutes and finally in Milli-Q for 5 minutes. This procedure should produce silver grains, which arrange around cells with a radiation source (Fig. 14B).

After turning on the lights, the slides were air-dried and the cover slip was removed from the slide and embedded in DAPI-Mix (Table B1, Supplementary materials) for microscopy.

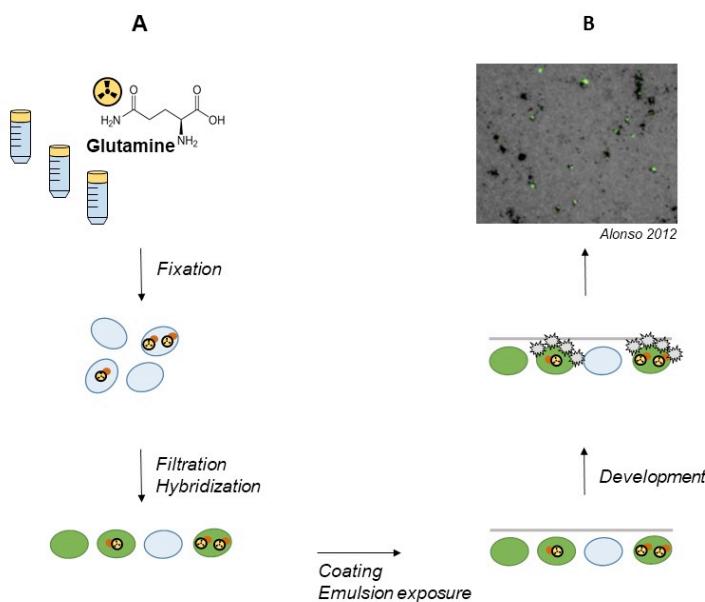


Figure 14: Representation of the MAR-FISH procedure. A) The sample is incubated with radiolabelled glutamine (^3H -Gln) and fixed afterwards. Some cells incorporated ^3H -Gln, some not. The lake sample is filtered onto small filters and hybridized with different probes. Some cells show a positive signal after CARD-FISH. B) The hybridized filtered are coated and exposed to autoradiography emulsion to form a latent image. Through a development process, silver grains are arranged around cells with a radiation source. The MAR-positive cells are counted with an epifluorescence microscope.

2.9.3 Microscopic evaluation

CARD-FISH

After CARD-FISH the hybridized filter sections were embedded in DAPI-mix (composition see Table B1, Supplementary materials) and automatically evaluated based on the method published by Zeder and Pernthaler (2009).

A fully automated high-throughput microscope platform (Zeiss Axio Imager.Z2) was used for image acquisition (Zeder and Pernthaler 2009) and three images were obtained on each filter sections with different excitations: UV excitation (358 nm) for DAPI-stained cells, blue excitation (488 nm) for CARD-FISH stained cells and green excitation (565 nm) for detecting cells with autofluorescence since they can produce false-positive FISH signals. The autofluorescence cells were later subtracted from the hybridized cells. Filter sections with at least 10 high quality images (maximum of 36 high quality images) were evaluated with an image analysis tool programmed (AICQ) by Michael Zeder (unpublished, <http://www.technobiology.ch>). The image analysis tool IMI5.4 (Zeder et al. 2010) was used to overlap the DAPI-, FISH-positive and autofluorescent cells.

MAR-FISH

After MAR-FISH the filter sections were embedded in DAPI-mix and automatically evaluated with the high-throughput microscope platform. Three images were obtained on each filter sections with different excitations: UV excitation (358 nm) for DAPI-stained cells, blue excitation (488 nm) for CARD-FISH stained cells and instead of green excitation (565 nm) for autofluorescence cells, bright field illumination was used to detect silver grains. Filter sections with at least 10 high quality images (maximum of 45 high quality images) were evaluated with an image analysis tool programmed (AICQ) by Michael Zeder (unpublished, <http://www.technobiology.ch>). The image analysis tool IMI5.4 (Zeder et al. 2010) was used to overlap the DAPI-, FISH-positive cells and silver grains. MAR-FISH slides, which could not be evaluated with the fully automated high-throughput microscope (due to resolution issues) were analysed manually at 1000x magnification with an epifluorescence microscope (Zeiss Axio Imager.M1). Per sample, at least 500 DAPI-stained cells were counted. Out of these cells the fraction of the hybridized cells with silver grain formation were calculated with a simple rule of three calculation.

3 Results

3.1 ISCA experiments

During the practical work of my master thesis six assays were performed (Table 2: Overview). The very first experiment aimed to find the most suitable substrate for the ISCA. The following assays were conducted to reduce variability within and between the plates and to discover the appropriate substrate concentration. Various additional experiments were performed to obtain a positive signal for a substrate. The final assay was performed with a lake water enrichment to prove the feasibility of the ISCA approach. However, the method was not as applicable for Lake of Zurich as it was for the ocean. None of the experiments showed an unambiguous positive signal for chemotaxis. Several additional chemotaxis assays were developed to test if the problem was technical or biological.

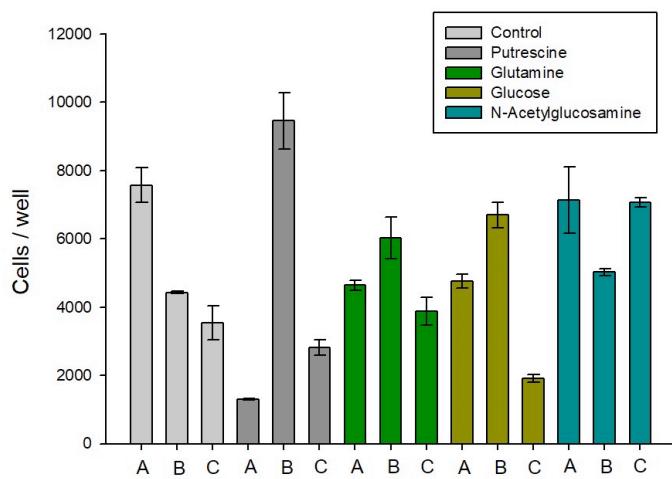
*Table 2: Overview of the different *in situ* chemotaxis assays (ISCAs) conducted over the course of the master project. The six experiments are listed with their aim, season/month, applied substrates, substrate-concentrations, water sample location and the filtration method used to filter the water sample. Used substrates: putrescine (Put), glutamine (Gln), glucose (Glc), N-acetylglucosamine (NAG), leucine (Leu), arginine (Arg), and 1- 20 % LB-medium.*

Aim	Month (2017)	Substrates	Substrate Concentration	Sample Location	Filtration Method
Suitable substrate	April	Put, Gln, Glc, NAG	10 mM	Lake Zurich Pelagial	PES filter system (0.2 µm)
Variability	June	Gln	10 mM		PES filter system
Substrate concentration	June	Gln, Glc, Arg, 1 % LB	10 µM 100 µM 1 mM		Hollow fibre (Spectrum Labs)
Positive signal I	June	Glc, Gln, Arg, NAG, Put, inorganic P	100 µM	Lake Zurich shore (Kilchberg)	PES filter system
Positive signal II	August	Glc, Gln, Put, NAG	100 µM		PES filter system
Enrichment	October	Gln, 5 – 20 % LB	100 µM		PES filter system

3.1.1 Suitable substrate

For this ISCA assay, the focus was placed on a substrate, that would evoke a bacterial chemotactic response. For this reason, a variety of different substrate groups were used: Polyamines (Put), amino acids (Gln), sugars (Glc) and amino-sugars (NAG), all in a concentration of 10 mM as suggested by Bennet Lambert. Previous studies could show that Put consistently dominated the pool of dissolved free polyamines in Lake Zurich and was utilized by *Alphaproteobacteria*, *Cyclobacteriaceae*, diatoms and cyanobacteria with short turnover times (Krempaska et al. 2017). Gln and Glc play an important role in bacterial physiology and previous studies could show that they were highly incorporated by *Alphaproteobacteria* and *Betaproteobacteria* (Salcher et al. 2013). High NAG concentrations were found during spring season and their uptake was mainly dominated by *Betaproteobacteria* and *Cytophaga-Flavobacteria* (Eckert et al. 2012). The water samples were taken from the pelagic zone at the regular sampling site of Lake Zurich (47°31' N, 8°58' E) (Posch et al. 2012; Yankova et al. 2017). We started the experiments in early spring since previous studies could show that the abundance of motile bacteria raised up to 70 % in spring and summer (Grossart et al. 2001). Lake water was filtered using a PES filter system. The cells were counted per well, which refers to 100 µl of content.

There was no big difference in terms of cell counts detectable between the treatment and the control (Fig. 15). Cell count variability was overall quite high in all treatments especially in the Put treatment with the highest counts of 9459 cells per well (Fig. 15, B) and the lowest counts of 1295 cells per well (Fig. 15, A). Cell abundances in the Gln-treatment displayed lesser variability between the three plates than the other tested substrates (Put, Glc, NAG). The Put-treatments displayed the most prominent cell count variability between the plates. Cell counts and their variability in the Gln-treatment (from 3877 to 6024 cells / well) are comparable with the Glc-treatment (from 1913 to 6694 cells / well) but the latter showed less cell counts in the third plate (1913 cells / well) (Fig. 15, C). The cell counts of NAG were variable between the plates (from 5030 to 7065 cells / well), the highest count was revealed in the third plate (Fig. 15, C). The central issue was the relatively high control in all three plates (\varnothing 5180 cells / well) compared to the control from the August 2016 experiment (425 cells / well). Fresh lake water and the filtrate were not measured with flow cytometry; the focus of this assay was on finding the suitable substrate. For more details see Table A2 in the Supplementary materials.



*Figure 15: Comparison of three *in situ* chemotaxis assay (ISCA) plates (A, B and C) conducted in April with cell counts (cells / wells) of the control, putrescine, glutamine, glucose, N-Acetylglucosamine. Error bars show standard deviations.*

3.1.2 Variability

The goal of the next experiment was to minimize the high variability detected in the first trial in April. To achieve this and also to improve the manipulation of the ISCA plates Eugen Loher, the engineer of the Limnological Station built a small crane (Fig. 16) with an attached motor to slowly deploy the plates at a controlled velocity and to minimize turbulence caused by clumsy hands.

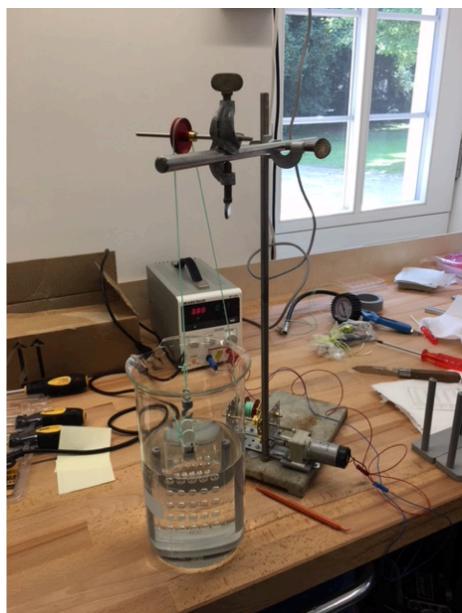


Figure 16: Picture showing the crane with an attached motor to deploy the plates at a controlled velocity into a beaker with fresh lake water.

Lake water was again collected from the shore of Lake Zurich (like the experiment in August 2016) and only Gln was used as a substrate in order to track variability more consistently. The assay was conducted in early summer (June) to ensure the presence of motile, chemotactic bacteria (Grossart et al. 2001). Lake water was filtered using a PES filter system.

The cell counts in the control-wells were quite low (\varnothing 496 cells / wells), and comparable to the control values of the ISCA assay from August 2016. Gln-treatment did not show any positive signal of doubled cell counts if compared to the control (Table 3).

Table 3: Overview of the cell counts (\varnothing Cells / well) for three in situ chemotaxis assay (ISCA) plates (Plate 1, 2 and 3). The average was calculated from the cell counts of each well. Glutamine (Gln) was used as the only substrate.

Treatment	\varnothing Cells / well		
	Plate 1	Plate 2	Plate 3
Gln	595 ± 200	391 ± 103	538 ± 169
Ctrl	591 ± 240	438 ± 90	422 ± 161
Gln	743 ± 316	1518 ± 2289 (outlier)	360 ± 87
Ctrl	674 ± 181	438 ± 12	416 ± 56
Gln	739 ± 283	458 ± 193	358 ± 37

3.1.3 Substrate concentration

The goal of this experiment was to score a positive signal with substrates to confirm the positive results in August 2016. Additionally, the aim was to discover the most matching substrate concentration. We used 10 μ M (as in previous experiments), 100 μ M and 1 mM of Gln, Glc and Arg. A negative (FLW) and positive control (1 % LB) were used in this experiment. This time, lake water was filtered with a hollow fibre (Spectrum Labs, Waltham, Massachusetts, USA) to reach a better filtration. Lake water was again taken from the shore of Lake Zurich and three plates (A, B, C) were used in total. Each plate was loaded with the respective substrate and slowly deployed in one beaker with fresh lake water (see chapter 2.3.2, Fig. 5).

Before starting the ISCA experiment, the contents of the syringes with the filtered lake water amended with the respective substrates were analysed by flow cytometry to estimate the

potential background in the syringes before the incubation (Fig. 17). Overall, there were higher cell counts present than in the previous ISCA (4700 – 6700 cells / well). The lake water sample revealed counts of around 2.7×10^6 cells ml⁻¹. For more details see Table A3 in the Supplementary materials.

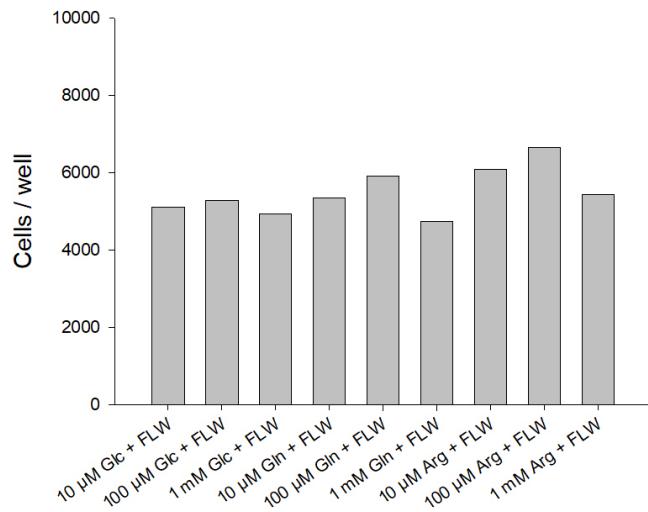


Figure 17: Background / content of syringe: Filtered lake water (FLW) amended with the according substrate before the incubation. Cell counts (cells / well) from the treatments with substrates: Glucose (Glc), glutamine (Gln) and arginine (Arg) in different concentrations (10 μ M, 100 μ M and 1 mM).

The controls in plate A and plate B (Fig. 18) showed high cell counts (5669 and 9012 cells / well) whereas the control in plate C (Fig. 18) was quite low (170 cells / well). 1 mM Glc revealed more cell counts followed by 1 mM Arg and 1 mM Gln (6242, 4606 and 3927 cells / well). 10 μ M Glc, Gln and Arg showed similar results in all plates (5100 – 5450 cells / well). 100 μ M Glc, Gln and Arg displayed more cell counts than the substrates with concentrations of 1mM and 10 μ M (6500 – 6800 cells / well) (Fig. 18). The positive control (1 % LB) did not show a positive chemotactic response (259 – 1175 cells / well). For more details see Table A4 in the Supplementary materials.

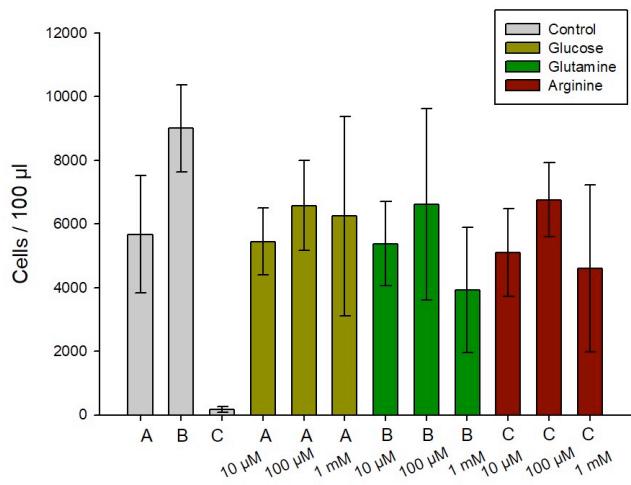


Figure 18: Comparison of three *in situ* chemotaxis assay (ISCA) plates (A, B and C) conducted in June with cell counts (cells / well) of the substrates glucose, glutamine and arginine in different concentrations (10 µM, 100 µM and 1 mM) and the corresponding control (Ctrl A, B, C). Error bars show standard deviations.

3.1.4 Positive signal I

The aim of this experiment was to deploy the ISCA again and see if we could obtain a positive signal with the right substrate concentration tested in the previous ISCA experiment (see chapter 3.1.3). Lake water was taken from the shore and filtered with the PES filter system. The filtration system of the first two ISCA experiments was resumed, since the filtration with the hollow-fibre did not seem to work efficiently (see chapter 3.1.3).

Six different substrates were tested at a concentration of 100 µM: Glc, Gln, Arg, NAG, inorganic phosphate (P) and Put. Additionally, inorganic P was used since previous studies have shown that diverse populations of lake water bacteria also exhibit chemotaxis towards inorganic nutrients (Dennis et al. 2013). The number of plates was increased to raise the chance of a positive signal. Six plates were used in total. Each plate was loaded with the according substrate and slowly deployed in one beaker with fresh lake water (see chapter 2.3.2, Fig. 5). Since we increased the number of plates, two were placed in one beaker with fresh lake water.

Before starting the ISCA experiment, the contents of the syringes with the filtered lake water amended with the according substrates were analysed by flow cytometry to estimate the potential background in the syringes before the incubation. The cell counts of the syringes were acceptable; it did not show contamination (100 – 250 cells / well), they were much smaller than in the previous experiment (Fig. 17). The FLW was low as it should be (1023 cells / well) and the substrates alone showed no contamination (Fig. 19), they displayed very

low cell counts up to almost nothing (0 – 80 cells / well with Arg: 343 cells / well as an outlier). The lake water sample revealed counts of around 2.8×10^6 cells ml⁻¹. For more details see Table S5 in the Supplementary materials.

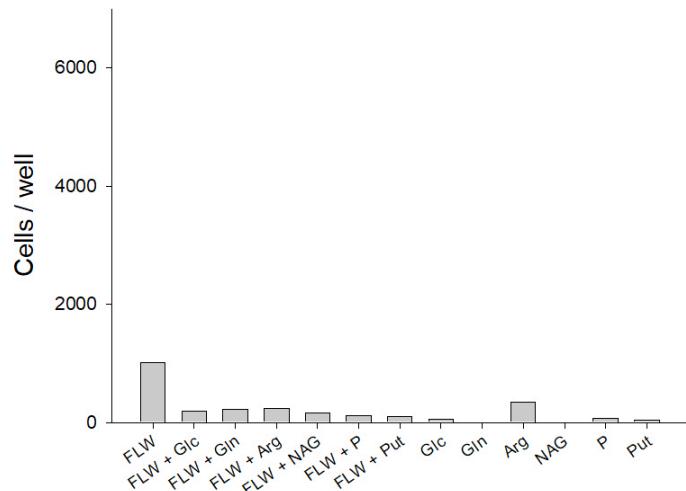


Figure 19: Background (content of syringe) before the incubation. Cell counts (cells / well) of filtered lake water (FLW) alone, FLW amended with glucose (Glc), glutamine (Gln), arginine (Arg) N-acetylglucosamine (NAG), inorganic phosphate (P) and putrescine (Put) and substrates alone are shown.

Cell count variability was low (mind the y-axis, FLW with the highest cell count showed 1023 cells / well) within and between plates and there were only slight differences distinguishable between the substrates (Fig. 19). The first ISCA plate with Glc-treatment displayed no big differences between the treatment and the control (\emptyset Glc: 222; \emptyset Control: 248 cells / well) (Fig. 20). The plate with Gln-treatment did not reveal the minimum increase of doubled cell counts in the treatment compared to the control, which is needed for a positive signal (\emptyset Glc: 254; \emptyset Control: 160 cells / well) (Fig. 20). Likewise, the plate with Arg did not show a positive signal for the treatment (\emptyset Arg: 174; \emptyset Control: 134 cells / well) (Fig. 20). Cell counts of the NAG-treatment and the corresponding control were almost the same (\emptyset NAG: 313; \emptyset Control 318 cells / well) (Fig. 20). Inorganic P-treatment revealed more cell counts than its control but the abundance was too low for a positive signal (\emptyset inorganic P: 309; \emptyset Control 183 cells / well) (Fig. 20). The last plate showed again no differences between the treatment and the control (\emptyset Put: 187; \emptyset Control 159 cells / well) (Fig. 20). Cell counts were quite low in all of the six plates. For more details see Table A6 in the Supplementary materials.

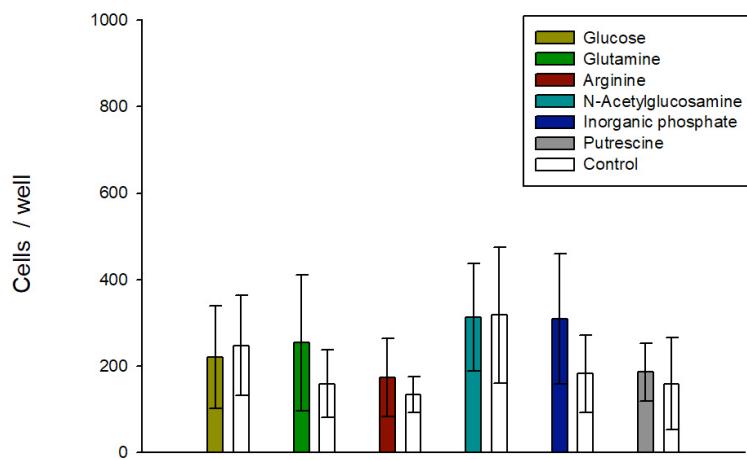
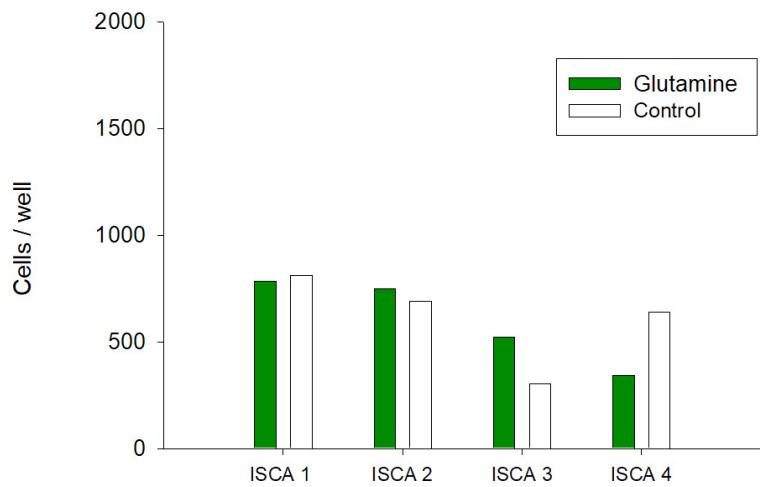


Figure 20: Comparison of six in situ chemotaxis assay (ISCA) plates conducted in June with average cell counts (cells / well) of glucose, glutamine, arginine, N-Acetylglucosamine, inorganic phosphate and putrescine as substrates and the corresponding control. The average was calculated from the cell counts of each well and treatment. Error bars show standard deviations.

3.1.5 Positive signal II

Since the only positive signal was achieved just in August 2016, it was decided to repeat the experiment one more time during the same time period in August 2017. The treatments with the same substrates were pooled after the incubation and analysed with flow cytometry. Two plates were used for a pre-experiment to choose which substrate obtained better signals at the very same experimental day. From Glc, Gln, Put and NAG as substrates, Gln was chosen for the actual experiment due to elevated cell counts compared to the other treatments. Four plates were deployed in total (ISCA 1, 2, 3 and 4) and the wells of each row with the treatment were pooled in the end for flow cytometry. However, no doubling of cell counts was reached in the Gln-treatment compared to the control. In ISCA 1 and 2 (Fig. 21) the cell counts of the Gln-treatment and the control were almost the same (ISCA 1-Gln: 785, ISCA 1-Control: 811; ISCA 2-Gln: 748, ISCA 2-Control: 690 cells / well). ISCA 3 displayed a small increase in cell counts after Gln-treatment (Gln: 523; Control: 305 cells / well) and ISCA 4 even a decrease (Gln: 343; Control: 641 cells / well) (Fig. 21). For more details see Table A7 in the Supplementary materials.



*Figure 21: Comparison of four *in situ* chemotaxis assay (ISCA) plates conducted in August 2017 with cell counts (cells / well) of glutamine as the substrate and its corresponding control (Ctrl).*

The FLW showed much more cell counts (Fig. 22) than in the previous experiments (2335 cells / well) but the FLW with amended Gln displayed surprisingly less (490 cells / well). The lake water revealed counts of around 4.7×10^6 cells ml⁻¹.

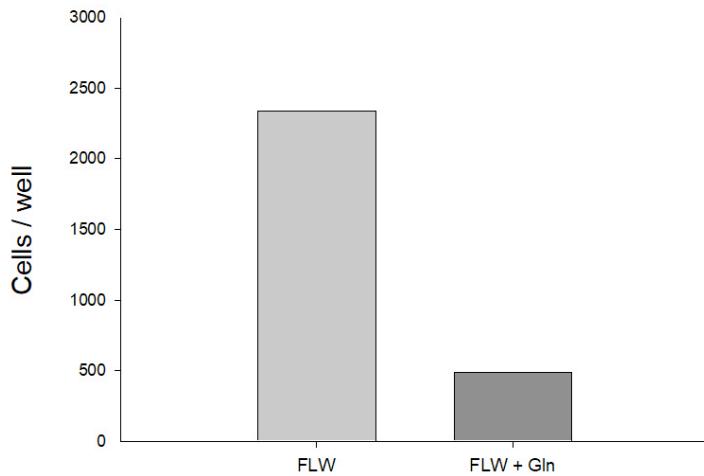


Figure 22: Syringe background with filtered lake water (FLW) alone and with added glutamine (FLW + Gln) before the incubation.

3.1.6 Enrichment

The aim of this experiment was to test the feasibility of the ISCA plate. Since the performed experiments did not show any positive signal, we reflected if there was a technical issue. In this case, the ISCA would probably not work under ideal conditions like in enriched lake water. In the event of functionality under ideal conditions the issue might be a biological one.

The filtration worked properly (56 cells / well) (Table A8, Supplementary materials) and the controls were low (3600 – 16'000 cells / well) compared to the treatments (Table A9 and A10, Supplementary materials). Two gates (G1 and G2, Fig. 24) were produced to detect the different bacterial communities. However, there was no change in abundance between the control and Gln-treatment in all three plates. Cell counts of Gln within the gated region of G1 in the first ISCA plate showed 3990 cells / well and the corresponding control revealed 3658 cells / well (Fig. 23A). Higher cell counts were observable in the gated region of G2, where Gln displayed 6932 cells / well and the corresponding control 5808 cells / well (Fig. 23A).

Cell counts within the gated region of G2 of the LB-treatment in the first plate (Fig. 23A) displayed an increase, as the LB-medium concentration elevated. 5 % LB medium showed cell counts of 3.7×10^4 whereas 20 % LB-medium revealed 7.7×10^4 cells per well. The second plate (Fig. 23B) displayed a decrease in the gated region of G1, as the LB-medium concentration enhances (Fig. 23B). 5 % LB-medium showed cell counts of 7.2×10^4 whereas 20 % LB-medium concentration revealed 4×10^4 cells per well. The third plate (Fig. 23C) revealed no specific pattern in both gates (Fig. 23C, G1 + G2). For more details see Table A9 and A10 in the Supplementary materials.

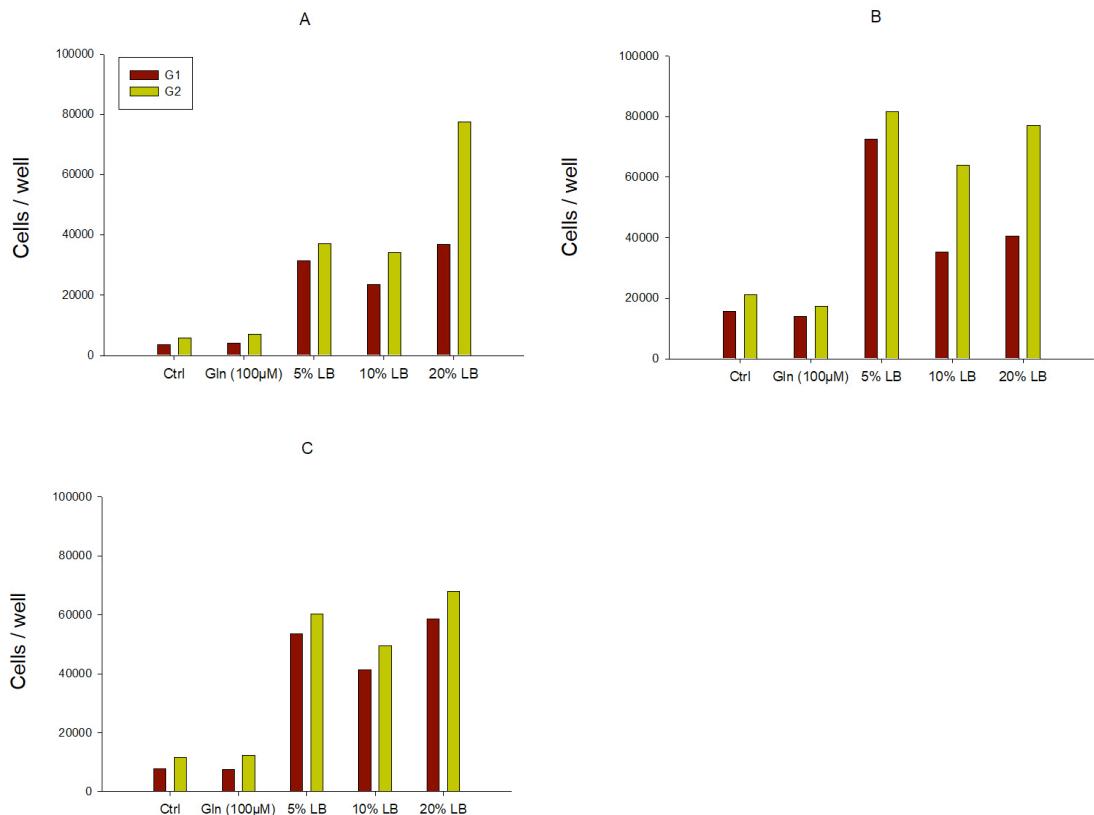


Figure 23: Comparison of three *in situ* chemotaxis assay (ISCA) plates (A, B, C) with cell counts (cells / wells) of the control (Ctrl), glutamine (Gln) and 5 – 20% LB-medium as treatments in an enrichment experiment.

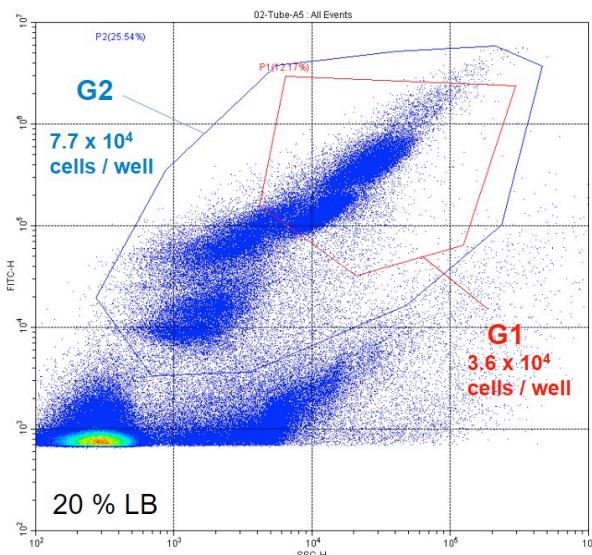


Figure 24: Example of a cytogram showing the two gates G1 (3.6×10^4 cells / well) and G2 (7.7×10^4 cells / well) for 20 % LB-medium.

3.2 Chemotaxis assay

Since we were not successful with the ISCA in Lake Zurich, the next step was to further investigate if the problem of the ISCA was biological or technical. To examine this, we wanted to use the ISCA plates on isolates from which we determined as chemotactic. If the ISCA works with these isolates, the problem probably might be biological. If not, a technical issue would be an explanation for the failure.

The further approach was to find good isolates, which are not only motile but also chemotactic. We cultivated *Paenibacillus* sp., *Rhodoferax* sp., *Modestobacter* sp., *Bradyrhizobium* sp. and *Flavobacterium* sp. and examined for motility under the microscope. It was decided to make further experiments with the *Modestobacter* sp. strain. To also define them as chemotactic bacteria we tried to develop chemotaxis assays, which should easily show the directed movement.

3.2.1 Multi-well assay

The aim of this experiment was to actually see the chemotactic movement of single bacterial cells towards an attractant under the inverted microscope. The idea was to observe the development of a gradient of bacteria towards the attractant. After some time, the well on the right side should be filled with motile bacteria and the left one should be less dense than before. In the control, no gradient should be observable due to the lack of the attractant. The expected gradient was not observable even after one hour of observation.

3.2.2 Capillary assay with petroleum jelly

The aim of this experiment was to observe chemotaxis in a capillary. The design was similar to the one with the multi-well microscopic slide but this time a capillary was used as a channel between the bacteria and the media. This assay could also not show any chemotactic movement due to the incapability of looking into the capillary.

3.2.3 Vertical capillary assay

In this experiment we used a classical capillary assay approach. With this assay we could indeed locate motile bacteria in the content of the capillary but they were not quantifiable.

3.3 Bacterial community in August 2017

3.3.1 Relative abundances

Different oligonucleotide probes with general and specific targets (Table B4, Supplementary materials) were used for CARD-FISH to obtain a first impression of the bacterial community present in Lake Zurich at 5 m depth in August 2017 with filters from the regular sampling campaign of Prof. Thomas Posch (Posch et al. 2012; Yankova et al. 2017).

On the sampling day, flow cytometry data of pre-filtered lake water revealed 2.9×10^6 cells ml^{-1} (Fig. 25). The cytogram (Fig. 25) shows gate P1 (manually drawn) for the total bacteria present in the sample. The rest refers to debris.

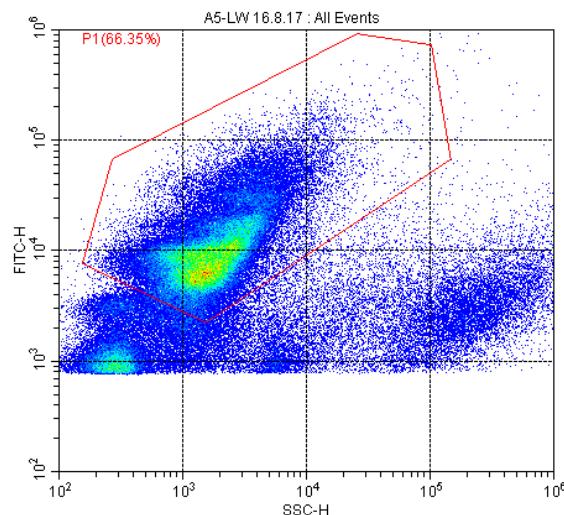


Figure 25: Cytogram of pre-filtered lake water on the sampling day in August 2017. P1 is the gate for the total bacteria (red) and revealed 2.9×10^6 cells ml^{-1} . Axes: FITC-H=525/40 nm; SSC-H: 488/8 nm.

The highest relative contribution was observed for LD12, a lineage which belongs to the *Alphaproteobacteria* and forms a tight monophyletic sister group with its correspondent marine genus *Pelagibacter* (Salcher et al. 2011), accounting for 21 % of the bacterial assemblage (Fig. 26). However, the *Alphaproteobacteria* targeted with the general probe ALF96 contributed with 5 % to the total bacterioplankton (Fig. 26). Following LD12, *Betaproteobacteria* targeted with the general probe BET42a displayed the second highest contribution, accounting for 13.5 % of the total bacterial assemblage (Fig. 26). Furthermore, substantial proportions of the *Betaproteobacteria* were identified by FISH with specific probes: Bacteria affiliated with the genus *Limnohabitans australis* and *curvus* (LimA) displayed only 1 %, *Limnohabitans* (R-BT) 2 % and *Polynucleobacter* (Bet2) 2.4 % (Fig. 26), revealing a small contribution to the *Betaproteobacteria* cluster.

Following the *Betaproteobacteria*, members of the acl lineage of *Actinobacteria* accounted for 10 % of the total bacterial assemblage whereas the two genera *Ca. Nanopelagicus* (acl-B1) and *Ca. Planktophila vernalis* (acl-A7) contributed on average with 3.5 % and 0.14 % (Fig. 25).

The *Cytophaga-Flavobacteria* targeted with the general probe CF968 accounted for 5.5 % and the *Cytophage-Flavobacteria* clade *Cyclobacteriaceae* (Cyc715) for 2 % of the total bacterial assemblage.

After all, the relative contribution of *Gammaproteobacteria* targeted with the general probe GAM42a was the lowest with 0.5 % (Fig. 26).

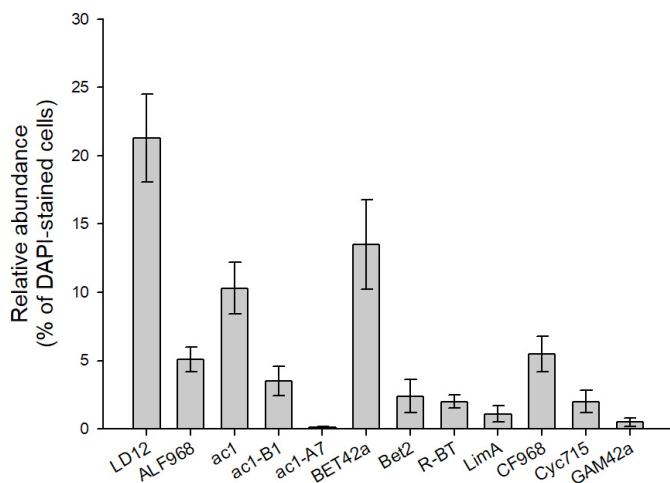


Figure 26: Relative abundance of FISH positive probes (% of DAPI-stained cells) showing the relative abundances to the bacterial community in August 2017. Probes (left to right): LD12 lineage of Alphaproteobacteria (LD12), Alphaproteobacteria (ALF968), the acl lineage of Actinobacteria (acl), the acl-B1 and acl-A7 sublineages of *Ca. Nanopelagicus* and *Ca. Planktophila vernalis* (acl-B1 and acl-A7), Cytophaga-Flavobacteria (CF968), Cyclobacteriaceae (Cyc715), Betaproteobacteria (BET42a), the Bet2 lineage of Polynucleobacter (Bet2), the R-BT lineage of the genus *Limnohabitans* (R-BT), the LimA lineage of the genus *Limnohabitans* (LimA) and Gammaproteobacteria (GAM42a). Values are means of triplicates. Error bars show standard deviations.

These results led to the decision of using only LD12, BET42a, R-BT, acl, ALF968 and LD12 for MAR-FISH analysis with the aim of investigating their radiolabelled Gln uptake activity. See Table B6 in the Supplementary materials for the absolute values of the bacterial abundances.

3.3.2 Incorporation of radiolabelled glutamine

We measured the Gln incorporation rate of a water sample from the incubation experiment in August (see chapter 2.5) with ^3H -Gln incubated for 1 and 4 h. The idea was to obtain a first

impression of the incorporation rate of ^3H -Gln by the present bacterial community and decide if MAR-FISH should be applied or not depending on the extent of incorporation. Incubation with ^3H -Gln displayed an incorporation rate of $282 \text{ pmol l}^{-1} \text{ h}^{-1}$ after 1 h and $204 \text{ pmol l}^{-1} \text{ h}^{-1}$ after 4 h with an absolute uptake of 282 pmol l^{-1} and $744.5 \text{ pmol l}^{-1}$, respectively.

MAR-FISH was conducted with the probes BET42a, R-BT, acl, ALF968 and LD12 for the two time points with MAR filters produced on the sampling day. BET42a, R-BT and LD12 were evaluated manually, acl and ALF968 with the automated microscope platform (see chapter 2.9.3). Of all counted DAPI-stained cells, LD12 and *Betaproteobacteria* targeted with BET42a revealed the highest contribution to the bacterial assemblage with 13.5 % and 13 %, respectively (Fig. 27). Members of the acl of *Actinobacteria* followed with 11 %, R-BT bacteria with 6 % and the lowest proportions of *Alphaproteobacteria* targeted with ALF968 accounted for 1.5 % (Fig. 27) of the total bacterial assemblage. Interestingly, the relative contribution of LD12 tested on FISH filters (see chapter 3.3.1) was higher (21.3 %) than the fraction obtained after the MAR-FISH assay (13.5 %).

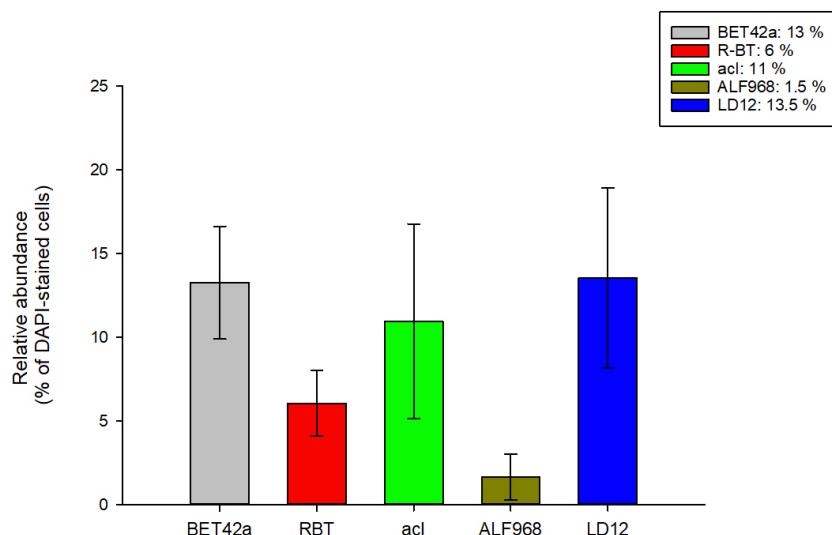


Figure 27: Relative abundances of FISH positive probes (as % of DAPI-stained cells) showing the relative contribution to the bacterial community in August 2017. Probes (left to right): Betaproteobacteria (BET42a), the R-BT lineage of the genus Limnohabitans (R-BT), the acl lineage of Actinobacteria (acl), Alphaproteobacteria (ALF968) and the LD12 lineage of Alphaproteobacteria. Values are means of triplicates. Error bars show standard deviation.

After 1 h of incubation, around 30 % of the total bacterial community ($2.9 \times 10^6 \text{ cells ml}^{-1}$) took up ^3H -Gln (Fig. 28; horizontal dashed line). Out of all the active cells, the *Betaproteobacteria* targeted with BET42a were above average with an incorporation of 33 %. Acl and LD12 showed an incorporation of 28 %. Furthermore, R-BT bacteria displayed 27 % incorporating cells slightly below average. *Alphaproteobacteria* targeted with ALF968 revealed the lowest incorporation of only 24 % (Fig. 28).

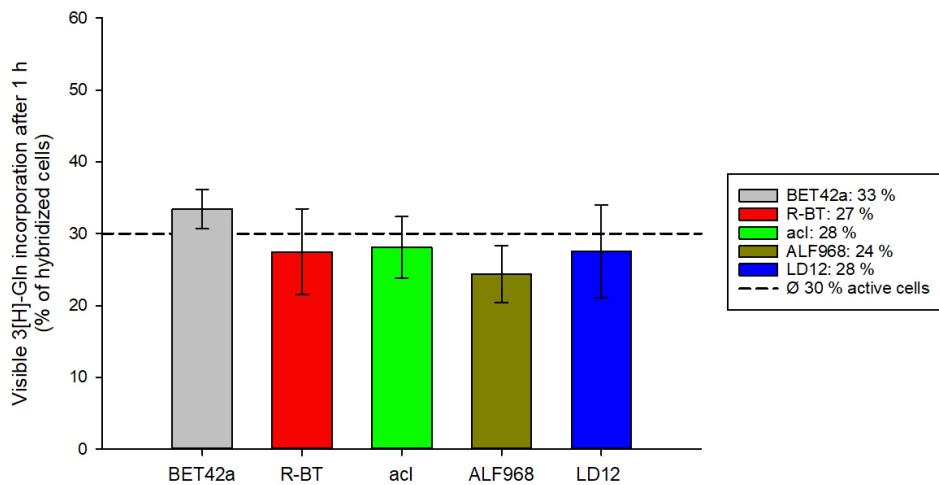


Figure 28: Fractions of cells with visible radiolabelled glutamine (${}^3\text{H}$ -Gln) incorporation (as % of hybridized cells) after 1 h affiliated with Betaproteobacteria (BET42a), the R-BT lineage of the genus Limnohabitans (R-BT), the acl cluster of Actinobacteria (acl), Alphaproteobacteria (ALF968) and the LD12 lineage of Alphaproteobacteria. Values are means of triplicates. Error bars show standard deviation. A horizontal dashed line indicates the average ${}^3\text{H}$ -Gln incorporation (as % of DAPI-stained cells).

After 4 h of incubation about 45 % of the total bacterial community were active in the uptake of ${}^3\text{H}$ -Gln (Fig. 29; horizontal dashed line). Once more the *Betaproteobacteria* targeted with BET42a, acl and LD12 displayed incorporations above average with 51%, 49% and 47 % of all active cells; followed directly by R-BT bacteria with 44 %. The *Alphaproteobacteria* targeted with ALF968 revealed again the lowest incorporation with 27 %, slightly higher than the incorporation after 1 h (Fig. 28 + 29).

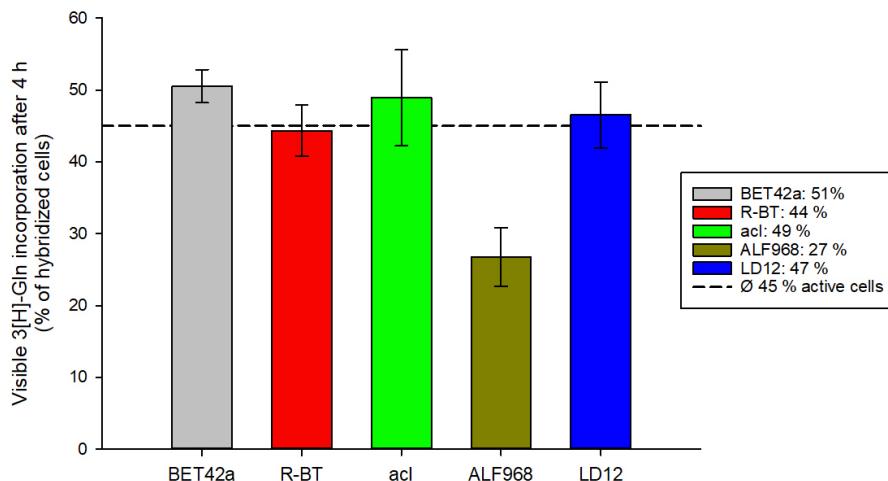


Figure 29: Fractions of cells with visible radiolabelled glutamine (${}^3\text{H}$ -Gln) incorporation (as % of hybridized cells) after 4 h affiliated with Betaproteobacteria (BET42a), the R-BT lineage of the genus Limnohabitans (R-BT), the acl lineage of Actinobacteria (acl), Alphaproteobacteria (ALF968) and the LD12 lineage of Alphaproteobacteria. Values are means of triplicates. Error bars show standard deviation. A horizontal dashed line indicates the average ${}^3\text{H}$ -Gln incorporation (as % of DAPI-stained cells).

All in all, there was a significant difference between the average Gln activity after 1 and 4 h (t-test, $t = -3.432$, p-value= 0.00892). A significant increase in ^3H -Gln after 4 h was shown for the *Betaproteobacteria* targeted with BET42 (t-test, $t = -12.171$, p-value= 0.00026), R-BT bacteria (t-test, $t = -5.567$, p-value= 0.01144), acl (t-test, $t = -3.703$, p-value= 0.02077) and LD12 (t-test, $t = -6.342$, p-value= 0.00793) whereas the *Alphaproteobacteria* targeted with ALF968 displayed no significant difference (t-test, $t = 0.503$, p-value= 0.6362).

For the absolute values of the bacterial abundances and radiolabelled glutamine incorporating cells see Table B7 in the Supplementary materials.

4 Discussion

There are many studies of chemotaxis which were performed successfully on marine bacteria in the past few years, one of the first even 40 years ago (Chet and Mitchell 1976). We tried to adapt the newly developed ISCA (Lambert et al. 2017) on freshwater bacteria of Lake Zurich but encountered some difficulties during the process.

4.1 ISCA production

The ISCA production was performed in the Stocker Lab with the instruction of the PhD student Bennet Lambert. Each step of the production was instructed in detail (Fig. 4, see chapter 2.1). One critical step was to clear any port obstructions of each well of the plate. To do this, a biopsy punch of the appropriate diameter was used (Lambert et al. 2017). Depending on how hard we pierced the biopsy punch through the PDMS, the ports were different in diameter or remained still closed. This issue led to different port sizes, which might resulted in different conditions in the same plate. The port of each well represents an independent connection to the external environment, which enables the chemoattractant to diffuse into. The chemotactic bacteria can respond by swimming into the well. If the port is still closed, it is most unlikely to create chemical microplumes that mimic transient nutrient patches (Stocker and Seymour 2012; Lambert et al. 2017). To control this, we examined the ports under the binocular after each time using the biopsy punch.

In some of the performed ISCAs we observed that the PDMS was not completely plasma-bonded to the sterile glass microscope slide. To optimize this, the sterilization was carried out much longer than the protocol suggested and the plate was also heated at 90°C for much longer to make sure that the covalent bonds were formed (Harrick Plasma 2014). However, a plasma activation of more than 5 minutes turned out to be counterproductive since it reverses the process of covalent bond formation, which makes the whole method even more difficult.

4.2 ISCA deployment

4.2.1 Variability of replicates

The first ISCA experiment of the practical work of my master thesis was conducted in April. The controls were too high (5180 cells / well) compared to the ones of 2016 (425 cells / well) and there was a lot of variability in the data between the ISCA plates (Fig. 15, see chapter 3.1.1). The controls in 2016 were representable since the filtration of the lake water worked, which was probably not the case in April 2017. Putrescine for example showed 1295 in the first, 9459 in the second and 2813 cells per well in the third plate (Fig. 15, see chapter 3.1.1). This variability was most likely caused by the manipulation. The position of the plates into

each beaker was done carefully by hand, which nevertheless might have caused a high level of turbulence. This could result in a rapid development of large nutrient gradients, whereas chemotactic bacteria could respond to these gradients and take advantage during uptake (Watteaux et al. 2015), which either benefits or prevents the chemotaxis response towards a substrate in the well of the ISCA (Stocker and Seymour 2012). To minimize turbulence, we used a small crane which could indeed minimize variability and good controls were produced (\varnothing 500 cells / well) (Table 3, see chapter 3.1.2). To prevent turbulence, Roman Stocker and his group secured each ISCA inside a deployment enclosure with a modified vice enabling the ISCA to be secured (Lambert et al. 2017). The enclosures were submerged into seawater. In contrast to our study, they did not focus on variability in the cell counts since they could indeed observe strong chemotaxis (3.6 ± 0.2 times greater than in negative control well) (Lambert et al. 2017).

4.2.2 Filtration methods

In the first few experiments, the Sartolab RF/BT Filter System with a PES membrane was applied to filter lake water. We were concerned about the plastic source of the filter tower, which might not be inert; meaning that it could act as an attractant or repellent. To test if better results could be achieved we decided to use an alternative filtration method in the next assay. The hollow fibre was applied, since it represents a gentle separation method and enables sterile processing (Jüttner et al. 1997). Surprisingly, the controls, treatments (Fig. 18, see chapter 3.1.3) and even the background itself (FLW, FLW + substrate) (Fig. 17, see chapter 3.1.3) showed high cell counts. If there was a response to one of the substrates, it probably was lost in the high background. These high numbers of cell counts (Table A4, see Supplementary materials) can be most likely explained by the overuse of the hollow fibre from previous experiments conducted in our laboratory. Nevertheless, we could demonstrate that 100 μM substrate concentration should be appropriate for future experiments since all three ISCAs showed more cell counts compared to the other concentrations (Fig. 18).

The experiment in August 2017 showed a strong background. The FLW was again very high, which explains the high controls in each plate after incubation (Fig. 22, see chapter 3.1.5). Flow cytometer data of the FLW prior to the incubation displayed high counts as well. An explanation might be that the FLW was contaminated with bacteria during the deployment of the ISCA plate. However, the FLW with added substrates was low (Fig. 22), which also strengthens the theory of contamination at a certain point of the experiment.

4.2.3 Biological reasons

Altogether, there was no visible difference observable between the treatments and controls in all of the conducted assays. There are several biological reasons and speculations why

the ISCA was not successful in lake water. First of all, to obtain a strong signal for the chemotactic response it is necessary to have a certain amount of motile, chemotactic bacteria present in the lake sample (Tout et al., 2015). In August 2016, where the experiment worked perfectly, more than 50 % of the original community (6233 of 11032 cells / well) responded to the chemoattractant (Fig. 3, see chapter 1.3). One possible explanation for not having a positive chemotactic response might be that Lake Zurich contains bacteria, which can inactivate their motility/chemotaxis. Signal transduction systems allow bacterial cells to adjust their motility in response to changes in chemoattractant concentrations (Bren and Eisenbach 2000). Furthermore, it is presumed that there is a motility-to-biofilm transition where the inhibition of motility enhances biofilm formation (Guttenplan and Kearns 2014). Another argument far more important to consider is the effect of dilution. If we argue, that Lake Zurich in general holds a very low fraction of motile, chemotactic bacteria, an effect of dilution cannot be avoided since only a small amount of lake water is used for deploying the ISCA. In the end, no motile, chemotactic bacteria were present in the beaker and therefore no chemotactic response could be measured.

A way to increase the amount of motile, chemotactic bacteria is to enrich the lake water with LB-medium. Previous studies have shown that the motile fraction of the bacterial community increased to approximately 80 % after 12 h enrichment with tryptic soy broth (Mitchell et al. 1996). The enrichment was performed in October with the help of Bennet Lambert. LB-enrichment overnight and incubation of the ISCA plate into LB-enriched lake water indeed led to a signal (Fig. 23, see chapter 3.1.6) and triggered a proliferation of a different bacterial community which was not previously present in the lake (Grossart et al. 2001). As the LB-medium concentration increased in 5 % steps, more distinct potential communities were observable (Fig. 23A). The Gln-treatment showed no signal, which was probably due to the fact that the LB-enrichment overnight already provided all the important nutrients (Fig. 23, see 3.1.6). LB-medium is composed of ingredients that promote growth like peptides, casein peptones, vitamins and other minerals (Sezonov et al. 2007). Casein is known to be a high source of nitrogen and Gln is known to be required for the biosynthesis of most nitrogen-containing compounds (Forchhammer 2007). If the bacteria had already taken up a high amount of nitrogen through the LB-medium, Gln might consequently not be such an attractive substrate anymore. All in all, cell abundances in the enrichment experiment were still very variable like in the previous assays. Enrichment studies are in general a good way to isolate certain bacterial strains (Mitchell et al. 1996) but are not representative for the composition of the lake therefore not a way to develop the ISCA for Lake Zurich.

Another reason why the ISCA did not work was probably because the substrate concentration was not high enough to attract the chemotactic bacteria, even though several concentrations were tested (see chapter 3.1.3). For future experiments we recommend to

use higher concentrations than the ones suggested from the Stocker Lab (10 and 100 µM) (Lambert et al. 2017).

The essential question is if there are enough motile, chemotactic, hypothetically copiotrophic bacteria (Smriga et al. 2016) present in Lake Zurich. If yes, could such an eminent fraction of them have inactivated their motility temporarily just at the experimental time (Bren and Eisenbach 2000).

After a discussion with Roman Stocker, one can say that if there is no signal in the ISCA, there are probably no motile bacteria present. His scientific group deployed the ISCA not only in the ocean but also in the Hudson River, which is a 507 km river that flows through New York in the United States. They could not achieve a positive signal for chemotaxis except for one time after rainfall (unpublished). The experiment from August 2016 (Fig. 3), which showed a positive chemotaxis response, was also conducted after rainfall. Could rain probably wash in some chemotactic bacteria? In the end there are still a lot of open questions and the method could not be developed for Lake Zurich.

4.3 Chemotaxis assays

4.3.1 Multi-well assay

The reproducibility of this experiment was a big issue. The channel was scratched open with a razor blade, which means that the width was always different and thus propagating potential variability. Sometimes the channel was not continuous which prevented the bacteria to cross between the two wells. Another problem was the implementation of the experiment. An inverted microscope was used to observe the bacterial chemotaxis in real time. After putting a cover slip over the microscopic slide, it triggered a flow between the culture and the media. It was not possible to distinguish between the bacteria which were actually motile and the ones that were just washed into the well through the flow caused by the cover slip. Furthermore, there were motile and non-motile bacteria present in the culture, which indicates that *Modestobacter sp.* had either lost or inactivated its ability to move.

4.3.2 Capillary assay with petroleum jelly

With this assay there was an uncertainty about the bacteria being inside the capillary, underneath it or both. However, the inverted microscope could not resolve the content of the capillary, therefore the motility of bacteria towards the opening of the capillary could not be observed in this way. Also, petroleum jelly might be a repellent for the bacteria we used for these chemotaxis assays. Since petroleum jelly was not sterile we could not completely exclude that external bacteria would migrate into the capillary. Further tests are necessary to assess the reaction of the bacterial strains upon petroleum jelly exposure. This experiment was quickly rejected since quantification of the bacteria inside the capillary was not possible

and using petroleum jelly would either prevent the chemotaxis response or lead to false-positive signals.

4.3.3 Vertical capillary assay

There were motile and non-motile bacteria present in the capillary consequently it was difficult to distinguish if the bacteria went into the capillary because of diffusion and capillary forces or due to actual chemotaxis. Furthermore, petroleum jelly was probably not dense enough to prevent the air coming into the capillary. Also, one bacterial strain that we used had cells, which were motile and some were not. Furthermore, the capillary revealed a relatively low number of cells due to the concentration gradient which became smaller over time until it completely disappeared (Mao et al. 2003). However, we could not find a chemotaxis assay for our purpose of defining *Modestobacter sp.* as chemotactic.

It is difficult to design an experiment for a system, which is still unknown. In literature there are many chemotaxis assays performed with bacteria like *E.coli* or *Vibrio fischeri*, which are nice model organisms to study chemotaxis due to their well-known life cycle (Adler and Dahl 1967). Compared to them, the isolates from Lake Zurich are harder to understand. It was observable that one part of the community even lost their motility since they were cultivated in a medium which already provided enough nutrients (Tittsler and Sandholzer 1936) and thus did not motivate them to be motile. The chemotaxis assays from the literature generally seem easy without a lot of effort mostly using capillary tubes (Adler and Dahl 1967; Bakker et al. 2007). Developing chemotaxis assays was difficult because it was almost impossible to define parameters which were quantifiable (Rivero-Hudec and Lauffenburger 1986). For instance, the distance of a bacterium from its starting point until reaching its destination was not measurable in a quantitative way. To improve this, a better imaging system, which can record bacterial movements should be applied (Molaei and Sheng 2014). All in all, the chemotaxis assays, which were performed during the practical work of the master thesis were not reproducible and quantifiable.

These difficulties resulted in putting the whole topic aside. The ISCAAs did not work with Lake Zurich after several trials. In order to produce some results for the master thesis we decided to lay focus on methods, which are well established like for example CARD- and MAR-FISH.

4.4 Bacterial community present in August 2017

The second aim of the master thesis was to investigate what taxa of free-living bacteria respond to radiolabelled Gln after two incubation time points. The idea was to obtain a first impression of the bacterial community at a certain time period (August 2017) and to analyse how active they were in ^3H -Gln uptake.

Filters from the regular sampling campaign of Thomas Posch were utilized for CARD-FISH assays with different oligonucleotide probes. In our experiments, the relative abundance of the acl cluster of *Actinobacteria* accounted for 10 % to the total bacterial assemblage (Fig. 26, see chapter 3.3.1). It is known from previous studies, that the acl cluster is amongst the most abundant bacterial species in freshwater systems (Warnecke et al. 2005). In September 2009, the warm epilimnion (0 – 10 m depths) of Lake Zurich showed the highest abundance of heterotrophic bacteria. The majority (77% of all bacteria) belonged to the low nucleic acid fraction which includes the acl cluster of the *Actinobacteria* and the LD12 lineage of the *Alphaproteobacteria* (Salcher et al. 2013). Likewise, the relative contribution of the LD12 lineage to the bacterial community displayed a high fraction of 21 % (6.2×10^5 cells ml^{-1}) of all DAPI-stained cells in our studies (Fig. 26). In addition, Salcher et. al. also identified high numbers (up to 0.4×10^6 cells ml^{-1}) of the LD12 ultramicrobacteria which were only present in the lower depths (Salcher et al. 2011, 2013). Therefore, our findings are consistent with the previous studies conducted by Salcher et. al.

Alphaproteobacteria targeted with the general probe ALF968 showed a relative contribution of 5 % to the total bakteriobankton (Fig. 26). It is known, that this probe targets the majority of the *Alphaproteobacteria* (Amann and Fuchs 2008) but it does not include the LD12 freshwater SAR11 cluster (Salcher et al. 2011), accordingly 21 % (detected for the LD12 in our experiments) are not covered. Furthermore, our analysis showed a relatively low contribution of *Gammaproteobacteria* targeted with GAM42a to the total bacterial assemblage (Fig. 26). Likewise, *Gammaproteobacteria* contributed < 1 % to the total assemblage in September 2009 (Salcher et al. 2013). Other *Alphaproteobacteria*, as well as *Gammaproteobacteria* are known to be ubiquitous but less frequent in freshwater systems (Newton et al. 2011), which is also consistent with our data.

Moreover, we could demonstrate that hybridization with the general probe BET42a targeted around 13.5 % of the *Betaproteobacteria* (Fig. 26). More specific probes (R-BT, BET2-870 and LimA) were used to identify the population of *Betaproteobacteria* but 9.5 % remained non-identified. In September 2009 Salcher et. al. detected *Betaproteobacteria* and *Bacteroidetes* throughout the whole water column and they managed to identify 84 % of all *Betaproteobacteria*. The epilimnion was mainly populated by *Limnohabitans* sp. (R-BT), followed by the LD28 tribe of *Betaproteobacteria*, *Polynucleobacter acidiphobus* and *P.*

difficiles (PnecB) while *Polynucleobacter necessarius* ssp. *asymbioticus* (PnecC) were only present in low densities (Salcher et al. 2013). Furthermore, small clusters within bet I-B (closely related to *Rhodoferax* sp. and *Abidiferax* sp.), bet VI (similar to bet I-B) and the whole bet III tribe (related to *Parapusillimonas granuli*) (Salcher et al. 2013) were detected. Comparison of our results with these findings indicates that further CARD-FISH assays with probes like LD28, PnecB, PnecC and possibly bet I-B, VI, III are necessary to identify the final fraction.

4.5 Incorporation of radiolabelled glutamine

4.5.1 Kinetics of radiolabelled glutamine uptake

Bacterial bulk incorporation rate of ^3H -Gln was higher after 1 h ($282 \text{ pmol l}^{-1} \text{ h}^{-1}$) than after 4 h ($204 \text{ pmol l}^{-1} \text{ h}^{-1}$). This implicates that the uptake rate does not increase linearly therefore closely follows Michaelis-Menten kinetics (Johnson and Goody 2012). At some point, saturation might be reached for ^3H -Gln uptake (Fig. 30). A model of reaction kinetics has long been described by Michaelis and Menten (1913) where the relationship between the concentration of available dissolved organic substrate and the rate of substrate uptake by bacterial communities was demonstrated (Wright and Hobbie 1966; Venrick et al. 1983). The uptake of dissolved organic substrates by bacteria in aquatic systems is generally assumed to obey Michaelis-Menten kinetics (Wetzel 1983) where a saturation of the enzyme or transporter is reached at high substrate concentrations (Logan and Fleury 1993).

One possible explanation for non-linearity is rapid tracer recycling where the label gets separated and metabolised (Taylor and Sullivan 1984). Therefore, uptake could not reach 100 % of activity like we demonstrated in our experiment.

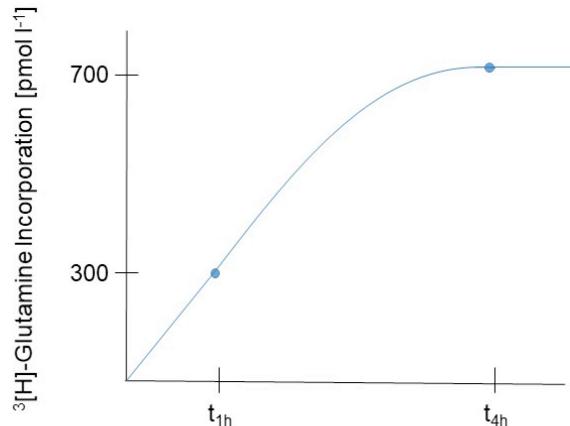


Figure 30: Conceptual curve of the absolute radiolabelled glutamine (^3H -Gln) incorporation for two time points. It displays an uptake of ~300 after 1 h and ~700 pmol l $^{-1}$ after 4 h.

4.5.2 Substrate incorporation

For the MAR-FISH assays, we decided to choose Gln as the substrate since it takes a universal role in bacterial metabolism. Bacteria are exposed to the frequently changing nutrient availability in the environment and even the slightest change has a high impact on their metabolic state (Forchhammer 2007). Gln is either synthesized by the cells or taken up from the environment. It is one of the building blocks for the 20 standard amino acids needed for protein synthesis, required as nitrogen donor for a vast range of biosynthetic reactions and as primary product essential for ammonium assimilation (Merrick and Edwards 1995; Forchhammer 2007).

MAR-FISH assays were conducted for the probes BET42a, R-BT, acl, ALF968 and LD12. All bacteria targeted with these probes showed similar radiolabelled Gln incorporation activity after 4 h except for the *Alphaproteobacteria* targeted with ALF968 (Fig. 31). *Betaproteobacteria* targeted with BET42a, R-BT bacteria, members of the acl and LD12 cluster displayed an incorporation-increase of 17 – 21 %, whereas the *Alphaproteobacteria* targeted with ALF968 only incorporated 3 % more after 4 h. Given that *Alphaproteobacteria* consist of different clusters (Salcher et al. 2011) it is most likely that some have taken up Gln and some not. The ones that took up Gln might have already reached saturation after 1 h.

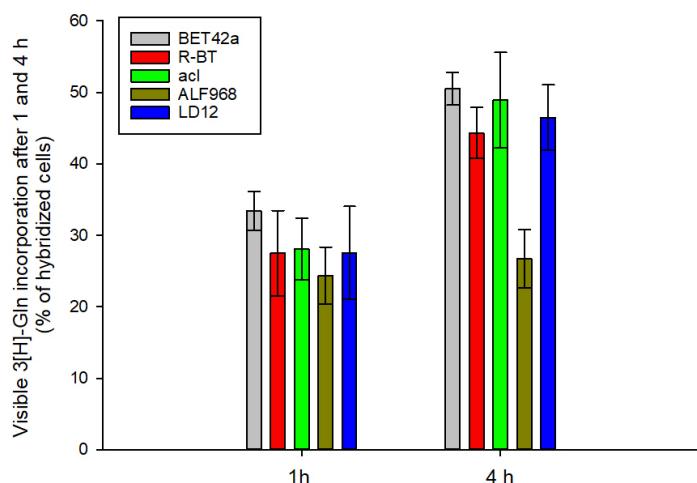


Figure 31: Comparison of the radiolabelled glutamine (^3H -Gln) incorporation activity after two time points ($t_{1\text{h}}$ and $t_{4\text{h}}$).

Previous studies have shown that the acl *Actinobacteria* and LD12 *Alphaproteobacteria* exhibited distinct substrate preferences. The examined acl cluster showed a specialization for Glc and Leu in combination with thymidine (Salcher et al. 2013) and did not display any pronounced preference for Gln whereas the LD12 appeared to be highly specialized for Gln and glutamate (Glu) (Salcher et al. 2011). This difference in substrate preferences suggested

that the acl and LD12 might coexist in the water column without competing for DOM compounds (Salcher et al. 2013). Both lineages are non-motile (Pernthaler 2017) though display genes for amino acid transporters. Several genes of the LD12 lineage of the *Alphaproteobacteria* encode for ABC transporters with narrow substrate specificity (Hosie and Poole 2001) like glutamine or glycine/proline transport (Salcher et al. 2011) as well as high-affinity transporters for branched amino acids like leucine (Giovannoni 2005; Salcher et al. 2011). As for the members of the acl lineage of *Actinobacteria* previous studies have shown that they displayed differences related to membrane transporters for low molecular weight organic compounds which were present in a species-specific manner (Kang et al. 2017; Neuenschwander et al. 2017).

In contrast, *Betaproteobacteria* seemed to follow a generalist life style by having preferences for a vast range of substrates and also represent true opportunistic strategies due to their fast growth and division (Šimek et al. 2005; Salcher et al. 2013). The assumption that members of the acl and LD12 lineage are not competing for ^3H -Gln and the *Betaproteobacteria* are not having a distinct substrate preference could be an explanation why they were similarly active. Total activity never exceeded 50 % (Fig. 31) which turned out to be universal for ^3H -Gln uptake. Focusing on LD12, this pattern was also discovered in previous incorporation assays of Salcher et al. The assay conducted in September 2009 included incorporation time points of 15 – 240 minutes with added ^3H -Gln which revealed an adaptation of LD12 bacteria to an oligotrophic life style (Salcher et al. 2011). They showed a slower uptake kinetics of ^3H -Gln and reached a saturation plateau only after 2 – 4 h of incubation. Furthermore, LD12 bacteria appeared to be highly active when phosphorous concentration was low, which implicates seasonal adaptation (Salcher et al. 2011). Therefore, saturation due to oligotrophic adaptations might be a reason why total incorporation activity never passed 50 % in our study.

Other substrate incorporation assays revealed an even higher uptake activity. Previous studies in spring 2014 investigating Leu and Glc uptake (Marty 2015) displayed a high proportion of active cells for the tested probes acl and R-BT. The relative Leu-incorporation of the acl cells was 59 % after 0.5 h and up to 96 % after 4 h. For R-BT there was an incorporation of 93 % after 0.5 h and 97 % after 4 h. For Glc uptake, both acl and R-BT showed a similar relative incorporation after 0.5 h with 27.6 % and 26 %, respectively. In contrast, after 4 h the pattern changed: R-BT cells showed a relatively high Glc-incorporation of 89.4 % whereas acl only displayed 57 % (Marty 2015). It seems that the similar uptake behaviour of Gln might not be the case for other substrates. Gln functions as a signalling molecule in bacteria, which is mainly related to metabolic regulation (Kan et al. 2015). There is a conserved sensory system present in bacteria as the central control unit in nitrogen metabolism and glutamine signalling (Forchhammer 2007; Kan et al. 2015). Gln gets

incorporated and metabolized quickly (Feehily and Karatzas 2013) given that it takes a ubiquitous role in the bacterial metabolism.

Furthermore, cells differ in their vital rates and response to the environment which determines population demography (Hole 2000). Hence, not all of the cells grow exponentially and are able to reach 100 % of uptake activity. However, to find the underlying cause more experiments with different substrates and oligonucleotide probes need to be conducted.

4.6 Evaluation of radiolabelled glutamine incorporating cells

Image acquisition and analysis of ^3H -Gln incorporating cells by the automated microscope platform was tedious. First, it was decided to evaluate the MAR-FISH filters with the automated microscope, which permits a fast, accurate and high throughput evaluation of samples (Alonso 2012). This fully automated process for image acquisition makes use of a program for image analysis, which was developed by M. Zeder (Zeder 2009). This process worked nicely with some of the samples, for example bacteria targeted with ALF968 since *Alphaproteobacteria* were not too small and had unambiguous fluorescent signals. However, ultramicrobacteria like LD12 for example are very small and the fluorescent signal was sometimes too weak. Moreover, the silver grains in close proximity to the substrate incorporating cells covered the cells themselves when images were acquired. This led to erroneous cell counts. Given that the MAR-FISH signal might have weakened the FISH signal, it was hard to obtain accurate data from the automated microscope since the values were very variable (Fig. 26, see chapter 3.3.1). Therefore, some probes (BET42a, R-BT and LD12) were manually evaluated even though it was a time consuming process and normally not recommended when many samples need to be assessed (Alonso 2012).

To demonstrate that a strong MAR-FISH signal can weaken the FISH-signal, a CARD-FISH assay was conducted by hybridizing the same MAR-filters with the LD12 probe. A relative contribution of 16 % was detectable (2.5 % more than in the previous MAR-FISH assay; see chapter 3.3.2).

4.7 Chemotaxis of freshwater bacteria

The topic of my master thesis was to investigate the response of free-living bacteria to a variable substrate field in Lake Zurich. For this purpose, two main aims were aspired. On one hand, we tried to develop the ISCA to sort the bacteria, which show a chemotactic response to a given substrate. On the other hand, we aimed to identify and investigate the groups of the freshwater bacterial community. Hence, metabolic processes of a distinct group present in freshwater could be linked to its genetic background with the goal to gain information about the dimension of chemotaxis in these bacterial communities.

In our study, we were able to identify some groups of the pelagic bacterial community of Lake Zurich, which actively took up ^3H -Gln. The acl cluster of *Actinobacteria*, the LD12 lineage of *Alphaproteobacteria* and the R-BT lineage of the genus *Limnohabitans* displayed radiolabelled Gln incorporation. At the present time, we cannot make any conclusions about their life strategies and chemotactic behaviour since there is little known about their chemotactic genes. Nevertheless, chemotactic genes in bacteria like *E. coli* and *Vibrio sp.* are well studied (Shah et al. 2000; Briegel et al. 2016) and it is likely that freshwater chemotactic genes are similarly structured. *E. coli* is considered to be the model organism for chemotaxis and there has been a broad study on the genes needed for flagellar formation (Fitzgerald et al. 2014), motility (Armstrong 1966) and chemotaxis (Morehouse et al. 2005). It was discovered that *E. coli* contains membrane-spanning, methyl-accepting chemotaxis proteins (MCPs) that sense the concentration of chemoattractants and transmit an activating signal to proteins which are part of the chemotaxis signal transduction pathway (Mowbray and Sandgren 1998).

4.8 Chemotaxis: Marine versus freshwater systems

Marine and freshwater environments vary in many aspects such as salinity, temperature, depth and nutrient content but both provide appropriate habitats for bacteria (Aryal et al. 2015). On one hand, marine bacteria have a great impact on environmental dynamics by affecting largescale processes like primary production. On the other hand, they interplay with the ocean at the microscale (Stocker 2012). In marine as well as freshwater systems nutrients are far from homogeneous, thus chemotaxis could be an advantageous adaptation in nutrient uptake (Blackburn et al. 1998; Seymour et al. 2008). Efficient exploitation of nutrient patches and gradients by the motile can result in an accumulation of opportunistic bacteria (Azam 1998; Barbara and Mitchell 2003). Therefore, motile bacteria which perform chemotaxis should occur either in marine and freshwater systems. Assaying chemotaxis with ISCA has been documented to work in marine environments (Tout et al. 2015; Lambert et al. 2017) whereas for freshwater systems it was harder to assess. One explanation of this observation might be that freshwater environments are variable in the resources available for microbial growth and therefore do not provide ideal conditions for motile bacteria to accumulate (Aryal et al. 2015). Estuaries of oceans display higher nutrient levels than lakes, resulting in inhabiting larger microbial populations (Aryal et al. 2015) and therefore the occurrence of motile bacteria increases.

Previous studies on Lake Zurich have shown that the concentration of dissolved free amino acids were rather low due to the re-oligotrophication of the lake during the past decades (Posch et al. 2012; Horňák et al. 2016) which consequently led to a higher occurrence of oligotrophic bacteria. Non-motile bacteria are found amongst the oligotrophs and passively

float throughout the water column instead of actively exploiting chemical gradients through chemotaxis like some members of copiotrophic bacteria. (Stocker et al. 2008; Stocker 2012). These non-motile bacteria seem to be adapted to nutrient depleted water layers which allows efficient uptake of amino acids at low concentrations (Corno et al. 2008). Thus, this adaptation of being non-motile could be advantageous for bacteria present in Lake Zurich (Salcher 2014). In the end we can only speculate about Lake Zurich not having the ideal conditions for using the mentioned *in situ* chemotaxis assays.

5 Conclusion and Outlook

We could demonstrate that the newly developed microfluidic-based ISCA is probably not applicable to Lake Zurich. The cause of turbulence during deployment, the effect of dilution and the absence of sufficient motile, chemotactic bacteria argue against an implementation of the device. Furthermore, the technical component of the method is not well engineered, which makes the process of development quite arbitrary. It is not clear yet how chemotaxis can be investigated otherwise in oligotrophic lacustrine systems. To assess this topic further, there is a need for deeper knowledge about the motility behaviour of the bacteria present in Lake Zurich.

During August 2017, we could show that the LD12 lineage of the *Alphaproteobacteria* and the acl cluster of the *Actinobacteria* were the most abundant species and active in glutamine uptake. For the *Betaproteobacteria* we could identify R-BT as one part of the bacterial assemblage, which also actively incorporated glutamine but there are still groups, which remain undiscovered. For glutamine incorporation, we detected a universal pattern for all the tested bacterial strains after two time points except for *Alphaproteobacteria* targeted with ALF968. There was an increase in the uptake and it reached saturation after some time since the incorporation rate did not change linearly. In the future, more probes need to be tested to discover how active other bacterial groups are in glutamine uptake and it would be interesting to test for other substrates to see what pattern they display.

The idea for future experiments is to reproduce these results and ideally link them to their genetic background of chemotaxis. A full incubation experiment is planned in early summer 2018 with more replicates, MAR-FISH data and a well-elaborated RNA isolation protocol. The data obtained in this master thesis can be used as a reference for further studies and be compared to future analysis afterwards. If it is successful, conclusions about the chemotactic behaviour of freshwater bacteria can be drawn and the topic can be placed into a broader context of variability of the substrate field in Lake Zurich.

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Statement of authorship

I declare that I have used no other sources and aids other than those indicated. All passages quoted from publications or paraphrased from these sources are indicated as such, i.e. cited and/or attributed. This thesis was not submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Kilchberg, 24. April 2018

Anahita Yazdanfar

Supplementary materials

Appendix A

Table A1: Overview of the average cell counts (\emptyset Cells / well) and standard deviation shown for the experiment in August 2016 with glutamine (Gln), serine (Ser), glucose (Glc) and aspartate (Asp) as substrates as well as fresh lake water (LW, unfiltered), filtered lake water (FLW) and control (Ctrl). The average was calculated from the cell counts of each well and treatment.

Treatment	\emptyset Cells / well
LW (unfiltered)	11032
FLW	130
Gln	7611 \pm 652
Ser	448 \pm 140
Glc	4854 \pm 533
Asp	338 \pm 76
Ctrl	425 \pm 41

*Table A2: Overview of the average cell counts (\emptyset Cells / well) and standard deviation for the three *in situ* chemotaxis assay (ISCA) plates (Plate 1, 2 and 3) with putrescine (Put), glutamine (Gln), glucose (Glc) and N-Acetylglucosamine (NAG) as substrates. The average was calculated from the cell counts of each well and treatment.*

Treatment	\emptyset Cells / well		
	Plate 1	Plate 2	Plate 3
Ctrl	7575 \pm 505	4423 \pm 35	3543 \pm 501
Put	1295 \pm 18	9459 \pm 824	2813 \pm 230
Gln	4646 \pm 146	6024 \pm 610	3877 \pm 405
Glc	4764 \pm 200	6694 \pm 362	1913 \pm 115
NAG	7137 \pm 972	5030 \pm 99	7065 \pm 135

Table A3: Cell counts (Cells / well) and standard deviation of the syringe background. Glucose (Glc), glutamine (Gln) and arginine (Arg) in different concentrations with added filtered lake water (FLW) are listed.

Treatment	Cells / well
10 µM Glc + FLW	5123
100 µM Glc + FLW	5290
1 mM Glc + FLW	4945
10 µM Gln + FLW	5360
100 µM Gln + FLW	5925
1 mM Gln + FLW	4745
10 µM Arg + FLW	6087
100 µM Arg + FLW	6658
1 mM Arg + FLW	5436

*Table A4: Overview of the average cell counts ($\bar{\sigma}$ Cells / well) and standard deviation for three *in situ* chemotaxis assay (ISCA) plates (ISCA A, B and C) with glucose (Glc), glutamine (Gln) and arginine (Arg) in different concentrations (10 µM, 100 µM and 1 mM). 1 % LB-medium was also tested as a positive control for each plate. The average was calculated from the cell counts of each well and treatment.*

Treatment	$\bar{\sigma}$ Cells / well		
	ISCA A: Glc	ISCA B: Gln	ISCA C: Arg
10 µM substrate	5449 ± 1055	5382 ± 1315	5101 ± 1387
100 µM substrate	6574 ± 1412	6613 ± 3008	6784 ± 1171
1 mM substrate	6242 ± 3123	3927 ± 1971	4606 ± 2627
Ctrl	5669 ± 1844	9012 ± 1368	170 ± 92
1 % LB	1175 ± 1620	431 ± 491	259 ± 79

Table A5: Average cell counts (\emptyset Cells / well) of the syringe background. Syringe contents are filtered lake water (FLW) amended with glucose (Glc), glutamine (Gln), arginine (Arg), N-Acetylglucosamine (NAG), inorganic phosphate (P) and putrescine (Put). Cell counts of FLW and substrates alone are also listed. The average was calculated from triplicates used for flow cytometry measurements.

Treatment	\emptyset Cells / well
FLW	1023
FLW + Glc	193
FLW + Gln	220
FLW + Arg	247
FLW + NAG	167
FLW + P	120
FLW + Put	103
Glc	63
Gln	3
Arg	343
NAG	0
P	77
Put	47

Table A6: Overview of the average cell counts (\emptyset Cells / well) and standard deviation for six in situ chemotaxis assay (ISCA) plates with glucose (Glc), glutamine (Gln), arginine (Arg), N-Acetylglucosamine (NAG), inorganic phosphate (P) and putrescine (Put). The average was calculated from the cell counts of each well and treatment.

Treatment	\emptyset Cells / well					
	Glc	Gln	Arg	NAG	P	Put
Treatment	222 ± 119	254 ± 157	174 ± 91	313 ± 125	309 ± 151	187 ± 67
Control	248 ± 116	160 ± 79	134 ± 41	318 ± 158	183 ± 89	159 ± 107

Table A7: Overview of the cell counts (Cells / well) for the four in situ chemotaxis assay (ISCA) plates with only glutamine (Gln) as substrate and the corresponding control (Ctrl). The wells of each row of the ISCA plate were pooled for flow cytometry.

Treatment	Cells / well			
	ISCA 1	ISCA 2	ISCA 3	ISCA 4
Gln	785	748	523	343
Ctrl	811	690	305	641

Table A8: Cell counts (\emptyset Cells / well) for fresh, enriched and filtered lake water (FLW). G1: First gate, G2: Second Gate

Treatment	G1 \emptyset Cells / well	G2 \emptyset Cells / well
Fresh Lake Water	3607	186319
Enriched Lake Water	385038	920806
FLW	56	565

Table A9: Cell counts (\emptyset Cells / well) for the control (Ctrl), glutamine (Gln) and 5, 10, 20 % LB- medium as treatments for gate G1.

Treatment	G1 \emptyset Cells / well		
	A	B	C
Ctrl	3658	15709	7811
Gln	3990	13973	7555
5 % LB	31313	72463	53506
10 % LB	23651	35144	41394
20 % LB	36964	40528	58662

Table A10: Cell counts (\emptyset Cells / well) for the control (Ctrl), glutamine (Gln) and 5, 10, 20% LB- medium as treatments for gate P2.

Treatment	G2 \emptyset Cells / well		
	A	B	C
Ctrl	5808	21167	11500
Gln	6932	17305	12389
5 % LB	37143	81598	60255
10 % LB	34082	63914	49395
20 % LB	77541	77134	67884

Table A11: Media used for chemotactic assays. Chemicals and corresponding concentration of the inorganic basal medium (IBM) are listed.

Inorganic basal medium (IBM)	
Chemical	Concentration in medium
MgSO ₄ · 7H ₂ O	750 mg l ⁻¹
Ca(NO ₃) ₂ · 4H ₂ O	430 mg l ⁻¹
NaHCO ₃	160 mg l ⁻¹
KCl	50 mg l ⁻¹
K ₂ HPO ₄ · 3H ₂ O	37 mg l ⁻¹
„Eisen-Stammlösung“: 100 x 367 mg l ⁻¹	100 ml
„Spurenelementlösung“: 100x	100 ml

Table A12: Media used for chemotactic assays. Ingredients and corresponding concentrations are listed for NSY medium.

Liquid NSY medium (3 g l⁻¹)
Add to the inorganic basal medium
Nutrient broth, 1 g l ⁻¹
Soytone peptone, 1g l ⁻¹
Yeast extract, 1g l ⁻¹
pH 7.2

Appendix B

Table B1: Solutions for DAPI-mix. The pH was adjusted by the addition of either NaOH or HCl.

DAPI – mix	DAPI-MIX, pH 9 5 x Citiflour (Glycerol/PBS solution, Linaris) 1 x Vectashield (Linaris) 1 x PBS DAPI (1 µg ml ⁻¹ final concentration, Sigma) Adjust to pH 9 Store at 4°C in the dark
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Table B2: Solutions applied for CARD-FISH. The pH was adjusted by the addition of either NaOH or HCl

0.1 % Agarose: 200 ml	0.2 g Agarose 200 ml MQ Stir and heat until agarose is completely dissolved Storage @ RT, melt it before use (microwave)
1 x PBS, pH 7.6: 1 l	8 g NaCl 0.2 g KCl 1.44 g Na ₂ HPO ₄ 0.24 g KH ₂ PO ₄ 1000 ml MQ Adjust pH to 7.6 Autoclave Store @ RT
10 x PBS, pH 7.4:	Ten times more, for example 500ml: 40 g NaCl 1 g KCl 7.2 g Na ₂ HPO ₄ 1.2 g KH ₂ PO ₄
Lysozyme solution: 10 ml	100 mg Lysozym (10 mg ml ⁻¹) 1 ml 0,5 M EDTA 1 ml 1 M Tris/HCl 8 ml MQ Prepare fresh
Achromopeptidase stock (30 KU)	40 mg (100 KU) 3.3 ml MQ Aliquote Store @ -20°C

NaCl-Tris buffer, pH 8: 50 ml	100 µl 5 M NaCl 500 µl 1 M Tris/HCl Fill to 50 ml with MQ Adjust pH to 8 Filter sterile Store @ RT
0.5 M EDTA, pH 8: 100 ml	14.612 g EDTA 100 ml MQ Adjust pH to 8 Stir until it is completely dissolved (after pH adjustment it's more easy) Autoclave Store @ RT
1 M Tris-HCl, pH 7.4 / pH 8: 100 ml	15.76 g Tris-HCl 100 ml MQ Adjust pH to 7.6 or 8 Autoclave Store @ RT
5 M NaCl: 400 ml	116.884 g NaCl 400 ml MQ Autoclave Store @ RT
0,01% PBST, pH 7.6: 500 ml	500 ml 1 x PBS 50 µl Triton X-100 Store @ RT
0.15% H₂O₂	1 ml 1 x PBS (or MQ) 5 µl 30% H ₂ O ₂ Prepare fresh
10 % Blocking reagent:	Prepare buffer 0.15 M NaCl (0.438g) 0.1 M maleic acid (0.5805g) 50 ml MQ Adjust pH to 7.5 Filter sterile 50 ml buffer 5 g Blocking reagent Stir & heat until it is completely dissolved Store @ -20°C or +4°C (several days)
Amplification buffer:	4 ml 10 x PBS 16 ml 5 M NaCl 4 g dextran sulfate Stir & heat (~40°C) until it is completely dissolved Cool down to +4°C 0.4 ml blocking reagent Fill up to 40 ml with MQ Store @ +4°C (several days)

Hybridization buffer (20 ml) for BET42a, acl-853, -A7, ALF968, R-BT065, GAM42a, CF968, Bet2-870	3600 µl 5 M NaCl 400 µl 1 M Tris/HCl (pH 7.4) 2 g dextran sulfate Stir & heat (~40°C) until it is completely dissolved Cool down to +4°C 3 ml MQ 11 ml formamide 2000 µl Blocking reagent 20 µl SDS Aliquote Store @ -20°C
Hybridization buffer (20 ml) for Cyc715, acl-B1	3600 µl 5 M NaCl 400 µl 1 M Tris/HCl (pH 7.4) 2 g dextran sulfate Stir & heat (~40°C) until it is completely dissolved Cool down to +4°C 2 ml MQ 12 ml formamide 2000 µl Blocking reagent 20 µl SDS Aliquote Store @ -20°C
Hybridization buffer (20 ml) for LimA and LD12	3600 µl 5 M NaCl 400 µl 1 M Tris/HCl (pH 7.4) 2 g dextran sulfate Stir & heat (~40°C) until it is completely dissolved Cool down to +4°C 1 ml MQ 13 ml formamide 2000 µl Blocking reagent 20 µl SDS Aliquote Store @ -20°C
Washing buffer	500 µl 0.5 M EDTA 1000 µl 1 M Tris/HCl x µl NaCl (according to the probe) fill to 50 ml with MQ 50 µl SDS

Table B3: Solutions applied for Microautoradiography (MAR).

2 % Agarose (200 ml)	4 g Agarose 200 ml MQ Stir & heat until agarose is completely dissolved Store @ RT, melt it before use
0.2 % Agarose (200 ml)	0.4 g Agarose 200 ml MQ Stir & heat until agarose is completely dissolved Store @ RT, melt it before use
Autoradiography emulsion	Dilute with 0.2 % agarose 1:1 (in the dark!) Fill in storage tubes for slides (20 ml) Wrap tubes with aluminium foil Store @ +4°C (several months)

Table B4: Overview of all the oligonucleotide probes used for CARD-FISH experiments. Name of the probe, specificity, sequence, formamide concentration and reference are listed.

Probe	Specificity	Formamide in HB [%]	Reference
GAM42a	<i>Gammaproteobacteria</i>	55	Manz et al. 1992
GAM42a -C	Competitor for GAM42a	55	Manz et al. 1992
CF968	<i>Cytophaga-Flavobacteria</i>	55	Acinas et al. 2015
LimA-23S-1435	<i>Limnohabitans australis & curvus</i> (NON-RBTs)	65	Shabarova et al. 2017
LimA-23S-C	Competitor for LimA	65	Shabarova et al. 2017
R-BT065	Cluster within beta I (Beta)	55	Šimek et al. 2001
Bet2-870	<i>Polynucleobacter</i> (BET II)	55	Burkert et al. 2003
Cyc715	<i>Cyclobacteriaceae</i>	60	Eckert et al. 2012

Cyc715C	Competitor for Cyc715	60	Eckert et al. 2012
ALF968	<i>Alphaproteobacteria</i>	55	Neef et al. 1997
LD12-115	Modified LD12	65	Neuenschwander et al. 2015
acl-853	acl lineage of <i>Actinobacteria</i>	55	Warnecke et al. 2005
acl-853-H-mix	Helper for acl-853	55	Warnecke et al. 2005
acl-B1-23S-2669	acl-B1, 23S probe	60	Neuenschwander et al. 2017
acl-B1-comp	Competitors for acl-B1	60	Neuenschwander et al. 2017
acl-B1-H-mix	Helper for acl-B1	60	Neuenschwander et al. 2017
acl-A7-23S-1420	acl-A7, 23S probe	55-60	Neuenschwander et al. 2017
acl-A7-Comp	Competitors for acl-A7	55-60	Neuenschwander et al. 2017
acl-A7-H-mix	Helper for acl-A7	55-60	Neuenschwander et al. 2017

Table B5: Overview of all the oligonucleotide probes used for MAR-FISH experiments. Name of the probe, specificity, sequence, formamide concentration in hybridization buffer (HB) and reference are listed.

Probe	Specificity	Formamide in HB [%]	Reference
BET42a	<i>Betaproteobacteria</i>	55	Manz et al. 1992
BET42a-C	Competitor for BET 42a	55	Manz et al. 1992
acl-853	acl lineage of <i>Actinobacteria</i>	55	Warnecke et al. 2005
acl-853-H-mix	Helper for acl-853	55	Warnecke et al. 2005
R-BT065	Cluster within beta I (Beta)	55	Šimek et al. 2001
ALF968	<i>Alphaproteobacteria</i>	55	Neef et al. (1997)
ALF968	<i>Alphaproteobacteria</i>	55	Neef et al. (1997)
LD12-115	Modified LD12	65	Neuenschwander et al. 2015

Table B6: Output of the image acquisition program IMI5.4. Listed are the absolute numbers of DAPI, FISH, autofluorescent cells (AF) and the relative values (%) of the FISH- and AF-positive cells. The numbers of the FISH-positive (%) cells are adjusted by subtracting the amount of autofluorescent cells. The absolute FISH numbers were counted with the flow cytometer data as a reference (bacteria present in Lake Zurich: 2.9×10^6 cells ml $^{-1}$).

Probe	DAPI	FISH	AF	% FISH (+)	% AF (+)	%FISH (+) (adjusted)	Abs. FISH (+)
	D	P	X	PD/D	XPD/D	PD/D – XPD/D	[cells / ml]
CF968	11750	851	286	7.2	1.7	5.5	1.6×10^5
Cyc715	10153	429	239	3.9	2.0	2.0	5.8×10^4
GAM42a	9463	275	258	2.8	2.3	0.5	1.5×10^4
BET42a (MAR-filter)	4529	611	-	13.5	-	-	3.9×10^5
LimA	12033	476	385	3.9	2.8	1.1	3.2×10^4
R-BT065	9400	551	465	6.2	4.3	2.0	5.8×10^4
Bet2-870	8724	392	188	4.3	1.9	2.4	7×10^4
ALF968	7912	707	362	9.6	4.6	5.1	1.5×10^5
acl	10166	1169	128	11.3	1.0	10.3	3×10^5
acl-B1	12333	488	79	3.6	0.1	3.5	1×10^5
acl-A7	11131	187	335	1.3	1.2	0.1	2.3×10^3
LD12-115	11831	2583	121	22.4	1.1	21.3	6.2×10^5

*Table B7: Overview of the proportions of FISH, MAR and MAR-FISH cell counts of all DAPI cells. The absolute numbers were counted with the flow cytometer data as a reference (bacteria present in Lake Zurich: 2.9×10^6 cells ml $^{-1}$). The numbers for the probe *acl* and ALF968 were evaluated by the automated microscope platform and BET42a, R-BT and LD12 manually with the epifluorescence microscope.*

BET42a	Abs. DAPI	%FISH (+)	Abs. FISH (+) [cells / ml]	%MAR (+)	%MAR-FISH (+)	Abs. MAR-FISH (+) [cells / ml]
Control	1005	12.3	3.6×10^5	0	0	0
1h 3 [H]-Gln	1798	13.5	3.9×10^5	37.9	33.4	3.7×10^5
4h 3 [H]-Gln	1726	13.1	3.8×10^5	57.1	50.5	8.4×10^5
R-BT065						
Control	1185	4.9	1.4×10^5	0	0	0
1h 3 [H]-Gln	3114	5.5	1.6×10^5	28.9	27.4	2.3×10^5
4h 3 [H]-Gln	2119	6.3	1.8×10^5	47.1	44.4	6.1×10^5
acl						
Control	4603	10.8	3.1×10^5	3.2	1.2	1.1×10^3
1h 3 [H]-Gln	10292	11.7	3.4×10^5	30.3	28.1	2.5×10^5
4h 3 [H]-Gln	11509	10.3	3×10^5	46.5	48.9	6.6×10^5
ALF968						
Control	4127	2.4	6.9×10^4	0.4	0	0
1h 3 [H]-Gln	20054	1.3	3.8×10^4	27.8	24.4	2×10^5
4h 3 [H]-Gln	11981	1.9	5.5×10^4	45.8	26.7	3.5×10^5

LD12-115						
Control	1103	16.1	4.7×10^5	0	0	0
1h ^3H -Gln	1100	13.8	4×10^5	31.4	27.6	2.5×10^5
4h ^3H -Gln	1887	11.0	3.2×10^5	51.6	46.5	7×10^5