Master thesis report

Assessment of a new method in protists research: NanoSIMS analysis

with Lake Taihu as a model system

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Abstract

The hypereutrophic Lake Taihu in China was used as a model system for the development of a new method in protist research. The possibilities and challenges of nanoscale secondary ion mass spectrometry (NanoSIMS) analysis were assessed for the study of carbon transfer by protists. Lake samples were incubated with ^{13}C-labelled DIC (^{13}C-$NaHCO_3$) to study the uptake and transfer of carbon in the Taihu food web. The NanoSIMS 50L instrument (Cameca) was used to measure stable carbon isotope ratios in protists and the cyanobacteria *Microcystis sp*. Individual organisms were studied to assess spatial heterogeneity within the cells. Precipitated crystals interfered with the signal of the organisms. A way to remove crystals or to prevent crystal formation must be developed in the future. Regions of interest were identified and analyzed. Significant differences were found between a light and dark uptake experiment indicating the importance of autotrophs for the food web. $^{13}C/(^{12}C+^{13}C)$ values increased over the timepoints of the incubation experiment. The high $^{13}C/(^{12}C+^{13}C)$ values of protists indicated autotrophy. Although several challenges in the methodology remain, the NanoSIMS approach was promising for single-cell analysis of protists.

1 Introduction

Protists, also called protozoa, tend to be avoided by microbiologists and ecologists. Protist studies have been performed by some groups of researchers but compared to bacteria, fungi and phytoplankton, protists research has been limited. The group of protists is an extensive and important group of organisms. Protists are hard to treat as one group in research: the differences within the group are large. Protists can be heterotrophs or autotrophs, with a size range from 0.1 µm to >100 µm and many different feeding mechanisms and cell shapes occur. Several definitions are used to describe protists. Here we define protists as single-cellular eukaryotic organisms that are not animals, plants or fungi. Changing systems of protist classification and nomenclature in the past decade may be one of the reasons that protists are often considered too complex and thus unattractive group to investigate[1].

Protists are found in many natural environments – from the gut to soils to both marine and freshwater bodies. Heterotrophic protists can play a key role in the energy and nutrient transfer between trophic levels in aquatic food webs[2]. Protists can graze on bacteria and algae by filter feeding or phagotrophy. Protistan grazing can limit algal blooms and stabilize aquatic ecosystems[3]. Prey particles can actively be selected, concentrated and ingested. Via their oral groove filter feeding protists ingest water and particles. From the oral groove food vacuoles are created inside the protist cell. These food vacuoles help digest and transport food through the cell. The food vacuoles contain digestion enzymes. Particles inside the food vacuole can be digested or egested through a fusion of the food vacuole with the cell membrane. The average lifetime of a food vacuole in a ciliate is twenty minutes[4]. Phagotrophic amoebae enclose food particles. Analysis of food vacuole content provides information on food sources of protists.

Although the role of protists in food webs is large, protists are still neglected in biodiversity and food web studies. This is primarily due to a number or practical constraints that limit the applicability of methods for protist research. For example, the size range of protists overlaps with that of bacteria and phytoplankton such as algae. Separation based on size is not sufficient. Protist cells are fragile, identification by microscopy is difficult and methods that are well established for other microbes such as phospholipid fatty acid (PLFA) analysis are not well developed for protists. The current stage of protist research methods will be discussed in the first part of this report.

The main objective of this thesis is to assess the use of NanoSIMS as a possible new analytical method to study the role of protists in food webs. Following the work of de Kluijver et al. (2012), we chose the hyper-eutrophic lake Taihu (surface area 2250 km^2) as a model system. This lake is located in the Yangtze Delta in East China. It is located near the city of Shanghai, and is the primary drinking water source for around 30 million people. In 2007, a drinking water crisis left approximately two million people in the city of Wuxi without drinking water for one week. The cause was a massive bloom of the toxin-producing cyanobacteria *Microcystis spp*[5]. *Microcystis* is generally assumed to be an unattractive food source for zooplankton, allowing massive bloom development. In 2009 de Kluijver et al. conducted a 13C-carbon labeling experiment combined with PLFA analysis to show that organic matter produced by *Microcystis* was taken up by bacteria and then transferred to zooplankton. However, due to the limitation of their approach and other practical obstacles, the role of other microorganisms – namely protists – was not investigated. Thus, the broader aim of this thesis was to assess this role. I hypothesized that protists are an important intermediary in the transfer of carbon from lower to higher trophic levels and thus play an important role in the food web in the lake Taihu.

2 Established methods in protist research

In this section I review the most important methods used in protist research, focusing on the main advantages and drawbacks. Due to the large diversity within the group of protists scientific papers generally focus on either ciliates, flagellates or amoebae. When I refer to literature, often one of these groups is addressed to illustrate the results of a specific procedure or technique.

2.1 Microscopy and sample preparation

Microscopy can be used to identify protists, count protists (enumeration) or to count ingested prey in protist food vacuoles. To increase the visibility of protists in a water or sediment sample several sample preparation methods are available. These are not suitable for observation of live organisms. Advantage of microscopic observation of live protists over fixated protists is the easier discrimination between protists, detritus and other material. Precise identification and counting is however difficult due to the movement of the protists and masking by other material. Fast moving, small and rare species can often be missed[5] leading to biased results. Another drawback of using live samples is that samples need to be analyzed quickly after sample collection. Environmental conditions in the samples change over time and can lead to the loss of fragile protists. Protist-matrix separation, fixation and staining can solve part of these problems. However, it has its own specific drawbacks as discussed in this section.

2.1.1 Protist-matrix separation

Centrifugation of natural samples is generally insufficient to separate protists from detritus, mineral particles and phytoplankton. The challenging characteristics of protists limit the amount of methods that can be used. Decanting and preserving with formaldehyde failed because protists were damaged too easily and protists could not be separated when they were attached to sediment[6]. The coverslip method as published by Webb in 1956 is selective: larger protists are not extracted[7]. The seawater-ice method and other flushing methods are not general enough for the large diversity of protists: the differences in reactions to temperature gradients makes this method unsuitable to address the whole protist group[6,8].

Silica gel-based density gradient centrifugation has frequently been used in recent papers. It is preferred over other methods because of its high yields and relatively fast protocol. The recovery rate for both small and large ciliates is over 94%[9]. Silica gel is a low viscosity density gradient medium composed of colloidal silica coated with polyvinylpyrrolidone (PVP) in the size range of 15 to 30 nm diameter. It is known under brand names Percoll and Ludox. For over two decades Percoll has been the main silica gel in use but the 20-40 times cheaper Ludox HS 40 is the main gel now. Ludox also has a higher, more suitable density of 1.3 g/l allowing the use of 10 mL tubes in contrast to Percoll, which is not suitable for samples larger than 2 ml due to its low density[9]. The silica gel is mixed with freshwater sample in a sample tube and centrifuged (between 200 to 1000*g*) to establish a density gradient and separate all solid material in the sample based on its density (figure 1). Protists-matrix separation techniques can be used for preparation of microscopic slides but also as sample preparation for other analysis techniques like fluorescence in situ hybridization (FISH) or NanoSIMS analysis.

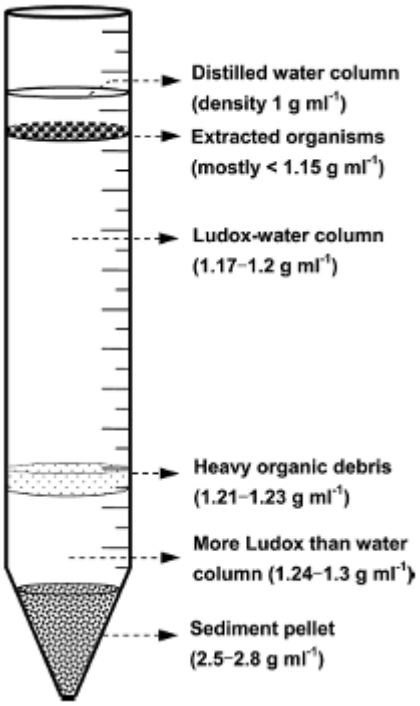

Figure 1. Silica-based density gradient centrifugation by Ludox. The working principle of Percoll is similar. Figure taken from Xu et al. 2010.

One of the drawbacks of silica-gel density separation is the strong size and shape distortion of protists. The use of density gradient centrifugation for separation of organisms is limited. Diatoms, meiobenthos and protists were found to end up all in the same density layer by Du et al. (2009)[10]. Silica-gels cannot be used in samples with a pH <5.5 or >10 and above a salinity of 5 because the density of the silica gel increases and becomes too dense for use[8].

2.1.2 Protist Fixation

Fixatives generally used for protists are formaldehyde, glutaraldehyde and Bouin solution. All are common nonspecific fixatives used in microbiology. Bouin solution is strongly acidic. The silica gels Ludox and Percoll form a thick, unfilterable gel when below a pH of 4.5 and can thus not be used combined with Bouin solution[11].

2.1.3 Non-specific protist staining

General protist stains are 4',6-diamidino-2-phenylindole (DAPI), 5-chloromethylfluorescein diacetate (CMFDA) and 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF). All are non-specific fluorescent stains often used in microbiology. Stains can be observed with a fluorescence microscope. The use of a fluorescence microscope also allows direct recognition of heterotrophic and autotrophic organisms due to chlorophyll a, b and c autofluorescence in autotrophs[12].

Lessard et al. (1996) used DAPI to stain protists to study the food web relationship between protists and fish. The fluorescence signal of the stained protists could be detected in the guts of fish. They stained twelve different protist species. Four bacterivorous (bacteria consuming) and four herbivorous (algae consuming) species were successfully and brightly stained after four or more hours of staining. Chlorophyll autofluorescence made the signal of two out of four autotrophic species less bright[13]. Interestingly, DAPI-stained protists showed unaffected growth rates and unaffected moving and swimming behavior[13]. A possible problem of this method was the transfer of the fluorescence stain to non-target cells when stained cells were mixed with natural cells, due to cell lysis. This transfer could be prevented by fixation of the stained protists with formaldehyde or glutaraldehyde[13].

CMFDA is a stain similar in use to DAPI. Its bright green or blue fluorescence (excitation wavelength 492 nm) is easy to recognize and distinguish from the red autofluorescence of chlorophyll[14,15]. Protist movement and reproduction are unaffected. Additionally fluorescence is kept over several generations of protists which allows multigenerational analysis. The fluorescence signal remains visible for at least 72 hours if the organisms are kept in the dark. CMFDA cannot be used for incubations with autotrophic organisms because it fades rapidly in light. Also microscopic observation time is limited due to the rapidly fading fluorescence signal[14]. CMFDA is labeled a possible human hazard but its toxicity to protists is low. DAPI is preferred over CMFDA as both have similar advantages but CMFDA has larger drawbacks.

The quantitative Protargol stain (QPS) method is a combined protocol for fixation and staining. It was first published by Montagnes and Lynn in 1987 and has been adjusted and improved several times[5,9–11,16]. Wickham et al. (2000) combined the QPS method of Montagnes and Lynn with Percoll density gradient centrifugation by Starink et al. (1994) into a highly effective method to study benthic protists. Du et al. (2008) adjusted the protocol of Wickham et al. to the use of Ludox instead of Percoll. The extraction efficiency of this Ludox-QPS method was 97.6±0.8% for ciliates[10]. The detection of small, rare and fast moving species is larger for QPS treated samples than for direct microscopic observations of live samples. 60% of all species identified in a QPS treated sample could not be determined by microscopy of live organisms[5]. The QPS method is based on staining with Protargol and silver or gold permanganate. The protocol of QPS has been improved from a 15-27 hour protocol to a 4 hour protocol by Skibbe et al. in 1994. The full protocol holds over twenty steps but can be subdivided into 7 major operations: fixation of protists on a filter which is then embedded in agar, hardening of the agar by formaldehyde, bleaching with potassium permanganate, impregnation in warm Protargol, a brief stain with gold chloride, fixing with sodium thiosulfate, and dehydration of the cells with alcohol series[11,16]. Protists are stained on both sides and cilia and the internal structures of ciliates are all intensively stained, allowing identification of protists up to genus level and identification of sexual processes and division stages[11,16]. The protocol is labor intensive, time consuming and requires expensive laboratory equipment and toxic chemicals but achieves great detail. The microscopic analysis takes one to two hours per filter. Normal light microscopy can be used but allows no direct identification of heterotrophs and autotrophs[5]. Ciliate biodiversity can be expressed from the results by using the Shannon-Wiener diversity index, as done by Wickham et al. (2000). Samples can be studied and stored for several months after QPS fixation[5,16]. Staining and visibility by QPS can vary in effectivity between protist species. QPS holds best results for ciliates and less for flagellates. Samples rich in organic matter are stained less than organic matter poor samples because permanganate has a high affinity to adsorb or react with organic matter. This results in less effective bleaching of ciliates[11]. QPS has the same tendency as Ludox and Percoll to cause shrinkage and shape changes in protists[17,18]. No biovolume and biomass of the protists can be determined, in contrast to counts of live specimen. Jerome et al. (1993) proposed the use of a conversion factor of 0.4 between the original cell volume and the volume found after QPS[18]. The paper by Pfister el al. (1999) however suggests different shrinkage rates for different protists species. One conversion factor would therefore be inaccurate. For more accurate biovolume determinations species-specific conversion rates should be determined. In general the shrinking rate found by Pfister el al. (1999) was higher than the factor of 0.4 found by Jerome et al. (1993).

2.1.4 Conclusion on staining and the use of microscopy

Because protist separation, fixation and staining are combined in Ludox-QPS this method allows for simultaneous enumeration and taxonomic determination. The extraction efficiency is very high but the protocol is long and expensive. In his recent update paper Foissner (2014) published updated protocols for various variations on the above mentioned QPS silver-staining methods. No method can be considered the universal best method because the effects are different on different protists. A table

with the suitability of several methods for 28 ciliate taxa is available in the update paper of Foissner. Even if the right method is used and protocols can be followed silver-staining and QPS are not suitable methods for relatively inexperienced protist researchers. The results of the stains can vary and translating silver-stained characteristics to characteristics of live protists is not straightforward. Even for scientists experienced in protist determination identification of silver-stained protist is difficult. Foissner states that identification on silver-stained protists must always be combined with live observation. Species descriptions based on solely silver-stained protists can be considered incomplete and of doubtful value[19].

The high level of expertise needed for microscopic enumeration and identification of protists makes it a method unsuitable for new researchers in the field of protists. The sample size that can be analyzed within reasonable time is low. Heterogeneity and rare species can be missed if small samples are used as representatives of a large water body. These difficulties combined with the labor-intensive and time-consuming procedures make microscopic analysis unattractive for enumeration studies. Newer, faster methods are expected to replace the role of microscopy for enumeration. Microscopic observations are still of high value for the discovery and description of new protist species. Staining and separation methods like Ludox, QPS and silver-staining are important to improve results. Only a small part of all expected protist species is described at this point in time and the identification of new species will be material for many publications in the future that use microscopic observation of live and fixated protists.

2.2 Analysis of grazing by microscopy

Grazing preferences and grazing rates of protists can be assessed by analyzing their food vacuoles. This can be done by microscopy. Certain prey species have a distinct autofluorescence and do not need staining, such as cryptophytes[14]. Most species have a less distinct autofluorescence that can be hard to distinguish in fluorescence microscopy due to the background fluorescence of the protist[15]. Prey visibility can be increased by staining alive or heat-killed prey prior to ingestion by the protist. Both algae and bacteria can be stained. Stained prey is added to the protist incubation in excess to outnumber unstained prey to ensure a high amount of visible prey inside protist cells. Drawback is that prey density is enhanced, possibly influencing grazing rates[14]. The grazing rate will increase in case the system was prey-limited. For systems that were not naturally prey-limited the absolute grazing rate will not be influenced. The relative grazing rate would be an underestimation[2]. This can be of importance if the capability of the specific protist to keep up with natural blooms is estimated. Ingested stained prey is counted by fluorescence microscopy. Masking of stained prey by protist organelles or other particles in the food vacuoles can occur[15]. The staining of prey is fast and relatively easy and prey motility and reproduction is unaltered[14,15]. Uptake of prey by protists takes 30-60 minutes so incubations can be short. Microscopic counting of the prey particles inside food vacuoles is, however, time-consuming and labor-intensive. The stains mentioned in the earlier chapter on protist staining are also used for prey staining. An additional stain for prey particles is Hydroethidine (HYD). It was proposed in 1991 by Putt as a vital stain for prey[20]. Due to its high toxicity and its chlorophyll-like red fluorescence HYD is not frequently used. Genetic modification of bacteria is a relatively new and promising method for prey staining. However, it can only be used for bacterial strains that can be cultured. Bacteria are modified to contain enhanced green fluorescence protein (EGFP). The EGFP stain is produced within the bacteria itself and is brighter and longer lasting than stains of other techniques. Bacterial cells are not affected in behavior or movement by the EGFP treatment and ingestion by protists is not different from normal bacterial ingestion[21]. During digestion inside protists food vacuoles the stain remains visible for a longer time than DAPI stained cells. The location of the stain inside the bacterial cell might be the reason for this. EGFP is stable for long-time preservation and storage up to 140 days[21].

Analysis of food vacuole content by microscopy can only be done on stained prey. Prey must be stained separate of the protists, or protists will also be stained. Naturally mixed samples can thus not be analyzed by this method. For the analysis of protist uptake of cultured prey it could be used as it is an easy and cheap method. The estimated grazing rate may however not be a good representation of the natural grazing rate.

2.3 Grazing preferences

Protist grazing is active and selective. Selection is size-based and viable, growing bacterial cells are preferred over starved or dead bacterial cells[22,23]. Size-selectivity seems to be of bigger importance than species-selection[24]. Grazing preferences on the viability of algal cells have not been tested. Studies on grazing rates make use of stained live prey, stained heat-killed prey or artificial (inert) prey particles. Often no effect of the prey type on the grazing rate is assumed. Heat-killed and inert prey have the advantage to be used off the shelf. Selection of a prey type that does not match the specialized way of grazing of the studied protist can result in biased estimations of grazing rates[25]. The design of the grazing experiment should be adjusted to the grazing preferences of the target protist species. Difficulty is that grazing preferences of many protists are unknown. Sibbald and Albright (1987) compared grazing preferences on free-living and aggregated bacteria by the microflagellates *Bodo sp.* and *Paraphysomonas*. *Bodo sp.* showed a preference for aggregated bacteria whereas *Paraphysomonas* showed a preference for free-living bacteria. Sherr et al. (1991) showed both flagellates and ciliates have a preference for heat-killed labeled algae over inert particles, except for the smallest particle size tested (1.1 µm). Jezbera et al. (2005) found prey preference can shift during incubation. Flagellates showed preference for a specific bacteria first and avoidance later. This shift was induced by size preference. After all bacteria with the preferential size and morphotype were consumed the flagellates showed preference of another bacteria with the right size over the earlier preferable bacteria with the wrong size[24]. The strength of avoidance or preference is complex and dependent on many factors. Best results are secured if natural grazing conditions are mimicked.

2.4 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a method that enhances visibility of target microorganisms when studied under a fluorescence microscope. The principle of FISH is illustrated in figure 2. It is a molecular technique originating from the medical sciences based on the use of fluorescently labeled rRNA-specific probes[26]. The probes used can be adapted to the purpose, from more general rRNA probes to species-specific. The probe holds a DNA/RNA code and therefore binds specificly to the target organisms or group of organisms.

Many fluorescence signal-enhancing techniques for FISH have been published over time. Cells in the stationary growth phase and cells with a thick gram-positive wall can have low fluorescence signals[24,27]. Autofluorescence of non-target cells and background fluorescence can cause difficulties in separating these weak-signal target cells from non-target cells. FISH-TSA is a method based on tyramide signal amplification (TSA)[28,29]. TSA enhances the intensity of the fluorescence signal of target organisms. Catalyzed reported

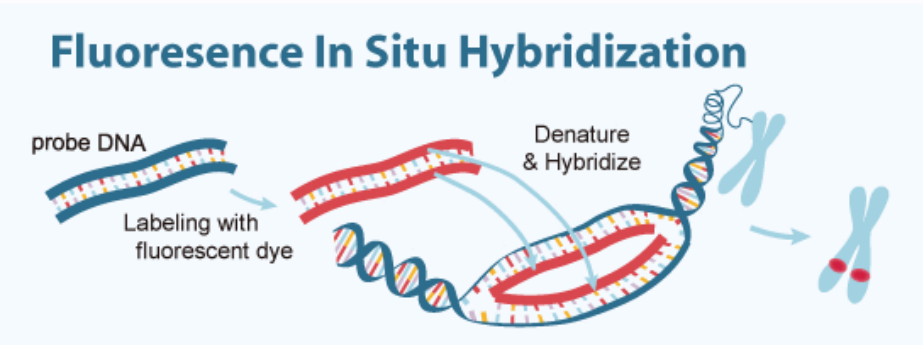

Figure 2. FISH procedure strongly simplified. Figure from abnova.com.

deposition-FISH (CARD-FISH) is adapted to improve the detection of small bacteria with a low ribosome content. Using this method, Medina-Sanchez et al. (2005) were able to produce high quality results of ingested prey in protist food vacuoles which allowed detection of nearly 100% of all ingested planktonic prokaryotes and archaea[30]. Detection efficiency for sediment samples using FISH is considerably lower than for water-column samples (59%)[31]. With the current databases on probe information, FISH can be considered an a-posteriori technique as no information on the sample composition is needed prior to FISH staining.

2.4.1 FISH to identify and enumerate protists

FISH can be used for accurate identification and enumeration of protists in natural samples. The misidentification error by FISH microscopy is considerably lower than by direct microscopic identification of unstained cells or cells stained by non-specific dyes. FISH microscopic counting is also more accurate than direct microscopic counts[32]. Lim et al. (1996) found that total nanoplankton counts in natural samples were twice as high by FISH as by direct microscopic counts of cells stained with DAPI or acridine orange (AO). For culture samples no significant difference was found. FISH can reveal the presence of protists inside detrital aggregates, resulting in the higher counts for natural samples[32]. More or less specific probes can be used dependent on the expectations of the sample by the researcher. Diversity counts performed with FISH can therefore be biased by probe selection. Species-specific probe availability has strongly increased since the first use of FISH in biological samples. Not et al. (2002) successfully used FISH-TSA to identify protist diversity. For FISH-TSA it can be necessary to test the permeability of the cell walls of target cell prior to analysis. The staining molecule of FISH-TSA is large and cannot enter through the cell walls of all protists without the need for enzymatic permeabilization[27].

2.4.2 FISH analysis of food vacuole contents

Gunderson and Goss (1997) were the first to use FISH to identify prey particles in food vacuoles of protists[33]. Hybridization of bacterial, algal and archaeal cells can be done directly inside the protistan food vacuoles[30]. Quality of rRNA of the ingested prey decreases relatively slowly. When a protist is fixated and analyzed, part of the food vacuole contents will already be digested. Stains attached to other parts of the protist cell than the rRNA will be degraded faster and will be harder to detect. The FISH signal remains visible for a relatively long time[33]. From the microscopy results after probe addition grazing rates can be determined by estimations of differences in food vacuole content between timepoints. Protists must be fixated prior to FISH probe addition so subsamples of a sample must be used to compare timepoints. If different types of prey were present in the sample, prey selection can be observed from the relative abundance of prey types in the food vacuoles. Jezbera et al. (2005) used a non-specific probe to detect all ingested bacteria in the food vacuoles first and then species-specific probes to detect the target prey organism, making it possible to quantify the relative abundance of the target prey. This method is most suitable for small or medium sized protists. The analysis of individual food vacuoles in large ciliates is difficult due to masking by the organelles, macronucleus or other material in the food vacuoles.

Drawbacks of the FISH method are the possible loss of sensitive species by the procedure, the possible bias due to probe selection and the labor-intensive procedure of fluorescence microscopy counting. Identification of species is much faster than by non-specific staining of protist and prey and can also be done by less trained persons. The drawback of the low through-put due to the time-consuming procedure of microscopic counting is still present, but less than for non-FISH stained or unstained protists and prey. An advantage over QPS is that if no fixatives are used, FISH does not change the size or shape of protists and all main cell features are kept, allowing biomass estimations.

Because FISH is an a posteriori method grazing rates and ingestion status can be obtained from natural samples without change of prey densities. Samples do not have to be incubated in the lab prior to

analysis. Prey and protist can be identified simultaneously under the microscope if several probes are used.

2.5 Phospholipid-Fatty-Acid analysis (PLFA)

Phospholipid fatty acids (PLFA's) are used as biomarkers in food web studies. Assuming that fatty acids that protists or zooplankton ingest are built into the cell unchanged, the full fatty acid profile can indicate the food source of the organism. The fatty acids of prey can only be recognized if the fatty acid profile of prey and predator are known. PLFA analysis is already used extensively for food source identification of zooplankton. The PLFA profiles of many bacteria and algae are known. In contrast only a few PLFA profiles of protists are presently known.

PLFA extraction is a long and labor-intensive process, followed by gas chromatography (GC). Phospholipid fatty acids are the main fatty acids used as their presence is genetically determined and thus little affected by environmental factors or feeding behavior of the organism[34]. To obtain fatty acid characteristics of an organism the organism must be isolated extremely pure. Fatty acids can then be extracted and the fatty acid profile is revealed by GC analysis. Protists need to be cultivated with one specific food source to remove all other prey PLFAs from their food vacuoles. Protists can be difficult to cultivate. Additionally, a clean fatty acid profile of protists is practically very difficult to obtain as their food vacuoles contain bacterial or algal fatty acids.

Vera et al. (2001) detected branched chain fatty acids in protists' PLFA profiles. Branched fatty acids are bacterial biomarkers. Mauclaire et al. (2003) determined the PLFA profile of flagellate amoebae[35]. The amoebae were cultivated on bacteria with a known PLFA profile. The protists PLFA profile was obtained by subtraction of the known PLFA profile of the bacteria from the total PLFA profile of the mixture. A relatively clean profile could be obtained because no other species but the bacteria and the protist were present in the culture. A scaling factor was needed to compare both PLFA profiles. The protist PLFA profile showed 16:0 and 18:0 as dominant fatty acids. These FA are however ubiquitous and not specific to protists. ω-6 series polyunsaturated fatty acids showed to be dominant in protists whereas the abundance of ω-3 series is low. The ω-3/ω-6 ratio could be used as a protist tracer. Similar results were found by Desvilletes et al. in 1997[34]. The typical ω-3/ω-6 ratio was found to be characteristic to all studied protists but tintinnids. It is unknown whether protists PLFA is (partly) metabolized when ingested by the predators. If the PLFA's are altered due to ingestion they may be difficult or impossible to find back in the predators PLFA profile. More research is required to clarify this.

Tetrahymanol (gammaceran-3β-ol) is used as a specific biomarker for marine ciliates that feed on bacteria. Tetrahymanol is found in both field samples and cultures of bacterivorous ciliates[36]. It has also been found in the fatty acid profile of copepods that were fed on bacterivorous ciliates[37].

Vera et al. (2001) found the food source of bacteria can strongly influence the composition of their fatty acid profile[38]. Bacterial polyunsaturated fatty acids (PUFA's) were strongest influenced by the different substrates. The production of fatty acids by the bacteria was altered due to limited availability of necessary substrates. The altered fatty acid profile was found in the higher trophic level of the protists.

Also environmental factors such as temperature and oxygen availability can influence the FA profile[39]. Bec et al. (2011) questions the reliability of PLFA analysis for species identification and carbon transfer studies in food webs[40]. FA are not always as specific as thought. Phytoplankton PLFA profiles can be altered by aggregate formation. Not all endmembers are known well enough to determine the food source of the fatty acids in zooplankton accurately. The effect of terrestrial carbon as a food source on the PLFA profile of microorganisms needs more research. The effect of trophic transfers on the FA

profile differs per species and per dietary mode. If more PLFA profiles of protists are discovered, PLFA combined with ^{13}C labeling can be a useful tool to study the transfer of carbon from bacteria to protists.

One major methodological difficulty in protist research is the difficulty in separation of protists and other cells like bacteria or phytoplankton. Obtaining a clean protist sample can only be done by creating cultures of many generations with many cleaning steps. To obtain information on the PLFA profile of a protist the sample must be clean from all other biological material. If the PLFA profile of the food source of the protist is known and the sample is clean, a PLFA profile of the protist can be obtained. A relatively large amount of biomass is needed for PLFA analysis, so many protist should be separated. Separation could be done by silica-gel density gradient centrifugation. But protist cells should be washed intensively to get rid of the bacteria on the outside. Many protist cells are fragile and could break by this washing. The procedure is very long and difficult. A great amount of time and money for PLFA research would be needed to find PLFA profiles of enough protists to draw a conclusion on general biomarkers for protists. Therefore I see little future in the use of PLFA analysis for protist research.

2.6 Flow cytometry

Flow cytometry (FCM) is an automatic cell sorting method. Sorting can be based on many characteristics such as size, autofluorescence, side scatter or fluorescence by staining. In the context of protist research, FCM can be used for determination, enumeration and identification of protist species and their food vacuole contents.

Kenter et al. (1996) were the first to use flow cytometry for protist analysis. Grazing rates of a freshwater ciliate were investigated. The algal prey and ciliates were stained with DAPI 30 minutes prior the experiment. After incubation FCM was used to distinguish between bacterial aggregates, fecal pallets, starved ciliates, ciliates with ingested algae, and ciliates that had recently digested food by using combined red and blue fluorescent signal intensities. Protists retained algal fluorescence for a limited time after digestion of algal prey allowing discrimination between recently fed and starved protists[41]. FCM allows large sample analysis within a relatively short time frame. Commercially available cytometers are relatively mobile devices and can therefore be taken on a boat or taken to a field station as long as electricity is available. Natural samples must be analyzed within hours after collection to prevent loss of vulnerable species and changes in the sample. FCM is often used during incubation experiments to obtain direct results. Measurement intervals can be as short as 2-3 minutes. After FCM the protists can be placed back into incubation. Lindstrom et al. (2003) found three out of four ciliate species that were studied showed growth after DAPI staining and FCM cell sorting[42]. Enumeration of phototrophic protists by FCM is done by using red fluorescence and side scatter. Phagotrophic protists are more difficult to distinguish from detritus and other plankton due to the lack of phototrophic pigments. Lindstrom et al. (2002) tested several stains to increase the visibility of the protists and to improve the identification of ciliate species. TO-PRO-1 (Molecular Probes Europe, Leiden, The Netherlands) was the best stain and resulted in a clear distinction between ciliates and other material despite their overlapping size[43]. Lindstrom et al. found a highly significant correlation between FCM and microscopy counts (r^2=0.956) with the ideal agreement between the methods falling into the 99% confidence interval of the regression line.

Due to its much higher analysis speed FCM allows enumeration and identification of a larger amount of cells and can therefore offer a much higher statistical power. The fast analysis allows almost simultaneous analysis of many samples of an experiment, lowering the error caused by a changing environment in the samples. FCM's have different detection limits but in general the protists density

must be in the range of 200 to almost 30,000 cells per ml. Several cell characteristics can be determined at the same time.

Flow cytometry allows analysis of a large amount of samples in a short time frame, in strong contrast to microscopy. Flow cytometry can be combined with FISH. Up to now no papers are published on using both FISH and FCM together for protists, but for bacteria and phytoplankton it is performed routinely [44,45]. The accuracy of FISH can then be combined with the fast and efficient cell sorting of FCM.

2.7 Conclusion on protist research methods

Protist enumeration can be done automatically by flow cytometry (FCM). The reliability of FCM is optimized and established to a point where the advantages over microscopy are so large that microscopic enumeration might be substituted. However, more research is needed on the use of FISH for protist samples. The accuracy for FCM and microscopy is similar but FCM analysis is much faster allowing larger sample sizes. Analyzing enough sample to get results that are representative for a waterbody is too time consuming when using microscopy. A large advantage of FCM is that simultaneously several characteristics can be measured. If one is interested in counts of a specific protist species and also in its mean size, one only has to do FCM analysis as both can be tracked during one analysis. FCM also is the ideal method for analysis of incubation experiment samples, also when fragile species are used of samples that quickly change in condition. Because FCM is rapid samples can be measured with a small timeframe in between. This helps to limit measurement artifacts because samples can change while being stored until analysis. In contrast, FISH combined with microscopy analysis is much slower. Also sample destruction during analysis is an issue for FISH because fragile protist species can break due to the heat of the microscope.

Grazing rate estimations by FISH combined with microscopy are more reliable than grazing rate estimations by incubations with stained prey. Another advantage of FISH is that prey don't have to be labelled prior to ingestion. The grazing, ingestion, digestion and egestion processes can be studied as in the natural situation. Drawback is that protists cannot be put back into the sample after FISH staining and subsamples must be taken. If the larger sample is sufficiently homogeneous taking subsamples doesn't have to be a problem. If protists and prey are not stained before microscopy the same protists can be observed at different timepoints to study grazing rates. Accurate analysis of food vacuole content is however very difficult in live protists. FCM combined with different FISH probes that mark protists and the prey inside their food vacuoles is the best method for both culture and natural samples. The largest drawback of FCM with FISH is the possible bias due to probe selection. This can partly be solved by using more general probes. Future research should not be done on PLFA analysis. The amount of missing information is too large and the method is far from ideal even if the missing information were all gained in the near future because fatty acid extraction is time-consuming and requires relatively large sample amounts. Future research should focus on FISH probe selection for both prey and protists and on expanding the characteristics and stains that can be used for FCM.

3 NanoSIMS: presentation of a new method

Single cell analysis by the NanoSIMS Cameca 50L instrument was performed on samples from Lake Taihu, China. To place this research into perspective a small literature overview on biogeochemical research with NanoSIMS analysis is given first. Then individual results of NanoSIMS analysis of the Taihu samples are described, interpreted and discussed, with the aim to demonstrate the possibilities offered by NanoSIMS for protist research but also to identify drawbacks. In the last part of this report I attempt to place the results into the larger picture of the incubation experiment. At this point in time only part of the collected samples has been analyzed by NanoSIMS. The amount of data is insufficient for performing sound statistical analysis or drawing strong conclusion. But as a thesis project is also a training project, I tried to analyze the available dataset in a way that was possible.

3.1 NanoSIMS literature overview

3.1.1 General working principle of SIMS

The working principle of Secondary Ions Mass Spectrometry (SIMS) analysis is illustrated in figure 3. Primary ions are sputtered at the sample surface. Atoms and small molecules are ejected into the vacuum, mostly as neutral particles. A small part is ionized. Only the ionized particles can be transferred to the mass spectrometer and counted. These ionized particles are called secondary ions[46]. Secondary ion intensity can be increased several orders of magnitude by Cs^+ surface implantation[47]. The Cs^+ ion concentration at the sample surface must be at saturation level to reach steady state and to get a constant secondary ion count rate. Cs^+ ions are deposited on the sample surface. When steady state is reached, the concentration of Cs^+ on the surface is constant. The Cs^+ incorporation and erosion of the sample surface are in balance and the ionization probabilities are constant. This results in a constant secondary ion yield. Signal intensities are then representative for elemental composition of the sample. If image acquisition is started before ionization probabilities are constant the secondary ion intensities are not a reliable measure of the presence of atoms and molecules at the sample surface[47]. A depth gradient in Cs^+ concentration can lead to a difference in ionization probability with depth.

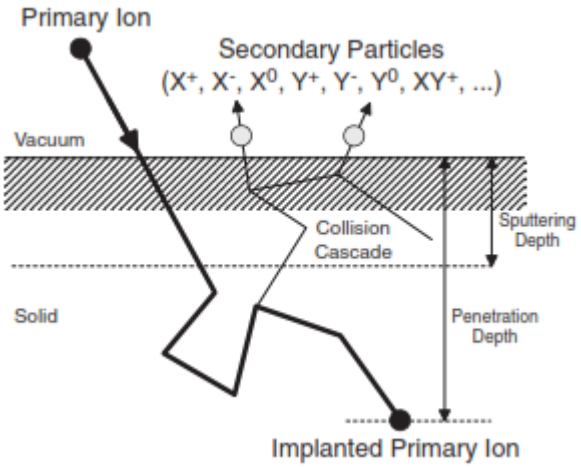

Figure 3. Principle on SIMS techniques. Primary ions erode the surface and release atoms and small molecules from the sample surface. Part of the particles is ionized and detected. Figure from Hoppe et al. (2013).

3.1.2 Introduction to the use of NanoSIMS analysis

SIMS is a combination of high-resolution microscopy and isotopic analysis. The basis for this technique was already introduced in the 1960s[48]. In the past SIMS techniques faced problems with spatial inaccuracy and too aggressive sample destruction[49]. These and other technical problems have been resolved in the design of the NanoSIMS 50L instrument, which is commercially available from (Cameca (Paris))[48]. Other advantages of the NanoSIMS 50L are the improved spatial resolution, the increased sensitivity and the possibility to analyze the full periodic table and isotopes of elements. NanoSIMS 50L uses an energetic ion beam of positive cesium ions or negative oxygen ions to ionize and liberate atoms and molecules from the upper layer. The primary ions hit the sample perpendicularly to the sample surface, allowing for the minimum spatial resolution of ~50 nm and ~250 nm for the Cs^+ and O^- primary ion beams, respectively. The secondary ions are accelerated and focused in the electrostatic part of

the instrument and then mass-analyzed by a magnet. Simultaneous detection of up to seven elements or isotopes is possible. However, the efficiency of detection depends strongly on the element. For example, the formation of pure nitrogen ions has extremely low probability; thus, nitrogen is typically detected as the molecular ion CN^-. Also elements from the group VIII cannot be detected[44]. The efficiency of detection additionally depends on the sample matrix. The range of masses that can be measured simultaneously is limited. For example, if the first detector is set to detect mass 1 (^1H), detector 7 cannot measure mass higher than 11.8[43]. The obtained NanoSIMS data are counts of each ion per pixel. The intensity of the received secondary ions is dependent on the concentration in the sample, but the relative sensitivity factors, such as practical ion yield and total sputtering field and their effect need to be investigated further[48]. Isotopic ratios can be calculated and can be used quantitative.

NanoSIMS 50L allows operation in three scanning modes: raster-scanning and line-scanning provides information on lateral heterogeneity whereas continuous measurements in one point allow detection of changes in composition with depth.

NanoSIMS analysis is expensive and time-consuming. Accurate selection of regions of interest (ROI) can help minimize the amount of scans. Electron microscopy or fluorescence microscopy on the NanoSIMS sample can be used to select ROIs prior to NanoSIMS analysis. Because NanoSIMS is a destructive technique electron of fluorescence microscopy should be performed prior to NanoSIMS analysis. Before samples are brought into the analysis chamber of the NanoSIMS instrument they are brought to high vacuum in the "intermediate-chamber" where outgassing of the sample takes place. If samples are embedded in resin the resin must be selected with consideration of the amount of outgassing expected. Samples should be thin and dry[48]. Establishment of the vacuum can be time-consuming. The vacuum in the analysis chamber is approximately 10^{-10} Torr. Samples are analyzed under this high vacuum to prevent interference of atmospheric ions. Samples must be flat. Variation cannot be more than on a nanometer level. Topography of samples can influence the measurements. An increased ion yield at the edges of topographical features was observed by Clode et al. (2007)[50]. The ratio between stable carbon isotopes ^{12}C and ^{13}C ions is not strongly affected by topographical effects; however, an effect can be detected in very precise measurements that require per-mil precision. For natural samples with a risk of topographical effects it is recommended to use the ratios of elements or isotopes instead of absolute values[50]. Samples must be conductive in order to dissipate the charge of the positive primary ion beam. A conductive layer can be created by the deposition of conductive material (for example, gold) on the filter or slide prior to sample deposition, or by conductive material deposition after the sample is put on the filter or slide. For samples with a strong topography the use of a conductive layer may not be sufficient and the sample can get charged, leading to a strong decrease in signal. An e-gun can then be used to lower the charging and increase the signal if Cs^+ ions are used.

Samples for NanoSIMS analysis must fulfill many requirements and sample preparation is time consuming. Resin embedded samples must be polished prior to analysis and samples in the aqueous phase cannot be analyzed. The price of the NanoSIMS 50L instrument and the running costs are however the main reasons why NanoSIMS analysis is not yet extensively used in science. The semiconductor industry is a big user of NanoSIMS analysis. Most scientific literature available is on case studies. I will therefore present a number of case studies to demonstrate techniques, challenges and results as found by other researchers using NanoSIMS for biogeochemical purposes.

3.1.3 Case studies

Herrmann et al. (2007) investigated the possibilities to use NanoSIMS for soil bacterial research. Sand and a bacterial culture were mixed, embedded in resin and then studied by NanoSIMS analysis. The

used resin contained carbon with natural isotopic composition. This could lead to dilution of the 13C-signal of an enriched sample[51]. Also in 2007 Herrmann et al. reviewed the current and future role of NanoSIMS analysis for soil science. Key factors for NanoSIMS analysis according to this review are ROI selection and selection of the right resin that allows both stable isotope analysis and outgassing of natural samples[48].

Grovenor et al. (2006) published a review on sample preparation for NanoSIMS analysis. The challenge addressed in this review is to find a method of sample preparation that does not influence the chemical composition and chemistry of the sample. Grovenor et al. state sample preparation is a key step in NanoSIMS analysis and the main problem to face is that 'loss or redistribution of easily diffusible ions is frequent, occurs rapidly and is difficult to prevent after sectioning and during preparation.' Samples preparation could be done chemically or cryogenic (freeze-based). Cryogenic methods less change the chemistry and diffusible ions of the sample but it must be done very fast and accurate. Slow freezing can cause ice crystal formation and can damage the sample[52]. Resin embedding resulted in ion transfer from sample to the resin and altered the natural situation. The importance of this error is strongly depended on the goal of the research. The resin must allow sufficient outgassing of the sample.

SIMS analysis can be combined with other methods of cell detection like FISH[53,54]. FISH can be used for a pre-NanoSIMS selection of regions of interest if that is not easily done by light microscopy or autofluorescence. Fike et al. (2008) used FISH to find regions with high densities of sulfate reducing bacteria in microbial mats. NanoSIMS analysis was performed after FISH. Element labeling FISH (EL-FISH) and Halogen in situ hybridization (HISH) are hybridization techniques that are applied simultaneous with NanoSIMS analysis. Both are based on the addition of horseradish peroxidase (HRP) labeled oligonucleotide probes to the sample that bind to specific places on the rRNA of target cells. Fluorine or Bromine containing tyramides are added to label the rRNA of the target cells, followed by NanoSIMS analysis. Isotopes and elements of interest and the fluorine and bromine are detected simultaneously. The fluorine or bromine signal can be used for automatic ROI dectection[54,55]. Problems can occur when fluorine and bromine attach to non-target material. For further reading on this subject I would like to refer to a review paper written by Musat et al. (2011). This review contains a comprehensive overview table of relevant papers on SIMS-combined methods.

The only research done on protists with the Cameca NanoSIMS is a study by Carpenter et al. (2013) on protists and other microbes in termite hindguts[56]. The results revealed that the spatial heterogeneity of the $^{12}C/^{13}C$ ratio was much higher inside the cell than at the cell surface. To analyze the internal structure part of the protists was top-cut by focused ion beam scanning electron microscopy (FIB-SEM) or sliced and sectioned by ultramicrotome prior to NanoSIMS analysis. Other protists were analyzed by a high-intensity NanoSIMS beam that was used to create a depth profile. The used method was of big influence on the results. Protists that were sectioned prior to analysis showed 5 times higher ^{13}C enrichment than high-intensity NanoSIMS beam "drilled" protists. The cutting and sectioning of protists prior to analysis also helped to prevent charging problems and low ion yields of larger protists, and precipitated minerals and bacteria were removed. Drawback of sectioning was the difficulty in discrimination of protist cells from other material. Cutting by FIB-SEM showed most promising results but was slow and costly with only four to five large cells to be top-cut within one full day.

3.2 Materials and methods

3.2.1 Site description

Taihu is located in the Yangtze (Changjiang) river delta in the province of Jiangsu, China (figure 4). It is the third largest freshwater lake of China with a surface area of 2338 km^2. The lake is shallow with an average depth of 2 m. It is a main drinking water source for approximately 10 million people and fishing, shipping, tourism and recreation make it of large economical value. The Taihu watershed is inhabited by approximately 40 million people[57]. Large cities in the vicinity of Taihu are Shanghai, Wuxi and Nanjing. Taihu is used as a repository for human, agricultural and industrial waste and the rapid economic growth of the past decades have led to a very strong increase in nutrient load. The lake is hypereutrophic and blooms of the toxin producing cyanobacteria *Microcystis* threaten its use as drinking water and fishing resource. In May 2007 a massive bloom of *Microcystis* led to a drinking water crisis that left 2 million people without drinking water for one week[58]. Temperature and wind direction and intensity have a large effect on the *Microcystis* density in various parts of the lake[58]. Experiments were performed at the Taihu Laboratory for Lake Ecosystem Research (TLLER) field station, located in the Northern area of the lake (figure 4).

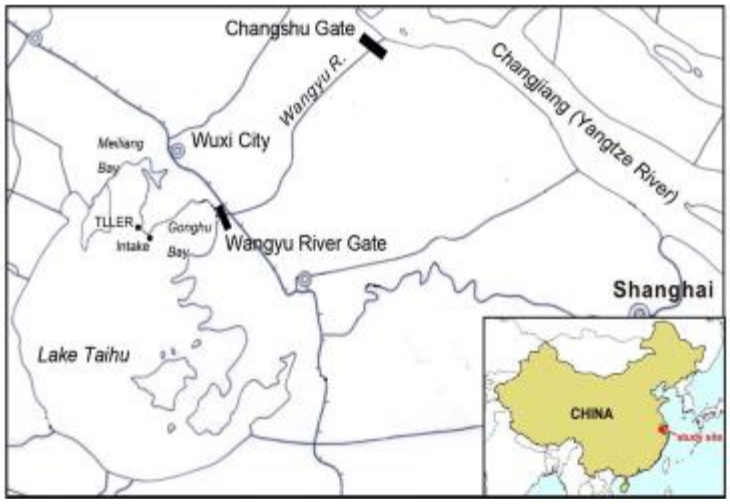

Figure 4. Lake Taihu, China. The experiments were performed at the TLLER field station in April – May 2015. Figure taken from Qin et al. 2009.

3.2.2 Incubation experiments

Samples were collected at the TLLER/NIGLAS field station near the city of Wuxi (figure 4) in April and May 2015. Lake water was collected in the early morning approximately 50 m from the shore. One liter of lake water was put in a 3 L plastic bottle. 55 mg of 13C-$NaHCO_3$ was added. The bottle was placed outside, floating on the lake surface. A 0.5 liter bottle was filled with 100 mL of the 13C-enriched lake water. It was covered and placed next to the other bottle for a dark control. Subsamples were taken from the incubation bottle after 6, 12, 18 and 24 hours (Table 1). The dark incubation was only sampled after 24 hours.

Table 1. Timepoints of the incubation experiments. Sunset was at 7 pm, sunrise at 6 am.

Time from start of experiment (h)	Local time	Light incubation	Dark incubation
0	8.00 AM	t_0	t_0
6	2.00 PM	t_1	
12	8.00 PM	t_2	
18	2.00 AM	t_3	
24	8.00 AM	t_4	t_4

3.2.3 Sample preparation

The aim was to make sample preparation as simple as possible. NanoSIMS protist samples were created by deposition of a drop of protist-rich sample water onto a gold-covered glass slide. In some cases microscopy was used during drying to localize the protists on the slide. Sample slides were air-dried and stored at room temperature until analysis. Slides were viewed by scanning electron microscopy (SEM) prior to NanoSIMS analysis. Part of the samples was washed with demineralized water to remove precipitated crystals.

3.2.4 Microscopy

Fluorescence microscopy was used on live samples to find the excitation of protists. Live protists were found to be excited by blue light (450-490 nm). Protists on dried, one month old samples did not emit light in any of the fluorescence wavelengths used. Light microscopy was used to locate protists. Scanning electron microscopy (SEM) was used to image the protists prior to NanoSIMS analysis and to select the most suitable protists for analysis. After NanoSIMS analysis protists could not be observed by SEM because the conductive surface was lost.

3.2.5 ROI selection

Regions of interest (ROIs) were selected and classified for all NanoSIMS images using Look@NanoSIMS[59]. Classes are listed in table 2.

Table 2. Region of interest (ROI) classes. ROI selection and classification were done by hand.

Class letter	Type of material
P	Protist material
M	*Microcystis* material
S	Diatom material
Y	Filamentous cyanobacterial material

4 NanoSIMS: Results and discussion

NanoSIMS imaging is a time consuming process. At this point in time several protists have been imaged. To analyze and compare results as I originally meant to do in the sense of the experiment the dataset is too small. This is unfortunate but the scans of individual protists already give us a lot of information. In the first part of this section I will focus on the individual protists. In the second part I will attempt to address the bigger picture.

4.1 NanoSIMS results: individual protists

4.1.1 Sample washing

Scanning electron microscopy (SEM) images of sample slides showed the presence of relatively large (~5 µm) crystals on the slides. Crystals may interfere with NanoSIMS analysis as they were found to lay on top of protists. Samples were soaked in demineralized water for 2 to 15 minutes in an attempt to remove the crystals. As shown in the SEM images of figure 5, most large crystals were removed whereas smaller crystals remained on the slide or on the protists. More intensive washing lead to removal of biological material. Some slides showed no effect of the washing (figure 6) Washing can have led to removal of bacteria from the slide and protists. The slide surface and the protist of this sample are visibly covered by relatively small unidentified particles in figure 7A. These particles are probably filamentous bacteria. The post-washing SEM image of this slide shows no filamentous particles. The washing could have removed the particles from the slide. If this is true, washing would be promising to increase the slide quality by bacteria removal. Another possible explanation for the absence of the particles on the SEM image could be the different focus by the SEM on the post-washing image. For cleaner images new methods for washing should be explored to find a method that will keep the target material on the slide but will remove precipitated crystals.

4.1.2 Crystal precipitation

On most of the SEM images (figure 5, 6, 7, 9) many relatively small bright spots can be observed on the protists and on the slide background. We assume these spots are crystals because of their general rectangular shape.

Table 3. Number of ROIs per timepoint and class.

	Protists	*Microcystis*	Filamentous cyanobacteria	Diatoms
t_1	-	45	-	-
t_2	18	183	-	-
t_3	6	69	17	-
t_4	5	10	1	21
t_4 dark	5	96	3	-

Three main groups can be observed in crystal composition: crystals high in 12C, ^{13}C and ^{16}O; crystals high in ^{12}C and ^{13}C but low in ^{16}O; and crystals low in ^{12}C and ^{13}C but high in ^{16}O (Figure 5 and 6). The more exact composition and origin of these crystals is unknown. The crystals high in ^{13}C and ^{16}O can be ^{13}C-HCO_3^- containing crystals that were added to the samples at the start of the experiment. Crystals of this kind are visible on top of the protist of figure 6. Protists that are exceptions are shown and discussed here.

Figure 8 shows a slightly different distribution of crystals than the other figures. The bright spots on the SEM image look similar to the crystals on other slides but are present only on top of the protist and not on the slide. One possible reason is that the water on the slide concentrated around and on the protist while evaporating. Dissolved crystals moved along with the water and precipitated on the protist. Another possible reason is that the spots are inside the protist. It could be local enrichments in the cell membrane or food vacuoles inside the protists. For food vacuoles the spots are however relatively small and present in large amounts.

Three spots that are high in ^{13}C and in $^{13}C/(^{12}C+^{13}C)$ are visible on the protist on figure 9. Because these spots are not visible on the SEM their origin is difficult to discover. They can be crystals located beneath the protist – if the protist is thin it can be sputtered away completely and the signal of the crystals beneath it can be picked up. The signal intensity of these spots is however decreasing with depth (figure 10). If the protist is heterotrophic the spots can also be ingested food particles that are (temporarily) stored at that location inside the protist. However, the $^{13}C/(^{12}C+^{13}C)$ signal of the spots is relatively high (0.085-0.1) compared to *Microcystis* food particles on the slide $^{13}C/(^{12}C+^{13}C)$.

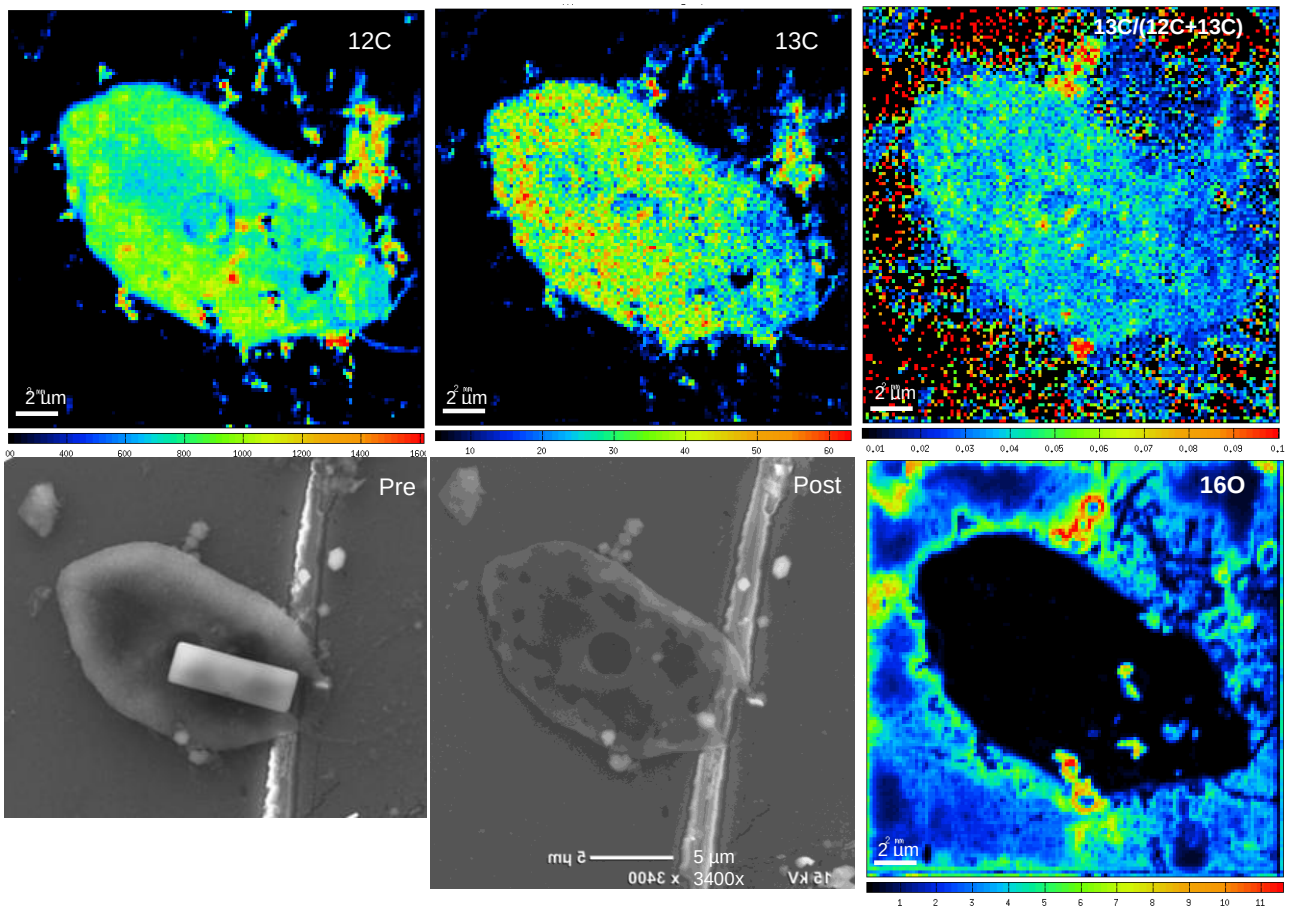

Figure 5. NanoSIMS and SEM images of a protist. Pre = pre-washing SEM. Post = post-washing SEM. NanoSIMS analysis was done after washing. A large crystal was removed by washing. The smaller bright crystals remained on the slide. The crystals are low in ^{12}C and ^{13}C but high in $^{13}C/(^{12}C+^{13}C)$ and ^{16}O. The signal of ^{16}O was divided by 1000 before imaging.

4.1.3 Topographical effects

NanoSIMS analysis is very sensitive to topographical effects. As mentioned above samples may show no more than nm-level variations. The large spherical shape of protists can cause charging and topographical effects such as ion shadows. An e-gun was successfully used to prevent charging. Topographical distortion was observed in several samples. No digital correction was performed on topographical distortion. Both figure 6 and 8 show a signal gradient for all elements. In figure 8 also the glass slide background signal shows spatial heterogeneity for ^{16}O. This may also be a result of topographical effects. Clode et al. (2007) stated that topographical effects and ion shadows are mostly observed at the edge of a topographical surface[50]. The background ^{16}O signal could also be caused by local precipitation of crystals. Water tended to concentrate around larger particles while the slides were drying. Crystals could have precipitated here while the water was evaporating. Clode et al. mentioned that the $^{13}C/^{12}C$ ratio is the most reliable variable to extract from the data because it is not affected by topographical distortion. When looking at the ^{13}C images of figure 6 and 8 we see that this also holds for these measurements.

4.1.4 Ionization probability

As mentioned in 'working principle NanoSIMS' insufficient Cs^+-sputtering and image acquisition before steady state is reached can influence secondary ion intensities. The ionization probability of different atoms can be different and the signal intensities are not fully representative for the concentration in

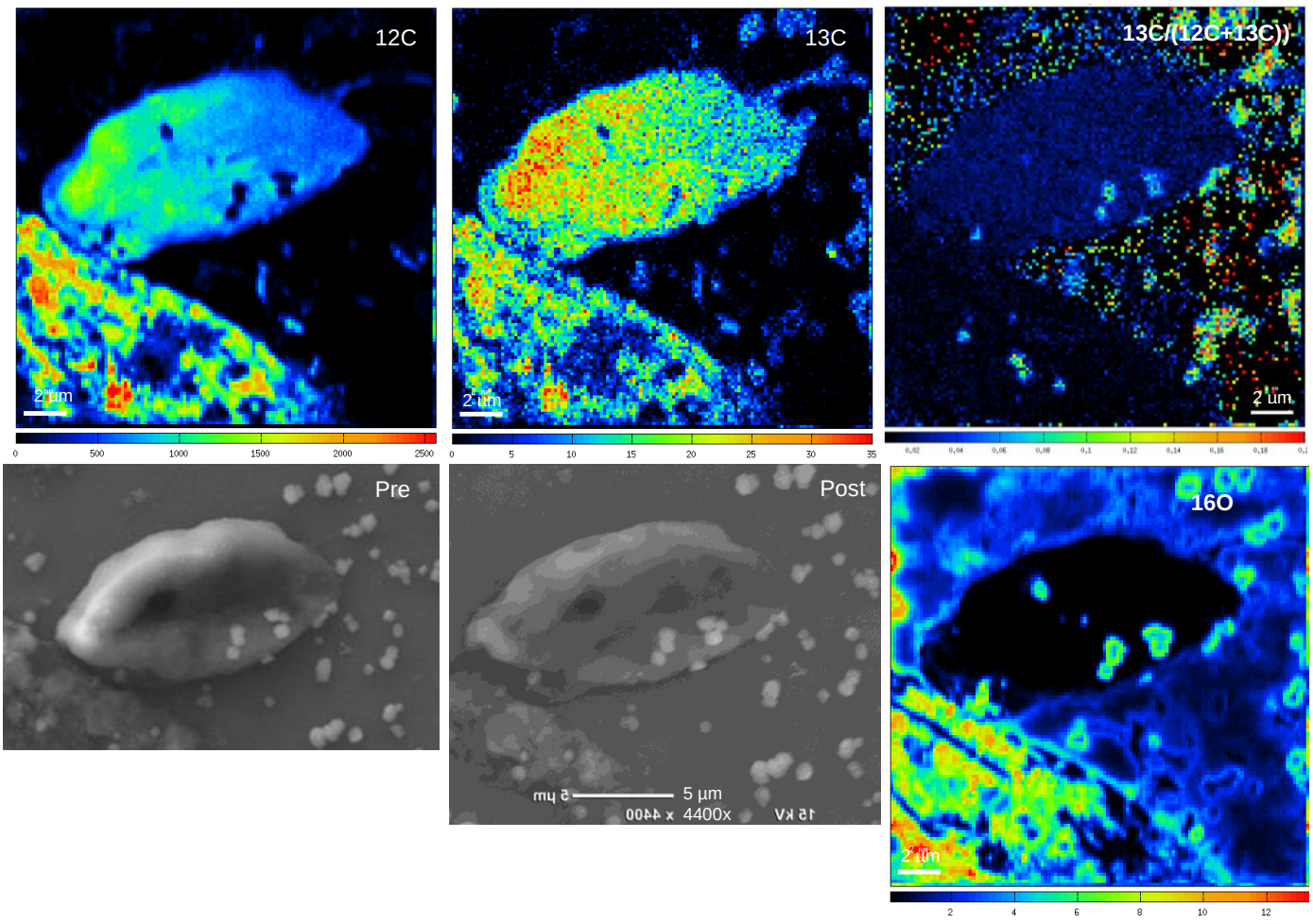

Figure 6. NanoSIMS and SEM images of a protist. Pre = pre-washing SEM. Post = post-washing SEM. No differences are visible between the pre and post-washing SEM images. NanoSIMS analysis was done after washing. A gradient in signal intensity is visible from the right to the left on all NanoSIMS images. Crystals on the protist and slide surface are visible as bright spots on the SEM and NanoSIMS images. The crystals have a low ^{12}C and ^{13}C signal but a high signal for $^{13}C/(^{12}C+^{13}C)$ and ^{16}O. The ^{16}O signal was divided by 1000 before imaging.

the sample. I expect this might play a role in the imaging of the crystals on some of the protists. In figure 5 crystals are present around the protist. They are visible on both the ^{16}O and ^{13}C images, but the shape of the crystals is different. The size in the ^{16}O image is slightly bigger and the shape is like a ring, with low signal in the center. In the ^{13}C image the crystals look slightly smaller and are not ring shaped. The images make it look like the inside of the crystal is high in ^{13}C whereas the outside is rich in ^{16}O. A possible explanation for this feature is a difference in ionization probability between oxygen and carbon. This can be caused by a gradient in the Cs^+ concentration with depth.

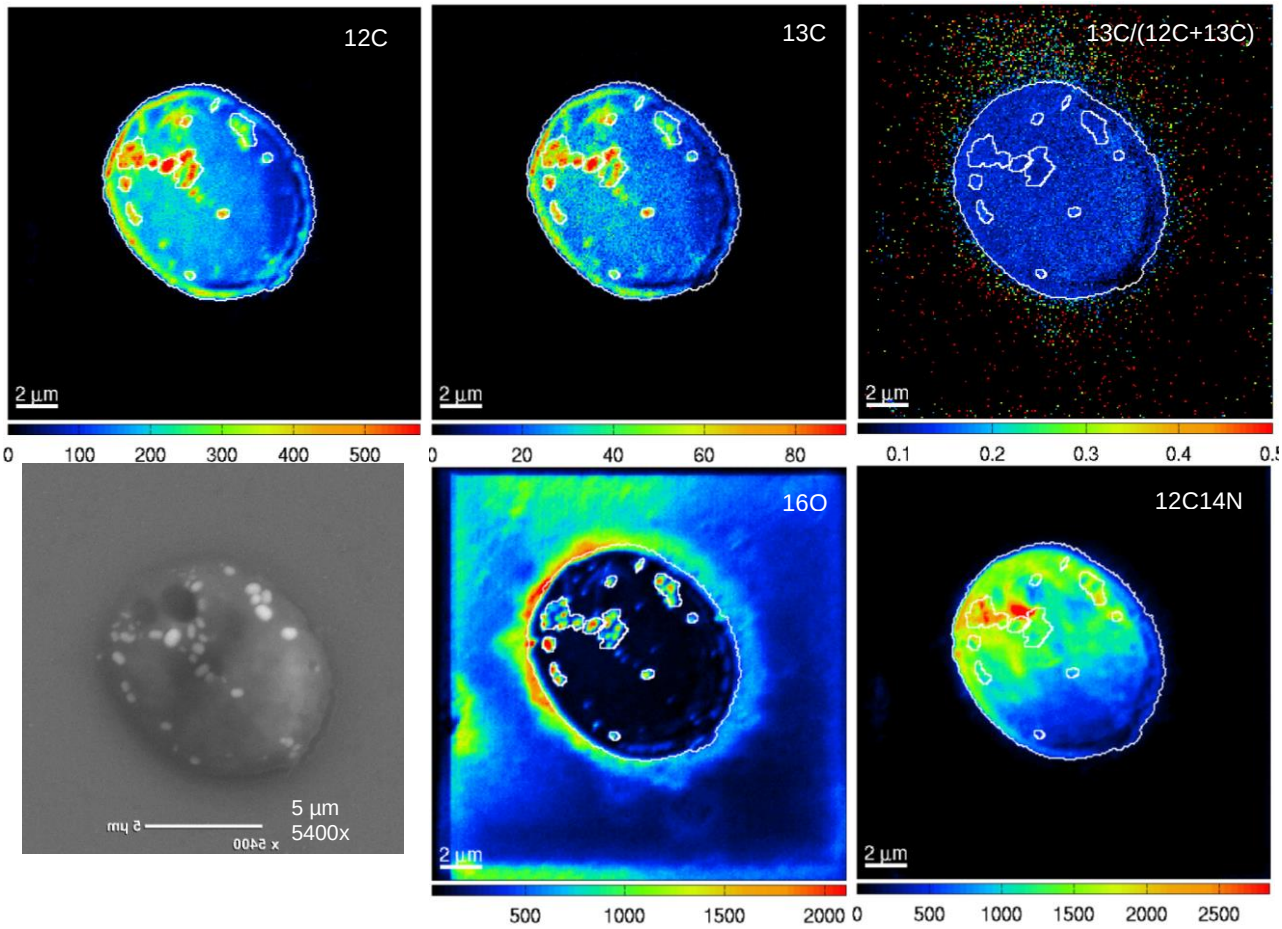

Figure 8. SEM and NanoSIMS images of a protist. A gradient in signal intensity from the lower-right to the upper-left part is visible for all elements. Also heterogeneity of the background in visible on the ^{16}O image. The SEM images shows bright spots on the protist. These bright spots are visible in all elements. $^{13}C/(^{12}C+^{13}C)$ is homogeneous over the whole protist.

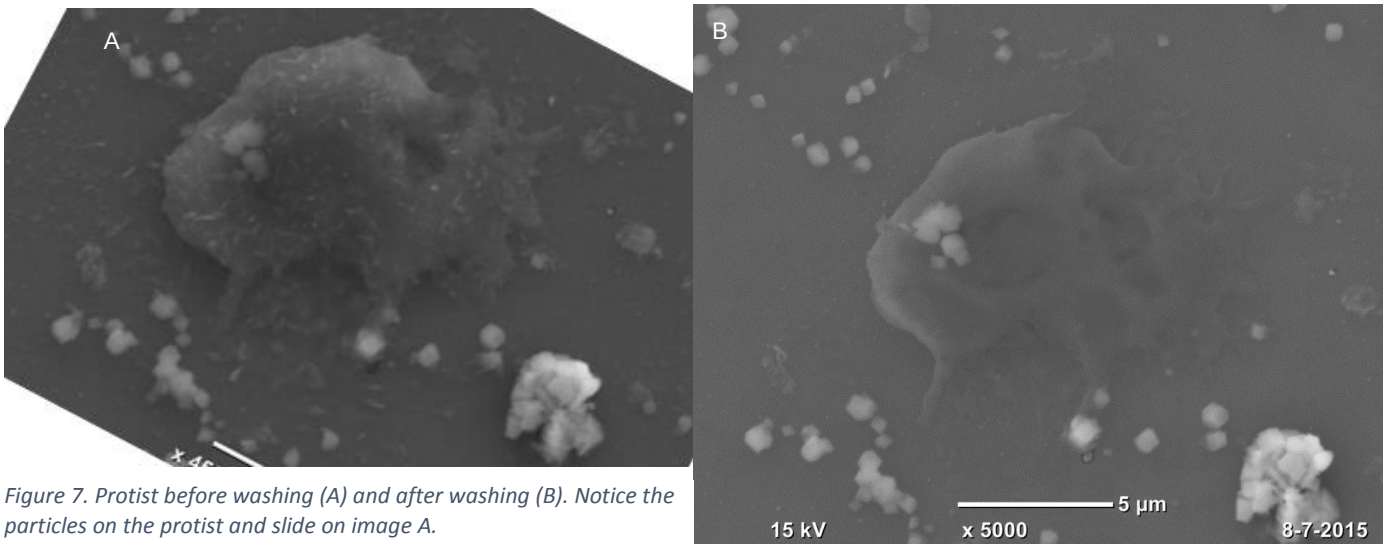

Figure 7. Protist before washing (A) and after washing (B). Notice the particles on the protist and slide on image A.

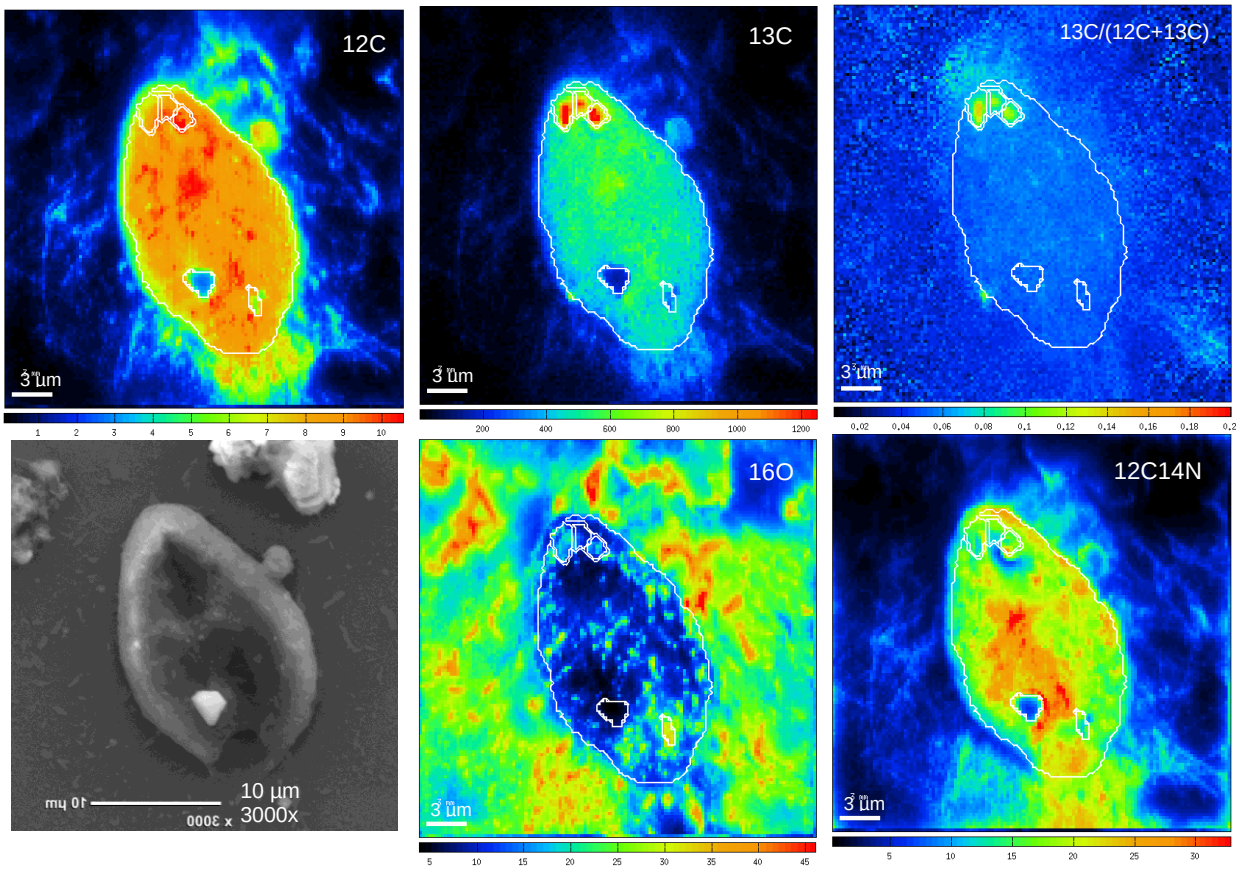

Figure 9. SEM and NanoSIMS images of a protist. A bright crystal is visible on the SEM image. All elements show low intensity for that spot. Three spots high in ^{13}C are visible on the ^{13}C and $^{13}C/(^{12}C+^{13}C)$ images but not on the SEM image. The spots have no high ^{16}O or $^{12}C^{14}N$ values. Signal counts of ^{12}C, ^{16}O and $^{12}C^{14}N$ were divided by 1000 before imaging.

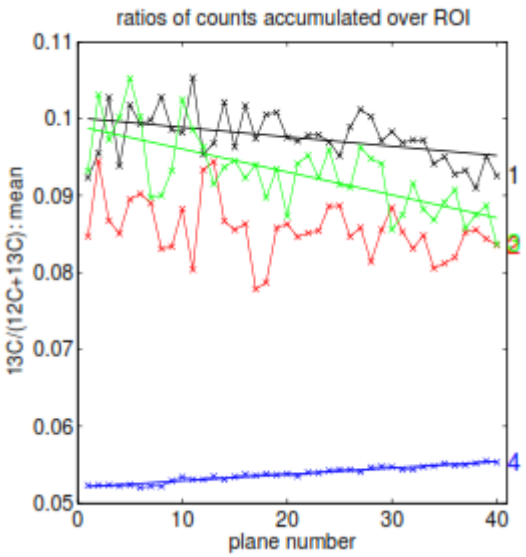

Figure 10. Signal intensity of $^{13}C/(^{12}C+^{13}C)$ with depth over 40 planes, for the protist shown in figure 9. ROIs 1, 2 and 3 (black, green and red line) represent the ^{13}C-rich spots in the protist. ROI 4 is the crystal on top of the protist.ROIs 1, 3 and 4 have a significant trend with depth.

4.2 NanoSIMS results: the bigger picture

At this point in time seven glass slides have been analyzed by NanoSIMS, representing 5 different water samples. 80 areas have been scanned, containing protists, *Microcystis*, filamentous cyanobacteria, diatoms or only crystals. ROIs were defined and classified on each slide. The number of ROIs per relevant cell class is shown in table 3.

4.2.1 ROI selection

ROI selection is a key step in NanoSIMS analysis. ROIs and ROI classifications are used by Look@NanoSIMS for data imaging and statistical tests. ROI selection with Look@NanoSIMS was done with the use of NanoSIMS results. This easily leads to a strong bias because regions are selected based on the results, not independent of the results. There is however no way to work around this. Also the ROI classification is biased. The researcher can decide to classify only ROIs high in ^{13}C as protists, because he or she believes the protists must be enriched. Protists areas low in ^{13}C are thus missed because they are overlooked, and the overall enrichment of the protists as found by analysis of the data is higher than the true enrichment. The use of SEM images of the imaged areas can help as the SEM does not show elemental composition and information is independent of NanoSIMS results. Drawing of the ROIs however always has to happen in NanoSIMS images.

4.2.2 Results

All NanoSIMS $^{13}C/(^{12}C+^{13}C)$ data from the light incubations is presented in the box-and-whisker plots of figures 11A-D. The number of data points can be found in table 3. Limited data is available for the filamentous cyanobacteria and diatoms. For *Microcystis* and protists more data of several timepoints is available. The range in the values is large for all four groups. The data range of *Microcystis* is smaller than the data range of protists. For protists the difference between the timepoints was not significant (Kruskal-Wallis test). A trend over the timepoints could be observed from the plots. The average value of timepoint 3 is higher than the average value of timepoint 2 for both *Microcystis* and protists. Between timepoint 1 and 2 a decrease in $^{13}C/(^{12}C+^{13}C)$ is found for *Microcystis*. For *Microcystis* the value of timepoint 2 was significantly different from timepoint 3 and 4. The differences between other timepoints were not significant. The mean $^{13}C/(^{12}C+^{13}C)$ values of protists are higher than the mean values of *Microcystis* for all timepoints. This difference is not significant but visible in the plots.

Figure 12 shows the data from the dark incubation experiment. Also data from the light experiment is shown. Both light and dark measurements are done at t_4. The data range for the dark incubation is smaller than for the light incubation and smaller for *Microcystis* than for protists. The $^{13}C/(^{12}C+^{13}C)$ values for the dark incubation for *Microcystis* are around 0.015. The $^{13}C/(^{12}C+^{13}C)$ values for dark incubation of the protists are around 0.02, with a range from approximately 0.015 to 0.03. The difference between the light and dark $^{13}C/(^{12}C+^{13}C)$ values is significant for both protists and *Microcystis*.

Figure 13 shows NanoSIMS images of *Microcystis*, filamentous cyanobacteria and diatoms. These images are illustrative. Spatial heterogeneity within cells of *Microcystis*, filamentous cyanobacteria and diatoms is not discussed in this report due to time limitations.

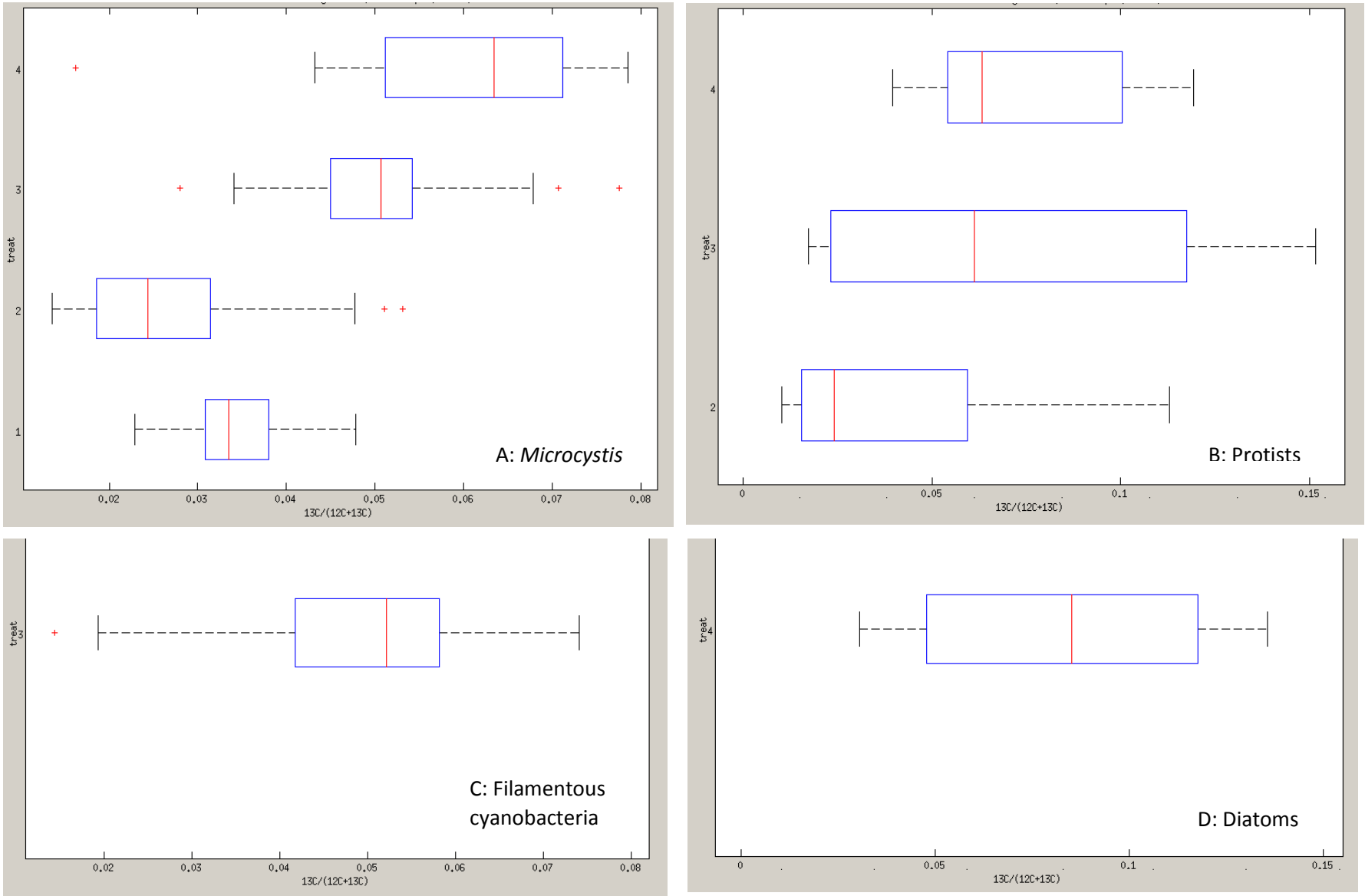

Figure 11A-D: NanoSIMS data of $^{13}C/(^{12}C+^{13}C)$ for ROIs defined as Microcystis, protists, filamentous cyanobacteria and diatoms. Timepoints are indicated on the y-axis. The number of datapoints for each timepoint is shown in table 3. At timepoint 0 $^{13}C/(^{12}C+^{13}C)$ is at natural abundance.

4.2.3 Discussion

The data ranges for all four groups (*Microcystis*, protists, filamentous cyanobacteria and diatoms) were large. The range in *Microcystis* data points was smaller than the range for the protists, filamentous cyanobacteria and diatoms. The ROIs classified as *Microcystis* all represented the same species, being *Microcystis* spp. The ROIs classified as protists are all organisms belonging to the group of protists but can represent different species. The data points could show interspecies variation. This is also a possible explanation for the wide range of data for filamentous cyanobacteria and diatoms. These ROIs could also represent different species of the same group.

The $^{13}C/(^{12}C+^{13}C)$ value of all groups was expected to increase due to substrate uptake. Autotrophic organisms can take up the 13C-enriched DIC directly. *Microcystis* sp, filamentous cyanobacteria and most diatom species are autotrophs. Protists can be either heterotrophic or autotrophic. From the microscopic observations we could not determine the protist species. Heterotrophic organisms consume other biological material and get enriched via consumption of enriched autotrophic or heterotrophic organisms. The enrichment of heterotrophs within 24 hours is lower than the enrichment of autotrophs, because the enrichment of the food source of heterotrophs is lower than the enrichment of the food source of autotrophs. Our objective was to study the carbon transfer from

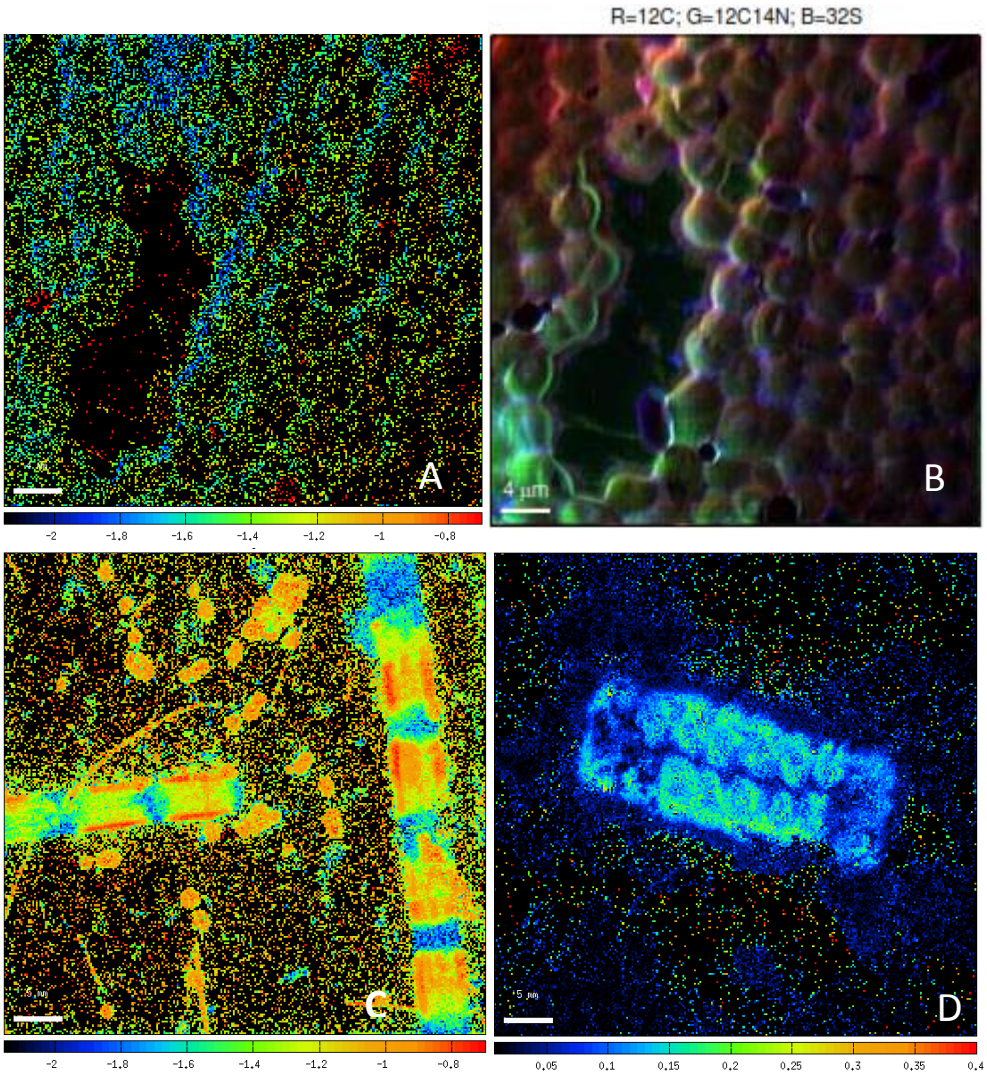

Figure 13. A: Microcystis cells, log $^{13}C/(^{12}C+^{13}C)$. B: Microcystis cells R= ^{12}C, G =$^{12}C^{14}N$, B=^{32}.. C: Filamentous cyanobacteria, log $^{13}C/(^{12}C+^{13}C)$. D: Diatoms, $^{13}C/(^{12}C+^{13}C)$.

autotrophic organisms to heterotrophic protists. The observed mean $^{13}C/(^{12}C+^{13}C)$ values indicate the analyzed protists are autotrophic. Heterotrophic protists cannot have a higher enrichment than the autotrophs in the incubation. The observed mean $^{13}C/(^{12}C+^{13}C)$ value of the protists was higher than the mean $^{13}C/(^{12}C+^{13}C)$ value of *Microcystis* for all timepoints. For none of the timepoints the difference between protists and *Microcystis* was significant. The higher $^{13}C/(^{12}C+^{13}C)$ value of the protists could be explained by higher DIC uptake rates of autotrophic protists than of *Microcystis* spp.

For both *Microcystis* and protists a trend can be observed over the timepoints with the strongest increase in $^{13}C/(^{12}C+^{13}C)$ between timepoint 2 and 3. Timepoint 2 was at 6 pm, just before sunset. The highest uptake of DIC would be expected between timepoints t_0 and t_2, during daylight. Autotrophic organisms perform photosynthesis during daylight. A strong increase in $^{13}C/(^{12}C+^{13}C)$ between 6 pm (t_2) and 12 pm (t_3) is unexpected. The effect of light on the uptake of DIC is supported by the results of the dark incubation. The $^{13}C/(^{12}C+^{13}C)$ values of the light incubations are significantly higher than the dark incubations for both *Microcystis* and protists. The $^{13}C/(^{12}C+^{13}C)$ value of *Microcystis* (0.015) is close to natural abundance (0.012) indicating little to no uptake of DIC by *Microcystis* in the dark incubation. The $^{13}C/(^{12}C+^{13}C)$ value for protists is slightly elevated (mean 0.02, range 0.015-0.03) indicating limited uptake of DIC. This could indicate the presence of a small amount of heterotrophic protists that have taken up ^{13}C enriched biological material such as chemotrophic bacteria. The $^{13}C/(^{12}C+^{13}C)$ of bacteria has not been measured yet but filters with bacteria are available for NanoSIMS analysis.

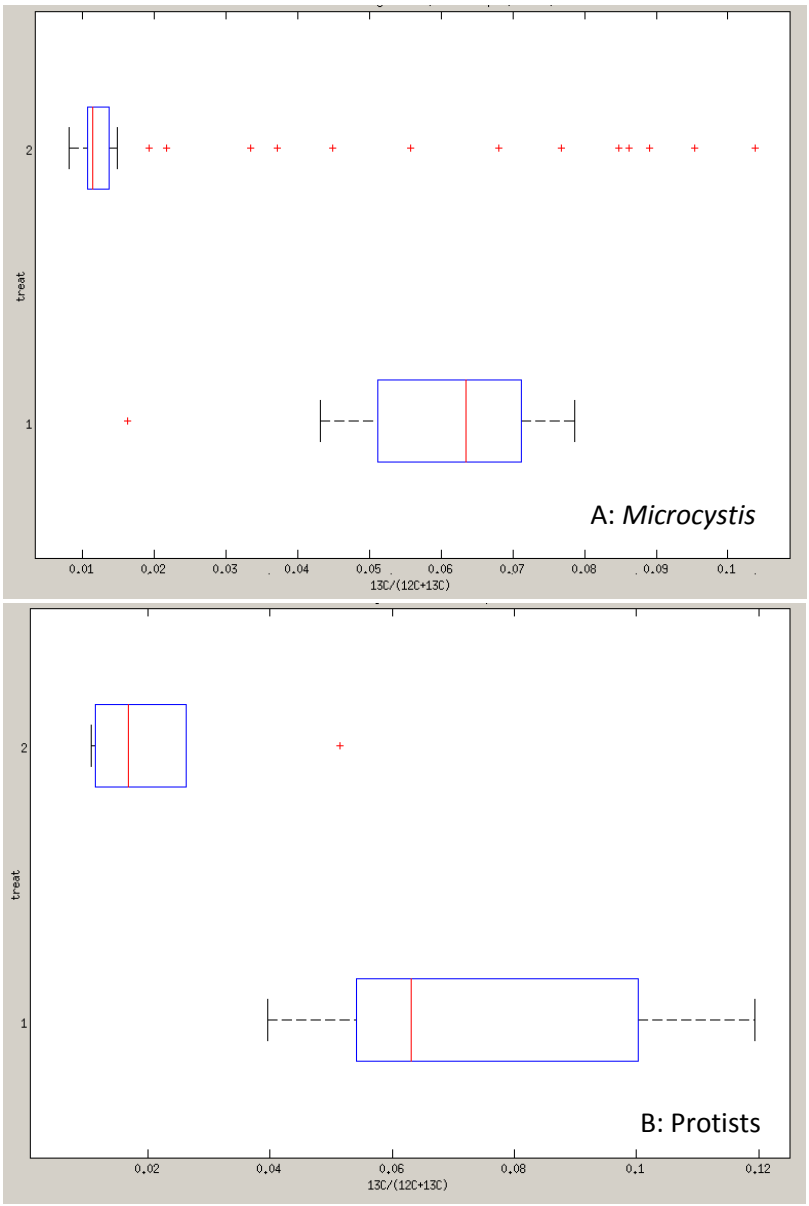

Figure 12: Light-dark incubation experiment $^{13}C/(^{12}C+^{13}C)$ values. Treatment 1 = dark incubation. Treatment 2 = light incubation. The data used for the box-and-whisker plot of the light incubation is the same data as used for figure 11.

4.3 Conclusion

The two objectives of this research were to assess the role of protists in the carbon transfer of the Taihu food web and to investigate the possibilities to study carbon transfer by protists by NanoSIMS analysis. Our first objective was not met because the protists that were analyzed turned out to be autotrophic. Autotrophic protists are primary producers and do not transfer carbon from the lower trophic levels to higher trophic levels. The relative abundance of heterotrophic protists could have been enhanced by dark incubation for a longer time span. This was done for a parallel experiment performed at Taihu. An increase in the protist abundance was observed over the first four days of incubation. Because in dark conditions only the heterotrophic protists are expected to reproduce the relative abundance of heterotrophic protist is expected to be increased. For future fieldwork on this subject it would be useful to include a person in the team that can identify protist and can immediately determine whether the protists in the samples are autotrophic or heterotrophic.

The work on the second objective was successful but there are still many challenges remaining. NanoSIMS analysis was successfully used for isotopic analysis of protists. Analysis of accumulated data of ROIs is promising. ROI classification is biased because it is done on data interpretation of the researcher, but if distinct groups are used like in this research (*Microcystis*, protists, filamentous cyanobacteria, diatoms) misidentification is very limited. For this research only $^{13}C/(^{12}C+^{13}C)$ values were used for analysis, but all 7 measured elements or isotopes can be used for other purposes. Analysis of spatial heterogeneity is still facing problems and challenges that limit analysis now. Interpretation of results is difficult because there are still a lot of questions on technical causes of heterogeneity. As long as these cannot be excluded as possible reasons, the results cannot be interpret as actual heterogeneity in the protist. Also the effect of precipitated crystals needs to be limited to increase analysis reliability. A proposed method for this is sectioning by focussed ion beam-SEM (FIB-SEM). Top-cut protists can be observed for true spatial heterogeneity. Comparison of those results with the current results of intact protists can give information on the reliability of intact protists analysis. Washing methods should also be explored further to maximize crystal removal by pre-NanoSIMS washing.

In comparison with other methods NanoSIMS analysis has shown to be a useful tool in analysis of spatial heterogeneity within samples. Intraspecies variability is often overlooked in both bulk analysis and single-cell techniques but can be assessed by NanoSIMS analysis. Drawbacks of NanoSIMS analysis are the low cell through-put and the high running costs. NanoSIMS analysis is time consuming, but this is a general characteristic of single-cell techniques.

I see an important future role for automatic ROI selection techniques such as halogen in situ hybridization (HISH-SIMS). This can limit labor-intensity and classification bias. For research on heterotrophic protist grazing preferences and rates NanoSIMS analysis can be combined with FISH-flow cytometry to gain information on both isotopic composition of ingested material and the identity of prey species. This creates possibilities for full-chain food web studies on both heterotrophic protist grazing and autotrophic DIC consumption.

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