FISHing for sulfate reducing microbes in Siders Pond, a meromictic lake

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Abstract

Protist and bacteria abundance was quantified in oxic, microaerophilic, and anoxic zones of Siders Pond, a density stratified meromictic lake. Protist species richness was also determined. The oxic zone exhibited greatest protist species richness and abundance, and lowest bacterial abundance. Protist richness and abundance decreased with depth as bacterial abundance increased. The microaerophilic zone exhibited greatest abundance of green sulfur bacteria, while the anoxic zone showed greatest abundance of sulfate reducing bacteria and greatest total bacterial abundance. Chlorophyll $a$ and bacteriochlorophyll were measured and yielded expected results showing Chlorophyll $a$ highest in oxic zone and bacteriochlorophyll highest in microaerophilic zone. We measured protist grazing rates using fluorescent microbeads, but results were inconclusive. Fluorescence in situ hybridization (FISH) was performed to search for sulfate reducer endosymbionts inside protists. Endosymbiotic relationships were suggested by FISH results, but further study is required to determine whether the microbes were situated inside the food vacuole or had been incorporated into the cytoplasm as a respiratory organelle.

Key words: meromictic, sulfate reducing bacteria, protist, grazer, endosymbiont, anoxic, fluorescent microbeads, bacteriochlorophyll, fluorescence in situ hybridization, FISH

Introduction

Coastal lakes that receive inputs of saltwater and freshwater, like Siders Pond, create unique ecosystems to study aquatic microorganisms across redox gradients. Siders is a meromictic lake in Falmouth, MA that contains a salinity gradient where the water becomes increasingly saline with depth (Figure 1). Because salinity affects water density, a density gradient also forms and prevents the yearly mixing typically undergone by most lakes. The lack of mixing causes the lake to stratify into distinct horizontal layers (Anderson 1958). Stratification has implications for the lake’s physical and chemical gradients, including temperature, salinity, and, particularly relevant to this study, dissolved oxygen. Siders Pond has three major zones: the oxic zone (0-3 m), the microaerophilic zone (3-8 m), and the anoxic zone (8 m and down). Below the oxic zone, protists struggle to survive on limited oxygen, but sulfate reducing microbes thrive by respiring sulfate ($SO_4^{2-}$) and consuming organic matter. Sulfate is one of the most prevalent anions in seawater and the most oxidized form of sulfur, providing sulfate reducing bacteria with an alternative electron acceptor to oxygen. Sulfate reducing bacteria are classified in the subclass deltaproteobacteria and reduce sulfate to hydrogen sulfide ($H_2S$) (Dubilier et al. 2001; Madigan et al. 2009). The role of sulfate reducing bacteria in the food web is not yet completely understood.
Food webs of aquatic systems originally emphasized carbon flowing from phytoplankton to fish through a series of intermediate consumers and assumed that microbes had little impact on the ecosystem as a whole. However, discoveries starting in the 1980s have shown that the microbial loop, or the microbial food web, plays an important role in aquatic whole-ecosystem food webs. The microbial loop begins with microbial consumption of dissolved and particulate organic matter. Microbes are then consumed by grazers, which are protists that feed on microbes. Grazers themselves are then preyed upon by larger grazers or zooplankton, and so on up the food web. Microbes are small and have large surface to volume ratios, which allows them to sequester nutrients at low concentrations. In this way, they provide an alternate food source for consumers in addition to phytoplankton (Azam et al. 1983).

Not only do microbes provide nutrients to consumers; some species can evade digestion and form symbiotic relationships with their grazers (Gong et al. 2016). This is of particular importance in anaerobic systems. Many grazers lack the ability to respire anaerobically, and are thus usually restricted to the aerobic zone. Here, they rely on phytoplankton to serve as a food source. However, microbes also serve as a major food source, and if grazers can leverage anaerobic respiration, they would have greater access to food (Oikonomou et al. 2015). Endosymbioses benefit both protists and microbes; a microbe confers its respiratory capabilities to the protist while acquiring a safe habitat in return.

Researchers have explored several kinds of anaerobic protist-microbe symbioses, including those with ciliates and methanogens or ectosymbiotic sulfate reducers (Embley and Finlay 1994; Fenchel and Ramsing 1992). To date, there is little information about the microorganism community of Siders Pond, much less the possibility of sulfate reducer symbioses. In this report, microorganism communities are characterized in oxic, microaerophilic, and anoxic regions of the pond. Bacteria and protist abundance is quantified, and protist species richness and size are measured to investigate how the protist community changes with depth. Sulfate reducers in samples were visualized using fluorescence in situ hybridization (FISH), a molecular technique used to detect specific bacteria. FISH entails hybridizing a DNA probe with a fluorophore to ribosomal RNA in the target bacteria (Amann et al. 1995). In this study, FISH and DAPI staining were used to visualize microbes and identify deltaproteobacteria, including potential sulfate reducers, both free-living and inside protists.

Methods

Site description: Siders Pond is an oblong-shaped lake about 700 m long and about 300 m wide located in Falmouth, MA (Figure 1). Its deepest points are 12 m and 15 m deep holes. Saltwater enters from Fresh River and freshwater enters via groundwater flow, making Siders a brackish water body. The high population density surrounding Siders promotes eutrophication by heavy nutrient loading from septic tanks (Caraco 1986).
**Sampling:** Water and chlorophyll samples were collected from Siders Pond in Falmouth, MA on November 13 and 18, 2017. On both days, samples were taken at stations within the 15 m deep basin of Siders Pond. On November 13, a Hydrolab Sonde DS5 (OTT Hydromet, Kempten, Germany) was used to measure where the water turned anoxic as well as other water column properties including salinity, pH, temperature, conductivity, PAR (photosynthetically active radiation), and dissolved oxygen. Water samples were collected at 3 m (oxic), 7 m (microaerophilic), and 10 m (anoxic) in 60 mL and 300 mL glass BOD bottles using a Geopump (Geotech, Loveland, CO). One 300 mL BOD bottle for each depth was filled to 150 mL and fixed with 7.5 mL of 25% glutaraldehyde for an approximately 20:1 fixative dilution for later bacteria counts.

On both November 13 and 18, six 60 mL BOD bottles were filled at each depth for a total of 36 BOD bottles, 12 from each depth. Around 2-4 mL of headspace was left in the oxic BOD samples to allow for oxygen dissolution to prevent anoxia. To ensure that oxygen was not introduced to the microaerophilic and anoxic samples, the bottles were overfilled from the bottom and capped quickly. Both 60 mL and 300 mL BOD bottles were stored in a 4°C refrigerator. Two one-liter plastic Nalgene® bottles were filled with 444 mL of 95% ethanol beforehand and filled with 400 mL of sample water in the field at the three sampling depths to give a final ethanol concentration of 50%. Chlorophyll samples were taken by filtering approximately 1 L of water through a 47 mm Whatman GFF filter at each depth. The filters were then folded and wrapped in aluminum foil for transport back to the lab. Both chlorophyll samples and ethanol-fixed sample were stored in a -20°C freezer.

**Bacteria and protist abundance:** Bacteria and protist densities were determined for each depth by counting individuals in 10 fields of view from fixed samples not incubated with beads. A 15 mL subsample was poured into a millipore filter tower and vacuum filtered on a blackened 5 μm 25 mm polycarbonate filter until approximately 2 mL remained. Samples were stained with 100 μl of 20% DAPI and incubated in the dark for 10 minutes. Abundance was calculated using the following equation:

$$\rho = \frac{n^{8000} \times 2}{\pi^{75}} \times (X)$$

Where \(\rho\) = average individuals/mL, \(X\) = average number of individuals per field of view at 100x objective and 10x eyepiece, and \(V\) = volume filtered (mL). For protists, size (μm) and morphological features were also recorded to quantify species richness.

**Grazing experiments:** Fluorescent microbeads (0.5 μm) at a concentration of 2.26 x 10⁷ beads/mL were vortexed for one minute and 600 μl was pipetted into a 60 mL BOD bottle filled
with a sample from the oxic water. Solution was gently swirled to give a final concentration of 2.7 \times 10^6 \text{ beads/mL}, similar to the method of Pace and Bailiff (1987). The same was done for microaerophilic and anoxic water samples, taking care not to introduce oxygen. Samples were incubated for 30 minutes, one hour, and two hours in the dark to find the optimal incubation time. After incubation, samples were combined with 60 mL of cold 4% glutaraldehyde and gently swirled to fix. Subsample (15 mL) was poured into a millipore filter tower and vacuum filtered on a blackened 5 μm 25 mm polycarbonate filter until 2 mL were left. To stain, 100 μl of 20% DAPI was added and incubated for 10 minutes in the dark. The rest of the sample was filtered, and the filter was placed on a slide for epifluorescent counting of beads and protist density (Porter and Feig 1980). A Zeiss compound microscope with long and short bandpass DAPI filters was used to count the number of grazers and beads inside each grazer. The grazing rate was calculated using the following equation:

$$\varphi = \left( \frac{y}{H} \right) \cdot (G + B) \quad (2)$$

Where \(\varphi\) = average grazing rate (beads consumed grazer\(^{-1}\) hour\(^{-1}\)), \(y\) = average number of beads consumed, \(H\) = length of incubation time (hours), \(G\) = bead concentration, and \(B\) = bacteria concentration determined from previous DAPI counts on a 0.2 μm PC filter.

**Chlorophyll:** Chlorophyll samples were each combined with 35 mL of 90% acetone and extracted overnight in the dark. Chlorophyll \(a\) and bacteriochlorophyll concentrations in oxic, microaerophilic, and anoxic portions were measured at 663, 668, and 850 nm on a spectrophotometer UV 1800 (Shimadzu, Kyoto, Japan) to quantify primary producer biomass (Henderson 2015; Caraco and Puccoon 1986).

**Fluorescence in situ hybridization (FISH), imaging, and analysis:** Subsamples of 2 mL were taken from material that settled by gravity to the bottom of sample bottles fixed in 50% ethanol. Approximately 100 μL was applied to slides. FISH hybridization, imaging, and image analysis was performed according to Mark Welch et al. (2016). A universal Eub338 probe labeled with a Rhodamine Red X fluorophore was used to detect all bacteria (Amann et al. 1990). Three probes synthesized and fluorescently labeled by Biomers (Germany) were used to detect 16S rRNA of deltaproteobacteria: Delta495a (sequence AGTTAGCCGTTGCTTCCT), Delta495b (sequence AGTTAGCCGCGCTTCCT), and Delta495c (sequence AATTAGCCGCGCTTCCT) (biomers.net). All Delta probes were labeled with a Dy490 fluorophore. Samples were also stained with DAPI to visualize nucleic acids including nuclei of protists. As a negative control for the probes, samples of oral bacteria *Streptococcus oralis* (strain ATCZ 9811) were hybridized
with the Eub338 and Delta495 probes. A control for nonspecific binding of the fluorophores was carried out using the same samples and same fluorophores attached to different oligonucleotides: a MIB probe for mouse intestinal bacteria (Salzman et al. 2002), labeled with Rhodamine Red X and Capn371 (Zijnge et al. 1990) labeled with Dy490. Slides were viewed using a Zeiss LSM 780 hyperspectral imaging confocal microscope using 561, 488, and 405 nm wavelengths. Linear unmixing was performed to separate signals from each fluorophore using Zeiss Efficient Navigation (ZEN) software. FIJI was used to add false color to unmixed images (Jahr et al. 2015). Slides were also imaged using a Zeiss widefield microscope equipped with four bandpass filters: a Cy5 filter, which allows signal similar to that of chlorophyll, filters for broad and narrow DAPI signal, and Dy490 signal. Delta proteobacteria would be expected to hybridize to both the Delta probe and the Eub338 probe, so where the colors overlap is where a delta proteobacterium may be situated. Where all colors are present and the morphology doesn't look like bacteria, there is probably nonspecific fluorescence from a salt crystal, piece of silica, or other object. Image analysis was carried out in FIJI for three fields of view from each depth, by segmenting cells from background using the Bernsen local thresholding algorithm (radius 15), then inverting the segmented image and using the “analyze particles” function to measure the mean intensity of each particle in both the Dy490 and the RRX images. Only particles larger than 400 pixels were included, as smaller particles were considered to be noise. Background intensity for each fluorophore channel was estimated from cell-free regions of images, and particles whose mean intensity was more than 10 times the background intensity were considered to show positive signal.

Results

Hydrolab data: The Hydrolab yielded results that were generally expected for Siders Pond. Dissolved oxygen (DO) began decreasing at 2 m but remained oxic at 3 m where the first samples were taken (Figure 2). DO declined until it reached below 1 mg/L at 6 m and reached 0 mg/L at about 9 m (Figure 2b). Strangely, the data showed DO increasing from 0 mg/L at 9 m to 0.5 mg/L at 10 m, probably due to hydrolab malfunction (data not shown). Salinity increased in the 2 m to 8 m region and indicated the presence of the pycnocline. The thermocline was evident from 2 m to 7 m where temperature increases rapidly, then plateaued before gradually decreasing by 3°C at 10 m. pH stayed relatively constant with depth. Photosynthetically active rations (PAR) data showed that water 7 m and above was illuminated (Figure 3).

Chlorophyll: Chlorophyll a was highest near the surface, at approximately 25 μg/L at 3 m, then decreased to 0 μg/L in the microaerophilic zone (Figure 4). Bacteriochlorophyll was lowest at 3 m, at about 2 μg/L, then increased to around 43 μg/L at 7 m. At 10 m, Chlorophyll a slightly increased and bacteriochlorophyll slightly decreased. However, there is likely some errors associated with extracting bacteriochlorophyll and chlorophyll signals from mixed samples, which may explain the low Chl a at 7 m (Henderson 2015).
**Bacteria and protist abundance:** Bacterial abundance in the oxic zone was $7.0 \times 10^5$ bacteria/mL, while abundance in the microaerophilic zone was $7.4 \times 10^5$ bacteria/mL (Figure 5). In the anoxic portion, bacterial abundance greatly increased to $1.1 \times 10^7$ bacteria/mL. Protist and microalgae species richness and abundance decreased with depth as bacterial abundance increased. The oxic zone exhibited great morphological diversity with six major species (Table 1, Figures 6, 7, 8). The microaerophilic sample had three dominant species and the anoxic sample had one dominant species (Table 1). Protist species were zone specific, except for the circular dinoflagellates that were found at all three depths (Figures 8, 9, 10). Average protist size decreased with depth; average protist size in the oxic sample was 7.3 μm, larger than average size from the microaerophilic sample (3.7 μm) (Table 1, Figures 11, 12). Average size found in the microaerophilic sample was only slightly larger than average size from the anoxic sample (3.2 μm) because a few organisms measured in the anoxic sample were unusually large and drove up the average (Table 1). Some protists were able to be identified to phylums Diatom and Dinoflagellate, and one microalgae was identified to the genus *Coelastrum* (Lee et al. 2000, Ward and Whipple 1959).

**Grazing experiments:** Grazing results were inconclusive because the grazers did not consume the microbeads as expected.

**FISH:** Images taken with laser wavelengths suited to the maximum absorbance of the probes’ fluorophores revealed the existence of sulfate reducers and other bacteria (Figure 13). The negative control samples showed minimal fluorescence (Figure 14). Linear unmixing results yield images showing DAPI, chlorophyll false colored in magenta, deltaproteobacteria false colored in green, and universal probe signal false colored in red (Figures 14, 15). The bacteria density increased with depth, especially from the microaerophilic zone to the anoxic zone. The oxic zone contained $6.99 \times 10^5$ bacteria/mL, then increased to $7.43 \times 10^5$ bacteria/mL in the microaerophilic zone, and increased again to $1.06 \times 10^7$ bacteria/mL in the anoxic zone. The proportion of bacteria that were deltaproteobacteria also increased with depth: 398 out of 4,265 bacteria (9%) in the oxic zone were deltaproteobacteria, 709 out of 3,241 in the microaerophilic zone, and 732 out of 2,842 (25%) in the anoxic zone (Figure 16). All bacteria that were clearly identified were free-living although some fluorescent signals were observed that could represent endosymbiotic bacteria (Figure 17).

**Discussion**

**Protist diversity:** The strong salinity-based density gradients in Siders Pond creates radically different environments at different depths in the water column. Variations in characteristics such
as temperature, oxygen, and PAR have implications for the aquatic life that can survive in each zone. PAR seemed unusually low due to the cloudy weather and time of year, and on average PAR values can be much higher and extend deeper into the water column than observed in this study (see Caraco and Puccoon 1986). The oxygen gradient is likely a large influencer on what kind of organisms can survive where. Oxygen as an electron acceptor in respiration liberates the most energy because it has the greatest electronegativity of any biologically relevant atom, meaning that aerobic organisms produce more energy per electron transfer than anaerobic organisms produce by using other electron acceptors. These differences in energy production have ramifications for the size and morphology of aerobic and anaerobic organisms. Protists in the oxic zone from 0 to 3 m, where oxygen from the atmosphere mixes freely, have a much higher degree of diversity in terms of size and morphology than do protists in other zones. Organisms in any environment may occupy unique niches, thereby potentially reducing competition. In aerobic environments, organisms produce enough energy to diversify and fill more niches than in environments where oxygen is limited. They are also able to maintain a larger body size and become more effective hunters. A study characterizing protist communities of meromictic Lake Alatsee in Germany using high throughput sequencing found that the most diversity occurred at the oxic zone, and the least occurred at the microaerophilic zone, or sub-oxic interface, unlike what we observed (Oikonomou et al. 2015). This research differed from the present study in that genetic diversity was investigated in the spring, summer, and fall in all three zones. Because the present study took place only in the fall, it may not provide the most accurate picture of what is happening in the Siders Pond throughout the year. Oikonomou et al. (2015) found that the protistan community in the oxic zone experiences the most structural change as seasons transition from one to the next. The oxic zone is exposed to the atmosphere and is most affected by seasonal changes in temperature and the availability of sunlight, while the anoxic zone exhibited the most stability (Oikonomou et al. 2015).

**Chlorophyll:** The trends observed in Chlorophyll \(a\) and bacteriochlorophyll measurements were expected according to the type of primary producer known to dominate at each zone. Aerobic photosynthetic organisms, such as cyanobacteria and algae, generally live in the oxic zone and use Chlorophyll \(a\) to capture photons. Bacteriochlorophyll is the pigment used by green sulfur bacteria, a phototrophic anaerobe that tends to live in environments characterized by limited oxygen, such as the microaerophilic zone at 7 m (Caraco and Puccoon 1986, Madigan et al. 2009).

**Anaerobic endosymbiosis:** In this study, the anoxic zone contained the least protistan diversity and only one dominant protist species, a small flagellate, was found here. Interestingly, Oikonomou et al. (2015) found that the anoxic zone in Lake Alatsee contained the second highest diversity of the three zones, and found two Ciliophora classes unique to the anoxic zone, the Armophorea and Karyorelictea. Anaerobic ciliates have been found in various other studies,
which often attribute their anaerobic nature to symbiosis with microbes. Certain anaerobic marine ciliates rely on sulfate reducing ectosymbionts for respiration, which in return receive a habitat and portion of the ciliates’ metabolites (Fenchel and Ramsing 1992). Because sulfate reducer ectosymbionts have a food source coming from their host, they can constitute 10-15% of the host’s weight, while methanogen endosymbionts commonly constitute 1-2% of their host’s weight (Fenchel et al. 1977; Fenchel and Finlay 1992). The few FISH images taken in the present study that show deltaproteobacteria signal inside grazers are ambiguous as to whether the green signals are inside or outside the grazer (Figure 17). If it could be confirmed that the microbes were inside the grazer, one would also need to distinguish whether they were in the food vacuole prior to digestion, or part of the cytoplasm. This could be achieved by staining food vacuoles. Microbes meant for digestion would probably be clumped in the food vacuole, while endosymbionts would likely be distributed throughout the protists’ cytoplasm.

Sulfate reducing endosymbionts have yet to be discovered and sulfate reducers are thought to be unable to survive inside protists (Dubilier et al. 2001). While the FISH results in this study do not rule out the presence of sulfate reducer endosymbionts, one must consider the limitations of the probes. The Delta probes detect all members of deltaproteobacteria, not specifically sulfate reducers. Many, though not all, of the species in deltaproteobacteria are sulfate reducers, meaning that the probe could have been labeling other functional groups.

It was surprising that deltaproteobacteria were detected in the oxic zone. It is possible that sulfate reducers get transported to the oxic zone or exploit microenvironments contained detrital debris that permit maintenance of anoxic environments in an otherwise aerobic water column. Alternatively, Siders could contain aerobic deltaproteobacteria. The probes may be overstating the presence of sulfate reducers in some cases, but underestimating it in others; since the probes hybridize with ribosomal RNA, deltaproteobacteria with fewer ribosomes may not have been detected.

Conclusions

Stratified lakes offer fascinating places to study how redox gradients create biological gradients. The inputs of saltwater and freshwater generate a salinity gradient that in turn creates a strong density gradient, which prevents oxygen from mixing sufficiently through the entirety of the water column. The specific oxygen concentration in each zone of the pond dictates what kind of organism can survive there, and thus each zone contains a unique community structure. Each microenvironment is produced by a combination of the aforementioned physical factors, as well as its unique biological activity. Siders Pond shows an inverse correlation between microbe and protist species richness and abundance; microbe abundance is low in the oxic zone and high in the anoxic zone, while protist abundance is high in the oxic zone and low in the anoxic zone. Factors contributing to this phenomenon are unavailability of electron acceptors and grazing. FISH results show the presence of deltaproteobacteria that increases down the water column, as
well as suggest the presence of sulfate reducer endosymbionts. However, confirming these findings would require further research. Future analyses for this project could include sequencing the Delta-labeled bacteria from each depth to determine whether they are sulfate reducers, and staining for the protist food vacuole to differentiate endosymbiont from food.

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Tables and Figures

Table 1 Species abundance and average protist and microalgae size varies throughout the water column. The average size in anoxic sample may not be representative; a small total number of species was found, and average size was heavily weighted by a few larger individuals.

<table>
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<th>Oxic</th>
<th>Microaerophilic</th>
<th>Anoxic</th>
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<tbody>
<tr>
<td>Species/sample</td>
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<td>3</td>
<td>1</td>
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<tr>
<td>Average size (μm)</td>
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<td>3.7</td>
<td>3.2</td>
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Figure 1  

a Location of Siders Pond in Falmouth, MA; b contour map (Caraco 1986).
Figure 2 Temperature, salinity, dissolved oxygen, and pH data collected at 10 depths by a Hydrolab on November 13, 2017.

![Graph showing DO (mg/L) vs depth]

Figure 2b Close up of dissolved oxygen levels. Water remains slightly aerobic down to 9 m.

Figure 3 Conductivity and PAR data collected at 10 depths by a Hydrolab on November 13, 2017.

![Graph showing Conductivity (mS/cm) and PAR (mE m-2 sec-1) vs depth]
Figure 4 Chlorophyll $a$ and bacteriochlorophyll follow expected trends according to the type of primary producers in each zone.

Figure 5 Bacterial abundance remains relatively constant in the oxic and microaerophilic zones, but increases by an order of magnitude in the anoxic zone.
Figure 6  Protist and microalgae diversity in oxic sample. a dinoflagellate; b unidentified tetrads; c diatoms; d and e microalgae *Coelastrum* *spp.* with green sulfur bacteria behind them in red; f chain of diatoms; g and h unidentified phototrophic protists.
Figure 7 Many rectangular protists, possibly diatoms, appeared in groups in the oxic sample.
**Figure 8** Abundance of the six major species found in the oxic zone displays the morphological diversity of aerobic aquatic organisms.

**Figure 9** Compared to the oxic zone, species diversity of major species found in the microaerophilic zone is cut in half, and abundances are also much lower.
**Figure 10** Abundance of the only major protist in the anoxic zone is much lower than abundances in the other two zones.

**Figure 11** Average sizes of the six major morphologies found in the oxic zone. In most cases, size inversely correlated with abundance.
**Figure 12** Average sizes of the three major morphologies found in the microaerophilic zone.

**Figure 13** Fluorescence emission spectra of the three fluorophores employed (DAPI, Cy490, Rhodamine Red X) plus a Cy5 filter whose spectrum is similar to that of the chlorophyll observed. Plotted using SpectraViewer tool (Thermo Fisher).
Figure 14 Images false colored in FIJI showing universal probe labeled with Rhodamine Red X (purple) and deltaproteobacteria probe labeled with Dy490 (green). A control test was performed with probes consisting of Rhodamine Red X and Dy490 attached to different oligonucleotides that were not expected to hybridize to environmental bacteria, in order to ensure that the signal was due to specific hybridization of the probe oligonucleotide rather than nonspecific binding of the probe fluorophore.
Figure 15 Oxic sample. Images created by linear unmixing in Zeiss software and false colored in FIJI. a DAPI, chlorophyll, universal probe, and deltaproteobacteria probe; b deltaproteobacteria probe; c universal probe; d universal and deltaproteobacteria probes.
Figure 16 Percentage of bacteria from each zone labeled as deltaproteobacteria.
**Figure 17** Deltaproteobacteria-labeled dots (green) suggest endosymbiotic relationship with grazer.