Representative monitoring of the calcifying alga *Phacotus lenticularis* (Chlamydophyceae) in lentic ecosystems

Sebastian Lenz, Uta Raeder, Juergen Geist*

Technical University of Munich, Aquatic Systems Biology Unit, Limnological Research Station Iffeldorf, Germany

ABSTRACT

The biogenic carbonate precipitation by the freshwater alga *Phacotus lenticularis* may play a role in long-term carbon (C) fixation that has not yet been quantified. This is partly due to the absence of a standardised methodology to representatively sample and assess the cell density and sedimentation of *P. lenticularis* in lakes. The objective of the present study was to define an adequate sampling methodology taking into account the spatiotemporal variation of *P. lenticularis* as well as the sedimentation and dissolution of calcite shells. Simultaneous measurements in three different lake sub-basins of Lake Grosser Ostersee, Germany, showed that the spatial cell density of *P. lenticularis* was similar in each sub-basin. At all sites, the vertical *P. lenticularis* cell density maxima corresponded with the slowly downshifting thermocline from depths of 2 to 6 m. During the entire growth period, composite samples from 0 to 7 m included 89% of the total *P. lenticularis* population. Lake bathymetry, as well as external factors like wind exposure, did not appear to affect the abundance of these calcifying algae. Sediment traps at a depth below the thermocline (at 7 m) and 1 m above the lake bottom recorded sedimentation rates of *P. lenticularis* shell halves (sh) between 1.1×10^6 and 1.1×10^8 sh m^{-2} d^{-1}, while mean cell concentrations in the water column were between 1.1×10^8 and 1.7×10^9 shells per m^2. Sinking velocity ranged between 3 and 4 m d^{-1}. Sediment from traps installed at a depth of 7 m did not reflect mean shell concentrations in the water column above. Dissolution of carbonates reduced the number of shells in sediment traps at the lake bottom and during the storage of samples. A laboratory experiment showed that even distilled water used for dilution during microscopic analysis led to dissolution of *P. lenticularis* shells. In conclusion, combined sampling of *P. lenticularis* from open water and sediment traps close to the lake bottom delivers a most representative assessment of biogenic carbonate precipitation. Due to dissolution effects, high temporal resolution along with appropriate sample preservation are crucial, whereas spatial representativeness was already achieved with low number of sampling sites per lake.

INTRODUCTION

Carbon fixation by planktonic algae is considered to be an important CO_2 sink in marine environments, and has even led to fertilisation experiments to investigate enhancing algal growth for carbon sequestration (Blain *et al*., 2007; Morris and Charette, 2013; Smetacek *et al*., 2012). Biogenic carbon sequestration has been less intensely studied in freshwater systems, yet there have also been documented occurrences of algae that form crystalline calcite structures in these environments. Among these algae, *Phacotus lenticularis* has drawn great attention since this species is particularly widespread in calcareous lakes and thus may be one of the most important freshwater algae species involved in carbon sequestration.

Phacotus lenticularis, hereafter referred to as *Phacotus,* is a unicellular, bi-flagellated green alga, belonging to the Chlamydophyceae. It forms regularly-shaped shells (loricae) of crystalline calcite plates that may significantly influence carbonate chemistry and sinking carbon fluxes in lakes (Cărăuş, 2002; John *et al*., 2011; Menezes, 2010; Sánchez *et al*., 1998; Wehr *et al*., 2001). *Phacotus* is widespread in the temperate, subtropical and tropical climatic zones of both hemispheres, appearing predominantly as planktonic algae in various morphometric and ecological states in stagnant inland waters (Schlegel *et al.*, 1998). In particular, mass developments of *Phacotus* may have a considerable influence on the $CaCO_3$ budget of lakes (Krienitz *et al*., 1993), and the importance of these calcifying algae is demonstrated by the long-term preservation of carbon in the form of remarkable shell deposits in sedimentary records dating back to the Miocene, 120 ka BP (Lagerheim, 1902; Müller and Oti, 1981).

The largest body of literature in the context of *Phacotus* has focused on the genesis and properties of its shell. Koschel and Raidt (1988) studied the morphology of *Phacotus* shells, while Giering *et al*. (1990) and Hepperle and Krienitz (1996, 1997) examined shell calcification pat-

Corresponding author: geist@wzw.tum.de

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terns. Schlegel *et al*. (2000) described four morphotypes of *Phacotus* shells, which are suggested to reflect organismic calcification control. The abundance of *Phacotus* and its contribution to suspended autochthonous calcite content in hard-water lakes of northern Germany have been studied (Krienitz *et al.*, 1993). Also well understood are the shell growth responses of *Phacotus* to meteorological changes (Gruenert and Raeder, 2014) and the systematic assessment of natural variations in the general size and particular calcite mass of *Phacotus* shells, in order to quantitatively determine its role in carbonate sedimentation in hard-water ecosystems (Lenz *et al.*, 2018).

Despite the intense research into *Phacotus* and its potential importance in biogenic carbon sequestration, a representative sampling method that at the same time considers spatiotemporal resolution as well as sedimentation and dissolution is still lacking. Such a protocol would be highly beneficial in assessing the contribution of this species to calcite precipitation and carbon sequestration in lake ecosystems, as well as for improving the comparability between different studies. Thus, the primary goal of this study was to determine and validate a methodology that facilitates a reproducible and representative assessment of the actual *Phacotus* cell density in a whole lake, considering this organism's spatiotemporal variations. The following hypotheses were tested: (1) a single continuous mixed sample taken from the epilimnion sufficiently describes the *Phacotus* population in a small lake; (2) *Phacotus* shell sedimentation rates at different depths in the hypolimnion mirror the *Phacotus* population in open water; and (3) dissolution of sedimented cells does not play a role under hard-water conditions.

METHODS

Study site

Four Bavarian lakes (Tab. 1) with documented *Phacotus* occurrences during the last ten years (2005–2015) were investigated: Lake Grosser Ostersee (GOS), Lake Abtsdorfer See (ABS), Lake Igelsbachsee (IGS) and Lake Hopfensee (HOP) (Fig. 1). Lake Igelsbachsee, a Ca-rich artificial reservoir, lies in a basin of late-Triassic quartz sandstones surrounded by Jurassic carbonates. The other lakes are natural hard-water lakes in the pre-alpine basin in Southern Bavaria (Germany). All of the lakes have carbonate-dominated catchments and are fed by alkaline water from the Northern Limestone Alps. They are embedded in an open quaternary aquifer of predominantly fluvioglacial sediments with intercalated basal moraine tills that overlay a tertiary sandstone aquitard (Grimminger, 1982).

Study concept

Several experiments were performed to obtain a representative *Phacotus* population assessment. In 2015, during an exploratory study at GOS, relevant elements for the representative sampling of *Phacotus* were systematically analysed in two experiments (1, 2). During an intensive sampling campaign in 2016 at GOS, ABS, IGS and HOP, the monitoring methodology consisted of two further advanced experiments (3, 4). Carbonate dissolution was identified as a disruptive factor in IGS, not only in the lake but also after sampling, therefore a dissolution experiment (5) was performed.

1. Water column investigations at three sites with 1-metre resolution and a daily sampling frequency to assess vertical distribution and temporal variation of *Phacotus* in order to determine the optimal sampling depth.
2. Sediment trap experiments at two sites and two different depths to determine the sedimentation velocity as well as differences in the horizontal distribution of *Phacotus*.
3. Water column investigations with mixed samples and a weekly sampling frequency to simultaneously detect *Phacotus* population development in the epilimnion of the four lakes (Fig. 2a).
4. Sediment trap experiments in each lake, with cylindrical sediment traps attached to a buoy (Fig. 2 a,b) and square sediment traps at the lake bottom (Fig. 2c).

Tab. 1. Selected hard-water lakes in Bavaria, Germany, and geographic sampling positions at each lake's deepest point.

	Gr. Ostersee	Abtsdorfersee	Igelsbachsee	Hopfensee
Trophic state	Oligotrophic	Mesotrophic	Mesotrophic	Eutrophic
Mixis type	Dimictic	Dimictic	Polymictic	Dimictic
Surface area (ha)	118	78	72	186
Max. depth (m)	29.7	20.0	11.5	10.4
Volume ($10^6 \times m^3$)	14.0	9.4	3.9	8.9
Calculated residence time (d)	247	252	-/-	128
pH value*	8.2 (0.2)	7.9 (0.4)	8.6 (0.5)	8.2 (0.4)
Ca^{2+} conc. (mg L^{-1})**	69.2	78.2	37.6	69.3

**pH average values of surface layer (0-7 m) during June till August 2016, SD in brackets; **Ca^{2+} concentration from last sampling in August (Lenz et al., 2018).*

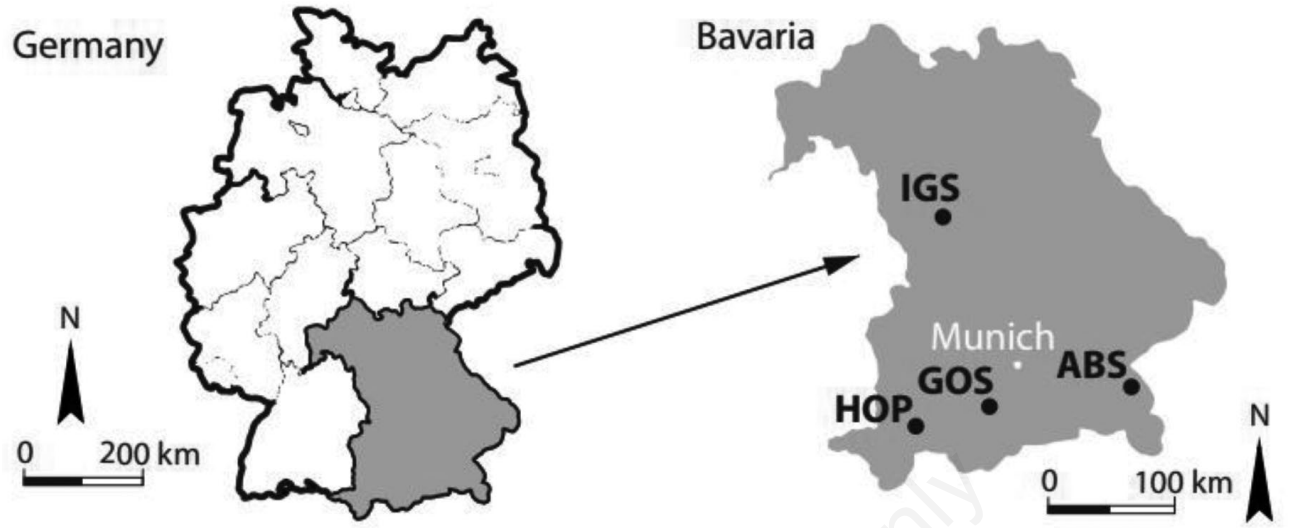

Fig. 1. Location of the four lakes investigated in Bavaria, Germany.

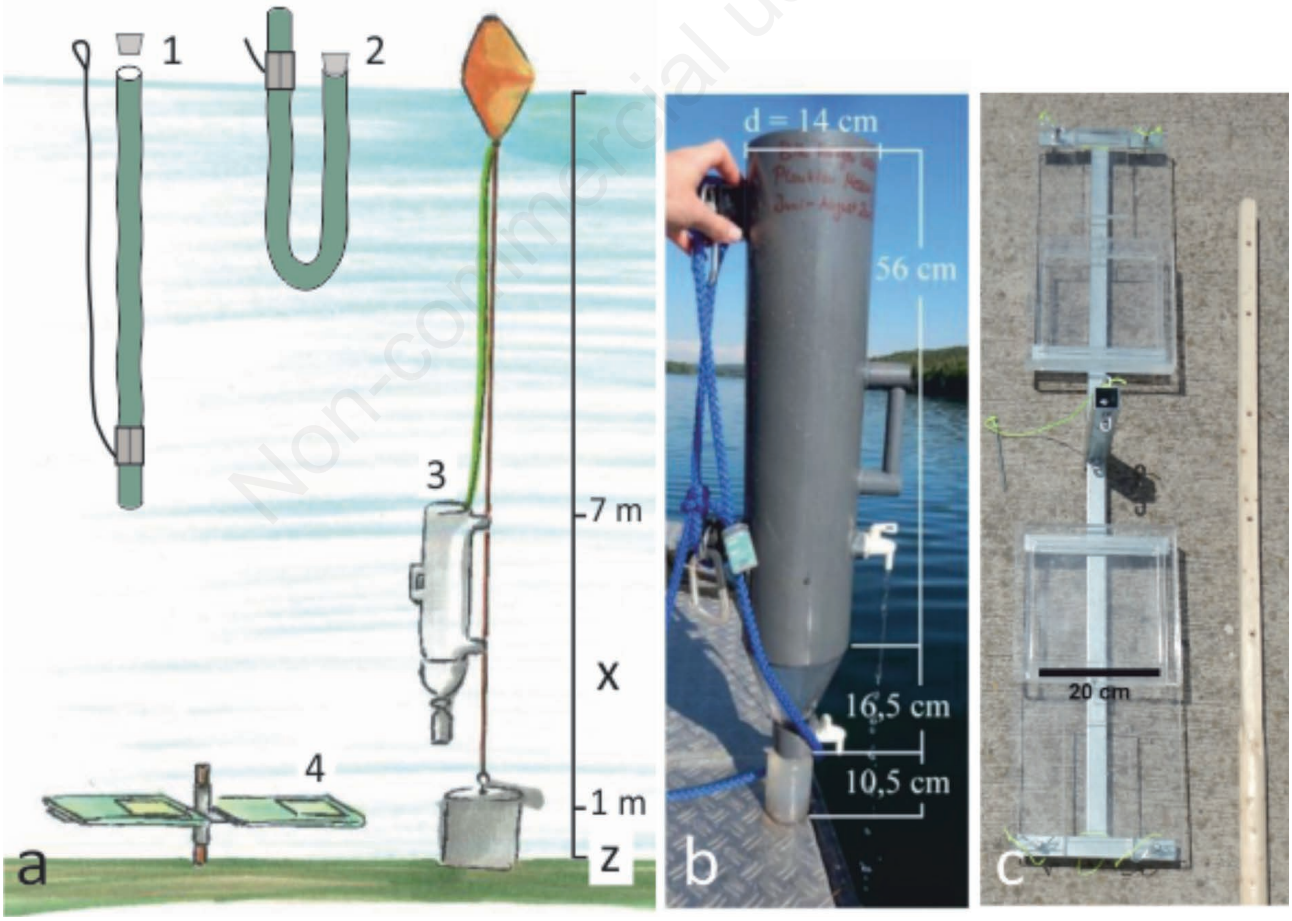

Fig. 2. a) Sampling equipment for representative *Phacotus* population monitoring: sampling hose insertion (1) and recovery (2), buoy with hanging cylindrical sediment trap (3) and square-type sediment trap 1 m above the lake bottom (4). b) Dimensions of the sediment traps: cylindrical-type for installation in the water column; c) square-type with a closable opening for installation and recovery on a wooden pole by divers at the lake bottom.

5. A laboratory experiment showing that the use of distilled water during microscopic examination leads to the dissolution of *Phacotus* shells.

Exploratory study

In 2015, an exploratory study was performed at Lake Grosser Ostersee (Fig. 3c), initially with a weekly sampling frequency, then a daily sampling frequency during the peak growth period of *Phacotus*. A field study was conducted to reveal spatiotemporal variations in *Phacotus* population density and the resulting sedimentation rates. From the lake surface down to a depth of 10 m, each metre was simultaneously sampled using a standard Ruttner water sampler at three sampling sites (GOS-W, GOS-N and GOS-E). Two cylindrical sediment traps (Hydro-Bios, Kiel, Germany), according to Saarso (1995), with an exposure area of 0.015 m², were installed at two of these sites (GOS-W and GOS-E) at depths of 16 and 26 m. During two weeks in June 2015, a total of 24 sediment trap samples were collected, with trap exposure times of one to two days. The sedimentation velocity of *Phacotus* shells, v [m d^{-1}], from the epilimnion to the sediment trap, was estimated. This velocity was derived from the time lag between the first increase in *Phacotus* cell density in the water column (0–10 m) and the resulting increase in *Phacotus* shell sedimentation rate at 16 m depth by dividing the sedimentation distance [m] by the time lag [d].

Monitoring methodology

The monitoring carried out during 2016 aimed to simultaneously assess *Phacotus* population development in the epilimnion of the four lakes. As a sampling methodology, we used the standard plankton sampling and analysis procedure of the European Water Framework Directive (Mischke and Nixdorf, 2008), with adaptions based to the results of our exploratory study. Special attention was paid to avoid variations in the sampling procedure, as the

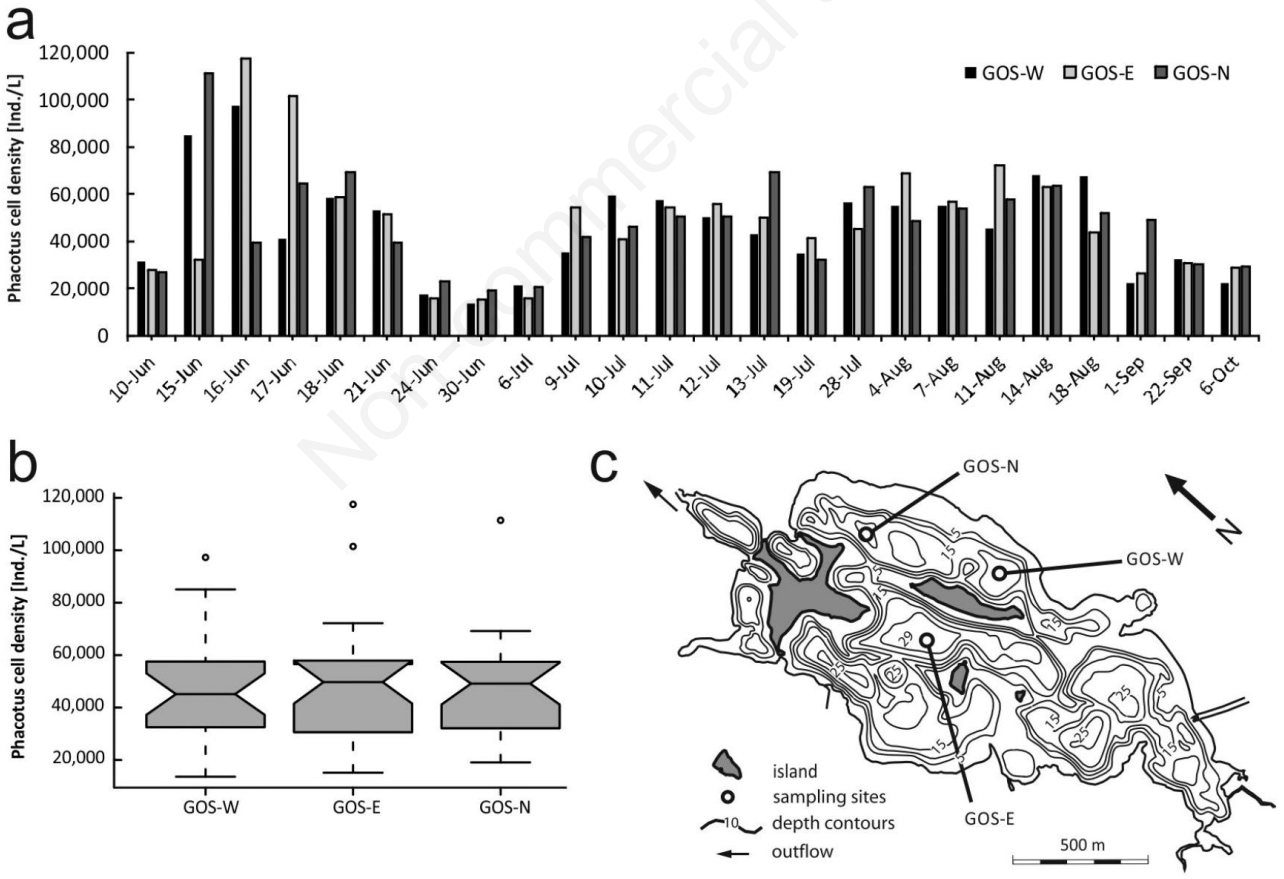

Fig. 3. Mean *Phacotus* population density at three different sampling sites in different lake basins from June to October 2015; n = 24 synchronized measuring days at depths of 0 to 10 m. a) Temporal variation during the growth period (the time axis is not linear). b) Boxplots of all data (boxes: 0.25 and 0.75 quartile, whiskers: 1.5 x interquartile range, notches, dots: outliers as defined as 3 x SD). c) Bathymetric map of Lake Grosser Ostersee with the three sampling sites shown.

largest errors in results typically originate from sampling errors in the field (Cairns Jr and Smith, 1993; Kelly, 1998). At the deepest point of each lake, a buoy and two different sediment traps (Fig. 2a) were installed. Personnel were trained in sampling techniques and hardware handling prior to beginning the fieldwork. Depth profiles of water temperature and pH values were measured using a WTW MPP 930 multiprobe (Weilheim, Germany), and the Secchi depth was determined using a Hydro-Bios visibility disc.

The beginning of the detailed sampling campaign was dependent on pH values of at least 7.8 and temperatures of more than 20°C at a depth of 0.5 m from the surface. These thresholds determine the expected onset of *Phacotus* growth. Weekly sampling was carried out during the growth period from June to September. To analyse *Phacotus* abundance, continuous mixed water samples from a depth of 0 to 7 m were taken using a 7-metre-long hose sampler (DIN Technical Committee Water analysis, 2015). To facilitate vertical sinking of the hose, a 3 kg weight was attached to the open end (Fig. 2a). The entire hose was slowly lowered into the water. After closing the top end of the hose with a plug, the open end was pulled up and its contents were emptied into a 6 L measuring bucket by lifting the hose from the top end. In order to rinse the sampling hose, the first sample was discarded. Brown-glass flasks were previously prepared with neutral pH Lugol's solution so that a 2% (v/v) concentration would ensure preservation during sample storage, transport and post-sampling. Procedures for plankton analysis of the epilimnion followed the method of Lenz *et al.* (2018). *Phacotus* shell concentration was determined according to Utermöhl (1958), using an inverted microscope (Leitz Labovert, Stuttgart, Germany) at 200-400x magnification. Samples were analysed immediately as far as possible to avoid carbonate dissolution during storage and at least 300 individual cells were counted per sample to ensure the accurate determination of shell numbers. Additionally, selected shells from the epilimnion were analysed using a Hitachi S-2300 scanning electron microscope (Tokyo, Japan).

During the detailed sampling campaign in 2016 at GOS, ABS, IGS and HOP, the monitored water column was reduced to the range of 0 to 7 m and the cylindrical sediment traps were set at a depth of 7 m, with exposure times of between 6 and 8 days. Additionally, square sediment traps we constructed ourselves (Fig. 2c) were used to investigate the dissolution of particulate carbonate in the hypolimnion. These traps were modified versions of the design of Kozerski and Leuschner (1999), with an exposure area of 0.040 m², and were designed to be installed and recovered by divers using a wooden pole, 1 m above the lake bottom. The exposure times varied from 6 to 16 days.

The sediment trap samples were recovered in 200 mL clear PVC bottles, transported at 20°C and stored with a 2% (v/v) concentration of neutral pH Lugol's solution for no more than two months in a dark room prior to analysis with an inverted microscope at 200-400x magnification, according to Utermöhl (1958). The sediment trap material from the bottom traps was analysed using a technique adapted from the coverslip method described by Koch and Young (2007). After weighing the freeze-dried samples, the organic sediment content was burned in a muffle furnace (550°C, 4 h). Thereafter, each loss on ignition sample was carefully crumbled in a mortar and 0.5 mg of the homogenised material was added to an Eppendorf tube containing 2 mL buffered suspension liquid comprising NH_3 and H_2O with a pH ≥8.5, (Bollmann *et al.*, 1999). As a calibration standard, 0.5 mg of borosilicate microspheres (Thermo Fisher Scientific #9015, Waltham, MA, USA) was added and the carbonate aggregates were destroyed in an ultrasonic basin (Bandelin Sonorex TK52, Berlin, Germany) for 60 s (150 W, 35 kHz), according to the method of Bordiga *et al*. (2015). Subsequently, the samples were mixed using a CHS Vortex homogeniser (Prague, Czech Republic) and 50 µL of the suspension was transferred using a pipette to the centre of a circular coverslip, according to the 'drop' method (Bordiga *et al.*, 2015; Koch and Young, 2007). The mass of the sediment, m_{cs}, on the coverslip was 50 ± 1.9 µg, and was calculated relative to the total amount of microspheres on the coverslip. Three microscope slides were made for each sample. Shell numbers on each coverslip were counted under light microscopy using polarised light at 200x magnification. A complete *Phacotus* housing was counted as two shells (sh). Single shells were counted as one shell.

Phacotus shell sedimentation rates, S [sh m^{-2} d^{-1}], were calculated according to equation 1, with an exposure time, t [d], and an exposure area, A, of 0.015 and 0.04 m² for the cylindrical and the square sediment traps, respectively. The total number of shells in a square sediment trap, N_{tr}, was calculated according to equation 2 using the difference between the total sediment dry mass, m_{tr} [g], and the sediment mass on the coverslip, m_{cs} [g], multiplied by the total number of shell halves, N_{cs}, on a coverslip.

$$S = N_{tr} A^{-1} t^{-1} \quad \text{(eq. 1)}$$

$$N_{tr} = N_{cs} m_{tr} m_{cs}^{-1} \quad \text{(eq. 2)}$$

Dissolution experiment

The effect of bi-distilled water on *Phacotus* shells during storage or sample treatment for microscopic analysis was examined. Representative samples from the field survey were diluted 1:10 with bi-distilled water. The 24 h experiment was performed under an inverted microscope at 400x magnification. Images were acquired using Kappa ImageBase camera software from Kappa Opto-Electronics and Accusoft 2007 (Gleichen, Germany). The temper-

ature (23.7±0.4°C) and pH values (8.4±0.3) were constantly monitored with a WTW Sentix pH electrode (Weilheim, Germany).

RESULTS

Spatial variability of *Phacotus* cell density

Over a time span of four months, the mean *Phacotus* cell densities from depths of 0 to 10 m at the three sites, GOS-W, GOS-E and GOS-N, were almost identical (Fig. 3a). No significant differences in *Phacotus* density (Fig. 3b) were observed, although the different sub-basins varied by depth and wind exposure (Fig. 3c). Even the main *Phacotus* growth period started at almost the same time, with a difference of only two days.

In contrast, the number of cells along the depth gradient showed a strong vertical variation between 0 and 10 m depth. From mid-June, an epilimnion developed and a strong, stable stratification formed, while the highest cell density of 475,000 individuals per litre (Ind. L^{-1}) was measured in 2 m depth during the first growth peak on 16th of June. The thermocline descended continuously and the maximum cell density of *Phacotus* also gradually moved downwards, from 2 to 4 m in June to 5 to 6 m in August. During August, increasing biogenic calcite precipitation led to concentrations of suspended carbonate >2 mg $CaCO_3$ L^{-1} in the upper water column. During this period average Secchi depth in Lake Grosser Ostersee was reduced to 1.5 m (SD=0.5) in comparison to a total mean of 3.3 m (SD=0.6 m). In August, the maximum cell density occurred at a depth of 5 m. The *Phacotus* cell densities were in the range of 4×10^5 Ind. L^{-1} (red), 2×10^5 Ind. L^{-1} (turquoise) and 0 (dark blue) (Fig. 4). Below a depth of 7 m, cell densities occasionally reached 5×10^4 Ind. L^{-1}, while the fraction of inactive and dead *Phacotus* individuals from the areas above increasingly adulterated the *Phacotus* shell density to an unknown degree. Over the monitored timeframe, the water column above a depth of 7 m contained 89% of all *Phacotus* individuals counted.

Phacotus shell sedimentation

Mean sedimentation velocities of 3 to 4 m d^{-1} for *Phacotus* shells were derived from the comparison of peak occurrences of *Phacotus* among water column and the sediment traps placed at different depths in the hypolimnion of Lake Grosser Ostersee.

The first and highest peak in *Phacotus* population growth (Peak 1) in June 2015 led to higher *Phacotus* shell sedimentation rates than the second peak (Peak 2) in July 2015. The two sites GOS-W and GOS-E showed sedimentation rates in a comparable dimension of 1.9×10^7 sh $m^{-2}d^{-1}$ at the same depths (Fig. 5b).

In 2016, the sedimentation rates of the cylindrical sediment traps at 7 m depth (dots) were generally lower than those from the bottom sediment traps 1 m above the lake bottom. *Phacotus* abundance and sedimentation rates corresponded in the four lakes investigated. In general, high *Phacotus* cell densities in the water column resulted in high quantities of shells in the sediment traps, but a significant correlation could not be established (Fig. 5a).

Carbonate dissolution

As indicated by the lowest cell densities and sedimentation rates of all four lakes, carbonate dissolution may have reduced the abundance of *Phacotus* shells in IGS despite of the alkaline pH in this lake. Observations of *Phacotus* shells from the water column and sediment traps suggest that dissolution took place in the epilimnion as well as at the lake bottom. Even massive calcite crystals showed clear signs of dissolution (Fig. 6 c,d) at the lake bottom. Microscopic analysis of samples from selected dates showed 28,640 Ind. L^- *Phacotus* shells in the epilimnion (0-7 m) shortly after sampling. In addition, following two months of storage, these previously measured cell densities could not be confirmed because the shell abundance was reduced to 60 Ind. L^{-1}.

The dissolution of carbonate particles during sample storage and microscopic analysis was also confirmed in the laboratory dilution experiment with bi-distilled water. The process which led to the complete dissolution of a *Phacotus* shell was observed and documented over 24 h inside an Utermöhl chamber (Fig. 7). Within the first 10 h, the rim edge of the *Phacotus* shell gradually became thinner and paler in appearance. After 12 h, the regular round outer line across the shell rim disappeared and an obvious loss of material could be observed. The shell rim had clearly been dissolved after 15 h, and the shell diameter was notably reduced from 15 to 11 µm. Eventually, the shell completely disappeared and only the exposed protoplast remained. At this stage, *Phacotus* would not have been recognised as such during microscopic analysis.

DISCUSSION

In order to determine an adequate sampling methodology for the representative assessment of the calcifying freshwater alga *Phacotus lenticularis*, several aspects of cell density distribution and shell sedimentation were examined. In Lake GOS, the horizontal heterogeneity (1) was negligible while the density changes of *P. lenticularis* throughout time showed a dynamic phenology (2) and the vertical distribution (3) could be representatively assessed by sampling an integrated water sample from

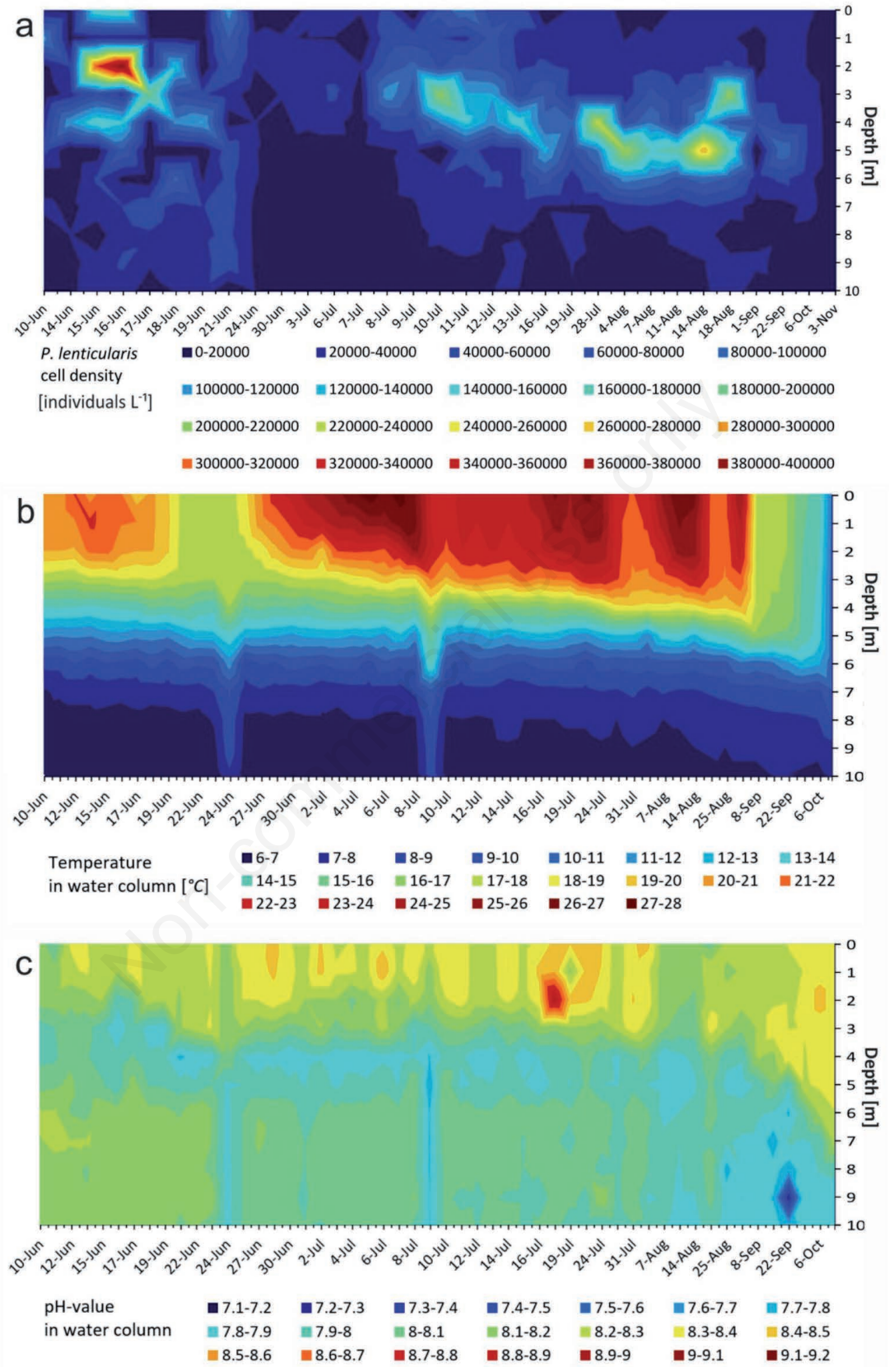

Fig. 4. Depth distribution and spatiotemporal variation of a) *Phacotus* cell density; b) temperature; c) pH-value at site GOS-W between depths of 0 to 10 m during 2015.

the epilimnion. With regard to temporal variation, an approach of assessing cell densities using sediment traps installed beneath the epilimnion (4) revealed that there was little correlation between sedimentation data and cell densities in the water column. One reason was carbonate dissolution during sedimentation (5), which also occurred during microscopic analysis and following storage of samples (6). Carbonate dissolution during microscopic analysis was further illustrated by a laboratory experiment (7).

In contradiction to the more general findings of van de Bogert *et al*. (2012), who suggested that single-location estimates can yield errors of more than an order of magnitude when it comes to estimating daily gross primary production and respiration, the horizontal variation (1) in *Phacotus* abundance at the different sites in Lake GOS was negligible. Apparently, the morphological characteristics of the lake basin (bathymetry), in addition to external factors such as wind exposure, did not control the horizontal distribution of *Phacotus* over a period of more than 10 weeks. It was verified that stable stratification provided conditions that enabled simultaneous *Phacotus* development in the epilimnion at three sites. Therefore, one single sampling site over the deepest point of a lake was sufficient for representative and effective *Phacotus* population monitoring in this lake being smaller 2 km². This finding is also in accordance with the standard plankton sampling procedure for the EU Water Framework Directive (Mischke and Nixdorf, 2008).

The density changes of *P. lenticularis* (2) over the vegetation period of five month in Lake Grosser Ostersee resulted in three successive growth peaks. A short and intense first one was followed by two temporally extended population peaks with lower cell numbers. This finding is quite similar to the descriptions of Krienitz *et al.* (1993) for Lake Haussee in northern Germany, suggesting that such a temporal dynamic may be characteristic for this species. During the first growth phase in mid-June, mean cell densities extended 100,000 Ind. L^{-1}. Within the following nine days, the population collapsed to the lowest cell number (17,000 Ind. L^{-1}) of the entire study period (Fig. 3). This can be explained by at least two factors or their combination: first, increased grazing of filtrating zooplankton that typically arises after the first phytoplankton bloom, and second, sev-

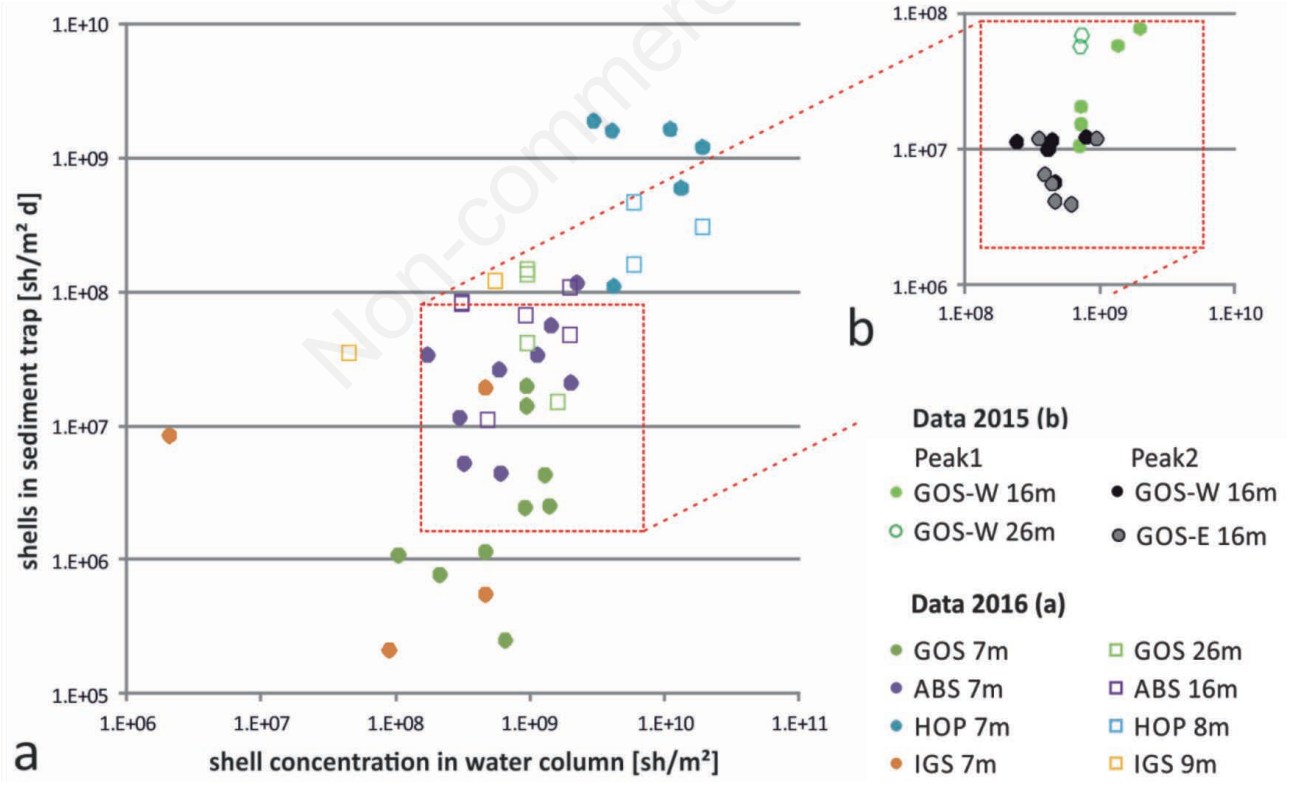

Fig. 5. Relationship between suspended epilimnetic *Phacotus* shell concentrations in the upper water column and corresponding *Phacotus* shell sedimentation rates in sediment traps exposed above (installation depth and place are indicated in the legend). a) Data from 2016 from depths of 0 to 7 m of four Bavarian lakes; b) data from 2015, from depths of 0 to 10 m of Lake Grosser Ostersee. The delay in sedimentation due to sinking was calculated using an estimated shell sedimentation velocity of 3 m d^{-1} for all lakes and all years.

eral windy days with rainfall leading to the breakdown of the epilimnion. With rising temperatures and the re-establishment of the stratification at the beginning of July, two further growth peaks developed with cell numbers around 30,000 and 65,000 Ind. L^{-1}. The second *Phacotus* growth phase was probably attenuated because it went along with the onset of intensified biogenic calcite precipitation leading to high amounts of suspended carbonate and consequently to a reduction of both light transmission as well as a depletion of dissolved phosphate and dissolved inorganic carbon (DIC) in the upper water column.

The measurement of the vertical spatial distribution (3) of *Phacotus* cell density along with water temperature revealed that the population maxima were always located above the thermocline, which developed from mid-June and descended continously from 2 to 6 m through the summer. This is to be expected since *Phacotus* actively moves near the surface where the light intensity is adequate and favourable for reproduction and photosynthesis (Gruenert *et al.*, 2016). Hydrographical parameters such as pH and high temperatures (Fig. 4) naturally result in carbonate oversaturation in the epilimnion (Bluszcz *et al.*, 2009), which is thought to be a relevant factor for the extracellular calcification of *Phacotus* shells (Hepperle and Krienitz, 1996). This implies that the vertical spatial distribution of *Phacotus* cell density occurs through a combination of active movement of the flagellate and passive accumulation due to the density gradient above the metalimnion, and can explain why 89% of *Phacotus* cells in the 0 to 10 m water columns were concentrated at depths in the range of 0 to 7 m. Therefore, this supported the hypothesis that an integrated water sample taken over the entire dimension of the epilimnion is sufficient to describe the *Phacotus* population in a small lake.

The sediment trap experiments (4) revealed that *Phacotus* shells contributed a maximum of 15 wt% to the total calcite at the bottom of Lake Hopfensee, where the highest *Phacotus* shell abundance in sediment trap material (Fig. 5a) was detected. High cell densities of *Phacotus* in the upper water column resulted in large amounts of shells in the sediment traps. However, in the majority of the lakes, sedimentation rates were higher at the lake bottom than in the depths up to 7 m. Cell densities only revealed weak temporal relationships with the sedimentation rates (Fig. 5a). For these reasons the second hypothesis, *i.e.* that sedimentation rates mirror the *Phacotus* population in the water column, was not supported. It would be interesting to explore this aspect further by investigating the depletion of phytoplankton by zooplankton inside the sedimentation traps, as suggested by Bloesch and Burns (1980), or whether *Phacotus* individuals living at a depth of 7 m are still fit enough to escape from the sediment traps.

In contrast to the last hypothesis, analysis of plankton from IGS revealed signs of calcite dissolution on the surfaces of *Phacotus* shells despite of the alkaline nature of all lakes. In the plane view, the *Phacotus* shells and their broad shell rim appeared even and compact (Fig. 6a), but viewing from an oblique angle revealed pronounced trenches between the edges of the calcite crystals at the shell rims (Fig. 6b). This was considered as indicator for carbonate dissolution of *Phacotus* shells already in the water column (5), which was surprising since the conditions in the epilimnion of a hard-water lake, with a Ca^{2+} ionic concentration of 37.6 mg L^{-1}, pH >8.6 (SD = 0.5) and temperature of 20.9°C (SD = 2.7°C) as described by Lenz *et al*. (2018), did not indicate carbonate undersaturated conditions. These parameters are within acceptable ranges indicated for the occurrence of *Phacotus* in lakes (Gruenert *et al.*, 2016; Schlegel *et al.*, 2000; Schlegel, 2001). A *Phacotus* mass occurrence was even documented at IGS in 2006 (Bavarian Environment Agency, 2016), which suggests suitable water conditions for *Pha-*

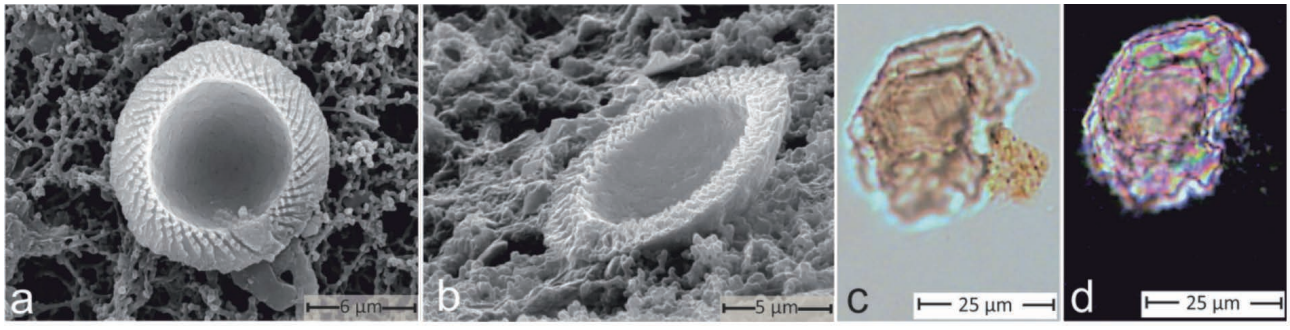

Fig. 6. a) Electron micrographs of *Phacotus* shells from the epilimnion in Lake Igelsbachsee: plane view of an open shell; b) diagonal perspective of the shell rim with deep trenches visible on the broad contact plane as evidence of dissolution; c) micrograph of a large blocky calcite crystal with dissolution structures indicating carbonate-dissolving conditions at the lake bottom; d) the same crystal under crossed nicols.

cotus in this lake. In addition, at the lake bottom, material from the sediment traps showed further features of dissolution on larger carbonate crystals (Fig. 6 c,d), which also indicated carbonate-diluting conditions in the lower water column – a process that has also been observed in other hard-water lakes (Bluszcz *et al.*, 2008; Ohlendorf and Sturm, 2001). The conditions in the hypolimnion of IGS, beyond a depth of 7 m, showed a pH value of 7.6 (SD = 0.1) and indicated anaerobic conditions and a marked increase in conductivity towards the bottom of the lake. Mineralisation processes, such as bacterial activity, are factors that can affect calcite sedimentation (Kelts and Hsü, 1978), therefore $CaCO_3$ undersaturated conditions may have developed in IGS. Calcite crystals, as well as *Phacotus* shells, potentially dissolved during sedimentation down the water column, undermining the sediment trap results from IGS. Consequently, in IGS, where dissolution cannot be excluded, sediment traps were not adequate for the representative assessment of the *Phacotus* population in the epilimnion.

Dissolution of carbonates continued in the IGS samples even in the brown-glass flasks (6) during storage. The evidence for this was the absence of *Phacotus* shells in stored samples which had demonstrably contained *Phacotus* shells prior to storage. Incorrect sample treatment can be excluded as a reason for this, since all samples were treated identically and IGS was the only lake which showed a reduction in *Phacotus* shell numbers following storage. The reasons for carbonate dissolution during storage remain unclear, because the pH was >7.7 in the overhead water of the sampling flasks. To avoid carbonate dissolution during storage it is recommended that Lugol's solution with a pH >7 is used and that plankton samples are analysed immediately.

A reduction in *Phacotus* shells also occurred during microscopic analysis. A dilution experiment (7) showed that it was not only decreased pH values in sampling bottles but also the use of distilled water during the preparation of samples for microscopic examination that led to dissolution of the calcite shells of *Phacotus* (Fig. 7). The experiment demonstrated that, following shell dissolution, no evidence of *Phacotus* remained, apart from the naked protoplast, which could possibly be misinterpreted as a single-celled chlorophyte flagellate such as *Chlamydomonas* sp. To prevent the unnoticed loss of *Phacotus* shells, the use of distilled water for sample preparation is not recommended. Instead, a buffered suspension liquid containing NH_3 and H_2O with a pH ≥8.5, after Bollmann *et al*. (1999), should be used.

All of the findings concerning dissolution suggest that dense sampling intervals of *Phacotus* as well as appropriate preservation and handling are essential to avoid a bias towards underestimating their abundance and contribution to biogenic carbon sequestration, even in alkaline lakes. This aspect appears equally important as an early start of sampling campaigns to capture the first and strongest population peak of the season.

CONCLUSIONS

In order to assess representative population densities of *Phacotus lenticularis* in a water column throughout the complete growth period, the timely commencement of monitoring is essential to assess what is the first and often largest population peak. For small lakes of <2 km², integrated water samples can be collected down to the metalimnion from one representative sampling site. The present study found that the epilimnetic range of 0 to 7 m provided optimum coverage of *Phacotus* individuals. Due to the rapid sedimentation of *Phacotus* shells, it is essential to homogenise plankton samples before transferring them into previously prepared sampling bottles. Fixation works well with 2% neutral pH Lugol's solution, which is recommended to be added to a sample at a four-fold concentration (Utermöhl, 1958). Cylindrical sediment traps are recommended to be installed at the bottom of a lake, at a depth of >10 m. To avoid carbonate dissolution during storage, microscopic analysis should ideally be completed immediately after sampling. As an indicator for carbonate dissolution, the ionic concentration of Ca^{2+} in the overhead water of the sampling flasks can be analysed. During counting under a microscope, it is essential to differentiate single shells from intact individuals accounting for two shells. During sample preparation, water with an adjusted pH value of >8.5 should be used for sample dilution. To track potential carbonate dissolution in the hypolimnion, sediment analysis can reveal whether all shells reaching the lake bottom are stored over geologic timescales or if dissolution in sediment or at the sediment surface reduces the amount of *Phacotus* shells.

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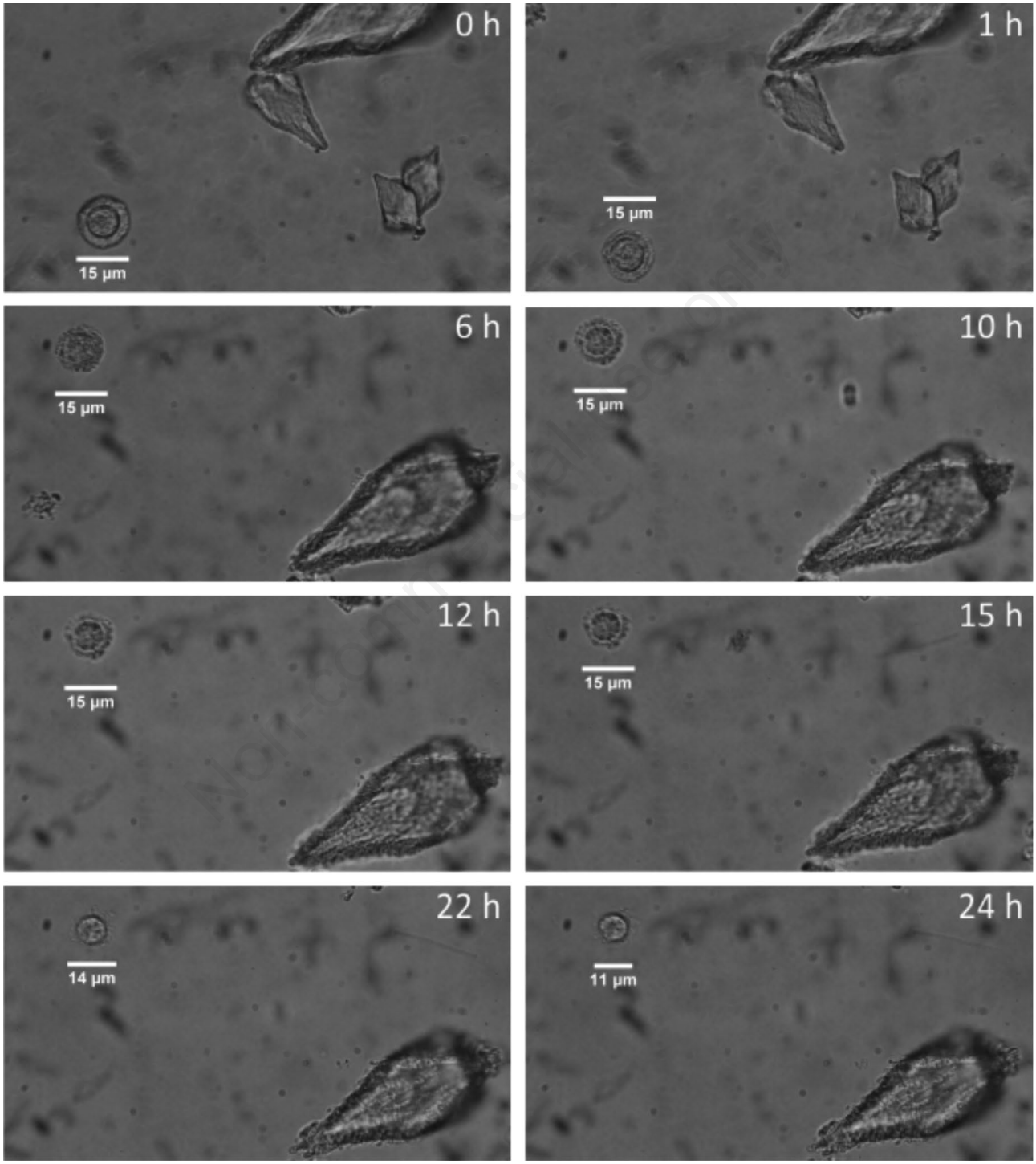

Fig. 7. Micrographs from a 24 h experiment to demonstrate carbonate dissolution on the circular shell rim of an individual *Phacotus* cell (left) and a wedge-shaped calcite crystal (right) showing slow dissolution; the experiment was performed under an inverted light microscope at 400x magnification in an Utermöhl chamber (Utermöhl, 1958), diluted 1:10 with bi-distilled water.

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