The queer Tetraëdron minimum *from Lake Kivu (Eastern Africa): is it a result of a human impact?*

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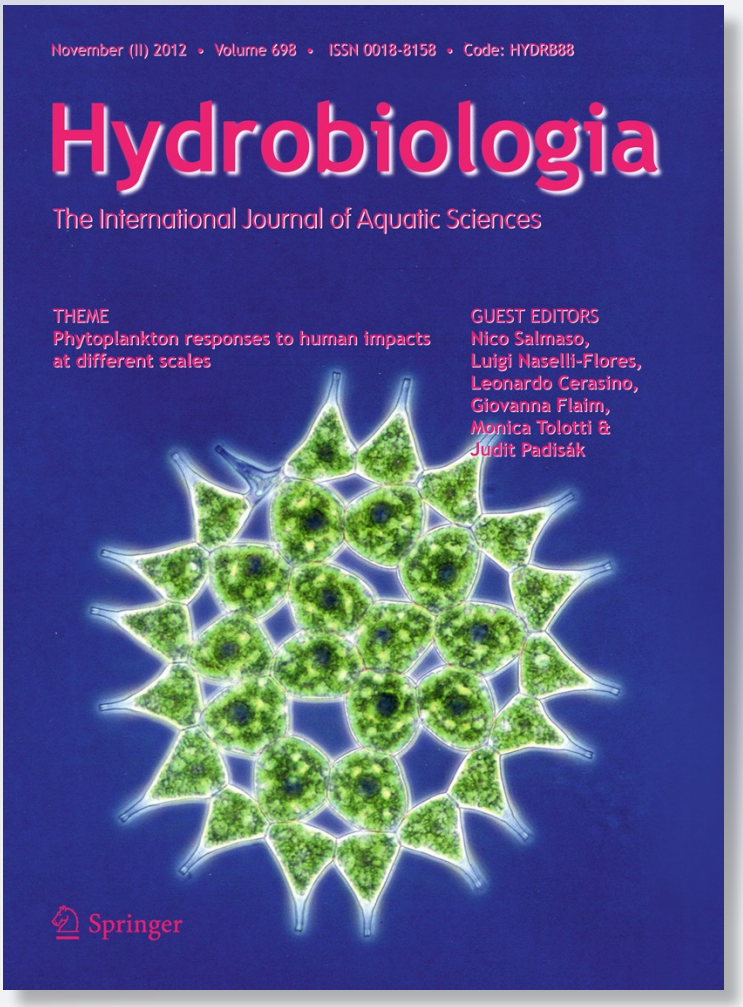

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PHYTOPLANKTON

The queer *Tetraëdron minimum* from Lake Kivu (Eastern Africa): is it a result of a human impact?

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Abstract The coccal unicellular green algal genus *Tetraëdron* Kütz. ex Korshikov, which can be easily identified by its typical polygonal shape, is a common member of freshwater plankton and metaphyton, frequently observed in lowland temperate and tropical waters. During the analysis of samples from tropical Lake Kivu (Eastern Africa), we found an interesting "lemon-shaped" alga, which, after observations in light microscope and scanning electron microscope, had been listed as *Tetraëdron* sp. Isolation in pure culture allowed a deeper study on morphology at different stages of the life cycle and the partial sequencing of the 18S rDNA. The results from the different combined approaches confirmed that it belongs to the species *Tetraëdron minimum* (A. Braun) Hansg. The unusual "lemon-shaped" forms predominant in Lake Kivu are young stages of the life cycle. This study contributes to the knowledge of the morphological variability, reproduction, and resting stages of *T. minimum* and discusses the reasons for the dominance of such unusual shape found in Lake Kivu, a lake strongly impacted by human activities as resulted by the large-scale biomanipulation following the introduction of the "Tanganyika sardine," *Limnothrissa miodon* (Boulenger, 1906), at the end of the 1950s.

Keywords Green algae · Phytoplankton · Zooplankton · Grazing · Tropical lake · Akinetes

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Introduction

"Although, from one point of view, *Homo sapiens* is just one more species among the millions, it is unique in its power to influence the environment of all the others" (Reynolds, 1997, p. 295). From this sentence, it is possible to turn to a special problem of a great concern—the human impact on the phytoplankton.

This peculiar community consists of organisms, and the distribution and success of each of them is a function of abiotic constraints and biotic processes with a hierarchical importance of different factors (Brönmark & Hansson, 2005). The abiotic environment of a water body often is altered by human-induced disturbances (Brönmark & Hansson, 2005), and the effects which some of them cause to the phytoplankton are generally well known. Among them are the consequences of eutrophication, acidification, and biomanipulation. However, in spite of the increasing number of reports on exotic species introductions and to the influence of invasive alien species on biological diversity and ecosystem integrity, it is possible to stand that the consequences of such introductions at the level of morphological variation of a given species, belonging to a different trophic level from that of the allochtonous species, are practically unknown.

This article shows the probability for the occurrence of unusual small-shaped cells of *Tetraëdron minimum* due to changes in the grazing pressure in tropical Lake Kivu, ca. 50 years after the introduction of the planktivorous endemic sardine *Limnothrissa miodon* from the lake Tanganyika in order to "improve" the food web and to become the basis of fisheries activities (Collart, 1960; Simberloff, 1995). With this article, based on microscopic observations on field and cultured material in combination with molecular methods, we would like to contribute also to the knowledge on the cytology, reproduction, and resting stages of *T. minimum.*

Materials and methods

Study site and sampling procedures

Lake Kivu, located between Rwanda and the Democratic Republic of the Congo (Kivu Province), is one of the Great Lakes of the East African Rift Valley and is formed by four main basins (Fig. 1). It is a deep (max. 489 m), meromictic lake, with an oxygenated epilimnion of about 70 m and a deep hypoliminion rich in dissolved gases (CO_2, methane). With an annual average of chlorophyll *a* in the mixed layer of 2.2 mg m^{-3} and primary production of 0.71 g C m^{-2} day^{-1} ($\sim$260 g C m^{-2} $year^{-1}$), the lake is clearly oligotrophic (Sarmento et al., 2006, 2009).

Phytoplankton samples were collected regularly from September 2002 till February 2004 twice a month in the southern basin, while northern, eastern, and western basins were visited twice a year (once in the dry season and once in the rainy season). The qualitative samples were collected by vertical plankton net (10 μm mesh size) in the 0–60-m layer, and the quantitative samples were collected with a Van Dorn bottle at different depths (surface, 5, 10, 20, 30, 40, 50, and 60 m). The samples were preserved immediately after collection with neutral formaldehyde (2–4% final concentration) and Lugol solution. Before observation, the samples were concentrated by settling. For scanning electron microscopy (SEM), samples were fixed with glutaraldehyde at 1–2% final concentration. More details on sampling sites and procedures can be found in Sarmento et al. (2007).

Strain isolation and cultivation

In September 2008, half a liter of subsurface water from Lake Kivu was shipped to the Institut de Ciències del Mar (CSIC) in Barcelona (Spain), within 48 h and then enriched with an equivalent volume of BG-11 culture medium. In sterile conditions, serial dilutions of the mixture were carried out in 12-well polystyrene plates. The plates were sealed with parafilm and incubated at 23°C under artificial photosynthetic active radiation (PAR) of 100 μmol photon m^{-2} s^{-1}, in a 16:8-h light:dark cycle for several weeks. Microbial growth was regularly checked directly on the plates without opening it, under an inverted microscope at 40× magnification. The wells in which the specimen of interest was found in large abundance were transferred to BG-11 culture medium agar plates for strain isolation. Individual dark green colonies were re-grown in fresh BG-11 liquid culture medium and filtered through a 0.2-μm filter (Durapore 47 mm), preserved in 750 μl of lysis buffer (40 mM EDTA, 50 mM Tris–HCl, 0.75 M sucrose) and stored at −80°C until nucleic acid extraction (Fig. 2).

In December 2008, a part of the material was transported to the Algal Collection of Sofia University (ACUS). There, the material was analyzed immediately by means of light microscopy (LM) and then transferred on new agar plates, enriched by Bold Basal medium (BBM). The transfer followed standard techniques (Ettl & Gärtner, 1995; Andersen, 2005). In an attempt to observe zoospore production, BBM

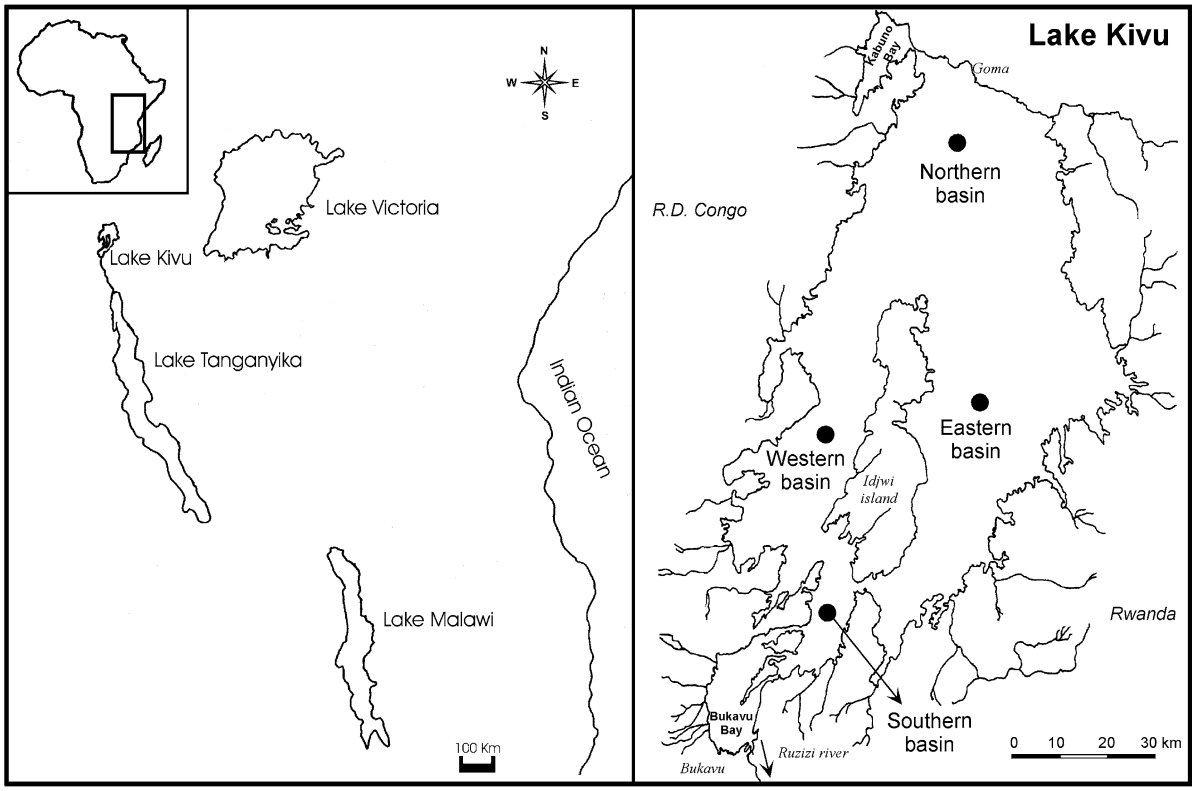


Fig. 1 Geographic situation of Lake Kivu with indication of its four major basins

liquid cultures were regrown several times, from the agar cultures. They were kept in darkness for ca. 16 h and then checked for zoospores.

LM and SEM processing with photo documentation of field and culture material

Light microscopic (LM) investigations were done on Olympus BX-50 (field material) and Motic BA 400 (culture material) microscopes with objectives 40× and 100× (oil immersion), both equipped with phase contrast. SEM study was done on Philips XL-microscope. Cell walls were stained with Gentian violet and Methylene Blue, and starch was colored with Lugol's solution (Ettl & Gärtner, 1995). Photomicrographs were taken with an Olympus Camedia digital camera (field material) and Moticam 2000 camera attached to the Motic BA 400 microscope with special adaptors (culture material). For processing of the photos, the computer software "Motic Images Plus 2.0" was used.

Nucleic acid extraction, amplification and sequencing

Nucleic acids were extracted by adding lysozyme (1 mg ml^{-1}) to the filter unit and incubating at 37°C for 45 min. Subsequently, proteinase K (0.2 mg ml^{-1}) and sodium dodecyl sulfate (SDS, 1%) were added, and the filter was incubated at 55°C for 1 h. The lysate was then extracted twice with an equal amount of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8) and once with an equal amount of chloroform–isoamyl alcohol (24:1). The aqueous phase was spun down in a microconcentrator (Amicon-100, Millipore), washed with 2 ml of sterile MilliQ water three times, and reduced to a volume of 100 µl. The recovered DNA was quantified using Nanodrop (Thermo Scientific). Nucleic acid extract was stored at −80°C.

One nanogram of DNA was used as template for PCR amplification of eukaryotic 18S rDNA. The reaction (50-µl volume) contained 200 µM of each of the deoxynucleoside triphosphates, 0.5 µM of each of the primers, 1.5 mM $MgCl_2$, 1× PCR-buffer and 1.25

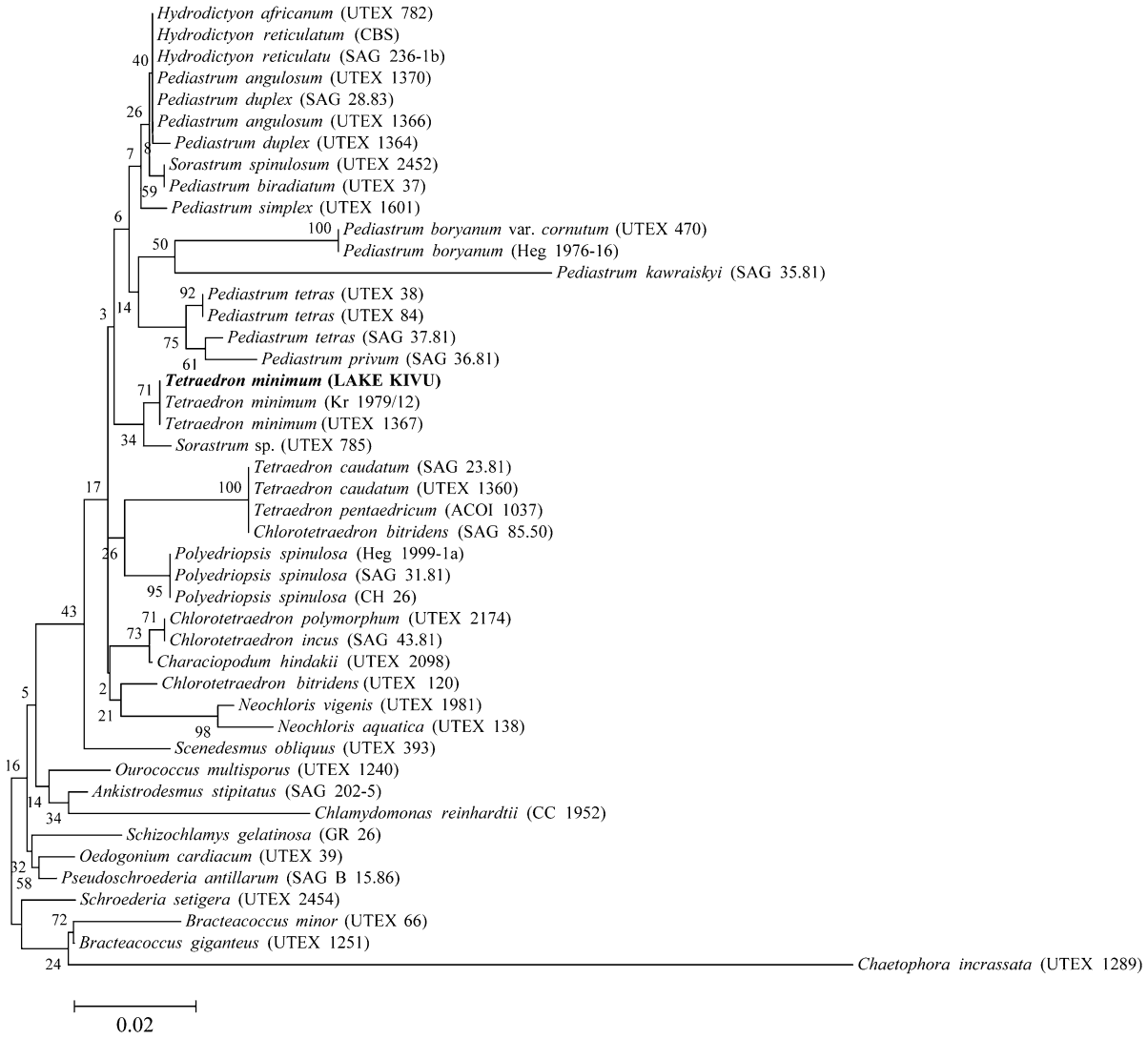


Fig. 2 Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred using the maximum likelihood method based on the data specific model (Nei & Kumar, 2000). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 64.2418% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 345 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011)

Units of Taq DNA Polymerase (Invitrogen). We used the eukaryotic specific primers EUK1F (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and 516r (5′-ACC AGA CTT GCC CTC C-3′). The PCR was performed with a thermal cycler (Bio-Rad) using the following program: initial denaturation at 94°C for 2 min 10 s; 30 cycles of denaturation (at 94°C for 30 s), annealing (at 56 for 45 s) and extension (at 72°C for 2 min 10 s); and a final extension at 72°C for 10 min. PCR products were verified and quantified by agarose gel electrophoresis with a standard in the gel (Low DNA Mass Ladder, Invitrogen).

Positive PCR products were purified and sequenced by Macrogen Sequencing Service (South Korea). The

nucleotide sequence was deposited in GenBank under accession number BankIt1523118 LKO1 JQ797441. The sequence obtained was aligned with the software MEGA 5.05 (Tamura et al., 2011) and compared with DNA sequences from algal culture collections used by Buchheim et al. (2005). Evolutionary analyses were conducted in MEGA5.05 (Tamura et al., 2011).

Results

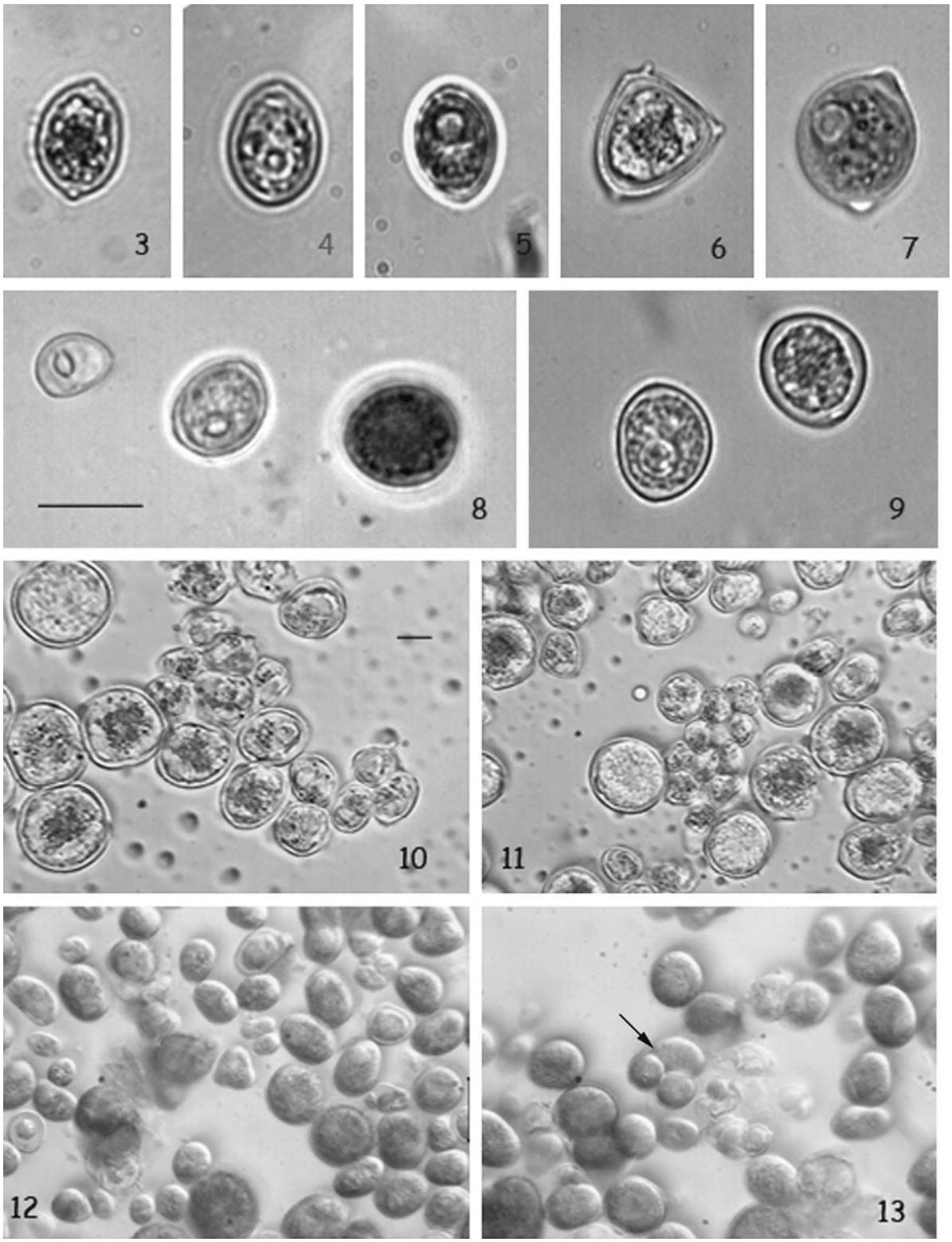
By means of LM in almost all phytoplankton samples from Lake Kivu, a free-floating alga with peculiar cell outline was found. The cells were solitary, ovoid, asymmetric to pyriform when seen in side view, and triangular (very rarely quadrangular) in front view, (5)–7–12–(14) µm in diameter. Each cell bears one or two, very rarely three or four, short-thickened polar protuberances, which were important for the "lemon-shaped" outline of the cell (Figs. 3–9, 20b). On higher magnifications, the rough character of the cell wall was visible, and by SEM, its scrobiculated character was confirmed (Figs. 14–17). Each cell contained a parietal, massive chloroplast, with a single pyrenoid (Figs. 5, 7–9) and oil droplets (Fig. 4). The pyrenoid bears a starch sheath, clearly visible after staining by iodine (Figs. 5, 8), thus confirming the disposition of the alga in the green lineage. The reproduction stages were rarely observed in the field material. They were represented by more or less developed autosporangia with 4 (8) autospores. Their release was preceded by cell wall rupture and its division in two parts. In the field material, the autospores have the peculiar "lemon-shape" of the free-floating cells, while some of the autosporangia showed a tetrahedral character. This was the first clear feature, which inspired the idea that the alga under investigation belonged to the genus *Tetraëdron.*

The next step—species identification—was more complicated. Finally, on the slides from the field material, we came to the conclusion that features observed overlap partially with the descriptions of two species, known for their polymorphism—*Tetraëdron regulare* Kütz. auct. post. (incl. var. *ornatum* Lemmerm.) and *T. minimum* (incl. var. *scrobiculatum* Lagerh.)—Sarmento et al. (2007). At the same time, we had to take into account that in less than 10% of the samples, in small amount, typical, and well-developed tetrahedral cells with pronounced protuberances of *T. regulare* were observed (Fig. 16 in Sarmento et al., 2007). In this case, the only possible correct solution was to postpone the final identification decision and to list the material as *Tetraëdron* sp. (Sarmento et al., 2007, Figs. 16, 47–50, 66) until we study it in pure cultures.

After the isolation of a clonal culture (in 2008), a part of the material was transferred for long-term cultivation on agar, and a small amount was immediately controlled for eventual zoospore production. However, zoospores were not observed. The first observations by LM of the material on agar plates did not reveal new features or significant morphological deviations compared with the data obtained from field material, except more abundant autosporangial and autospore production. In April 2009, on the original plate, sent to ACUS, a drying of the agar was detected, attended by the change in the color of algal stripes from green to yellowish-green. This was due to the abundant presence of akinetes—large (up to 25–30 µm in diameter) spherical cells with thick cell walls, which probably contained haematochrome (Figs. 10, 11). Some of them were in stage of division in two. The akinetes were immediately transferred to new agar plates.

In May 2010, PCR amplification and sequencing of the 18S rDNA of the material previously conserved from the first clonal cultures revealed the phylogenetic affiliation of the Kivu alga to *T. minimum* (Fig. 2). The partial sequence obtained was 100% similar to those of two *T. minimum* strains from other culture collections (Kr 1979/12 and UTEX 1367).

The culture material was studied again by LM from September 2010 to April 2011, after the algae in the new cultures, obtained from the akinetes, were developed more abundantly (Figs. 12, 13). Then an alteration in the abundance of the well-developed vegetative cells (some with typical for *T. regulare* shape) and autosporangia (Figs. 13, 18–28), and the smaller "lemon-shaped" cells was observed: the last ones dominated in February 2011 and then again in April 2011. All of the well-developed single vegetative cells were of bright green color and contained very large pyrenoids. Their starch sheath was clearly visible even without iodine staining (Fig. 7) and generally shows a bipartite character (Figs. 5, 8, 9). Again, a part of the material was transferred in BBM liquid and afterward checked by LM for zoospore formation. However, only autosporangia together with small "lemon-shaped" cells were recorded.

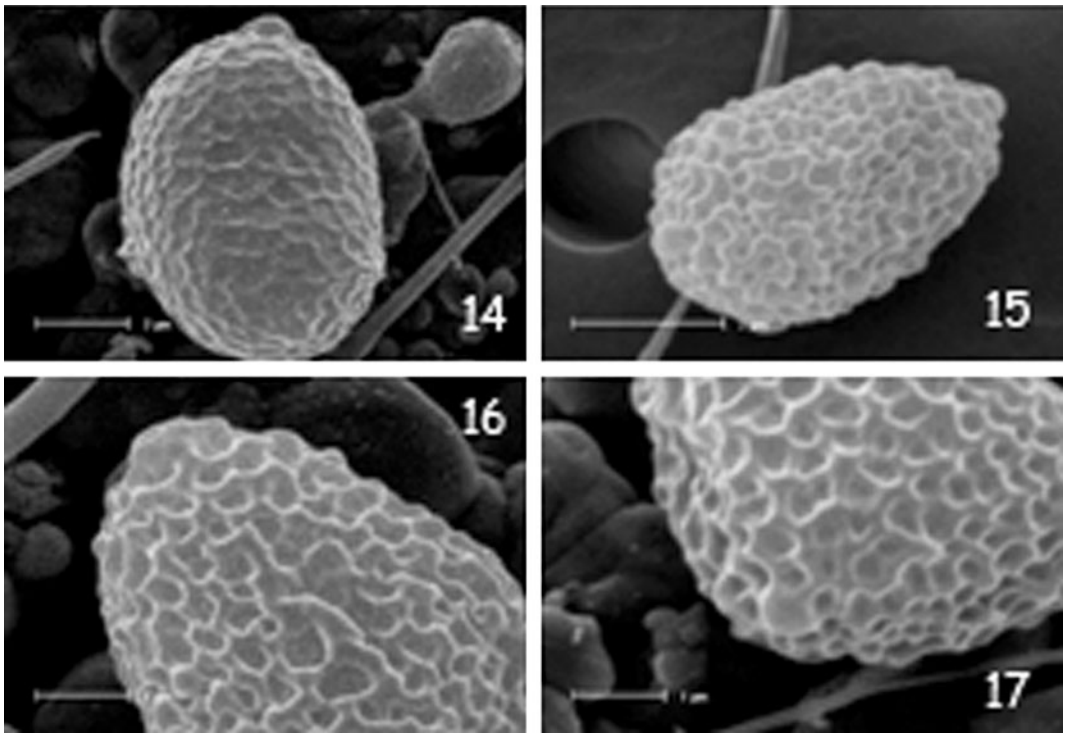


◀ **Figs. 3–13** *Tetraëdron minimum* in LM: 3–7, 9—single cells of the species; 8—single cells and initial autosporangium; 10, 11—akinetes with large *spots* or total content with resemblance to haematachromes and single cells in a drying culture; 12, 13—general view on a culture, developed from akinetes with new well developed vegetative single cells (some of them with typical triangular outfit like the cell in the centre of the photo, some more ovoid or spherical; among them smaller "lemon-shaped" cells could be seen), new young autosporangia and autosporangium with consecutive formation of autospores (arrow). Scale bar for Figs. 3–9—5 μm, for Figs. 10–13—10 μm

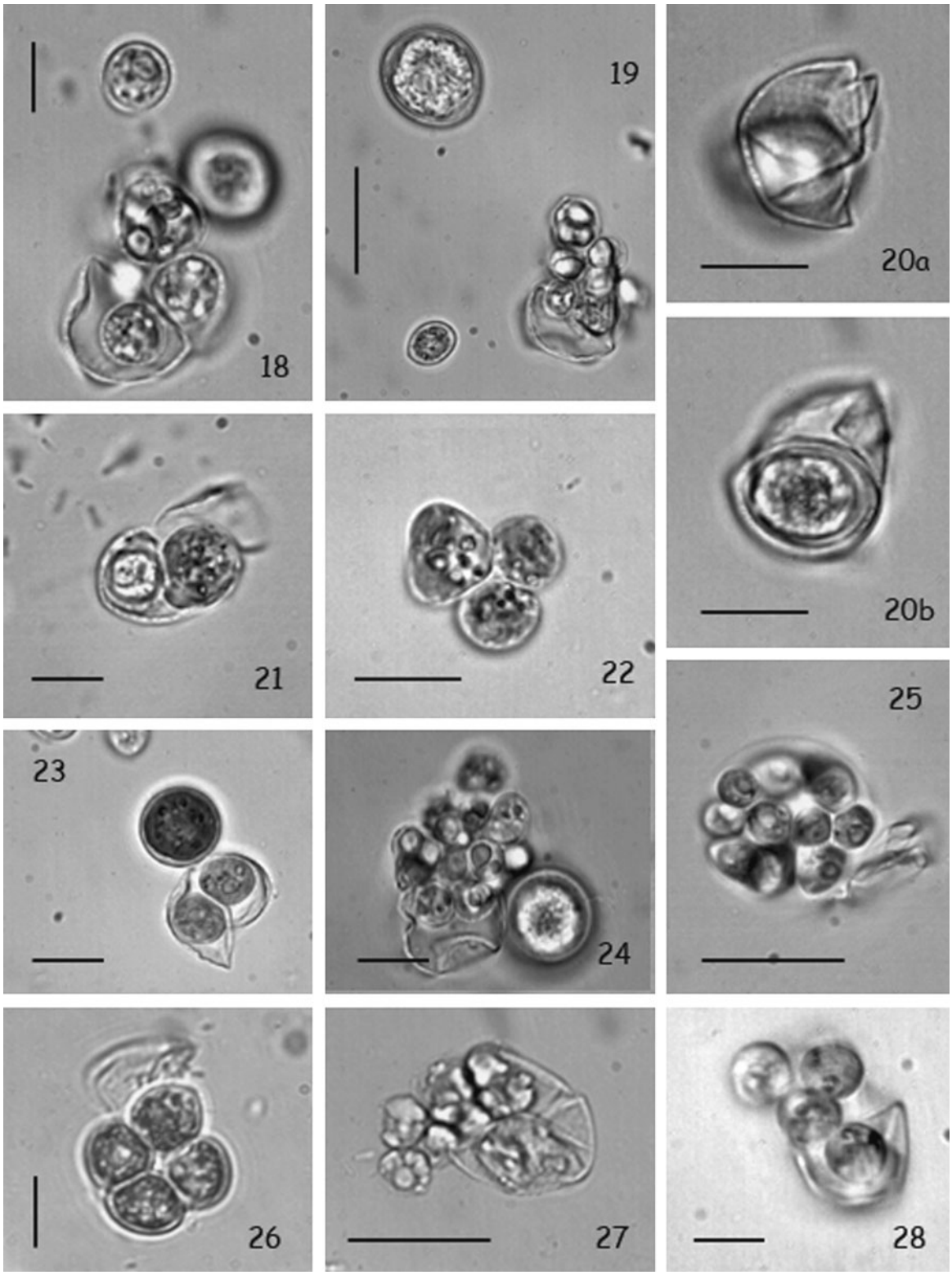
Discussion

The observations on the morphology and reproduction show that the peculiar alga, found in the Lake Kivu, belongs to the genus *Tetraëdron* and is able of asexual reproduction by autospores and, additionally, of akinete formation. Until recently, production of akinetes as a process of enlargement of cells under harsh conditions supplied by increase of dimensions and changes of the shape and coloration (from green to yellow or red) in *Tetraëdron* was described only by Troitzkaja (1933) for *T. minimum* and for *T. regulare*, and later by Korshikov (1953) for *T. incus* (Teiling) G. M. Sm. and by Davis (1966) for *T. bitridens* Beck-Managetta. All of the aforementioned authors noted their role as resting stages, but only Troitzkaja (1933) used the term "cysts" instead of "akinetes." Both terms could be applied to the "giant" cells, observed in our cultures, because they were formed in asexual way from vegetative cells through enlargement, supplied by cell wall thickening and change in cell protoplast toward production of reddish content (possibly haematochrome). Due to this asexual (vegetative) way of forming, which is not always clear when term "cyst" is used (Ettl, 1980) we prefer to refer the stages found as "akinetes." The vegetative division of akinetes, observed by us, is rarely reported in the physiological literature, but is a known process (Ettl, 1980).

The main cytological features observed in the vegetative cells (e.g., parietal chloroplast, single distinct pyrenoid with starch sheath, oil droplets) are on conformity with all former observations (see Kováčik, 1975 for details). Our records clarify the general bipartite character of the starch sheath around



Figs. 14–17 *Tetraëdron minimum* in SEM: 14, 15—general view on total cells; 16, 17—parts of cell surface with scrobiculated cell wall



◀ **Figs. 18–28** Autospores and autosporangia of *Tetraëdron minimum* in LM: 18, 22, 26, 28—autosporangia with four autospores (18 and 28—the release of the autospores after rupture of the autosporangium wall in two parts is seen; 22—autosporangium with one large and two smaller cells, showing the consecutive formation of autospores); 19, 24, and 27—autosporangia with more than four autospores; 20, 21, and 23—autosporangia with two autospores and ruptured autosporangium wall; 25—released autospores in a mucilage vesicle with remnants of the autosporangium wall beneath them. Scale bar for Figs. 18, 20–23, 26, and 28—5 μm, for Figs. 19, 24, 25, and 27—10 μm

the pyrenoid. The two parts of the sheath are distinct on the first photo from Plate 4 in the paper of Pickett-Heaps (1972) and on one drawing provided by Kovácik (1975, p. 365, plate 3c), but were not described or discussed by the authors. It could be supposed that the bipartite character of the pyrenoid sheath is typical for the genus. The cell wall surface in its outline by LM and its vision in SEM coincide with the data of Kováčik & Kalina (1975) on cell wall surface of *T. minimum*. The cell dimensions and mode of asexual reproduction by autospores only, as well as the data from molecular analyses, are on conformity with the same species.

The more frequent appearance of tetrahedral cells, resembling in outline *T. regulare*, than of more flat, quadrangular cells, widely known as typical of *T. minimum*, is on conformity with the data of Troitzkaja (1933), who underlined the simultaneous appearance of both types of cells in clonal cultures of *T. minimum*. However, the predominance of the peculiar "lemon-shaped" outline (with one or two small protuberances) of most of the cells found in the field and in some of the cultures provokes the question about the reasons which trigger the alga to appear in this form instead of its typical, widespread polygonal shape?

The change of the ratio of polyhedral and non-polyhedral cells in cultures of different age and observations of autosporangia and their development lead us to the idea that the "lemon-shaped" cells are just juvenile stages in the development of normal vegetative cells. This hypothesis finds support in the published details and illustrations on the development of different *Tetraëdron* species (e.g., Troitzkaja, 1933; Starr, 1954). After prolonged observations in cultures of *T. bitridens*, Starr (1954, p. 19) wrote that generally "each autospore is an exact replica of a mature vegetative cell, although, in some instances, where the spores are retained within a sporangial wall for a long time, the autospores may have less pronounced angular processes." Troitzkaja (1933) showed that in *T. minimum* the enlargement of the cell and increase in volume sometimes did not start from the central region (when a classical tetrahedral shape is formed), but from one of the sides, bringing to irregular, asymmetrical outline of the whole cell.

The data and conclusions of Kováčik & Kalina (1975) on cell wall surface, observed by SEM, are also on conformity with the idea that "lemon-shaped" cells found in Kivu waters and are young stages of *T. minimum*. The authors postulated that the network character of *Tetraëdron* cell wall surface, found by them in *T. caudatum* (Corda) Hansg. and *T. minimum*, is typical for the genus and the superficial undulation represents corrugation of two surface layers of the cell wall. Detailed analysis showed ontogenetical differences in the thickness and folding of the layers: in young cells the periphery of the middle layer is abundantly folded and the network is very dense, whereas in older cells the corrugated surface of outer layers evens out, the network thins out, and is composed of larger, often interlocked meshes. In old, large, rounded cells, the network is reduced or the cell surface is completely smooth (op. cit.). The photos of Kivu material, obtained by means of SEM, clearly show a well-developed mesh-network of the cell surface with deep folds (Figs. 14–17), which confirms that the "lemon-shaped" cells are young stages in development phase.

Alone, this result cannot explain the predominant occurrence of the immature stages of *T. minimum* in the oligotrophic Kivu waters. Lake Kivu phytoplankton composition is peculiar when compared to that of the other Rift Lakes (Sarmento et al., 2007): it is dominated by diatoms, cyanoprokaryotes (cyanobacteria/blue-green algae), and cryptophytes. Green algae, which, for example, in Lake Tanganyika are a dominant group, have here a secondary role in terms of abundance and biomass (Sarmento et al., 2006, 2008). Their diversity is also low, and the few taxa found are mainly colonial coccal green algae and desmids; unicellular forms are comparatively rare. This suggests that most green algae of Lake Kivu are grazing-resistant forms, as Stoyneva et al. (2007) supposed for a new *Eremosphaera* taxon in Lake Tanganyika.

Grazers in Lake Kivu are essentially three species: two cyclopoid copepods and one cladoceran (Isumbisho

et al., 2006). Although their grazing rates on algae are not known, their diet was studied using fatty acids (FA) as biomarkers: Masilya (2011) measured FA in several zooplankton size classes and found that small zooplankton (i.e., the 50–100 μm size class, comprising rotifers and copepod nauplii) fed essentially upon cryptophytes and diatoms. Copepods in the 100–300-μm size fractions also consumed chrysophytes, while the larger copepods (>300 μm) fed on cryptophytes, chrysophytes, and cyanoprokaryotes. FA from green algae were not found in zooplankton fractions, although they were present in the seston fractions. This indicates that green algae were either not ingested by zooplankters or that they were ingested but not assimilated.

Regarding *T. minimum* autospores, they are in the lower range of edible size for copepods (see, e.g., Sterner, 1989), and this could be a refuge strategy from grazing by the most abundant zooplankters in Lake Kivu. Small algae are more readily grazed by rotifers and by herbivorous protists. Rotifers are abundant in Lake Kivu, in contrast with the other oligotrophic Rift lakes (Isumbisho et al., 2006), and large ciliates are also present, although data on their abundance are lacking. Therefore, small algae, in order to survive in environments where grazing pressure is high and permanent, need to have traits that provide adequate refuge from grazers. In the case of *T. minimum*, fast reproduction rates with mass formation of autospores are a clear advantage for compensating grazing losses. Another trait which could be seen as a defense mechanism is the hard cell wall of *Tetraëdron* with high algaenans content, where the biopolymers are composed of long-chain even-carbon-numbered ω^9-unsaturated ω-hydroxy FA monomers varying in chain length from 30 to 34 carbon atoms (Blokker et al., 1998). This renders the cells and autospores resistant to digestive enzymes: several authors have reported that ingested phytoplankton cells may transit through zooplankton guts and be egested undamaged and able to grow (see a review of defense mechanisms in Van Donk et al., 2011).

Thus, grazing pressure in this tropical great lake may explain why these peculiar stages of *T. minimum* are so abundant and make the bulk of the population of this alga. In Lake Tanganyika, another green alga, *Eremosphaera tanganyikae* Stoyneva, Cocquyt, Gärtner, and Vyverman, efficiently escapes grazing thanks to large cell size (Stoyneva et al., 2007): this is a similar "strategy," at the other extreme of the size spectrum of the main grazers. A similar process was described for planktonic bacterial communities, who under high grazing pressure show a higher proportion of extremely small coccoid shapes or large filaments, out of the edible range for predators (Jürgens & Güde, 1994). The role of grazing in molding the size and shape of phytoplankters was recently summarized by Naselli-Flores & Barone (2011), who showed that inducible defenses in prey traits in response to predation risk are particularly common in natural systems and that these low energetic cost adaption reduces phytoplankton mortality due to herbivory.

As for the "human impact" issue, it may be indirectly involved in these morphological defenses of *T. minimum*. Indeed, Lake Kivu is an example of large-scale biomanipulation, which consisted in the introduction of the "Tanganyika sardine," *L. miodon*, at the end of the 1950s (Collart, 1960). This planktivorous fish induced important changes in the zooplankton structure, affecting both composition and abundance (Dumont, 1986). Thus, present zooplankton of Lake Kivu, different from that of the other Rift lakes in several respects, and the related grazing pressure, are the result of a major anthropogenic change. Due to the lack of detail in the knowledge of phytoplankton structure and composition before the sardine was introduced, the extent of the changes affecting the whole plankton is not easily evaluated. However, Sarmento et al. (2012) estimated that crustacean abundance may have declined by a factor of three as a result of the introduction; also, a major grazer, a large cladoceran has disappeared (Dumont, 1986). It is likely that a reduction in zooplankton body size distribution occurred from predation on large zooplankton (Brooks & Dodson, 1965), and that small zooplankton (small crustaceans, rotifers and protists) became more abundant, thereby resulting in an increased grazing pressure on small phytoplankton. In this context, phytoplankton taxa exhibiting traits providing efficient defense against grazing may have had an advantage, and this may explain the peculiar morphology of *T. minimum* in Lake Kivu as a device for reducing grazing losses, exploited consequently after the human impact on the food web of the lake.

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