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Biomonitoring of Lake Garda: Identification of ciliate species and symbiotic algae responsible for the "black-spot" bloom during the summer of 2004 ☆

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Abstract

At the end of July 2004, a "black-spot" appeared in the western portion of Lake Garda, an oligomictic lake classified as meso-oligotrophic. A few days later, this phenomenon spread throughout the lake. A first analysis by optical microscopy revealed that the origin of the black spot was a ciliated protozoan. Ciliates represent a small percentage of the total zooplanktonic community of Lake Garda and have never produced bloom episodes. Using morphological and molecular analysis, we characterized the protozoan responsible for the bloom as *Stentor amethystinus* and its symbiotic algae as a *Chlorella* sp. Continuous monitoring of the northeast of Lake Garda showed that the apex of the *S. amethystinus* bloom took place during the first 20 days of August, and the highest density of *S. amethystinus* occurred in the euphotic zone. During this period, high chlorophyll *a* values were obtained in water samples collected from the euphotic zone due to the presence of the endosymbiont *Chlorella*. After early September, the black spot completely disappeared, and the causative organism was detected at low concentration only in the southern basin of the lake. The results obtained on the progress of the black spot phenomenon led us to hypothesize that: (i) *S. amethystinus* was recently introduced in Lake Garda by anthropogenic activities or it was already a member of the zooplanktonic community but at a very low concentration; (ii) *S. amethystinus* blooms may have been driven by an unusual high availability of total phosphorous in the euphotic zone and (iii) Lake Garda is not the preferred habitat for *S. amethystinus*.

Keywords: *Stentor amethystinus*; SSrDNA; Ciliate density; Chlorophyll *a*; Photosensor

1. Introduction

The deep southern subalpine lakes Garda, Iseo, Como, Lugano and Maggiore together represent one of the largest freshwater supplies in Europe. Their waters are used for agricultural and industrial purposes, for fisheries and as sources of drinking water. These lakes were originally oligotrophic and have undergone a gradual deterioration since the 1950s and 1960s (Guilizzoni et al., 1983; Ambrosetti et al., 1992). The slow and progressive deterioration of the lake's trophic condition was indicated by the continuous increase of spring phosphorus concentrations in the water column (Ambrosetti et al., 1992), cyanobacterial blooms (Salmaso et al., 1994) and high phytoplankton density and bio-volume (Salmaso and Cordella, 1994).

Lake Garda is the largest ($49 \times 10^9\,m^3$) and most extensive ($368\,km^2$) of the Italian lakes. Based on chemical, physical and biological parameters, it can be classified as meso-oligotrophic (Provincia Autonoma di Trento, 2005). Lake Garda belongs to a distinct typology of lakes characterized by high depths (max. depth 350 m) and large

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volumes. Due to its morphometric features, Lake Garda has an oligomictic character, i.e. the water column undergoes complete mixing only in harsh winters. During the period 1992–1998, the hydrodynamic condition of Lake Garda remained stable with a consequent phosphorous accumulation in the deep layers of the water column. In contrast, during the first months of 1999, 2000 and 2004, Lake Garda waters underwent complete circulation, resulting in an increased accessibility of phosphorous in the euphotic zone, which induced blooms of cynobacteria in 1999 and 2000, and diatoms in 2004 (Provincia Autonoma di Trento, 2005). At the end of July 2004, a black spot appeared in the western portion of Lake Garda. A few days later, the phenomenon spread throughout the lake. A first identification by optical microscopy performed by APPA laboratories of Forte S. Nicolò (Riva del Garda) revealed that the organism responsible for the phenomenon was a ciliated protozoan (Provincia Autonoma di Trento, 2005). Ciliates represent a small percentage of the total zooplanktonic community of Lake Garda (Provincia Autonoma di Trento, 2005). With the exception of the phenomenon of the black spot of summer 2004, bloom episodes of ciliates have never occurred in this lake.

In this paper, we report the characterization of the ciliated protozoan responsible for the black spot formation. Using morphological and molecular analysis, we characterized this protozoan as *Stentor amethystinus*. Continuous monitoring of northeastern Lake Garda revealed that by the beginning of September, the black spot had completely disappeared, and *S. amethystinus* was detected at low concentration only in the southern basin of the lake.

S. amethystinus is a ciliate referred to as a heterotrich as it possesses different ciliary structures on different parts of the body. *S. amethystinus* and the other *Stentor* species are among the largest aquatic protozoa, with dimensions of approximately 250–500 μm. *Stentor* species usually inhabit freshwater environments. When free-swimming, they acquire an oval or pear shape; when attached to substrates, they assume a trumpet shape. Typically, they feed on bacteria, algae or other protozoa. *S. amethystinus* and *Stentor polymorphus* can form photosynthetic relationships with algae, causing them to have a green color. The presence of symbiotic algae allows them to grow even in the absence of food, so they can be defined as mixotrophic. The green pigmentation of *S. amethystinus* is masked by colored cortical granules, which confer amethyst-blue coloration. In *Stentor coeruleus*, these granules contain a substance named stentorin, used for photoreception (Lankester, 1973). A recent study showed that stentorin possesses toxic properties and is used by *S. coeruleus* not only as photosensor but also as a chemical defense against predators (Miyake et al., 2001).

S. amethystinus is common in lakes and ponds in central Europe (Foissner, 1980; Foissner et al., 1982, 1992, 1999) where it is responsible for bloom episodes. Generally, it inhabits oligotrophic lakes, with temperature and pH values lower than those of Lake Garda (Foissner et al., 1999; Macek et al., 2001). The closest lake to Lake Garda which is inhabited by *S. amethystinus* is Pressegger See, in Carinzia (Austria), where in 2002 a *S. amethystinus* bloom gave rise to the formation of large black spots (Foissner and Wölfl, 1994). The sudden appearance and proliferation of *S. amethystinus* in Lake Garda during the summer of 2004 may be attributed to uncommon environmental circumstances occurring during the first 6 months of that year.

2. Materials and methods

2.1. Water sampling, ciliate density estimation and species determination by morphological analysis

Water samples (1 l) were collected with a sampling bottle of 5 l at 0, 2, 5, 10 and 20 m of depth at the following five sites: Ponale torrent outfall (site no. 1); S. Nicolò harbor of Riva del Garda (no. 2); between Riva del Garda and Torbole (no. 3); Sailing Club of Torbole (no. 4); central lake, close to the provincial district border (no. 5) (Fig. 1). Samplings were performed weekly from August 9 to September 7.

Ciliate density was determined using a stereomicroscope Water samples were concentrated by filtration soon after collection on 45 μm membranfilters. The filtered water volumes varied from 50 to 400 ml, to be able to count at least 100 organisms. Ciliates retained by the filter were also estimated using a stereomicroscope.

Species determination by morphological analyses was carried out both *in vivo,* after slowing cilia motility by addition of methyl cellulose, or *in vitro* on fixed cells by Feulgen reaction (Lee et al., 1985), which reveals the nuclear apparatus in detail.

2.2. DNA purification, Polymerase Chain Reaction (PCR) DNA amplification, and sequencing

For DNA extraction, a water sample of 100 ml collected from Lake Garda at site no. 2 during the period of greatest proliferation of the black spot, *was used*. The water sample was concentrated by filtration on 60 mm membranfilter to final volume of 10 ml. The estimated density of *S. amethystinus* was 7000 ml^{-1}. DNA was purified as described in Pucciarelli and Miceli (2002). Before extraction, the organisms in the water sample were starved for 1 week. Subsequently, the sample was centrifuged at 16,000*g* and the resulting pellet was suspended overnight at 50 °C in lysing solution (0.5 M EDTA, 1% SDS, 10 mM Tris–HCl, pH 9.5), containing proteinase K (0.2 mg/ml, final concentration). DNA was purified with a phenol/chloroform extraction, and a final treatment of RNase A (50 μg/ml) was administered for 2 h.

For species identification of *Stentor* and its symbiotic green algae, PCR strategies were performed. In the former case with the following oligonucleotides: 5′-TATAAACTTGTATACGGTGAG-3′, as the forward primer, and 5′-TGCAGGTTCACCTACAGAT-3′, as the reverse primer, designed on a *Stentor* small subunit rDNA (SSrDNA) consensus obtained by the alignment of all *Stentor* SSrDNA sequences available in GenBank® (acc. nos. *S. coeruleus* AF357145; *S. polymorphus*, AF357144; *Stentor roeselii*, AF357913). In the latter case, PCR was performed with 5′-AGGCCTACCATGGTGGTA-3′, as the forward primer, and 5′-ATGCTTCCATTGGCTAGTCG-3′, as the reverse primer, designed on the alignment of some of the *Chlorella* SSrDNA sequences available in GenBank® (acc. nos. *C. sorokiniana*, EF030580; *C. vulgaris*, AM231734; *C. minutissima*, AB006046; *C. lobophora*, X63504; *C. emersonii*, AJ242761; *C. pyrenoidosa,* AB240151). To better discriminate between *Chlorella* and *Micractinium,* two PCR strategies were performed with the oligonucleotide 5′-CTGGCCTATCCTGTGGTCTGTA-3′, as a forward primer, designed on the SSrDNA sequence of the symbiotic algae obtained from the PCR strategy previously described, in combination with two different reverse primers. The first was the oligonucleotide