Production of transparent exopolymer particles (TEP) in the nitrogen fixing cyanobacterium *Anabaena flos-aquae* **OL-K10**

Waldemar Surosz[1,*]
Katarzyna A. Palińska[2]
Agnieszka Rutkowska[1]

[1] Institute of Oceanography,
University of Gdańsk,
al. Marszałka Piłsudskiego 46, PL–81–378 Gdynia, Poland;

e-mail: ocews@univ.gda.pl

*corresponding author

[2] Carl von Ossietzky University,
ICBM, Geomicrobiology,
PO Box 2503, D–26111 Oldenburg, Germany

Abstract

The aim of the study was to measure the transparent exopolymer particles (TEP) concentration in cultures of *Anabaena flos-aquae* OL-K10 and to determine the relationship between the quantity of particles produced and the light intensity, the age of the culture and the presence of nitrogen in the culture medium. This is the first time TEP production has been investigated in the *Nostocales*, an order of nitrogen-fixing phytoplankton species. The results showed that TEP production depends on the presence of nitrogen in the culture medium. The longer the culture is grown, the higher the correlation between its TEP content and its chlorophyll *a* concentration.

1. Introduction

The reason for undertaking the research described in this paper were observations of *Anabaena flos-aquae* OL-K10 cultures in other experiments

during which suspicious changes in slime production took place. Under certain culture conditions, *Anabaena flos-aquae* exuded large amounts of a sticky substance causing the culturing medium to jellify. After an extensive study of the literature concerning similar issues, we decided to test our culture for the presence of transparent exopolymer particles (TEP).

Organic matter, the product of photosynthesis, is the basis for the functioning of ecosystems. The components of 'marine snow' (macroscopic aggregates) are phytoplankton cells, matter exuded by zooplankton and detritus material that form a substrate of carbon compounds transformed by microorganisms (Hong et al. 1997). Marine snow formation in the sea is closely connected with the occurrence of transparent exopolymer particles (TEP) in the environment.

Alldredge et al. (1993) were the first to describe TEP. Consisting of polysaccharide compounds commonly occurring in ocean waters and algae cultures, the gel-like TEP are formed from dissolved carbohydrate polymers exuded by phytoplankton (Hong et al. 1997, Passow 2000) and bacteria (Passow & Alldredge 1994). The TEP concentration in the ocean has been found to increase during bloom periods (Long et al. 1996, Passow et al. 1998). TEP are used as a substrate and microhabitat by bacteria, alter the interactions between bacteria and their environment, play a major role in transforming organic matter, influence the transport of carbon from the surface layers of the oceans, and are a food source for zooplankton. The first studies of TEP were conducted using microscopic techniques. Quantitative measurements became possible only after the development of a spectrophotometric method in combination with Alcian blue (Passow & Alldredge 1995).

The aim of the present study was to measure the TEP content in *Anabaena flos-aquae* cultures and to determine the relationship between the quantity of particles produced and the light intensity, the age of the culture and the presence of nitrogen in the culture. Furthermore, the correlation between TEP concentration and the chlorophyll *a* content was evaluated in all the experimental treatments.

2. Material and methods

2.1. Research material

The research was conducted on the OL-K10 strain of *Anabaena flos-aquae* from the culture collection of the University of Oldenburg (Germany). The cyanobacterium was isolated from plankton collected in the Gulf of Gdańsk (Baltic Sea).

Anabaena flos-aquae belongs to the order *Nostocales*. A peculiar property of this order is that their representatives can fix nitrogen from the air, a process that takes place in heterocysts.

2.2. Culture conditions

The cyanobacterium strain was cultivated in BG-11 and BG-11° media prepared with redistilled water (Rippka et al. 1979). The BG-11 medium contained nitrate in a concentration of 1.5 g dm^{-3} (17.65 mM), whereas the BG-11° medium was nitrogen-free. *Anabaena flos-aquae* was acclimatized to the experimental conditions prior to the experiments and was in a logarithmic growth phase. The initial chlorophyll *a* concentration in all the experimental treatments was approximately 0.4 mg dm^{-3}. The cultures were grown in 2000 cm^3 Erlenmeyer flasks containing approximately 500 cm^3 of medium (64 days; 20°C; 16 h light – 16 h dark photoperiod; light source – Philips lamps). All the processes were conducted under sterile conditions in a laminar sterile airflow chamber. Four experimental treatments consisting of culture media with or without nitrogen at two different light intensities (40 and 120 μE m^{-2} s^{-1}) were prepared. Chlorophyll *a* and TEP concentrations in the cultures were measured once a week.

2.3. TEP measurement

1 cm^3 of the culture was passed through a polycarbonate filter (0.4 μm pore ø) under constant low-vacuum conditions (150 mm Hg). Then, 0.5 cm^3 of 0.02% Alcian blue solution (prefiltered through a 0.2 μm pore ø filter) in 0.06% acetic acid at pH 2.5 was added. Staining lasted for 2 seconds; afterwards the filter was rinsed in distilled water to remove excess dye. Finally, the filter was placed in a 250 cm^3 beaker; 6 cm^3 of 80% H_2SO_4 was added and left for 2 hours for extraction with gentle stirring. Absorption at a wavelength of 787 nm in 1 cm cuvettes with distilled water as reference was performed on a Shimadzu 1201 spectrophotometer.

The standard curve was prepared with 30 mg xanthan in 200 cm^3 distilled water. 0.5, 1.5 and 3 cm^3 of this solution were passed through polycarbonate filters (0.4 μm pore ø) and then subjected to the same procedural stages as in the cyanobacteria culture staining process (Passow et al. 1995).

2.4. Chlorophyll *a* content

The chlorophyll *a* content in the cultures was determined by the spectrophotometric method of Strickland & Parsons (1972). Samples of known volume were passed through Whatman GF/C filters and stored at −20°C in the dark until extraction. Next, the filters were placed in a glass

test tube and ground with a glass rod. The dye was extracted in 10 cm^3 of 90% acetone. After two hours of extraction (4°C, in the dark), the samples were centrifuged at 5000 rpm for 10 minutes. Extinction was measured spectrophotometrically at 630, 645, 665 and 750 nm in 1 cm quartz cuvettes.

The chlorophyll a content in the samples was calculated according to the formula of Jeffrey & Humphrey (1975):

$$\text{chl}\,a(\text{mg m}^{-3}) = [11.85 \times (E_{665} - E_{750})\ -$$
$$-\ 1.54 \times (E_{645} - E_{750}) - 0.08 \times (E_{630} - E_{750})]\,v\,L^{-1}\,V^{-1},$$

where
E – extinction at the given wavelength,
v – volume of acetone used for extraction [cm^3],
L – optical path length [cm],
V – volume of sample passed through the Whatman GF/C filter [cm^3].

2.5. Scanning electron microscope preparation

A 1.5 cm^3 aliquot of the culture was centrifuged and the pellet placed for 2 hours in 4% glutaraldehyde solution in 0.1 mol dm^{-3} sodium phosphate buffer (pH 7.5). The samples were then rinsed 3–5 times in the phosphate buffer (each time for 15 minutes), dehydrated through a series of ethanol-water solutions starting with 15% ethanol, then proceeding through seven steps to pure ethanol, remaining at each step for 20 minutes. The dehydration series ended with two rinses in pure ethanol (Surosz & Palińska 2004). Afterwards the samples were critical-point dried in a Balzers Union (CPD 010) apparatus prior to gold sputtering (SCD 030, Balzers Union). The objects were examined under a Zeiss DSM 940 or a Hitachi S-450 scanning electron microscope operated at 10 or 20 kV and a working distance of 7 to 9 mm. Photographs were taken on Agfapan APx25 or Ilford FP4 film.

3. Results

The tests consisted of four two-month experiments conducted under two different light intensities (40 and 120 μE m^{-2} s^{-1}) with (17.65 mM of nitrate) or without nitrogen in the culture medium. Fig. 1 shows the results of these tests: the growth of the *Anabaena flos-aquae* cultures is expressed by the chlorophyll a concentration.

Initially, the chlorophyll a concentration was approximately 0.4 mg dm^{-3} in all the experimental setups. It then increased gradually in all treatments during the first four weeks of the experiment. In treatments IB, IIA and IIB, the respective maximum chlorophyll a concentrations of 2.94 mg dm^{-3},
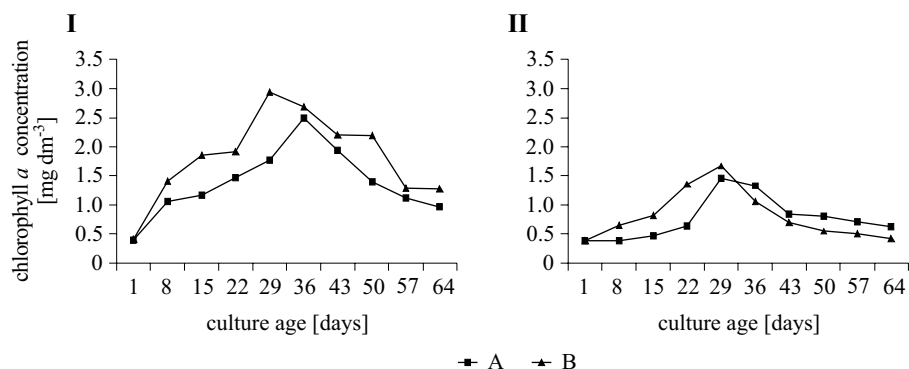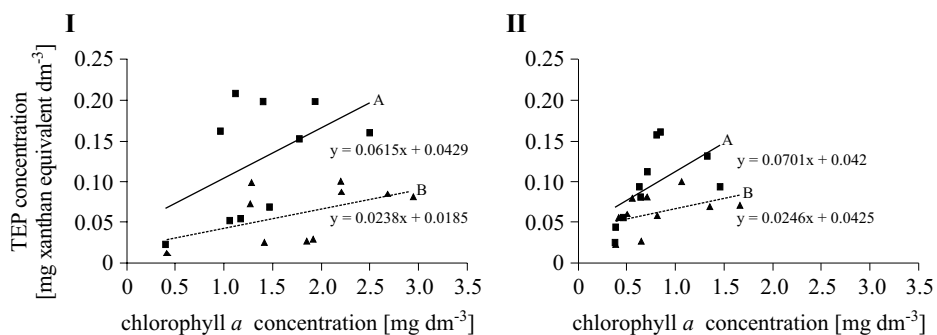
Fig. 1. Chlorophyll *a* concentration in *Anabaena flos-aquae* cultures growing in different conditions: I – light intensity – 40 μE m^{-2} s^{-1}; II – light intensity – 120 μE m^{-2} s^{-1}; A – nitrogen-free medium (BG 11^0); B – medium (BG 11) with a nitrate concentration of 1.5 g dm^{-3} (17.65 mM)

1.46 mg dm^{-3} and 1.66 mg dm^{-3} were recorded after 29 days of cultivation. In experiment IA, the maximum chlorophyll *a* concentration of 2.50 mg dm^{-3} was measured after 36 days. Chlorophyll *a* then gradually decreased until the end of the experiment. Fig. 2 illustrates the changes in TEP concentrations during the experiment.
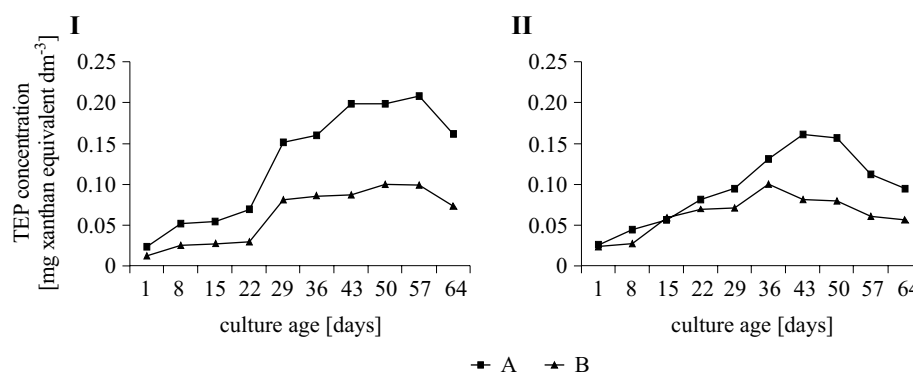

Fig. 2. Transparent exopolymer particles (TEP) concentration in *Anabaena flos-aquae* cultures growing in different conditions: I – light intensity – 40 μE m^{-2} s^{-1}; II – light intensity – 120 μE m^{-2} s^{-1}; A – nitrogen-free medium (BG 11^0); B – medium (BG 11) with a nitrate concentration of 1.5 g dm^{-3} (17.65 mM)

In treatment IA, the TEP concentration rose gradually but constantly to reach a maximum of 0.21 mg dm^{-3} on the 57th day of the experiment. In the final week of the experiment, the TEP concentration decreased. In treatment IB, similar changes in TEP concentration were noted: it reached

Fig. 3. Regression of chlorophyll *a* versus transparent exopolymer particles (TEP) in *Anabaena flos-aquae* cultures growing in different conditions: I – light intensity – $40\ \mu E\ m^{-2}\ s^{-1}$; II – light intensity – $120\ \mu E\ m^{-2}\ s^{-1}$; A – nitrogen-free medium (BG 11^0); B – medium (BG 11) with a nitrate concentration of 1.5 g dm^{-3} (17.65 mM)
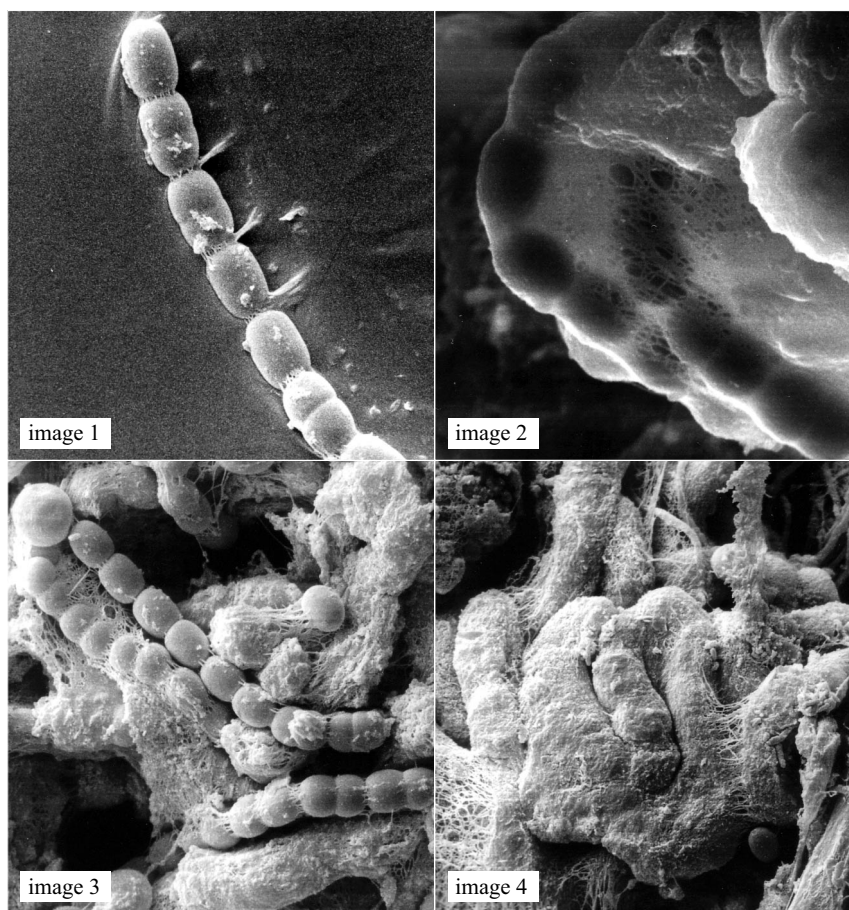
Photo 1. Scanning electron micrographs of *Anabaena flos-aquae* before (image 1) and after the experiment (images 2–4)

a maximum on the 50th day (0.1 mg xanthan equivalent dm^{-3}), after which it decreased. In treatment IIA, the maximum TEP concentration (0.16 mg xanthan equivalent dm^{-3}) was recorded on the 43rd day of cultivation, and in treatment IIB on the 36th day (0.1 mg xanthan equivalent dm^{-3}). TEP decreased in all treatments once the maximum had been reached.

Fig. 3 illustrates the regression of chlorophyll *a* versus TEP for each treatment. In all the treatments, the parameter reached a maximum at the end of the experiment. Scanning electron micrographs of *Anabaena flos-aquae* show its morphology before (Photo 1: image 1) and after the experiment (Photo 1: images 2–4).

4. Discussion

TEP concentrations in the ocean increase during bloom periods (Long & Azam 1996). In the pelagic environment, coagulation of suspended particles leads to the formation of macroscopic aggregates (marine snow), which provide food substrates for bacteria and are a source of dissolved organic carbon (DOC) and organic matter. A correlation was discovered between the disappearance of TEP from surface water layers and the increase in the amount of suspended carbon. It was also found that TEP and other particles were able to form larger 'floccules' at a TEP concentration of 500 particles dm^{-3} (Passow & Alldredge 1994). Tests on the production and distribution of TEP in the water column are crucial for a better understanding of the marine carbon cycle (Hong et al. 1997).

In the experiments conducted in this study, the TEP concentration was measured in *Anabaena flos–aquae* cultures grown on nitrate-containing and nitrate-free media at two different light intensities (40 and 120 $\mu E\ m^{-2}\ s^{-1}$). TEP concentrations were high in the nitrate-free cultures (BG-11° medium), but much lower in the nitrate-containing cultures (BG-11 medium).

The results of this research are similar to those obtained by Passow et al. (1995) in research conducted in the Santa Barbara Channel (USA). During the algal blooms, they recorded very high TEP concentrations, even in excess of 0.3 mg xanthan equivalent dm^{-3}. TEP concentrations beneath the layer containing high numbers of phytoplankton were > 0.050 μg xanthan equivalent dm^{-3}. In winter, TEP concentrations at all depths were very low; nonetheless, a small, residual amount of the substance was always present in the phytoplankton. According to environmental test data, the TEP concentrations in various environments ranged from 0.010 to 0.500 mg xanthan equivalent dm^{-3}. Yet much lower TEP concentrations were noted in the laboratory tests (Passow et al. 1995). Average TEP concentrations ranged from 1.0 μg xanthan equivalent dm^{-3} in *Chaetoceros gracilis* to 1.706 μg xanthan equivalent dm^{-3} in *Thalassiosira rotula* and 1.920 μg

xanthan equivalent dm^{-3} in *Emiliania huxleyi*. Hong et al. (1997), who conducted their research in the Ross Sea, Antarctica, present much higher values than Passow et al. (1995): their average TEP concentrations were 0.301 mg xanthan equivalent dm^{-3}, with maximum TEP values as high as 2.8 mg xanthan equivalent dm^{-3} in the case of *Phaeocystis antarctica*. The results obtained in the present research are consistent with the TEP concentrations given in the literature.

The results of our experiments with the *Anabaena flos-aquae* strain suggest that the light intensity does not substantially affect the amount of TEP produced. However, both the present study and previous ones have shown that TEP concentrations do depend on the presence of nitrogen in the culture medium (Corzo et al. 2000). The ability to bind nitrogen from the air may be related to a property specific to a number of cyanobacteria species, namely, the presence of heterocysts; *Anabaena flos-aquae* also has these structures. Specialized cells surrounded by thick walls, heterocysts form nitrogenase, an enzyme playing a major part in nitrogen assimilation, which functions in anaerobic environments. Heterocysts also degrade system II, the photosynthetic apparatus, thereby preventing the production of oxygen. Moreover, the oxygen produced during photosynthesis in the growth cells of cyanobacteria is unable to permeate the thick walls of the heterocysts. As a result, the environment within heterocysts is anaerobic, and nitrogenase activity is ensured (Szweykowska & Szweykowski 1994). It is probable that such culture conditions facilitate TEP production.

One of the aims of this study was to determine the correlation between the TEP concentration and the chlorophyll *a* concentration in cultures of the *Anabaena flos-aquae* strain. Therefore, along with determining the TEP concentration, the chlorophyll *a* concentration was measured in the cultures. The variability curve of the chlorophyll *a* concentration shows that this increased much more in the low light intensity cultures (experimental setups IA and IB). The measured chlorophyll *a* concentrations in these cultures were twice as high as those in the cultures cultivated at a high light intensity (IIA and IIB). The presence of nitrogen did not substantially influence culture growth. Counts of the number of TEP produced per chlorophyll unit (Fig. 3) showed that the maximum values of the parameter were achieved at the end of the experiment. This is probably related to the increase in the TEP concentration during the whole 2-month long cultivation. The decrease in the chlorophyll *a* content at the end of cultivation is connected with the death of the culture. At the same time TEP concentration increased. In their study, Hong et al. (1997) showed that the TEP production in algal cultures was a function of the coefficient of growth rate and photosynthetic activity. The TEP concentration measured during the

bloom period was considerably higher than in periods when there were no blooms; it was, moreover, correlated with the chlorophyll *a* concentration. Passow & Alldredge (1994) drew a similar conclusion on the basis of their measurements.

TEP in the environment are much more important than previously thought. As well as playing a crucial role in cell aggregation during algae blooming (Passow et al. 1995), they are essential in the food chain, microbiological processes and in the oceanic carbon cycle (Alldredge et al. 1993, Alldredge 1998). On the basis of the experiments conducted on *Anabaena flos-aquae* OL-K10 cultures in this study, it seems that TEP production does depend on the presence of nitrogen in the culture medium. The correlation between the TEP content and the amount of chlorophyll *a* shows that the longer the culture is grown, the higher the correlation describing the TEP concentration per chlorophyll unit tends to be. No correlation was found between light intensity and the TEP concentration in the investigated cultures. The higher TEP production in the nitrogen-free medium (where it grows more slowly) could be explained as being due to stress. Our laboratory results explained the previous data showing that *Anabaena flos-aquae* appears in the Baltic Sea in smaller amounts than *Nodularia spumigena* and *Aphanizomenon flos-aquae*, which can better acclimatize to the lack of nitrogen (Kahru et al. 1994). Unlike *Anabaena flos-aquae*, *Nodularia spumigena* and *Aphanizomenon flos-aquae* prefer nitrogen-free waters.

Acknowledgements

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