Structural heterogeneity in cyanobacterial mats is associated with geosmin production in rivers

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The structural characteristics and microprofiles of dissolved oxygen and redox potential were examined in benthic cyanobacterial mats, which developed in a nutrient-rich river. The mats were highly dynamic both in their structure and relative composition, and showed large spatial heterogeneity. The mats differed in the contribution of Oscillatoria limosa, which was higher in the free-floating than in the attached mats. Maximum dissolved oxygen concentration at saturating irradiances occurred within the first 2 mm in the attached mat, but was smaller and deeper in the free-floating mat. The free-floating mats showed heterogeneous profiles inside the same mat due to varying thickness in nearby zones of the mats. In these mats, some black micropatches (high proportion of Oscillatoria) showed anoxic conditions under low irradiances, whereas other brown micropatches (lower proportion of Oscillatoria) never became anoxic. After several hours in the dark, a negative redox potential (−400 mV) was detected inside the mat in the black micropatch, but redox potential was always positive in the brownish micropatches. The dissolved oxygen and redox profiles indicate that potential conditions for the production of geosmin could occur in the black micropatches. There, diffusion constraints within the mat could be associated with resource depletion. High peptidase activities in the black micropatch indicated a high demand for inorganic nitrogen. These mats with prevalence of Oscillatoria functioned as “hot spots” of limited diffusion, which probably caused low nutrient availability, defining the appropriate conditions for the production of geosmin.

INTRODUCTION

Massive growth of benthic algae and cyanobacteria in rivers is usually associated with anthropogenic influences, which modify both the flow regime and the nutrient input (Perona et al. 1998; Vilalta et al. 2003a). In nutrient-rich, slow-moving rivers the cyanobacterial masses may accumulate in the littoral and dead-flow areas and, not being tightly adhered may become free-floating and reach the water surface. Mass growth of cyanobacteria is able to produce remarkable impairments of water quality (Biggs & Close 1989). Among these, growth of filamentous cyanobacteria has been related to the occurrence and release of volatile organic compounds such as geosmin and 2-methylisoborneol (Izaguirre et al. 1982; Burlingame et al. 1986; Izaguirre & Taylor 1995; Jüttner 1995), which produce earthy and musty tastes and odours (Naes et al. 1985). Geosmin is a secondary metabolite derived from monoterpenene and sesquiterpene precursors within the isoprenoid biosynthetic pathway, where carotenoids and the phytol chain of chlorophyll-a are synthesized (Naes & Post 1988).

The adherence state of benthic cyanobacterial masses (attached or free-floating) as well as differences in the mat structure and composition have been related to the geosmin concentration within the mats (Sabater et al. 2003). Free-floating cyanobacterial masses had a higher content of geosmin than those attached. The working hypothesis in the present paper is that the different state of attachment of the cyanobacterial mat can be related to specific structural and physiological characteristics, which can either favour or not the production of geosmin. The functional structure of cyanobacterial mats that are potential producers of geosmin is approached by means of oxygen and redox microelectrodes. Microelectrodes have been useful in characterising the structure of biofilms (Santegoeds et al. 1998; Yu & Bishop 1998) and cyanobacterial mats (Epping & Kühl 2000; Kühl & Fenchel 2000) as well as sediments (Berninger & Huettel 1997). A shift in microelectrode profiles within a narrow region indicates a well-stratified mat where different metabolic processes could take place (Bishop et al. 1995). In the present study the physiological measurements derived from O2 and redox microelectrodes were combined with microscope and exoenzymatic measurements. The objective of the present study was to determine whether the microstructures within the mats were consistently different in attached and free-floating parts and how these could affect the physiological conditions associated with geosmin production.

MATERIAL AND METHODS

Sampling and experimental set-up

Cyanobacterial mats were collected in an open site of the Llobregat River at Navàs (Catalonia, NE Spain; Vilalta et al. 2003α) during a geosmin episode period in March and April 2003. Intact mats and accompanying river water were collected from the same site in the river using acrylic cores (inner diameter of 4.6 cm, 15 cm length), which were sealed with rubber stoppers at both ends after collection. Samples of attached and free-floating mats were collected separately. Typ-
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**Fig. 1.** $O_2$ concentration profiles for different measurements (represented by different symbols, $n = 3$), expressed as micromolars and as percentage of air saturation (%). The profiles for the attached (A) and free-floating (B) mats were obtained at light saturated conditions ($>1000$ µmol photons m$^{-2}$ s$^{-1}$) and temperature between 15°C and 17°C. Depth is in millimetres. Note that the horizontal black line indicates the surface of the mat in contact with the water (A) or with the air (B). Short horizontal solid lines indicate the position of the bottom part of the free-floating mat (B) being in contact with the water.

Geosmin content in the mats was analysed spectrophotometrically from frozen samples, after grinding and subsequent methanol extraction. Total carbon and nitrogen content was measured with a Carlo Erba C/N analyzer 1500, using vanadium pentoxide as the oxidation catalyst. About 5–10 mg DW of ground sample (three replicates) was used for each analysis. The potential extracellular enzyme activity of leucine-aminopeptidase (AMA) and alkaline phosphatase (APA) was measured using fluorescent-linked substrates amionomethyl-coumarin (AMC) for peptidase and methylumbelliferon (MUF) for phosphatase activities as described by Sabater et al. (2003). Activities were expressed in micromoles (of MUF or AMC released) per unit of AFDW and per milligram chlorophyll-$a$ per hour.

**Sample analysis**

Samples (three replicates) from the two different mat types were taken with a small polyvinylchloride (PVC) core (3.1 cm$^3$) in the field to measure algal composition and abundance, chlorophyll-$a$ concentration, geosmin content, C–N content and extracellular enzyme activities. Samples for algal composition were filled with 10 ml of river water and fixed with 4% formaldehyde and observed under a light microscope using a Reichert Polyvar at ×500. The total carbon concentration (number of cells per square centimetre of colonized substrata) was measured after dispersion of the sample using a sonication bath (Selecta 40 W power, 40 kHz ultrasound frequency) and determining an aliquot of 0.2 ml in an inverted microscope using the technique of Utermöhl (1958), giving the results in number of cells per square centimetre. Cyanobacteria were determined according to Geitler (1932), and the taxonomic identity of *Oscillatoria limosa* confirmed in the Czech Academy of Sciences. The chlorophyll-$a$ concentration was measured after three successive extractions with 90% acetone and sonication (4 min) to achieve total extraction of chlorophyll. Chlorophyll-$a$ concentration was determined spectrophotometrically (Lambda UV/Vis Spectrophotometer, PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) following Jeffrey & Humphrey (1975) after filtration (Whatman GF/F) of the extract. Chlorophyll-$a$ was expressed by surface area, dry weight (DW) and ash-free dry weight (AFDW).

**Microelectrode measurements**

**OXYGEN MICROPROFILES:** A miniaturized Clark-type oxygen sensor with an internal reference and a guard cathode (Uni-
Table 1. Community composition and total cell concentration of the algal and cyanobacterial community in the attached and free-floating mats. Values expressed as the percentage contribution of the different taxa of groups to the total concentration (with the exception of Vaucheria*, where abundance is expressed as mm filament cm$^{-2}$). Taxon presence is expressed by +.

| Taxon                        | Attached | Free-floating |
|------------------------------|----------|--------------|
| Total concentration [cells per square centimetre] $\times 10^6$ | 1.6      | 9.9          |
| Cyanobacteria                |          |              |
| Geitlerinema sp.             |          |              |
| Oscillatoria limosa (Ag.) Gom. | 17.3    | 76.1         |
| O. aff tenuis (morph.1) (Ag.) Gom. | 11.9 | 5.2          |
| O. aff tenuis (morph.2) (Ag.) Gom. | 1.4 | 8.7          |
| Phormidium sp.               | 0.1      |              |
| Pseudanabaena catenata       | 27.5     |              |
| Algae                        |          |              |
| Cladophora glomerata (Linn.) Kütz. | +    |              |
| Spirogyra sp.                | +        |              |
| Closterium sp.               | +        |              |
| Vaucheria sp.*               | 1143     | 222          |
| Diatoms                      | 41.7     | 9.7          |

sense A/S, Aarhus, Denmark), with a tip diameter of 10 μm for fine scale measurements, was used. The sensor was connected to a high-sensitivity PA2000 picoameter (Unisense A/S) and was mounted on a motor-driven micromanipulator (Oriel, Cambridge, Massachusetts, USA). The data were recorded on the computer data acquisition software ProfX 2.0 (Unisense A/S) after reading data from the microsensor amplifier via the A/D converter ADC-101 (Unisense A/S). ProfX software also controlled the micromanipulator. A prepolari
ing of the oxygen microsensor was done by immersing the tip of the microelectrode in continuously aerated water to consume the dissolved oxygen of the electrolyte by the sensing cathode and the guard cathode. After the sensor signal was stabilized during prepolari
ing, calibration was performed in a calibration chamber. Signals were read from well-aerated water (after 5 min of vigorous bubbling in 100% air saturation) and from oxygen-free water (after bubbling with N$_2$ gas, 0% air saturation). Steady-state O$_2$ microprofiles were measured at intervals of 300 μm vertical depth. Signals were read as partial pressure of oxygen (S) and were converted to the partial pressure at zero reading and Sa is the partial pressure at zero reading and Sa is the partial pressure of oxygen (S) and were converted to the equivalent concentration of oxygen (C):

$$C = \alpha (S - S_a)/(S_a - S_0)$$

where $\alpha$ is the atmospheric level solubility of oxygen, $S_a$ is the partial pressure at zero reading and $S_0$ is the partial pressure at atmospheric reading.

Net photosynthesis (P$_n$) of the mats was calculated as the diffusive flux of O$_2$ across the mat–water interface, using Fick’s first law of (one-dimensional) diffusion:

$$J_o = -D_o (dC/dz)$$

where $D_o$ is the free solution molecular diffusion coefficient of O$_2$, and (dC/dz) is the linear slope of the oxygen concentration profile in the diffusive boundary layer, where transport of solutes is dominated by molecular diffusion (Jørgensen & Revsbech 1983). In the attached mat and in the lower part of the free-floating mats, the considered oxygen diffusion coefficient was used that was that of gaseous oxygen, $D_o = 19.7 \times 10^{-2}$ cm$^2$ s$^{-1}$ at salinity (0%) and temperature (15°C) (Li & Gregory 1974). In the upper part of the free-floating mat, however, the diffusion coefficient used was that of gaseous oxygen, $D_o = 19.7 \times 10^{-2}$ cm$^2$ s$^{-1}$ at 15°C (Marrero & Mason 1972). In the two mat types, the net photosynthesis (P$_n$) at light saturation (defined as the irradiance > 300 μmol photons m$^{-2}$ s$^{-1}$; Boston & Hill 1991), was estimated as the relative O$_2$ export across the mat–water or air interface (600 μm of depth). Net photosynthesis was estimated both per area (P$_n$ are) and per chlorophyll-a (P$_n$*are).

REDOX MICROPROFILES: A miniaturized redox platinum electrode was used in combination with a reference electrode (Unisense A/S), a simple open-ended Ag–AgCl electrode with a gel-stabilized electrolyte, both with a tip diameter of 10 μm for fine scale measurements. They were connected to a high-impedance millivolt-meter (Unisense A/S) to measure poten
tometrically the oxidation–reduction potentials. A calibration was done using two points of calibration immersing the redox and the reference microelectrodes tip in quinhydrone redox buffers (pH 4.0 and 7.0). The calibration was done at the same temperature that the measurements were taken. The redox po
tential was standardized against a standard hydrogen reference electrode.

RESULTS

Composition, abundance and geosmin content of the communities

There were some differences between the different mat types, especially considering their cell concentration per surface area (Table 1). In the attached fraction, Vaucheria sp. and some diatom taxa were abundant and Cladophora glomerata was present. Oscillatoria limosa and O. tenuis accounted for 30.6% of the total cells cm$^{-2}$. Chlorophyll-a ranged from 27.7 to 33.4 g cm$^{-2}$ (Table 2). The free-floating mat had a higher

Table 2. Values of Chlorophyll-a per square centimetre and milligram DW, geosmin content per milligram DW and surface area, C/N (molar ratio) and carbon and nitrogen content per milligram DW from the attached and free-floating mats, as well as for the black and brown fractions of the floating mat. Mean values ± standard deviation. Not measured values are indicated by -.

|          | Chl-a (μg cm$^{-2}$) | Chl-a (μg mg DW$^{-1}$) | Geosmin (μg mg DW$^{-1}$) | Geosmin (μg cm$^{-2}$) | C/N | C (μg mg DW$^{-1}$) | N (μg mg DW$^{-1}$) |
|----------|----------------------|-------------------------|---------------------------|------------------------|-----|---------------------|---------------------|
| Attached | 31.46 ± 3.25         | 0.40 ± 0.08             | 0.1 ± 0.3                 | 6.3 ± 3.6              | 21.5 ± 0.61 | 83.89 ± 0.89 | 3.90 ± 0.12     |
| Free-floating | 32.99 ± 16.04     | 0.63 ± 0.07             | 1.03 ± 0.8                | 36.5 ± 23              | 15.99 ± 2.96 | 95.61 ± 11.99 | 6.24 ± 1.72     |
| Black    | —                    | 1.02 ± 0.14             | 4.03 ± 1.3                | —                      | 10.66 ± 0.08 | 111.20 ± 0.85 | 10.43 ± 0.15    |
| Brown    | —                    | 0.55 ± 0.11             | 1.64 ± 0.9                | —                      | 17.34 ± 0.16 | 97.70 ± 0.10 | 5.63 ± 0.06     |
cell concentration (Table 1) with the highest proportion of Oscillatoria spp. (90.0%), where 76.1% was O. limosa (Table 1). Vaucheria sp. and diatoms constituted a much lower proportion (9.7%). Chlorophyll-a ranged from 7.5 to 53.3 µg cm⁻². Geosmin in the different mats was also analysed. Whereas it was close to zero in the attached mat, the concentration was higher in the free-floating mat (Table 2).

Some micropatches were visually distinct within the free-floating mat. Black and thick micropatches were constituted by O. limosa and O. tenuis. Diatoms, sediment particles and a few Oscillatoria spp. filaments, formed a brownish micropatch. Sample analyses showed that chlorophyll-a concentration per milligram DW was higher in the black than in the brown micropatches (Table 2). Geosmin concentration was significantly higher in the black fraction than in the brown. The C/N ratio was lower in the black part than in the brown, reflecting the higher nitrogen fraction in the former (Table 2).

O₂ fluxes in the attached and free-floating mat

Some differences between the attached and free-floating mats were evident when the profiles of dissolved oxygen obtained at light saturation (> 300 µmol photons m⁻² s⁻¹) were compared (Fig. 1A, B; n = 3 in both cases).

A subsurface maximum in O₂ concentration occurred within the first 2 mm of depth in the attached mat (Fig. 1A). Maximum oxygen concentration (1177–1316 µM) corresponded to oxygen saturation of 365–409%. The maximum O₂ concentrations were lower in the free-floating mat (from 796 µM [247%] to 927 µM [290%]) and never occurred in the first few millimetres, but in the deepest parts of the mat (Fig. 1B). Heterogeneous profiles inside the same mat were due to varying thickness in nearby zones of the mats.

The net photosynthesis at light saturation (Pₐ) was similar in the lower part of the free-floating and in the attached mats (4.87 ± 1.81 and 4.85 ± 1.84 µmol O₂ cm⁻² d⁻¹, respectively). However, the net photosynthesis was much higher when the upper end of the free-floating mat was considered (24.984 ± 7720 µmol O₂ cm⁻² d⁻¹). Fluxes followed a similar pattern when the results were expressed per unit Chl-a. PₐChl of the lower part of the free-floating and the attached community were 0.127 ± 0.047 and 0.155 ± 0.059 µmol O₂ µg Chl-a⁻¹ d⁻¹, respectively, and the upper end of the free-floating mat showed a PₐChl of 653 ± 188.7 µmol O₂ µg Chl-a⁻¹ d⁻¹.

O₂ dynamics in the free-floating mat

The dynamics in the oxygen profiles of the free-floating mat were associated with the sequence of addition or detachment of parts of the mat. Initially the free-floating mat was relatively thin (7 mm) (Fig. 2A–C), but later become thicker (10–14 mm) (Fig. 2D–F) due to the incorporation of a partly collapsed part, which became free-floating. The thin free-floating mat reached a minimum O₂ concentration of 120 µM (35%) in the dark (Fig. 2B). At 800 µmol photon m⁻² s⁻¹, O₂ production increased again and concentration reached a maximum of 860 µM (267%) at 4 mm depth. The thicker free-floating mat, which was produced later, was completely supersaturated after midday (1300 µmol photon m⁻² s⁻¹). Maximum O₂ concentration at that time was of 927 µM (290%) (Fig. 2D). During the early afternoon, at lower irradiance, some parts of the free-floating mat partially sank and the mat had a loose appearance but a higher thickness (14 mm). In these conditions, some black fractions located in the lowest part of the mat became highly compacted and showed anoxic conditions, whereas the upper parts of the mat still had values over saturation (Fig. 2E, F).
Redox potential profiles in the free-floating mat

Redox profiles were measured in a thick (12 mm) free-floating mat (Fig. 3). During the early afternoon the cyanobacterial mat presented a positive redox potential (about +500 mV) (Fig. 3A). At that time the mat had some loose fraction in the lowest part, whereas the upper was more compacted. These conditions remained during the late afternoon and some hours of darkness. However, after 8 h in the dark, a negative redox potential (~400 mV) was detected inside the mat in a black compacted fraction (Fig. 3B, C, dark circles). However, redox potential was always positive in the brownish micropatches (Fig. 3, white circles). The negative redox potential inside the black fraction remained after midday when, coinciding with the highest irradiances, the redox potential became positive again (Fig. 3D, black circles). Negative redox potential in the black micropatch occurred again when irradiance decreased in the afternoon (Fig. 3E, F, black circles).

Metabolic activities inside the free-floating mat

Exoenzymatic activities were separately measured in the black and brownish micropatches. The black patch had higher peptidase and phosphatase activities than the brown patch, both related to surface area of the mat and to the chlorophyll. The phosphatase:peptidase ratio was similarly low in the two micropatches (Table 3).

DISCUSSION

The benthic cyanobacterial mats dominated by Oscillatoriales constitute a dense accumulation of filaments, particles and other organisms loosely attached to the sediment of rivers and lakes (Moss 1977). These mats may float, change their thickness or sink through their own variations in oxygen dynamics, provided they develop in quiet waters (Peterson 1996). Elevated irradiances, which lead to high productivity of dissolved oxygen, coincide with the maximum occurrence of free-floating masses of Oscillatoria (Burlingame et al. 1986). Throughout this process, the cyanobacterial mat experiences large changes in its structure and composition, favouring the isolation of cyanobacteria from accompanying algae and particles in the unattached fraction (Sabater et al. 2003).

Low water turbulence in areas where cyanobacterial mats accumulate may favour limited diffusion within the mat (Glud et al. 1994). The lower oxygen production in the attached

|          | Peptidase (µmol AMC g AFDW⁻¹ h⁻¹) | Phosphatase (µmol MUF g AFDW⁻¹ h⁻¹) | Peptidase (µmol AMC mg Chl-a⁻¹ h⁻¹) | Phosphatase (µmol MUF mg Chl-a⁻¹ h⁻¹) | Phosphatase/Peptidase |
|----------|-----------------------------------|-------------------------------------|------------------------------------|---------------------------------------|-----------------------|
| Black    | 107.37 ± 18.81                    | 13.13 ± 2.26                        | 106.3 ± 6.3                        | 13.2 ± 3                              | 0.122 ± 0.12          |
| Brown    | 44.22 ± 11.24                     | 5.91 ± 0.54                         | 77.9 ± 5.3                         | 10.7 ± 1.7                            | 0.134 ± 0.05          |
mats and in the submerged parts of the free-floating mats of the Llobregat indicates that diffusion constraints may be high. The free-floating mats are highly heterogeneous because the submerged parts show a different net productivity than the upper part of the mat. Furthermore, at the smaller spatial scale of the micropatches that are distinct within the cyanobacterial mats, the existence of small spots with different structural components is shown, mainly related to the concentration of Oscillatoria in the mats. The black micropatch (dominance of Oscillatoria) reached anoxia during the night, which could persist until midday. However, this did not occur in the brown micropatch (lower contribution of Oscillatoria). Whereas in the former the high oxygen concentration produced during the day was consumed by respiration during the night, in the latter oxygen diffusion between the bulk water and the mat was still possible. The difference between the oxygen environment in the two micropatches was most probably related to the much higher cell concentration in the black micropatch.

There are therefore a variety of situations that potentially affect the physiological responses of O. limosa mats. The extreme values in redox potential observed within the free-floating mats of the Llobregat were in accord with the extreme metabolic plasticity in Oscillatoria. When oxygen is present inside the mat during the dark, these cyanobacteria can obtain energy by respiring endogenous glycogen (Stal 2000). However, in anaerobic conditions in the dark, O. limosa is able to cover its energy demands by fermentation (Heyer et al. 1989). Production of energetically efficient fermentation products (Stal 2000) may be associated with the highly negative redox potentials found inside the free-floating mat. Oscillatoria limosa has been described as a potential nonheterocystous nitrogen-fixer (Villbrandt et al. 1990), in response to nitrogen deficiency. The oxygen depletion in the black micropatches of Oscillatoria produced a strongly negative redox potential, which indicates the appropriate conditions for nitrogenase activity (Stal & Krumbein 1987). Nitrogenase requires a very high-energy consumption and low-potential reducing equivalents because oxygen exerts a negative effect on this enzyme (Stal 2000). Higher peptidase activities in the black than in the brown micropatches of the free-floating mats also indicate that there is a more relevant demand for nitrogen in the former. High peptidase levels are evidence of inorganic nitrogen being obtained from organic sources, which is what occurs when inorganic nitrogen availability is low (Sala et al. 2001).

It is possible that the described physiological processes may be related to the preferential conditions for geosmin production by the free-floating mats. Geosmin production by cyanobacteria has been related to changes in their physiological state, which may be induced by altered light regimes (Naes et al. 1985; Paerl & Millie 1996) or resource limitation (Naes & Post 1988). Wu et al. (1991) found a negative correlation between the amount of geosmin produced and the growth rate of Anabaena sp. Saadoun et al. (2001) observed that high nitrate suppressed, whereas ammonium enhanced, geosmin production. Conditions of resource limitation (especially nitrogen imbalance) have been observed during the growth of cyanobacteria mats and geosmin production (Vilalta et al. 2003b). A major limitation of inorganic nitrogen and phosphorus during those periods was indicated by the remarkable peptidase and phosphatase activities, respectively, in the mats (Sabater et al. 2003).

The separate analysis of the black and brown micropatches has shown higher peptidase and phosphatase activities (both in terms of areal surface and chlorophyll) in the former, where the geosmin production was the highest. Additionally, the results derived from the microprofiles show that conditions inside the mat were those of limited diffusion, especially in the fractions where Oscillatoria was prevalent, and that these conditions could be appropriate for geosmin production by these organisms. The cyanobacterial mats in the Llobregat were highly dynamic both in their structure and relative composition, and showed a remarkable heterogeneity within the mat. The parts of the mats with prevalence of Oscillatoria could function as real “hot spots” within the mat. It is possible that low nutrient availability related to diffusion constraints could lead to the preferential production of secondary metabolites, such as geosmin. The cyanobacterial growth may attain a critical phase where cell concentration and agglomeration impede diffusion of gases and resources, this being more relevant in the free-floating mats, which then become the sites for the mass production of the metabolite in the river.

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