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RESEARCH ARTICLE

Antibiotic effects of three strains of chrysophytes (*Ochromonas*, *Poterioochromonas*) on freshwater bacterial isolates

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agar diffusion assay; antibiotic activity; *Flectobacillus*; growth inhibition; *Poterioochromonas*.

Abstract

We investigated the antibiotic effects of extracts of freeze-dried biomass and culture supernatants from the mixotrophic chrysophyte species *Ochromonas danica*, *Poterioochromonas* sp. strain DS, and *Poterioochromonas malhamensis* on bacterial strains isolated from lake water. Methanolic biomass extracts inhibited the growth of all tested strains, albeit to a different extent, whereas aqueous biomass extracts only affected bacteria of the genus *Flectobacillus*. The antibiotic action of supernatants from flagellate cultures could be mostly attributed to lipophilic substances, but the growth of bacteria affiliated with *Flectobacillus* and *Sphingobium* was also affected by hydrophilic compounds. A comparison of biomass extracts from light- and dark-adapted cultures of *Poterioochromonas* sp. strain DS showed that the growth-inhibiting factor was unrelated to chlorophyll derivatives. Supernatants from a dark-adapted, phagotrophically grown flagellate culture had stronger antibiotic effects and affected more bacterial strains than the supernatant from a light-adapted culture. Significant growth reduction of a *Flectobacillus* isolate was already induced by extremely low concentrations of lipophilic extracts from these supernatants. Our results show that metabolites of the studied flagellates – either released actively or during cell lysis – may selectively affect the growth of some aquatic bacteria even in very small doses and thus potentially affect microbial community composition. Moreover, the antibiotic potential of mixotrophic chrysophytes may change with their nutritional mode.

Introduction

The process of allelopathy involves secondary metabolites that are released into the environment by plants, microorganisms, viral lysis, and fungi and that affect competing organisms at natural concentrations (Willis, 1985). Allelopathy can influence many ecological processes in pelagic systems such as competition, community structure, or nutrient flow (Ervin & Wetzel, 2003), and it may also modulate the interactions between aquatic prokaryotes, or between eukaryotes and bacteria.

Mixotrophic flagellates are common members of freshwater planktonic microbial assemblages (Bird & Kalff, 1989; Bennett *et al.*, 1990) that are particularly abundant in oligotrophic or dystrophic water bodies (Šimek *et al.*, 1998; Medina-Sanchez *et al.*, 2004) and that can substantially contribute to total predation on prokaryotes in the spring plankton of temperate lakes (Comte *et al.*, 2006). The ecological role of mixotrophs appears to be related to their dominant mode of nutrition (phagotrophy or phototrophy) (Rothhaupt, 1996; Rothhaupt, 1997). Thus, they may be predators of bacteria, but at the same time compete with them for limiting nutrients (Thingstad *et al.*, 1996).

Mixotrophic *Ochromonadaceae* have long been known for the production of allelopathic secondary metabolites. Toxic effects of *Ochromonas* sp. and *Poterioochromonas* sp. have been described upon ingestion by different zooplankton species (Leeper & Porter, 1995; Boxhorn *et al.*, 1998; Boenigk & Stadler, 2004). The excretion of bioactive secondary metabolites by *Ochromonas* sp. (Halevy *et al.*, 1971; Hansen, 1973) may affect aquatic organisms even when the flagellates themselves are not ingested (Boxhorn *et al.*, 1998). Allelochemical compounds extracted from the biomass of an *Ochromonas* sp. have also been reported to inhibit the growth of microorganisms (Hansen, 1973). However, the so far studied strains (e.g. *Staphylococcus aureus*, *Bacillus megaterium*) are rather unlikely to coexist with the flagellates in their natural habitat. Moreover, in view of the

pronounced physiological differences of the flagellates during photo- and phagotrophic growth, respectively (Rothhaupt, 1997), it is conceivable that metabolic changes might also be reflected in a differential production of bioactive compounds.

Laboratory studies were conducted to evaluate the antibiotic impact of biomass extracts and culture supernatants from three strains of *Ochromonadaceae* on several gram-negative freshwater bacterial isolates. Allelopathic effects of lipophilic and hydrophilic fractions were assessed by agar diffusion assays and in bacterial batch cultures. In addition, one flagellate strain was grown in the presence or absence of heat-killed bacterial prey under different light regimes to investigate the influence of nutritional mode on the production of antibiotic compounds.

Materials and methods

Isolation and phylogenetic identification of bacteria

Bacterial strains were isolated in July 2006 from surface water of Lake Zürich, and in October 2006 from surface water of Egelsee (canton Aargau, Switzerland) (Table 1). Additionally, *Flectobacillus major* DSMZ 103 (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany) was used in the experiments. All bacterial strains were grown on DSMZ 7 medium ($1\,g\,L^{-1}$ yeast extract, $1\,g\,L^{-1}$ glucose, and $1\,g\,L^{-1}$ peptone) in Erlenmeyer flasks at 18 °C in the dark.

DNA from bacterial cultures was extracted (QIAamp DNA Stool Mini Kit; Qiagen GmbH, Hilden, Germany) and 16S rRNA genes were PCR amplified using the primers GM3F and GM4R (Muyzer *et al.*, 1993). The purified PCR products (QIAquick PCR Purification Kit, Qiagen GmbH) were sequenced on an automated capillary sequencer (ABI 3730, Applied Biosystems). Partial sequences were assembled using the freeware GAP4 (STADEN PACKAGE, http://staden.sourceforge.net/) and submitted to the European Molecular Biology Laboratory database (Table 1).

Axenization of flagellate cultures

Cultures of *Poterioochromonas* sp. strain DS [formerly termed *Ochromonas* sp. strain DS (Hahn & Höfle, 1998; Boenigk *et al.*, 2006), and kindly provided by T. Posch] were grown in 'undefined *Ochromonas*-medium' [yeast extract ($1\,g\,L^{-1}$), meat extract ($1\,g\,L^{-1}$), glucose ($1\,g\,L^{-1}$), and peptone ($1\,g\,L^{-1}$); Culture Collection of Algae (SAG) at the University of Göttingen, Germany]. Axenic cultures of the mixotrophic flagellate were produced by adding a mixture of antibiotics (chloramphenicol, streptomycin, and gentamycin; ratio 1 : 1 : 0.5) (Corno & Jürgens, 2006). The flagellate cultures treated in this way were kept in the dark at 18 °C and fed with heat-killed cells (1×10^7 cells mL^{-1}; preincubated at 70 °C for 2 h) of *F. major* DSMZ 103. Flagellate cultures without obvious growth of bacteria (as tested by microscopic inspection and plating) were subsequently transferred into 10 mL of Jüttner–Friz medium (Jüttner & Friz, 1974) in 25 mL Erlenmeyer flasks. The cultures were repeatedly inspected for bacterial contamination during the course of the study.

Two more chrysophyte strains (*Ochromonas danica* SAG 933-7 and *Poterioochromonas malhamensis* SAG 933-1a) already available in axenic culture [Culture Collection of Algae (SAG) at the University of Göttingen, Germany] were used in some of the experiments. All three flagellate cultures were grown on Jüttner–Friz medium at 18 °C at a light/dark cycle of 14 : 10 h without addition of heat-killed bacteria (subsequently referred to as 'light-adapted cultures'). Additionally, *Poterioochromonas* sp. strain DS was grown in the dark and fed with heat-killed *F. major* DSMZ 103 ('dark-adapted culture').

Preparation of extracts from light-adapted flagellate cultures

Flagellate cultures (stationary phase, 4 weeks old) were centrifuged (7400 ***g***, 10 min) under sterile conditions. The pellets were frozen for 1 h at −20 °C, and subsequently freeze dried overnight. Aqueous extracts were produced by resuspending 50 mg of the freeze-dried flagellate biomass in

Table 1. Phylogenetic affiliation and isolation conditions of the bacterial freshwater strains

Strain	Accession number	Most closely described isolate	Sequence identity (%)	Isolation conditions
JFB Z007*	FN293045	*Sphingobium rhizovicinus*	97	Surface water+DSMZ 7 medium (1 : 1/v : v)
JFB Z012*	FN293046	*Azospirillum massiliensis*	99	Surface water+DSMZ 7 medium (1 : 1/v : v)
JFB Z013*	FN293047	*Flectobacillus speluncae*	98	Surface water+DSMZ 7 medium (1 : 1/v : v)
JFB Z021*	FN293048	*Flectobacillus speluncae*	98	Surface water+DSMZ 7 medium (1 : 1/v : v)
JFB Z022*	FN293049	*Acidovorax temperans*	99	Surface water+glucose ($10\,mg\,L^{-1}$)
JFB E104†	FN293042	*Pseudomonas fluorescens*	99	Surface water+50 μL axenic *Poterioochromonas* sp. culture
JFB E106†	FN293043	*Janthinobacterium lividum*	99	Surface water+DSMZ 7 medium (1 : 1/v : v)
JFB E107†	FN293044	*Janthinobacterium lividum*	100	Surface water+DSMZ 7 medium (1 : 1/v : v)

*Isolated from Lake Zürich.

†Isolated from Egelsee.

1 mL of distilled water for 1 h. After mixing, the suspension was centrifuged (10 000 ***g*** for 10 min), the supernatant was collected, and kept at 0 °C until further use (3 h). Methanolic extracts were produced by resuspending 50 mg of freeze-dried cells in 100% methanol (MeOH), and treated as outlined above. Before harvesting dark-adapted cultures of *Poterioochromonas* sp. strain DS the flagellates were starved for 2 days. Distilled water, 100% MeOH, and aqueous and MeOH extracts of freeze-dried biomass of heat-killed *F. major* DSMZ 103 were used as controls.

Screening for antibiotic activity by agar diffusion assays

The assays for antibiotic activity were performed on square agar plates (12 × 12 cm) filled with 45 mL of yeast agar (1 g L^{-1} yeast extract, 1 g L^{-1} glucose, 1 g L^{-1} peptone, and 16.4 g L^{-1} agar). Twenty microlitres subsamples of each extract (equivalent to 2 mg of freeze-dried biomass) were added to sterile filter discs with a diameter of 6 mm (Schleicher & Schuell, Germany). Filter discs with MeOH extracts were stored at room temperature for 3 h before application on agar plates to allow for the evaporation of MeOH. The agar plates were first covered with a thin layer of 200 μL of fresh bacterial cultures (48 h at 20 °C in the dark). Subsequently, the filter discs were placed on the surface of the agar plates, and the plates were incubated at 20 °C for 4 days. The area of the inhibitions zones was calculated from the measured distance in millimetre between the edge of the filter discs and the margin of visible bacterial growth.

To evaluate the presence of antibiotic substances in the supernatants of the three flagellate cultures, 4-week-old cultures of the three light-adapted flagellate strains and of the dark-adapted *Poterioochromonas* sp. strain DS were centrifuged (7400 ***g***, 10 min) under sterile conditions. The supernatants were collected, and volumes of 50 or 100 μL were carefully pipetted on filter discs. Subsequently, the discs were air-dried, and their inhibitory activity on *F. major* DSMZ 103 was tested in agar diffusion assays as described above.

Separation of flagellate pigments, testing for antibiotic activity

Freeze-dried biomass of the light- and dark-adapted flagellate cultures was extracted with 100% MeOH. Separation of pigments was carried out by HPLC on a 10AVp system (Shimadzu, Switzerland) with a photo diode array detector and a C18 Gromsil ODS4-HE reversed-phase column (4.6 × 250 mm; 5 μm particle size; Stagroma, Germany). Solvent A was 80% MeOH and 20% UV-treated distilled water; solvent B was 80% MeOH and 20% acetone. The following gradient was applied: 10 min isocratic 100% solvent A, and then a linear increase of solvent B to 100% in 20 min and 100% solvent B for 10 min. The chlorophyll derivatives were identified from their typical absorption spectra (Jeffrey *et al.*, 1997), and quantified relative to the pigment area at 665 nm (corresponding to the second maximum of the chlorophyll *a* absorption spectrum).

MeOH extracts of a light-adapted *Poterioochromonas* sp. strain DS culture were injected into the HPLC system, and the pigment-containing fractions were collected separately. For the separation of extracts from the dark-adapted cultures (i.e. without pigments), fractions were collected at the same retention time slots as from the light-adapted cultures. After evaporation, the residues were resuspended in 100% MeOH, added to the filter discs and tested for their inhibitory activity against *F. major* DSMZ 103. All fractions were tested in triplicate and at identical relative concentrations (equivalent to 2 mg of freeze-dried biomass).

Growth inhibition assays in liquid culture

Thirty millilitres of centrifuged supernatants from light- and dark-adapted cultures of *Poterioochromonas* sp. strain DS were applied to a C18 cartridge (C18 ec; Mega Bond Elut, Varian, Switzerland). The eluate from the cartridge was collected and evaporated. The residue was then redissolved in fresh DSMZ 7 medium and used for the bioassay ('hydrophilic fraction'). Substances bound to the cartridge were separated into three fractions by washing out with 10% aqueous MeOH, followed by 50% aqueous MeOH and 100% MeOH. Only the 100% MeOH fraction was used for the bioassay. After evaporation of the solvent, the residue was redissolved in fresh DSMZ 7 medium ('lipophilic fraction').

The growth of five bacterial strains in these fractions was compared with their growth in unamended DSMZ 7 medium. Ten microlitres from overnight bacterial cultures (24 h) were added to 100 μL of each fraction (and another 100 μL of DSMZ 7 medium) or to 200 μL of DSMZ 7 medium (controls) in 96-well plates (Costar; Corning Inc., Lowell, MA) in triplicate. The growth of the cultures was measured using an absorption plate reader (Spectra Max; Molecular Devices Corporation, Sunnyvale, CA) at 585 nm at four time points. Six randomly distributed wells were filled with DSMZ 7 medium only. In addition, aqueous-MeOH fractions of the cartridge itself were tested for growth inhibition by accidentally eluted compounds, but no such effects were observed (data not shown). To determine the growth of the bacterial strains in all treatments, first, the mean ODs of the medium-filled cuvettes were subtracted from those of the inoculated ones for each measurement. Growth rates were then estimated from the slopes of the linear regression over those data points for which the cultures were in the exponential growth phase (log OD vs. time).

The lipophilic fraction of supernatants from the dark-adapted flagellate culture was diluted to various extends

with DSMZ 7 medium and tested in five replicates on *Flectobacillus* sp. strain Z013 and *F. major* DSMZ 103. The total biomass of bacteria generated over 48 h was calculated as the area underneath the growth curves and compared with the control treatment. Kruskal–Wallis nonparametric ANOVA was applied to test for overall differences between the total integrated biomass formed over 48 h in the various dilution treatments and the controls. Subsequently, pairwise differences between individual treatments and the controls were tested for significance by two *post hoc* approaches that do not assume equal variance of data sets (Tamhane's T2, Dunnett's C), and were only deemed significant if both tests were in agreement. All analyses were carried out using the SPSS software package (SPSS Inc.).

Results

Antibiotic activity of biomass extracts from light-adapted flagellate cultures

Aqueous extracts of freeze-dried biomass of all three flagellates inhibited the growth of the three bacterial strains related to *Flectobacillus* (strains Z013, Z021, and *F. major* DSMZ 103) (Table 1; Figs 1 and 2). In one assay, a small, but visible, inhibition zone was also observed for *Acidovorax* sp. Z022 (Fig. 2a). The strongest inhibition was caused by aqueous extracts of *O. danica*, particularly on strain Z021. In general, the antibiotic effect of the MeOH extracts was stronger than of the aqueous extracts, and almost all tested bacterial strains, showed growth inhibition to some extent (Fig. 2b). Again, the three *Flectobacillus* spp. were the most sensitive strains, and extracts of *O. danica* were most effective. *Sphingobium* sp. Z007, both *Janthinobacterium* sp. E106 and E107 and *Azospirillum* sp. Z012, showed less pronounced inhibition zones. *Pseudomonas* sp. E104 and *Acidovorax* sp. Z022 were hardly affected by extracts of freeze-dried cells, with only small inhibition zones $< 100\,mm^2$. No visible inhibition zones were caused by controls (data not shown).

Inhibitory activity of photosynthetic pigments (chlorophyll derivatives)

Quantitative HPLC analysis revealed that the distribution of photosynthetic pigments in MeOH biomass extracts was similar in all three light-adapted flagellate strains. The number of chlorophyll *a* and chlorophyll *a* derivatives was the highest in extracts from *O. danica* (Table 2). Both *Poterioochromonas* strains featured comparable concentrations of pigments. No difference in the distribution and quantity of the pigments was observed between extracts of living cells and of freeze-dried biomass (data not shown). Figure 3a depicts chromatograms ($\lambda = 665\,nm$) of MeOH extracts of the freeze-dried biomass of cultures of *Poterioochromonas* sp. strain DS under light and dark conditions. While the light-adapted strain possessed several pigments with $A_{665\,nm}$, the dark-adapted strain hardly contained any chlorophyll derivatives. Overall, nine different pigment-containing HPLC fractions were tested for their inhibitory activity on the sensitive strain *F. major* DSMZ 103 by agar diffusion assays (Fig. 3b). Altogether, four pigment-containing fractions showed growth inhibition, and the largest inhibition area ($225\,mm^2$) was induced by fraction 2 (containing two different chlorophyll *a* derivatives). However, all of these fractions (plus an additional one) also inhibited the growth of *F. major* DSMZ 103 (Fig. 3b) when collected from MeOH extracts of the dark-adapted, non-pigment-containing culture (Fig. 3a) of *Poterioochromonas* sp. strain DS. Moreover, the growth inhibition caused by the fractions from the dark-adapted culture was as pronounced as (or even stronger than) the inhibition by the pigment-containing fractions from the light-adapted cultures.

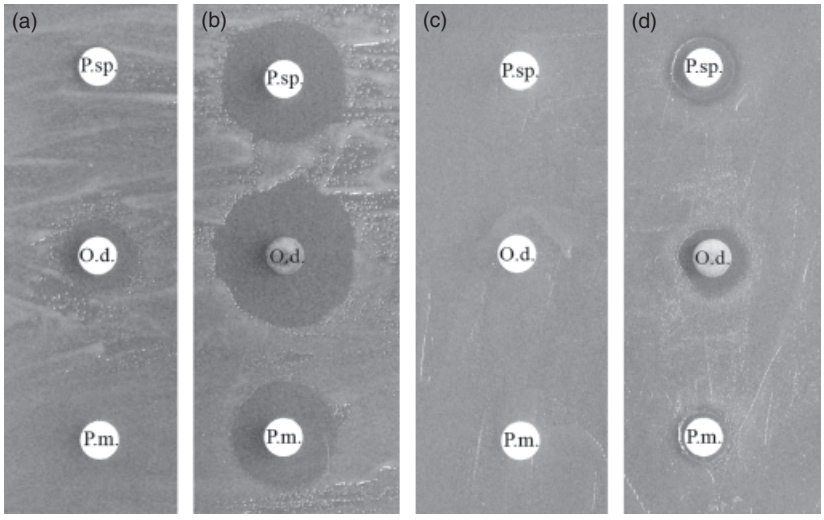

Fig. 1. Example of the antibiotic activity of aqueous (a, c) and MeOH (b, d) extracts of freeze-dried cells of *Poterioochromonas* sp. strain DS (P.sp.), *Ochromonas danica* (O.d.), and *Poterioochromonas malhamensis* (P.m.) on *Flectobacillus* sp. strain Z013 (a, b) and *Janthinobacterium* sp. strain E107 (c, d). The diameter of the sterile filter discs was 6.0 mm.

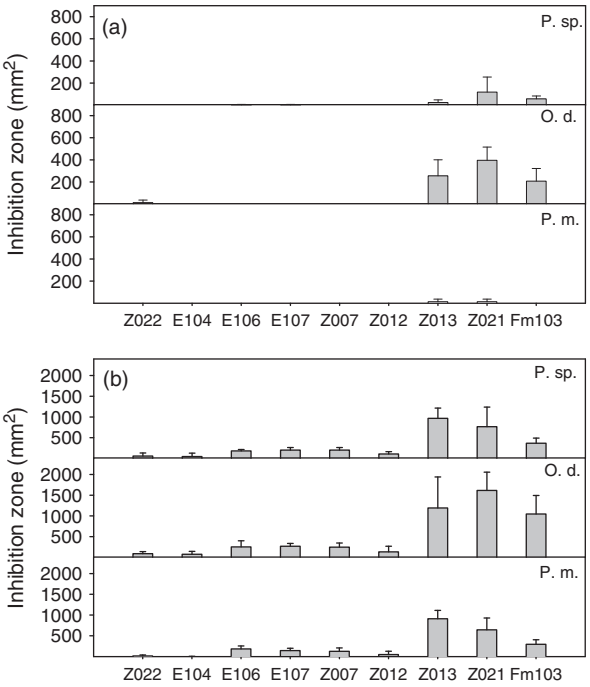

Fig. 2. Inhibition areas on agar plates (mm^2) induced by aqueous extracts (a) and MeOH extracts (b) of freeze-dried cells of *Poterioochromonas* sp. (P.sp.), *Ochromonas danica* (O.d.), and *Poterioochromonas malhamensis* (P.m.) using different freshwater bacterial isolates. The error bars indicate the SD of triplicate experiments.

Table 2. Relative concentrations of chlorophyll *a* and its derivatives in biomass extracts of the three flagellates normalized to the amount of chlorophyll *a* in *Ochromonas danica* SAG 933-7 (100%)

		Light adapted		Dark adapted	
Pigment	Rt (min)	P.sp.	O.d.	P.m.	P.sp
Chlorophyllide a	12.2	9.1	13.1	5.3	0.0
Pheophorbide a	15.5	5.5	8.8	2.1	0.0
Unknown chlorophyll *a* derivative	16.8	16.0	82.1	5.5	0.2
Unknown chlorophyll *a* derivative	19.6	1.4	3.8	4.5	0.3
Chlorophyll *a*	21.1	58.0	100	44.2	0.2
Pheophytin a	31.1	3.3	2.9	1.9	0.1

Rt, retention time; P.sp., *Poterioochromonas* sp. strain DS; O.d., *O. danica*; P.m., *Poterioochromonas malhamensis* SAG 933-1a.

Antibiotic activity of supernatants of light- and dark-adapted flagellate cultures

A potential inhibitory activity of the culture supernatants from the three flagellate strains was first studied in agar diffusion assays using the sensitive strain *F. major* DSMZ 103. In the light-adapted cultures, supernatants of *O. danica* induced the largest inhibition areas, followed by *Poterioochromonas* sp. strain DS (Fig. 4). Hardly any inhibitory effects were found for supernatants of *P. malhamensis*. Interestingly, the growth inhibition caused by 100 μL of supernatants of a dark-adapted culture of *Poterioochromonas* sp. strain DS was comparable with the antibiotic action of *O. danica* (Fig. 4).

The growth of five freshwater bacterial strains in liquid culture was then assessed using DSMZ 7 medium amended with the hydrophilic or lipophilic components of the supernatants of light- and dark-adapted cultures of *Poterioochromonas* sp. strain DS (Fig. 5). The growth of all tested bacteria, except for *Pseudomonas* sp. E104, was completely inhibited by the presence of lipophilic compounds from the supernatant of dark-adapted *Poterioochromonas* sp. strain DS culture. Lipophilic compounds from the supernatant of light-adapted cultures most strongly affected *Sphingobium* sp. Z007, to a lesser extent also *F. major* DSMZ 103, and caused some growth inhibition in *Janthinobacterium* sp. E106. The hydrophilic compounds of supernatants from both light- and dark-adapted cultures negatively affected the growth of *F. major* DSMZ 103 and *Sphingobium* sp. Z007.

In a second set of experiments, the growth of two sensitive bacterial strains was determined in liquid culture with different dilutions of the lipophilic fraction of the supernatant of the dark-adapted *Poterioochromonas* sp strain DS. Dilution of the lipophilic fraction by 1 : 20 or less significantly reduced biomass development of both *Flectobacillus* strains as compared with the controls either by inducing growth arrest or reducing growth rates (Fig. 6). At higher dilutions of the extracts, significantly decreased biomass formation was only observed in *Flectobacillus* sp. strain Z013.

Discussion

Species-selective antibiotic metabolites of ochromonads

Chemical interactions between bacteria and unicellular algae in freshwater environments have frequently been studied with a focus on the growth-promoting or -inhibiting impact of bacteria on algae (de Bashan *et al.*, 2002; Kang *et al.*, 2008) or on the beneficial effect of alga on coexisting 'satellite' bacteria (Bell *et al.*, 1974; Cole, 1982). By contrast, relatively few studies deal with antibiotic substances of freshwater phototrophs that are produced and possibly released into the surrounding medium (Jones, 1988). Such growth-inhibiting factors so far have been mainly reported from cyanobacteria (Pushparaj *et al.*, 1998; Jaki *et al.*, 2000).

The antibiotic effects of biomass or supernatants from ochromonads have mainly been tested on pathogenic strains (Jørgensen, 1962; Halevy *et al.*, 1971; Hansen, 1973; Chen *et al.*, 1994). Our results add an ecological dimension to

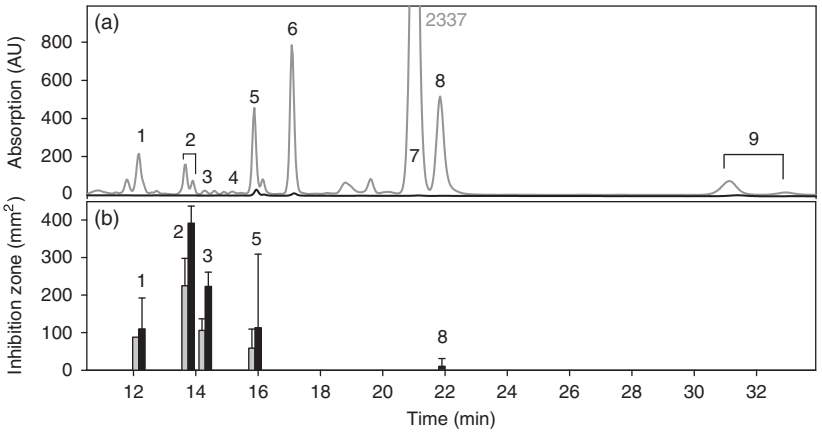

Fig. 3. (a) Chromatograms (665 nm) of MeOH extracts of freeze-dried cells of light-adapted (grey line) and dark-adapted *Poterioochromonas* sp. strain DS (black line). Numbers indicate the retention time slots of the individual fractions that were tested for antibacterial activity in agar diffusion assays. Fractions from the dark-adapted cultures were collected at the same retention times as from light-adapted cultures. (b) Inhibition areas (mm^2) of *Flectobacillus major* DSMZ 103 induced by HPLC fractions of biomass extracts from light (grey bars) and dark (black bars)-adapted *Poterioochromonas* sp. cultures. The error bars indicate the SD of triplicate experiments.

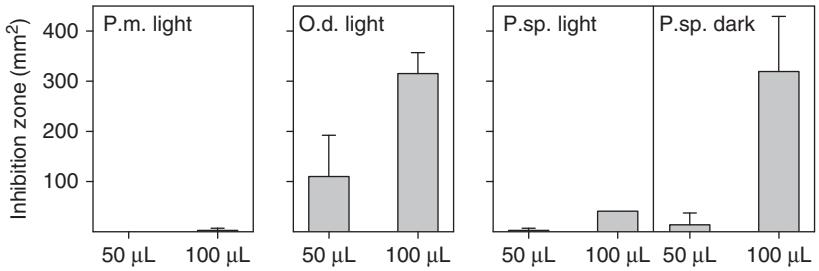

Fig. 4. Inhibition areas (mm^2) of *Flectobacillus major* DSMZ 103 induced by supernatants of different flagellate cultures: light-adapted *Poterioochromonas malhamensis* (P.m.), *Ochromonas danica* (O.d.), *Poterioochromonas* sp. strain DS (P.sp. light), and dark-adapted *Poterioochromonas* sp. strain DS (P.sp. dark). The error bars indicate the SD of triplicate experiments.

these findings by showing growth inhibition of bacteria from different phylogenetic groups that were isolated from lake water (Table 1, Figs 2 and 5). The antibiotic action of the hydrophilic fractions of freeze-dried biomass of the flagellate strains (Figs 1–3) implies storage of the bioactive compound in the flagellate cell. Numerous processes in the plankton might lead to a destruction of flagellate cells, for example viral lysis or bacterial attack (Imai *et al.*, 1993; Massana *et al.*, 2007), resulting in the release of metabolites that negatively affect bacterial growth. While such an allelopathic effect might be considered 'useless' for the individual flagellate cell, it could nevertheless be an effective strategy against bacteria at the population level (Pohnert *et al.*, 2007). Moreover, moderately lipophilic solvents such as MeOH extract low-molecular-weight compounds that can be transferred through intact cell membranes by direct cell–cell contact (Gross, 1999). Some bacteria are known to colonize and parasite senescent phytoplankton cells (Grossart *et al.*, 2005). They can actively track motile phytoflagellate cells (Barbara & Mitchell, 2003), attach to them, and reduce primary production by uptake of growth-limiting nutrients (Brussaard & Riegman, 1998). It is conceivable that flagellates may produce and store allelopathic compounds to defend themselves against direct bacterial contact. Such a production of membrane-permeable antibiotic agents has been shown previously in marine algae (Steemann Nielsen, 1955; Sastry & Rao, 1994).

It has been suggested that chlorophyllides might play an important role as antibiotic substances in natural waters (Jørgensen, 1962). In order to test this hypothesis, we compared the antibiotic effects of fractionated biomass extracts of light- and dark-adapted flagellate cultures (Fig. 4). An equivalent (or even more pronounced) inhibitory effect was observed by HPLC fractions from the dark-adapted culture that had the same retention times as the pigment-containing fractions of the light-adapted culture, but did not contain chlorophyllides (Table 2). Therefore, other substances that eluted at the same time as the pigments must have been responsible for the antibiotic effect. These substances might be produced in higher concentrations in the dark, as for example, indicated by the enhanced action of HPLC fractions 2 and 3 (Fig. 3).

A structural elucidation of the bioactive compounds would go beyond the scope of this study. Recently, the total synthesis of a chlorosulpholipid cytotoxin was published and the substance was discussed as one of the agents that contribute to seafood poisonings in humans (Nilewski *et al.*, 2009). Chlorosulpholipids are particularly intriguing because of their structural and stereochemical complexicity, but the mechanism of biological activity remains unknown. The chlorosulpholipid malhamensilipin A isolated from *P. malhamensis* UTEX L1297 has been suggested to be responsible for antimicrobial activity in pathogenic bacterial strains (Chen *et al.*, 1994).

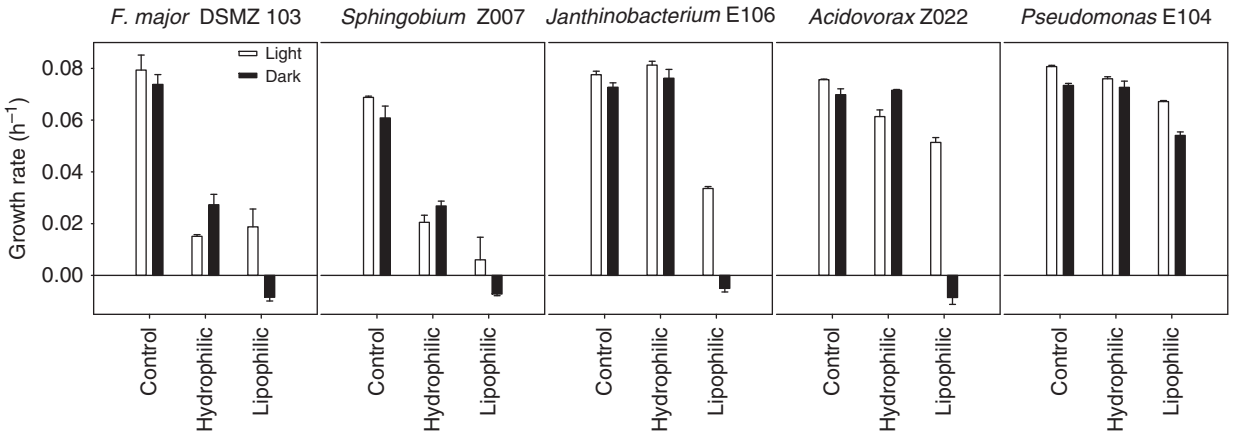

Fig. 5. Growth rates of five freshwater bacterial isolates in liquid medium (DMSZ 7) amended with hydrophilic and lipophilic fractions of culture supernatants from *Poterioochromonas* sp. strain DS. White bars, supernatants from a light-adapted culture; black bars, supernatants from a dark-adapted flagellate culture. Control, growth in unamended medium. The error bars indicate the SD of triplicates. See text for details of extract preparation.

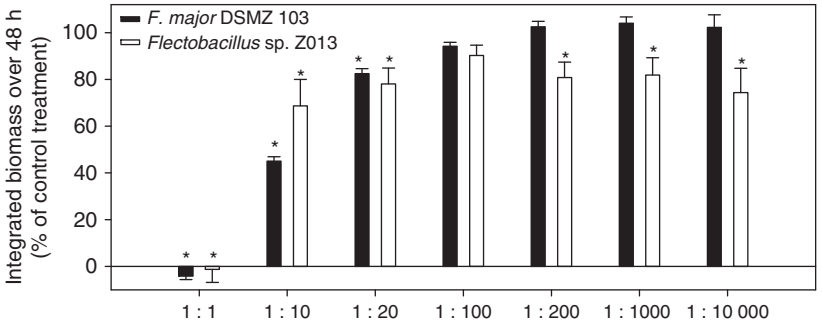

Fig. 6. Integrated biomass over 48 h of two *Flectobacillus* strains in liquid medium (DMSZ 7) amended with lipophilic and diluted lipophilic fractions of culture supernatants from a dark-adapted *Poterioochromonas* sp. strain DS compared with the growth in unamended medium. The error bars indicate the SE of five replicates, assuming additive error propagation. *Significantly lower than control treatment at $P < 0.05$.

Effects of supernatants related to flagellate trophic mode and growth condition

Supernatants from all three flagellates caused visible growth inhibition of *F. major* DSMZ 103, one of the most sensitive of the tested strains (Figs 2 and 3), albeit to a very different extent (Fig. 4). The release of antibiotic substances into the surrounding medium has been demonstrated previously for other *Ochromonas* strains (Jørgensen, 1962; Halevy *et al.*, 1971; Hansen, 1973). Aquatic algae compete with bacteria for limiting nutrients, for example, phosphate, and bacteria are typically superior competitors at low nutrient concentrations (Currie & Kalff, 1984; Mindl *et al.*, 2005). It is therefore conceivable that ochromonads might release antibiotic compounds into the surrounding medium (either actively or as a consequence of cell lysis) to inhibit bacterial growth and thus improve their own nutrient supply. In our agar diffusion assay, supernatants of *O. danica* produced the largest inhibition zones of all light-adapted flagellate strains. This species contained the highest concentrations of photosynthetic pigments (Table 2), and might therefore be regarded to gain more profit from autotrophy than the two *Poterioochromonas* strains. A more pronounced antibiotic activity of *O. danica* would thus be in agreement with an anticompetitor strategy.

Antibiotic effects of algae may be either 'constitutive' or 'induced', i.e., allelopathic substances may be constantly produced, or they may be synthesized and released into the environment only in the presence of bacteria (Safonova & Reisser, 2005). The supernatants from dark-adapted cultures of *Poterioochromonas* sp. strain DS had considerably more pronounced antibiotic effects and acted on more bacterial strains (Fig. 5) than supernatants from light-adapted cultures. This might merely be an upshift in the quantity of toxins. Alternatively, some metabolic pathways might have been active in the flagellates only during darkness or while feeding on bacteria, and other bioactive compounds might have been generated under such conditions than during phototrophic growth. A strain of *P. malhamensis* was only toxic for *Daphnia ambigua* if grown heterotrophically on bacteria in organic medium, but not if grown autotrophically in inorganic medium (Leeper & Porter, 1995).

Concentration-dependent bioactivity of supernatant extracts

In our experiments, we used Jüttner–Friz medium that was specifically designed for the cultivation of *Ochromonadaceae* and yielded cultures with very high flagellate numbers [up to 10^7 cells mL^{-1} as determined by flow cytometry (data not shown)]. Experiments were performed with stationary-phase flagellate cultures because the antibiotic activity of the green algae *Chlorella vulgaris* was also found to be maximal during this growth phase (Pratt *et al.*, 1945). The dense cultures provided sufficient amounts of biomass for an initial bioassay-guided fractionation and the search of novel bioactive compounds. At the same time, it is difficult to draw direct ecological conclusions, because the natural concentrations of these flagellates in freshwaters are much lower: densities between 80 and 7000 *Ochromonas* sp. mL^{-1} were found in acidic and alpine lakes (Nixdorf *et al.*, 1998; Callieri *et al.*, 2006). Dilutions of the lipophilic fraction of the supernatant of a dark-adapted *Poterioochromonas* sp. strain DS culture resulted in a small, but significant, reduction of biomass development of a *Flectobacillus* sp. isolate even at extremely low concentrations (Fig. 6). Thus, considering the dilution factors, the number of compounds produced at a density of 500 flagellates mL^{-1} might theoretically already be sufficient to reduce the growth of some bacterial strains. This high efficacy of antibiotic compounds produced by *Poterioochromonas* sp. strain DS emphasizes their potential ecological relevance, for example in affecting bacterial community structure. Interestingly, the *Flectobacillus* strain Z013 recently isolated from Lake Zürich (Table 1) was significantly more sensitive to the antibiotic compounds than another closely related strain (*F. major* DSMZ 103) that has been maintained in a culture collection for decades.

Simultaneous action of strain-selective antibiotic factors

At least two different types of antibiotic substances were produced by *Poterioochromonas* sp. strain DS: one or several compounds with hydrophilic properties specifically inhibited the growth of bacteria related to *Flectobacillus* (Figs 2 and 5). A second group of more lipophilic substances affected a wider range of bacterial isolates. Because the hydrophilic and moderate lipophilic substances extracted from the supernatants were redissolved in fresh medium and no artefacts due to the extraction procedure were observed, the specific effects on the five tested isolates (Fig. 5) could be unambiguously assigned to the respective chemical fraction and/or the flagellate cultivation condition.

Interestingly, the most sensitive of the strains studied were from bacterial groups that exhibit inducible morphological defence strategies against flagellate predation: a shift towards inedible filamentous cells of *Flectobacillus* spp. at high grazing has been described both in pure culture experiments (Hahn *et al.*, 1999; Corno & Jürgens, 2006) and *in situ* (Šimek *et al.*, 2007). *Flectobacillus* formed $> 25\%$ of the total bacterioplankton biomass if protistan grazing was experimentally enhanced (Šimek *et al.*, 2001). *Sphingobium* sp. Z007 may form large cell aggregates in the presence of *Poterioochromonas* sp. DS (J.F. Blom & J. Pernthaler, unpublished data), as has been observed previously for other waterborne bacteria (Hahn *et al.*, 2004). Phylogenetically closely related bacteria from the genus *Sphingomonas* are widely distributed in freshwater habitats, for example, on organic aggregates (Knoll *et al.*, 2001; Schweitzer *et al.*, 2001), and may also form high densities in the plankton (Piccini *et al.*, 2006). By contrast, both the completely unaffected *Pseudomonas* sp. strain E104 and the only partially affected *Janthinobacterium* sp. strain E106 proved to be toxic to the flagellate in direct feeding experiments (J.F. Blom & J. Pernthaler, unpublished data), as has also been shown for phylogenetically closely related strains (Matz *et al.*, 2004; Jousset *et al.*, 2006). While at present there is no general pattern emerging from such single observations, it is intriguing to speculate that the sensitivity of aquatic bacteria to allelopathic metabolites from mixotrophic unicellular eukaryotes might be related to their specific ecological interactions.

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