



Bacterial colonization of the freshwater planktonic diatom *Fragilaria crotonensis*

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ABSTRACT: In 2006, the seasonal course of bacterial colonization and silification rates of a colonial diatom *Fragilaria crotonensis* were studied at 2 distinct depths ('surface' 0.5 m and Z_{eu} , the depth of 1% remaining surface photosynthetically active radiation) in the eutrophic Římov Reservoir (Czech Republic). In the study period (July to September), *Fragilaria* dominated the phytoplankton and formed remarkable subsurface peaks of biomass at Z_{eu} . Diatoms living under light-limited conditions at Z_{eu} displayed lower silification rates measured with the fluorescence probe 2-(4-pyridyl)-5-[[4-(2-dimethylaminoethyl-aminocarbonyl)methoxy]phenyl] oxazole (PDMPO) and were significantly more colonized by bacteria than at the surface (seasonal means 7.8 and 5.0 bacteria per *Fragilaria* cell, respectively). In 3 additional experiments using double-stained *Fragilaria* cells (PDMPO and 4',6-diamidino-2-phenylindole [DAPI]), diatom cells with no PDMPO fluorescence were significantly more colonized by bacteria regardless of the depth, indicating that bacterial colonization reflects the physiological state of the diatoms. As an apparent consequence of a flood event in August, the *Fragilaria* assemblage was composed of 2 temporally isolated sub-populations differing markedly in the cell length, cell surface area and extent of bacterial colonization. Although both *Fragilaria* sub-populations showed similar silification rates, the late summer (post-flood) *Fragilaria* sub-population with smaller cells showed a significantly lower bacterial colonization, while the difference in bacterial colonization between the depths remained consistently significant throughout the study. Our results imply that bacterial colonization of diatom cell surfaces is modulated by both diatom growth and changes in population structure over the season.

KEY WORDS: *Fragilaria crotonensis* · Diatoms · Bacterial colonization · Physiological state · Silification · PDMPO

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INTRODUCTION

Phytoplankton cells represent an important bacterial habitat that is markedly distinct from the surrounding water (Bell & Mitchell 1972). Diatoms are frequently colonized by bacteria (Cole 1982, Vaqué et al. 1989, 1990, Bidle & Azam 1999, Grossart et al. 2005), sessile bacterivorous choanoflagellates (Šimek et al. 2004) or freshwater chytridiomycetes (Wurzbacher et al. 2010). Notably, diatoms are frequently the key component of phyto-

plankton assemblages (Armbrust 2009), responsible for 20 to 25% of global carbon fixation (Smetacek 1999), and serve as the basis for pelagic food webs. There is a multitude of possible interactions between planktonic diatoms and bacteria colonizing their frustules (Cole 1982, Grossart et al. 2005, Grossart & Simon 2007). The dynamics of bacteria and diatom assemblages are thought to be closely linked, and thus, their interplay markedly affects the functioning of the pelagic microbial food web (Cole 1982, Simon et al. 2002). The abundance and

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activity of bacteria colonizing diatom cells have been found to alter diatom sinking properties due to excessive production of exopolymeric substances, which increase diatom aggregation and thus the removal of organic matter from the water column (Grossart et al. 2006).

Variability in bacterial colonization appears to be closely dependent on diatom population dynamics and physiological state because increased numbers of attached bacteria coincide with late senescent stages of diatom blooms, while in early stages, bacterial colonization is generally low (Middelboe et al. 1995, Riemann et al. 2000, Grossart et al. 2005). However, there are some contradictory reports on the high abundance of attached bacteria on actively growing diatom cells (Smith et al. 1995) and low colonization in the late phase of a batch culture experiment (Kaczmarek et al. 2005). Current knowledge of bacteria–diatom interactions is based on indirect evidence without direct examination of the diatom physiological state and growth activity. Both might be highly variable at population or individual cell levels, even if these cells are in the same colony (Znachor & Nedoma 2008).

Various modes of bacteria–diatom interactions can be described in terms of general ecological concepts: (1) Competition—bacteria compete with diatoms for inorganic nutrients (Bratbak & Thingstad 1985); (2) Commensalism—bacteria benefit from diatoms without having any negative effect on them. Nonetheless, bacterial commensalism may switch to parasitism, particularly when diatoms grow under environmental stress (Cole 1982); (3) Parasitism—bacteria either invade the host cell or produce metabolites with algicidal activity, both leading eventually to cell lysis and death (Imai et al. 1993, Park et al. 2010). In contrast, diatoms can inhibit bacterial growth by producing antibiotic compounds (Leflaive & Ten-Hage 2009) (4) Mutualism—bacteria benefit from diatom exudates (Pete et al. 2010), whereas diatoms profit from bacterial products, such as vitamins or other growth factors or recycling of limiting nutrients (Croft et al. 2005, Droop 2007).

To date, studies addressing bacteria–diatom interactions have been performed either in marine systems or predominantly marine diatom cultures, while there are only a very few reports for freshwater diatoms. Under favourable conditions, marine diatoms dramatically increase in cell numbers, i.e. by several orders of magnitude in just few days (Tsuda et al. 2003, Armbrust 2009, Iluz et al. 2009), and thus, growth activity might be deduced from rapid changes in pigment concentration or diatom abun-

dance comparable to culture experiments (Grossart et al. 2005). However, in freshwater lakes or reservoirs, diatoms can dominate the phytoplankton for months (Middelboe et al. 1995, Znachor et al. 2008), and therefore, to assess diatom physiological status, algal growth activity must be directly examined (Znachor & Nedoma 2008).

Recently, the fluorescence probe PDMPO, i.e. 2-(4-pyridyl)-5-[[4-(2-dimethylaminoethyl-aminocarbonyl)methoxy]phenyl] oxazole, has been employed to measure silica deposition in the summer diatom assemblage at various depths in the eutrophic Římov Reservoir (Czech Republic; Znachor & Nedoma 2008, Znachor et al. 2008). During these experiments, the diatom assemblage was exclusively dominated by *Fragilaria crotonensis*, which formed pronounced peaks of biomass at the base of the euphotic zone (Z_{eu} , 2 to 6.5 m). At Z_{eu} , silica deposition was found to be significantly constrained by low light availability, while at the surface, it was limited by phosphorus deficiency; the effect of dissolved Si was not statistically significant (Znachor & Nedoma 2008). Extreme rainfalls were shown to have a crucial effect on the dynamics of the diatom assemblage: a marked increase in Si deposition rates was reported just after the summer storm event, consequently followed by an increase of *Fragilaria* abundance (Znachor et al. 2008). Because *Fragilaria* recurrently dominates summer phytoplankton in the Římov Reservoir, it has been also used as a model organism for studying phytoplankton osmotrophy (Znachor & Nedoma 2010). It was found, however, that the phytoplankton uptake of ^3H -glucose was very low, which contrasted with the high uptake rate observed for *Fragilaria*-associated bacteria (Znachor & Nedoma 2010).

In the present study, we investigate the relationship between the bacterial colonization and growth activity of diatoms on both individual cell and the whole diatom population levels. While a part of these data has been previously used in different context, e.g. as variables documenting seasonal changes in *Fragilaria* silification rates (Znachor et al. 2008) or factors affecting diatom silification at various depths (Znachor & Nedoma 2008), our present study has a quite distinct focus. Here, we provide compelling field evidence of an inverse relationship between the bacterial colonization of a freshwater diatom, which dominated the phytoplankton community during summer, and its growth activity deduced from silification rates. In addition, we found a significant relation between seasonal changes in bacterial colonization and variation in *Fragilaria* cell dimensions.

MATERIALS AND METHODS

Study site and sampling design

Our study was conducted in the dam area of the dimictic, meso-eutrophic Římov Reservoir (Czech Republic; 48° 50' 56" N, 14° 29' 26" E; 470 m a.s.l.; area, 2.06 km²; volume, 34.5 × 10⁶ m³; length, 13.5 km; max. depth, 43 m; mean depth, 16.5 m; mean retention time, 100 d). From July to September 2006, we took samples from the surface (0.5 m) and the depth of 1% surface irradiance (Z_{eu} , 2 to 6.5 m) at weekly intervals to assess the abundance of total and diatom-associated bacteria along with the phytoplankton composition and physiological state.

Background limnological data

Vertical profiles of photosynthetically active radiation (PAR) necessary for estimation of the euphotic-zone depth (Z_{eu}) were obtained using a LI-COR

LI-1400 datalogger with a spherical quantum underwater sensor LI 193 SA (LI-COR). Samples for phytoplankton cell counts were taken with a Friedinger sampler and preserved with a Lugol solution. Phytoplankton were enumerated employing the Utermöhl method on an Olympus IMT-2 inverted microscope (Olympus; Lund et al. 1958).

Assessment of *Fragilaria* growth activity

During the study period, the phytoplankton community was prevalently dominated by the colonial diatom *Fragilaria crotonensis* (Znachor & Nedoma 2008), which formed remarkable subsurface peaks of biomass at Z_{eu} (often identical with the depth of the metalimnion) due to cell accumulation above the density gradient (Znachor et al. 2008). To assess *Fragilaria* growth activity, we used a 24 h incubation with PDMPO, a specific tracer for biogenic Si deposition (Shimizu et al. 2001). PDMPO quickly enters diatom cells and is co-deposited with silica into the

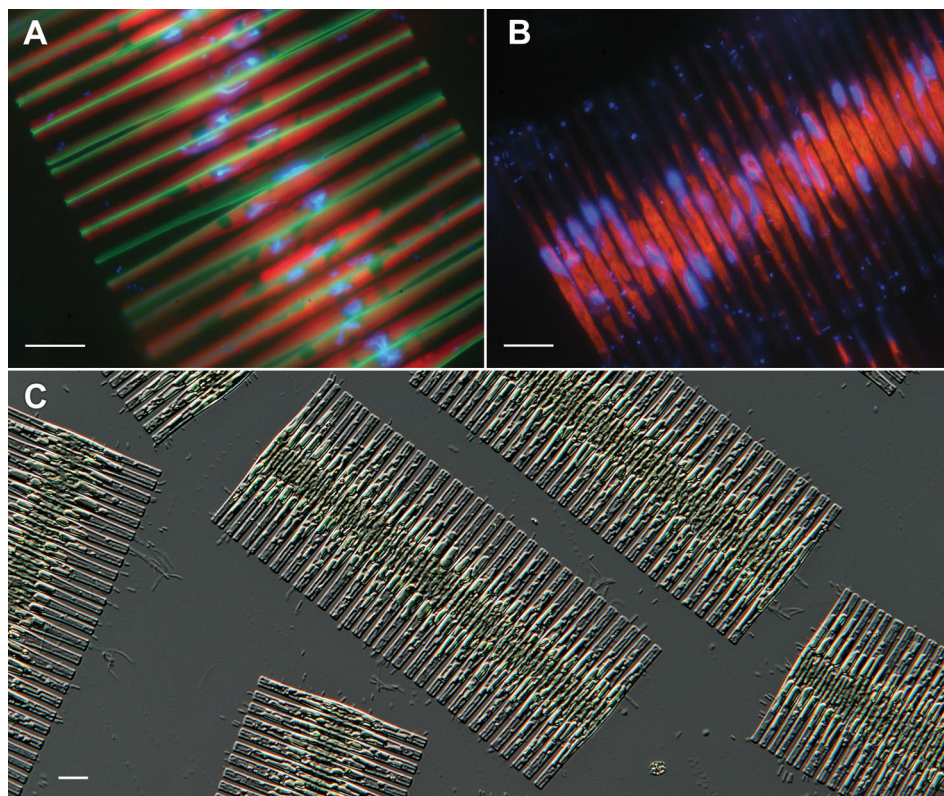


Fig. 1. Composite fluorescence image of double-stained diatoms. At the surface, (A) *Fragilaria* colonies actively depositing Si were without a substantial population of attached bacteria, while (B) those colonies inhabiting the metalimnion were often non-active and showed dense bacterial colonization. Green fluorescence is PDMPO incorporation indicating diatom growth, blue objects are DAPI-stained bacteria and diatom cell nuclei, and red is chlorophyll autofluorescence. Panel C provides an overall view of *Fragilaria* colonies using a Nomarski contrast technique. Scale bars = 10 μ m

newly synthesized frustule, yielding a bright green fluorescence (Shimizu et al. 2001). Application of this epifluorescence microscopy technique enables a simple distinction between cells within diatom colonies and populations that are actively depositing Si and those that are not (Leblanc & Hutchins 2005; Fig. 1). In addition, PDMPO fluorescence has been shown to be proportional to the amount of Si deposited (Leblanc & Hutchins 2005), and thus, it provides a useful indirect measure of the rate of recruitment of new cells and, therefore, may be used as a quantitative proxy for diatom growth (Znachor & Nedoma 2008). Both the PDMPO fluorescence intensity expressed as relative fluorescence units (FU) and the *Fragilaria* cell dimensions were quantified by image analysis as described by Znachor & Nedoma (2008). Briefly, samples were incubated for 24 h in 50 ml quartz glass bottles *in situ* at both the surface and Z_{eu} ; the PDMPO (final concentration of $0.125 \mu\text{mol l}^{-1}$; Molecular Probes) was added at the beginning of the incubation. Samples were filtered through $10 \mu\text{m}$ polycarbonate filters immediately after the incubation and transported to the laboratory for fluorescence measurements. Filters were rinsed with distilled water to eliminate any unbound PDMPO and inspected using epifluorescence microscopy. PDMPO fluorescence was measured 30 min after the filter preparation using image cytometry; ~50 diatom colonies were measured from each sample (magnification $200\times$). Each image file consisted of 2 meshed 8-bit individual monochromatic images of an identical microscope field taken with a PDMPO-specific filter-cube (excitation/emission: $360\text{--}370 \text{ nm}/520\text{--}540 \text{ nm}$) for quantification of the PDMPO fluorescence and a chlorophyll autofluorescence image (excitation/emission: $510\text{--}550 \text{ nm}/>590 \text{ nm}$) for localization of *Fragilaria* colonies and cell dimension measurements. An image analysis system consisting of a Nikon Eclipse 90i fluorescence microscope (Nikon; Nikon Plan Fluor $20\times$, Nikon Plan Apo $100\times$), a monochromatic digital integrating camera (VDF Voskühler) and an NIS-Elements 3.0 PC-based image analysis software (Laboratory Imaging) was used. The surface area of *Fragilaria* cells was calculated from mean cell dimensions by approximation to an elliptic prism (Hillebrand et al. 1999).

Bacterial enumeration

Seasonal changes in the numbers of total and *Fragilaria*-associated bacteria at the surface and Z_{eu} were obtained by bacterial counts after 4',6-diamidino-2-

phenylindole (DAPI) staining (Porter & Feig 1980) of PDMPO untreated samples on 0.2 (total bacteria) and $2 \mu\text{m}$ (diatom-attached bacteria) pore-size Poretics filters (Osmonics, Poretics), respectively. Both total and diatom-attached bacteria were counted under $1000\times$ magnification. The vast majority of the attached bacteria was clearly focused on several optical planes and counted at planes clearly different from surface of the $2 \mu\text{m}$ pore-size filter where the free-living bacteria might eventually be left after a gentle rinsing prior to counting. Careful focusing at different planes allows for counting of the attached bacteria on both sides of diatom frustules because the frustules are semi-transparent in the DAPI-stained preparations. At least 500 bacterial cells were counted for total bacterial counts, and 50 diatom colonies (200 to 400 individual cells) were inspected for diatom-attached bacterial abundance. Additionally, in 3 parallel experiments in July, bacteria attached on *Fragilaria* were counted after a 24 h incubation with PDMPO. This allowed for a direct estimation of bacterial colonization of *Fragilaria* cells with contrasting Si deposition rates at a particular depth (Fig. 1).

Statistical analysis

All measurements were made in duplicate. Statistical treatment of the data was done using Prism 5 (GraphPad Software). A 2-way ANOVA was performed to examine differences in the measured parameters with (1) depth and (2) sampling period. Unpaired *t*-tests were used to test differences in bacterial colonization of *Fragilaria* cells after double staining with DAPI and PDMPO.

RESULTS

The bacteria attached on *Fragilaria* at the surface and Z_{eu} represented only a small portion of the total bacteria (seasonal average 2.3 and 2.9%, respectively; Table 1). Temporal changes in bacterial colo-

Table 1. Total numbers of *Fragilaria*-attached and total bacteria per ml (seasonal mean values with ranges, $\times 10^6 \text{ ml}^{-1}$)

| Depth | <i>Fragilaria</i> -attached bacteria | | Total bacteria | |
|----------|--------------------------------------|------------|----------------|-----------|
| | Mean | Range | Mean | Range |
| Surface | 0.07 | 0.001–0.35 | 3.55 | 2.29–4.81 |
| Z_{eu} | 0.11 | 0.001–0.32 | 3.31 | 1.06–5.24 |

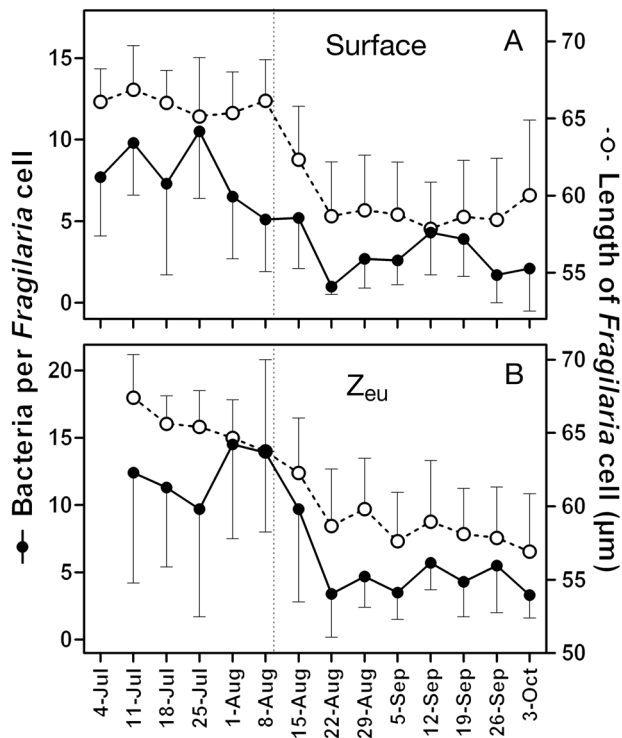


Fig. 2. Seasonal course of bacterial colonization of *Fragilaria* cells and their length at 2 depths. Over the season, 2 *Fragilaria* sub-populations developed, differing markedly in cell size and the extent of bacterial colonization. Seasonal courses of both parameters were highly correlated (surface, $r^2 = 0.70$, $p < 0.001$; Z_{eu} , $r^2 = 0.79$, $p < 0.001$, linear regression). Vertical dotted lines indicate flood event. Means \pm SD

nization of *Fragilaria* and diatom cell length at the surface and Z_{eu} are given in Fig. 2. The seasonal courses of colonization were characterized by (1) higher numbers of *Fragilaria*-associated bacteria at Z_{eu} than at the surface (7.8 and 5 bacteria per *Fragilaria* cell, respectively) and (2) an abrupt decrease in bacterial colonization in the second part of the study period observed at both depths. The depth-related difference reflected *Fragilaria* growth activity measured by PDMPO fluorescence, which was much lower at Z_{eu} than at the surface, while the seasonal decrease in bacterial colonization was related to a change in *Fragilaria* cell dimensions (Fig. 3). The effects of both depth and season were highly significant ($p < 0.0001$, 2-way ANOVA; Fig. 3A,C).

Both the silification rates and portions of *Fragilaria* cells actively depositing Si were markedly lower at Z_{eu} (PAR, 4 to 100 $W m^{-2} d^{-1}$) than at the surface (PAR, 1102 to 8586 $W m^{-2} d^{-1}$; 2-way ANOVA, $p < 0.0001$; Fig. 3E,F). To obtain direct evidence that bacterial colonization is negatively related to *Fragilaria* growth activity even under the same light conditions, DAPI-

stained bacteria were counted on diatoms previously incubated with PDMPO (Fig. 1). Regardless of depth, *Fragilaria* cells actively depositing Si showed a significantly lower bacterial colonization than slow-growing or senescent cells without any measurable Si deposition ($p < 0.001$; Fig. 4). This indicates that the depth-related differences in the per-cell colonization of *Fragilaria* by bacteria resulted from a variable proportion of Si-depositing diatom cells rather than from changing silification rates.

Fragilaria assemblages were composed of 2 temporally isolated sub-populations differing markedly in cell length, cell surface area and extent of bacterial colonization (Figs. 2 & 3). The reasons for such a dramatic mid-summer decrease in *Fragilaria* cell length are still unknown; however, a sudden disturbance in the form of a flood event might be a plausible explanation. An extreme rainfall (7–8 August) substantially increased the flushing rate, disrupted the thermal stratification, temporarily altered the nutrient and light availability (Znachor et al. 2008) and apparently also caused a discontinuity in the seasonal course of *Fragilaria* population dynamics. Although both *Fragilaria* sub-populations showed similar silification rates ($p > 0.35$, 2-way ANOVA; Fig. 3E,F), the late summer *Fragilaria* sub-population with smaller cells (pooled data from both depths: pre-flood 65.6 μm , post-flood 58.9 μm ; $p < 0.0001$, 2-way ANOVA; Fig. 3B) was significantly less colonized by bacteria ($p < 0.0001$, 2-way ANOVA; Fig. 3A,C), suggesting that other factors besides growth and physiological state can be involved in the bacterial colonization of diatoms. Notably, nutrient concentrations dropped within ~ 2 wk after the flood to previous values (soluble reactive phosphorus $\sim 4.5 \mu g l^{-1}$, nitrates $\sim 1 mg l^{-1}$; Znachor et al. 2008).

DISCUSSION

The depth-related differences in the bacterial colonization of *Fragilaria* cells obviously reflected the different physiological states and growth activity of the respective diatom populations. At Z_{eu} , the *Fragilaria* growth was constrained by low light availability; the diatom cells were narrower and contained more chlorophyll, and the cell number of the *Fragilaria* colonies was lower than at the surface, as reported in the parallel study (Znachor & Nedoma 2008). It has been shown that when diatom growth is slow, algal surfaces are exposed to bacterial colonization for a longer time, leading to higher bacterial colonization than on fast-growing cells (Vaqué et al. 1990).

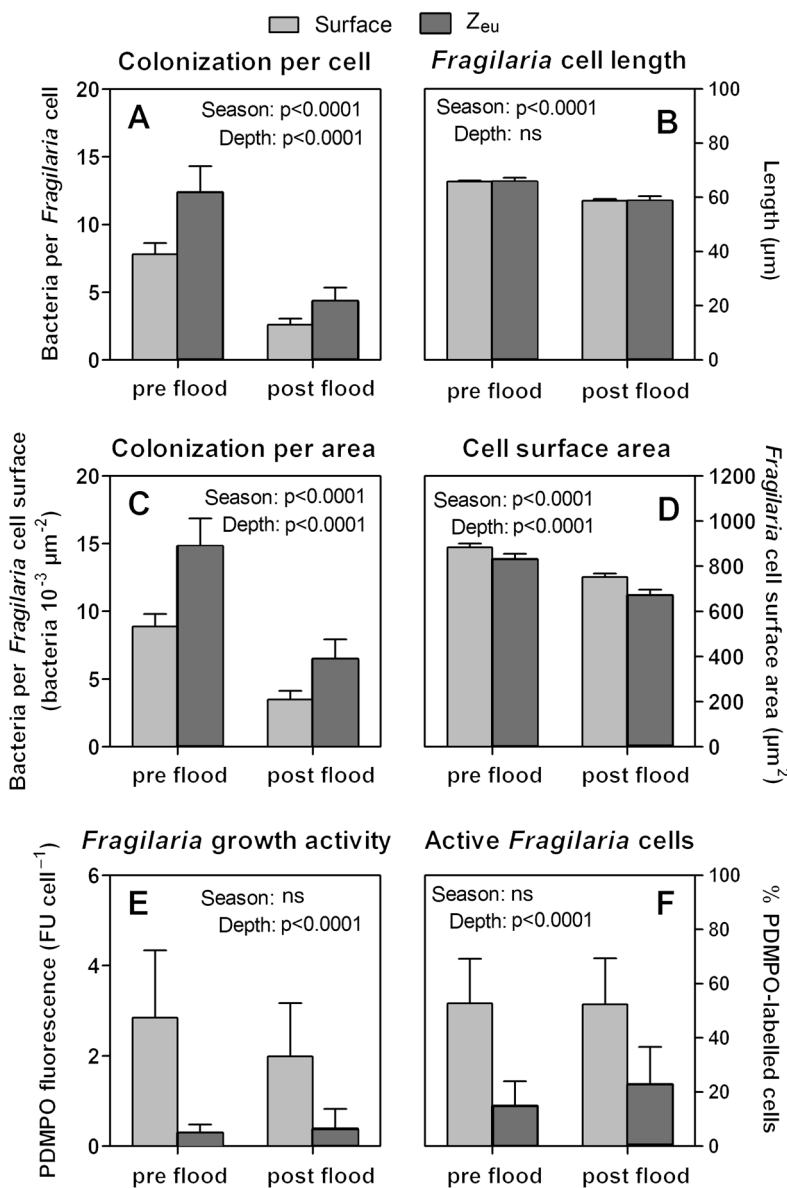


Fig. 3. Effect of flood event (season) and depth on (A,C) bacterial colonization of *Fragilaria*, (B,D) diatom cell dimensions and (E,F) growth activity estimated with PDMPO technique. Statistical significance was tested with 2-way ANOVA. Means \pm SD

Additionally, bacterial attachment to phytoplankton depends on encounter probability, which increases with the abundance of both bacteria and diatom cells in water and also with algal size (Vaqué et al. 1989). In the present study, we did not find any relationship between total bacterial abundance and the degree of diatom colonization at both depths. Larger diatom species have been reported to support more abundant bacterial colonisers than smaller ones (Vaqué et al. 1990). The same is true for bacterial colonization of dispersed water particles. In Lake Con-

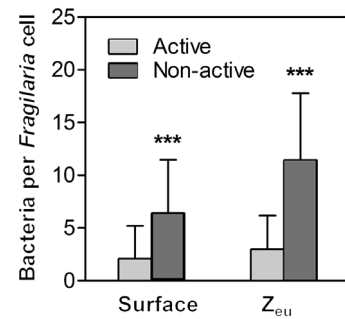


Fig. 4. Mean (\pm SD) numbers of bacteria per *Fragilaria* cell from 3 measurements conducted in July 2006. Bacteria were counted after double staining with PDMPO and DAPI, allowing direct estimation of diatom activity in Si deposition. *Fragilaria* cells actively depositing Si (labelled with PDMPO) were significantly less colonized by bacteria than non-labelled cells regardless of the depth. ***Significant differences ($p < 0.0001$, t -test) between active and non-active *Fragilaria* cells

stance, Grossart & Simon (1998) found that the abundance of particle-associated bacteria increased with depth, mainly due to larger aggregates. Generally, attached bacterial abundance tends to increase with increasing size of diatom aggregates (Ploug & Grossart 2000), detritus particles (Luef et al. 2007) or transparent exopolymeric particles (Pedrotti et al. 2009), which can be formed from precursors released by diatoms (Simon et al. 2002, Pedrotti et al. 2009).

In our study, the seasonal decrease in bacterial colonization (by ~60%, Fig 3A,C) observed after the flood event was not proportional to the reduction in *Fragilaria* cell length (by ~11%; Fig. 3B) and consequently cell surface area (by ~17%; Fig. 3D), and the total bacterial counts were virtually unaffected by the flood (pooled average from both depths: pre-flood $3.26 \times 10^6 \text{ ml}^{-1}$, post-flood $3.34 \times 10^6 \text{ ml}^{-1}$). This implies that bacterial colonization is modulated by intervening factors affected by the flood event and not only by the encounter probability. In the post-flood period, weak thermal stratification produced by cold and windy weather (Znachor et al. 2008) and a decrease in temperature (pooled data from both depths: pre-flood 21.2°C, post-flood 17.5°C; $p < 0.001$) might account for the low numbers of bacteria attached to *Fragilaria* cells. A similar pattern has been found for bacterial colonization of transparent exopolymeric particles in Lake Pavin (Arnous et al. 2010). Additionally, both *Fragilaria* sub-populations

may have differed in specific modes of interactions with bacteria, supposedly resulting from e.g. a seasonal shift in bacterial community composition (Grossart et al. 2005), the distinct quantity and quality of exudates (Pete et al. 2010), varying composition of the organic matrix protecting diatom frustules or from generally different susceptibility to bacterial colonization under changing environmental constraints. However, besides the season-related shift in factors such as temperature and light intensity, we have no direct indication of which controlling factors prevailed in the present study.

Despite a low relative abundance of *Fragilaria*-associated bacteria (Table 1), they supposedly played an important role in the transformation of organic carbon. This notion is supported by a combination of the data presented here with those of a follow-up study (Znachor & Nedoma 2010), where microautoradiography and size fractionation were applied to study carbon flows within pelagic food webs in the Římov Reservoir. The bacteria attached to *Fragilaria* accounted for 15% of the overall ^3H -glucose uptake, while free-living bacteria accounted for 75% (Znachor & Nedoma 2010). Thus, diatom-associated bacteria could account for a much higher fraction of total glucose uptake than predicted based on their abundance, indicating higher cell-specific glucose uptake rates than for free-living bacteria. Assuming similar proportions of free-living and *Fragilaria*-associated bacteria in 2006 and 2007, *Fragilaria*-associated bacteria could exhibit, on average, ~8-fold higher cell-specific uptake rates of glucose than free-living bacteria. This is in accordance with a commonly accepted concept that diatom colonies and their attached bacteria represent true microbial 'hot spots', which are also characterized by high bacterial production and ectoenzymatic activities (Simon et al. 2002).

Bacteria colonizing diatom cells might benefit from their host by several means. It is well known that diatom exudation, which markedly increases under growth-limiting conditions, supports bacterial growth (Pete et al. 2010). When environmental stress exceeds the ecological tolerance of the algae, diatom cells start to decompose, and as a result, organic-rich cell constituents are liberated (Franklin et al. 2006). Thus, bacteria directly attached to diatom cells can utilize the released organic compounds, allowing them to display generally higher growth rates than free-living bacteria (Simon 1987). Moreover, diatom-attached bacteria preferentially utilize the algal exudates, which limits the availability of the exudates for free-living bacteria. In addition, surface attachment and embedding into an organic matrix has been sug-

gested as an alternative mechanism for bacteria to avoid predation pressure by typical planktonic suspension-feeding flagellates (Pernthaler 2005). Such amorphous extracellular polymers were also observed in the present after DAPI staining; however, we were not able to accurately quantify them.

Besides these specific algal-bacterial interactions, almost all of the bigger diatom colonies in our experiments were observed to also anchor bacterivorous choanoflagellates (data not shown). When these diatom-associated bacterivores become abundant, they can significantly contribute to the overall mortality rates of free-living bacterioplankton (Šimek et al. 2004), creating an alternative pathway of organic carbon to higher trophic levels. Thus, on the micro scale, both the processes of diatom cell surface colonization and intensive bacterivory apparently create hot spots of microbial activities, accelerate nutrient recycling and, therefore, affect overall nutrient availability in aquatic systems.

In summary, using a sensitive, fluorescence-based approach, this study has brought new insights into factors affecting the bacterial colonization of *Fragilaria* studied at both single-cell and population levels in a natural aquatic system. Diatom growth activities and changes in population structure modulate the dynamics of bacterial populations on diatom cell surfaces. With respect to the large diatom biomass over a significant part of the season, diatom colonies represent hot spots of microbial interactions and activities in the water column of eutrophic freshwater reservoirs.

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