



Effects of microcurrents in the boundary layer on the attachment of benthic heterotrophic nanoflagellates

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ABSTRACT: Surfaces in running water are covered by a boundary layer. Virtually nothing is known about the importance of water currents in the microenvironment of nanofauna. Many questions have been partially answered concerning the effect of surface topography on the hydrodynamics in the vicinity of macrofauna; however, investigations of the 2 to 5 µm water layer where nanoprotoists live have been neglected. In the present study, we show that the flow velocity at a distance of a few micrometres from the substrate is high enough to be very effective regarding the detachment of nanoprotoists. We analysed the impact of flow velocity (detachment from substrate) on 8 nanoflagellate taxa (*Entosiphon*, *Cercomonas*, *Codonosiga*, *Anthophysa*, *Bodo*, *Neobodo*, *Apusomonas*, *Spumella*) with different abilities to crawl and attach to the surface. A Plexiglas disc was used to generate a defined flow velocity on the surface of a Petri dish microcosm. Laminar flow in the boundary layer was found between 0 and 700 µm above the substratum. The effect of 4 different flow velocities on heterotrophic flagellates was investigated (0.3, 0.6, 0.9 and 1.2 m s⁻¹ at 5 mm above the substratum, corresponding to flow velocities of 0.001 to 0.004 m s⁻¹ at 10 µm above the substratum). The colourless, gliding euglenid *Entosiphon sulcatum* showed the highest resistance towards high flow velocities. Another species, the crawling cercomonad *Cercomonas crassicauda*, had the weakest attachment. Small changes in the micro-topography of the substrate (e.g. *Ancylus* shells) may significantly influence spatial distribution of nanoflagellates.

KEY WORDS: Heterotrophic nanoflagellates · Boundary layer · Microcurrents · Biofilm · Flow velocity · Topography

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INTRODUCTION

It is commonly agreed that flow velocity can be an important factor for the structure and dynamics of aquatic ecosystems, especially in running waters and tidal regions (Battin et al. 2003a,b). It is assumed that the flow velocity decreases asymptotically from the surface towards the bottom. A few millimetres above the bottom, the flow velocity is very low (Ambühl 1959). This region is called the boundary layer and serves as a refuge for torrenticole macrofauna (Vogel 1981, Koehl 1984, Prandtl et al. 1984). Only a very few reports on the flow velocity in the microenvironment

and the diffusion rates around microfauna and bacteria exist (e.g. Silvester & Sleigh 1985, Lazier & Mann 1989, Shimeta et al. 2001, 2002, Vopel et al. 2002, 2005, Willkomm 2006). Virtually nothing is known about the importance of water currents in the microenvironment of nanofauna. All of the questions that have partially been answered concerning the effect of surface topography on the hydrodynamics in the vicinity of macrofauna. Although some information is available on the effect of surface topography on hydrodynamics in the vicinity of macrofauna (e.g. Ambühl 1959, Statzner & Holm 1989, Hunt 2004), the 2 to 5 µm water layer in which nanoprotoists live remains to be investigated. To

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understand microbial interactions on biofilms, several questions have to be answered, e.g. to what extent do water currents affect organisms in a size range of a few micrometres? Could the roughness of bacterial biofilms form a refuge for organisms of the nanofauna? Can meio- and macrofauna burrows and houses create hydrodynamically specific habitats for nanoflagellates?

We investigated the flow velocity in the micro-layer above the substrate and its impact on nanoflagellate species with different abilities to crawl and attach on the substrate in order to address at least some of the above-mentioned questions.

MATERIALS AND METHODS

Flow velocity measurements on a microscale. The effect of microcurrents on the attachment of heterotrophic flagellates was investigated in experimental microcosms. The microcosms consisted of Petri dishes with a diameter of 13.5 cm. These were filled with about 75 ml of flagellate cultures (WC medium is MBL; Guillard & Lorenzen 1972) to reach a water column with a height of 5 mm. By changing the current supply of a 12 V motor, the speed of a rotating Plexiglas disc on top of the fluid was regulated, generating a specific flow velocity in defined regions of the Petri dish (the regions had a distance of about 46 mm from the centre of the dish). To observe these regions, the bottom of each Petri dish contained 4 windows with cover slips (50 × 25 mm) to allow observation of the surface-associated flagellates by inverse microscope at high magnifications (up to 400×; Zeiss Axiovert 100; Fig. 1). The inner surface of the Petri dish (including cover slips) was flush to avoid any disturbances of the water current. For identification of the flow velocity in microcosms, the fluid was spiked with a suspension of 10 µm neutrally buoyant hollow glass spheres (cf. Røy 2003; kindly provided by Hans Røy, Max Planck Institute for Marine Microbiology, Bremen, Germany). Flow speed of particles was estimated from video image sequences of 50 frames s⁻¹. This is a classical method of visualising the flow fields under laboratory conditions (e.g. Riedl & Forstner 1968, Oakey et al. 2002). The flow velocity was determined in different layers of the water established by a calibrated fine drive of the microscope.

The flow velocities were determined in the different layers of the circular flow chambers between 0 and 5000 µm above the substrate (Fig. 1). At a disc velocity of 0.3 m s⁻¹, the flow velocity ranged between 0.001 m s⁻¹ (0 to 10 µm) and 0.3 m s⁻¹ (5000 µm). In the layer between 0 and 700 µm, a linear increase of the flow velocity was detected. A slight increase of flow velocity

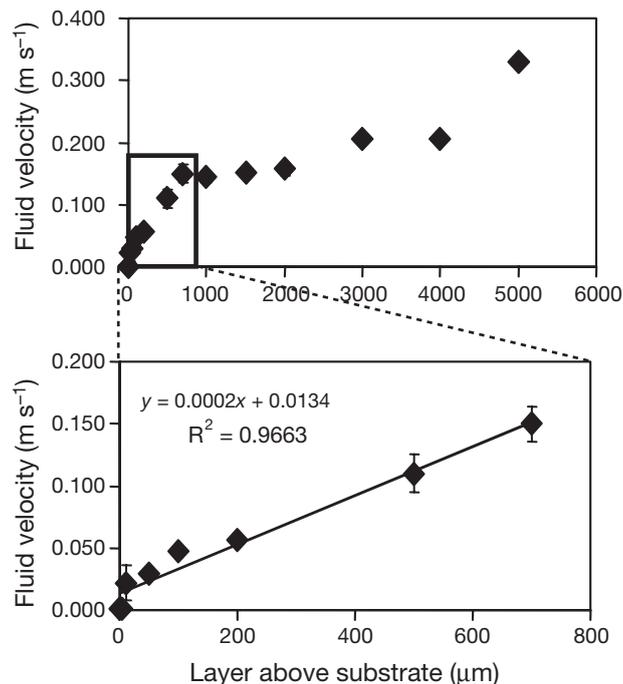


Fig. 1. Flow velocity at different layers of the microcosm (in µm above the substrate) at a speed of the rotating disc of 0.3 m s⁻¹. Error bars indicate the standard deviation. The lower graph is an enlargement for the region of linear increase of flow velocity (n = 2 to 5)

was recorded for the layer between about 1000 and 4000 µm above the dish surface, while a linear approach to the velocity of the disc was registered in the upper layer (4000 to 5000 µm above the dish surface).

Flagellate cultures. Eight different species of heterotrophic nanoflagellates (monoxenic cultures) were used in the experiments (Fig. 2). Two species formed colonies at the end of a stalk: *Codonosiga botrytis* (total length of the colony: 45 to 95 µm; Choanoflagellida; isolated from the Rhine River by M. Weitere) and *Anthophysa vegetans* (total length of the colony: 65 to 125 µm; Chrysomonadida; isolated from the Rhine River by M. Weitere). *Spumella* sp. (cell length: 3 to 7 µm; Chrysomonadida; isolated from Lake Schöhsee by A. P. Mylnikov) was attached to the substratum by a protoplasmic thread. The euglenid *Entosiphon sulcatum* (20 to 25 µm; obtained from CCAP 1220/1A), the apusomonad *Apusomonas proboscidea* (8 to 12 µm; isolated from a lake near Borok, Russia, by A. P. Mylnikov), the kinetoplastid *Neobodo designis* (4 to 7 µm; isolated from the Rhine River by M. Weitere) and the cercomonad *Cercomonas crassicauda* (15 to 20 µm; isolated from a lake near Borok, Russia, by A. P. Mylnikov) are tectic flagellates that glided over the surface of the Petri dish. The kineto-

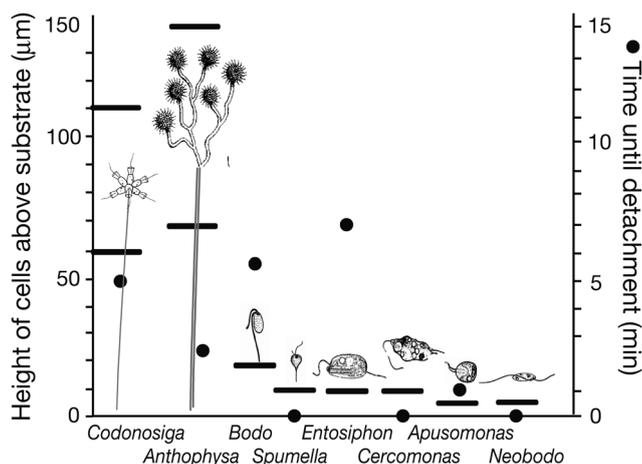


Fig. 2. Relationship between the morphology and the detachment time of 8 heterotrophic nanoflagellates. Horizontal bars indicate the mean distance of single cells from the substrate (for *Codonosiga* and *Anthophysa* a range is given for the colonies); black dots stand for the mean time until detachment at a disc velocity of 1.2 m s^{-1} (cf. Fig. 3)

plastid *Bodo saltans* (5 to 6 μm ; isolated from a lake near Borok, Russia, by A. P. Mylnikov) was attached to the surface of the Petri dish by the tip of the posterior flagellum.

Effects of flow velocity on smooth surfaces. The 8 species of heterotrophic flagellates mentioned above, with different morphologies and types of attachment to the surface, were exposed in separate experiments to different flow velocities on a smooth surface (Petri dish). Flagellate cultures were inoculated to the Petri dish microcosms 12 h before the start of the experiments. Preliminary studies showed that this pre-incubation served as an adaptation time, allowing a comparative study of the different species. The flow velocity was increased stepwise with intervals of 0.1 m s^{-1} every 5 s until the target velocity was reached. At this point the investigation period started. During this 10 min period comparative data for all species were collected. The time until detachment (passive pull off) of flagellates was determined at flow velocities of 0.3, 0.6, 0.9 and 1.2 m s^{-1} (measured at a height of 5 mm above the surface of the Petri dish). At the height of 10 μm above the surface of the Petri dish, the flow velocities were 0.001, 0.002, 0.003 and 0.004 m s^{-1} . Then, 5 to 8 replicate experiments with different cultures of the same flagellate strain were carried out. The height of organisms indicated (Fig. 2) was estimated at conditions without flow using a calibrated fine drive of the microscope.

Effects of flow speed on the distribution of flagellates depending on surface topography. Two of the above-mentioned heterotrophic flagellates, *Entosi-*

phon sulcatum and *Cercomonas crassicauda*, were introduced into Petri dishes with shells of the basomatophoran snail *Ancylus fluviatilis* (length: 3.1 mm, width: 1.9 mm, height: 1.1 mm; average dimensions) glued on the bottom of the dishes. The shells were used to mimic the roughness of the biofilm due to the presence of macrofauna. *A. fluviatilis* is a typical inhabitant of biofilms in rivers (Nielsen 1950, Elser 1999), where most of the flagellates used in this study were isolated. The spatial distributions of the 2 flagellate species were investigated in the area behind the shells. This area was divided into 20 fields ($92 \times 100 \mu\text{m}$). Experiments were carried out at a flow velocity of 0.3 m s^{-1} (at a height of 5 mm above the surface of the Petri dishes). The direction of the current and the flow velocity were determined in every field (methods see above). The abundance of the heterotrophic flagellates was determined every minute for each single field by an inverse microscope (videomicroscopy with a ZEISS Axiovert 100; $25\times$ magnification). The average abundance of flagellates in each field was determined for a period of 10 min. The maximum abundance in each experimental set up was considered as 100%. Four replicate experiments were carried out for each species. Pearson rank correlation was used to analyse the correlation between abundance and flow velocity.

RESULTS

Effects of flow velocity on nanoflagellates on smooth surfaces

When the disc velocity changed from 0.1 to 1.4 m s^{-1} , the flow velocity above the substrate changed from about 0.0003 to 0.0045 m s^{-1} . The question arose whether these changes of more than an order of magnitude in flow velocities might affect the behaviour of nanoflagellates living close to the substrate. The 8 different nanoflagellate species were exposed to 5 different flow velocities, and the time until detachment was measured. All species differed significantly regarding their type of attachment, the substrate and the mean distance from the substrate (Fig. 2). *Codonosiga botrytis* and *Bodo saltans* withstood the maximal target time (10 min) up to a velocity of $0.6/0.002 \text{ m s}^{-1}$ (at a distance of either 5 mm or 10 μm from the substrate; Fig. 3). At the highest velocity of $1.2/0.004 \text{ m s}^{-1}$, *Entosiphon sulcatum* (7:25 min) reached the longest attachment time of all 8 flagellates, followed by *B. saltans* (5:53 min), *C. botrytis* (5:24 min), *Anthophysa vegetans* (2:45 min), *Apusomonas proboscidea* (1:35 min), *Neobodo designis* (0:31 min), *Spumella* sp. (0 min) and *Cercomonas*

crassicauda (0 min). No species withstood the flow for the maximum target time of 10 min at the highest velocity. *Spumella* sp. and *C. crassicauda* showed the least resistance to the flow, numbers of attached specimens decreased very rapidly already when low flow velocities were applied (Fig. 3). There was no correlation between the distance of the individual flagellate cells from the substrate and the period of attachment (cf. Fig. 2).

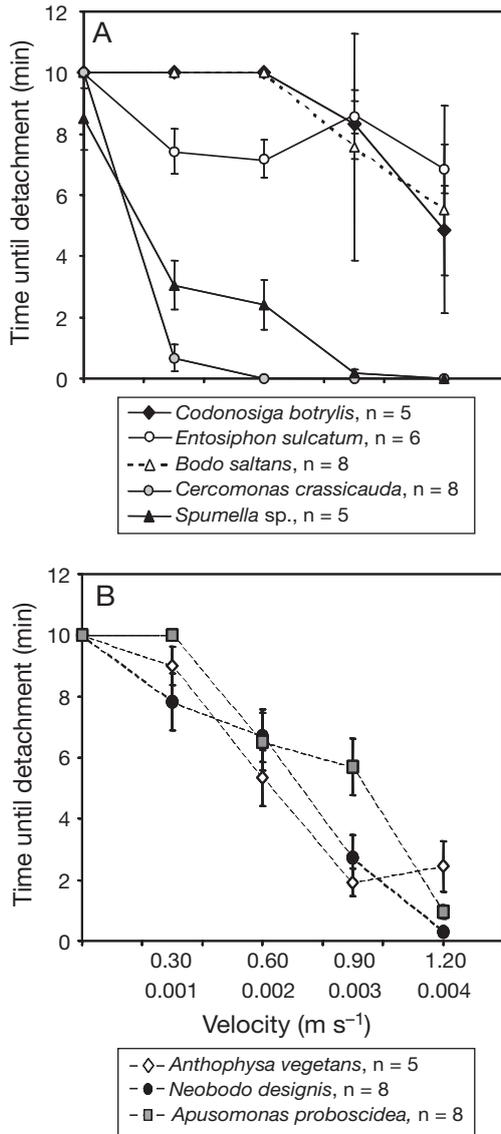


Fig. 3. Time until detachment of 8 different heterotrophic nanoflagellates at different flow velocities. The upper line of the abscissa gives the disc velocity (5 mm above the substrate), while the lower line shows the corresponding flow velocities above the substrate (0 to 10 μm). The maximum investigation time was 10 min: (A) *Codonosiga botrylis*, *Entosiphon sulcatum*, *Bodo saltans*, *Cercomonas crassicauda* and *Spumella* sp. and (B) *Anthophysa vegetans*, *Neobodo designis* and *Apusomonas proboscidea*

Effects of flow velocity on the distribution of flagellates in relation to surface topography

The effect of substrate topography in the microscale using shells of the snail *Ancylus fluviatilis* as a model was studied by analysing the distribution patterns of 2 flagellate species, *Entosiphon sulcatum* and *Cercomonas crassicauda*. The highest abundance of *Entosiphon* was found close to the shell, on the lee

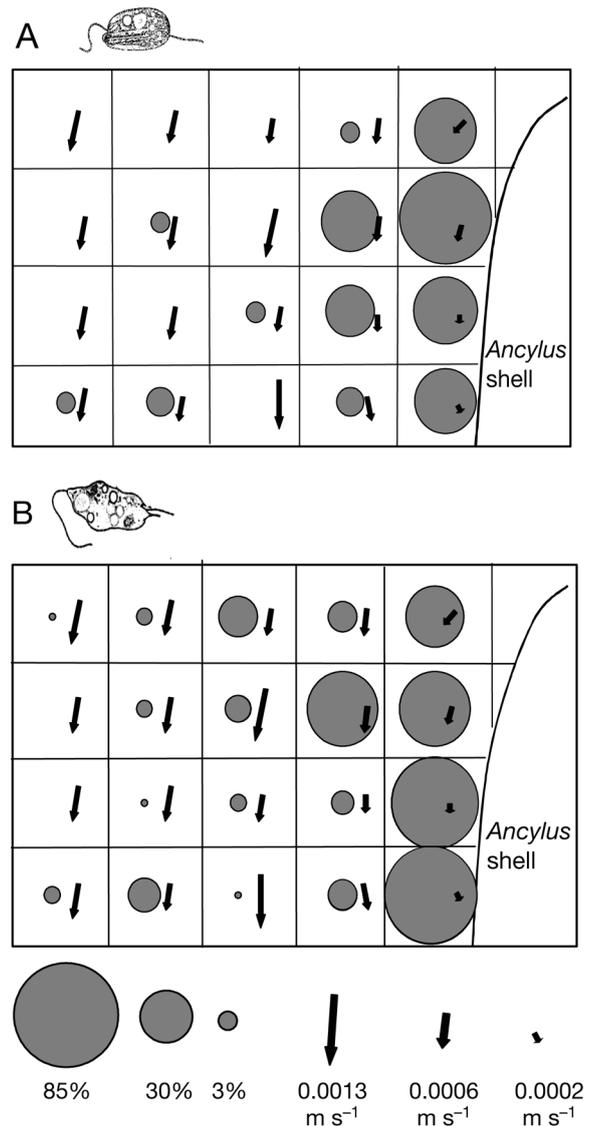


Fig. 4. (A) *Entosiphon sulcatum* and (B) *Cercomonas crassicauda*. Distribution behind an *Ancylus* shell exposed to flow for 10 min at a disc velocity of 0.3 m s⁻¹ (mean of 4 experiments for both species). The diameter corresponds to the mean abundance in the fields of observation (92 × 100 μm). The arrows indicate the direction of the water current and the flow velocity above the substrate (in the 0 to 10 μm layer)

side, in the region where flow velocities were lowest. *Cercomonas* showed a similar distribution pattern (Fig. 4). There was a significant negative correlation ($p < 0.05$) between the abundance in the investigated fields of observation and the corresponding flow velocity.

DISCUSSION

We used glass slides as an artificial substrate in order to compare the different behaviours of heterotrophic nanoflagellate species. The addition of snail shells gave an idea of the potential effects of natural microtopographies on biofilms.

It is known that boundary layer flow characteristics in the sub-millimetre range may control pathways and magnitudes of material and gas exchange in the surface micro-layer of sediments that depend on the topography of the sediment surface (e.g. de Beer et al. 1994, 1996, Huettel et al. 2003, Røy et al. 2005). Even today, only very few studies have considered the effects on microfauna (e.g. Jakobsen 2001, Vopel et al. 2002, 2005) and the influence of flow velocity on nano-fauna ($< 20 \mu\text{m}$) has not yet been studied. Our microscopic technique gave us the opportunity to study the effect of flow on heterotrophic nanoflagellates in the laminar layer at intermediate Reynolds numbers (0.91 to 12.42; e.g. Smith 1975, Vogel 1981). We found significant effects on the attachment of heterotrophic flagellates already at very low flow velocities directly above the substrate (0.0003 to 0.004 m s^{-1}). We hypothesised that when such small flow velocities affect the occurrence of different nanoflagellates, small changes in the surface topography significantly influence the distribution patterns of flow-sensitive flagellates. Our experiments with *Entosiphon* and *Cercomonas* supported this hypothesis.

Another important result was that flow speeds of only a few millimetres s^{-1} had significantly different effects on the attachment of diverse flagellate species. Heterotrophic nanoflagellates may differ significantly with regard to their ecology and behaviour (Fenchel 1987, Arndt et al. 2000). The large *Cercomonas* species showed the lowest resistance towards the flow. This species lives generally within thick sediment layers and is not adapted to attach closely to the surface. *Spumella* has a low resistance as well. The thin plasmatic thread by which the single cells are attached to the substratum is suitable for attaching to suspended particles, but it does not withstand strong turbulence well. This is in agreement with observations of field biofilms (e.g. from rivers: M. Willkomm unpubl. data), where *Spumella* forms only a minor part of the biofilm community, in contrast to its major contribution to the

plankton community. The calculation of drag according to Silvester & Sleigh (1985) revealed a force of $1.6 \times 10^{-15} \text{ Nm}$ to be strong enough to detach *Spumella* from surface, while *Bodo saltans* seems to be adapted to withstand a 2 to 3 times higher drag force. The other investigated colourless chryomonad *Anthophysa* is much better protected from high flow velocities. Colonies are attached via a flexible, excreted, extracellular polymer matrix. Though the flow velocity increases with increasing distance from the substrate, colonies are pressed by the flow towards the substrate, thus avoiding high flow velocities. Similar behaviour was described by Vopel et al. (2005) for peritrich colonies; when colonies are disrupted by high flow velocities, whole colonies or single cells break free and swim individually in the water column until they find more suitable habitats for attachment. *Codonosiga*, the other colony forming flagellates, has a much more rigid stalk made of proteinaceous substances, allowing this choanoflagellate species to withstand even high flow velocities. This species regularly populates biofilms of rivers in high densities (M. Willkomm & M. Esser unpubl. data). The speciality of the kinetoplastid *B. saltans* is its strong attachment by the tip of the posterior flagellum, which explains its attachment even at relatively high flow velocities. This species occurs at extremely high turbulences due to artificial aeration in waste water treatment plants. The colourless, gliding, euglenid *Entosiphon* showed the highest resistance towards high flow velocities. It has a dorsoventrally flattened pellicle and a shape reminiscent of the body shape of torrenticolous insects. The shape seems to be optimised for a current-sheltered life on surfaces.

Statzner (1981) and Statzner & Holm (1989) characterised the flow field around shells of *Ancylus fluviatilis*, a typical rheophilous organism of running waters (Elser 1999). Our experiments in the microscale showed that there is still water motion behind the shells, though at very low flow velocities. However, even these low flow velocities (0.0002 to 0.002 m s^{-1}) lead to characteristic micro-distributions of *Entosiphon* and *Cercomonas* accumulating in the region with lowest flow velocities (0.0002 to 0.0005 m s^{-1}). We conclude that the structure of biofilms and specific flow fields around objects and organisms inside the laminar flow region (e.g. sand grains, invertebrates, 3-dimensional structures of bacterial and/or algal biofilms) influences the micro-distribution and community structure of nano- and microfauna communities. We observed a behaviour typical of *Entosiphon* and *Cercomonas* in relationship to the flow velocity: at high flow velocities, the flagellates actively searched for areas of low flow velocity. Furthermore, in the absence of structures, both flagellates stopped movement at high flow velocities until detachment. For planktonic

ciliates, it is known that they may react to flow velocities created, e.g. by the filter current of copepods (Jakobsen 2001).

The bulk of microbial activity in streams occurs on biofilms (Bryers 1982), and colonisation processes seem to be strongly influenced by surface microtopography, which, in turn, should govern the community structure of nano- and microfauna in lotic environments. Understanding the interactions between physical and biological processes in the micro-scale seems to be an important prerequisite in order to analyse microbial life in the water.

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