Negative consequences of glacial turbidity for the survival of freshwater planktonic heterotrophic flagellates

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Heterotrophic (phagotrophic) flagellates are key components of planktonic food webs in freshwater and marine ecosystems because they are the main consumers of bacteria. Although they are ubiquitous in aquatic ecosystems, they were numerically undetectable in turbid glacier-fed lakes. Here we show that glacial particles had negative effects on the survival and growth of heterotrophic flagellates. The effect of glacial particles was concentration-dependent and was caused by their interference with bacterial uptake rather than by physical damage. These results are the first to reveal why establishment of heterotrophic flagellates populations is hindered in very turbid glacial lakes. Because glaciers are vanishing around the world, recently formed turbid meltwater lakes represent an excellent opportunity to understand the environmental conditions that probably shaped the establishment of lake communities at the end of the last glaciation.

One of the most prominent signs of climate change is the worldwide retreat of glaciers1,2. One consequence of vanishing glaciers is the on-going formation of many new proglacial lakes around the world3. Proglacial lakes are characterized by high water turbidity produced by minerogenic particles, so-called glacial ‘flour’. Glacial particles originate from the crushing and abrasion of bedrock by the glacier and, due to their small size, they can be suspended in the water column for long periods4. The recent climatic warming and associated melting of glaciers can further increase the turbidity of lakes connected to glaciers and extend the periods of high particle loads during the ice-free season. On the other hand, when lakes become disconnected from glacial influence, they turn clear.

Little is known about the effect of glacial ‘flour’ on lake biota, but filter-feeding species such as the water flea Daphnia are usually absent in oligotrophic lakes influenced by high glacial ‘flour’ concentrations5 and the vertical distribution and community composition of phytoplankton and zooplankton is affected by the discharge of turbid glacial meltwaters into clear lakes6. Paleoecological studies have also documented extensive collapse of lake ecosystems during the last glacial maximum7, and the discharge of glacial turbid water into coastal waters has been putatively associated with increased zooplankton mortality8. The presence of large amounts of suspended minerogenic particles probably has negative consequences for other taxa of planktonic organisms such as heterotrophic nanoflagellates (HNF). Together with viruses, this group of organisms, usually described as bacterivores or phagotrophic flagellates, is one of the main agents of mortality for prokaryotes. Heterotrophic nanoflagellates are ubiquitous in marine ecosystems, lakes, rivers, and estuaries9 where they typically reach abundances between 100 and 10,000 cells ml⁻¹. Because they channel organic matter to larger planktonic organisms10, and they are so abundant, they play a pivotal role in aquatic ecosystems.

In this work, we investigate whether suspended glacial particles have a negative effect on HNF in order to explain their apparent absence in turbid glacial alpine lakes. We compare the effect of glacial ‘flour’ on a natural flagellate community, as well as on experimental model flagellate species having different nutritional strategies. Further, we test the potential of glacial particles to interfere with HNF feeding. Since we expected the glacial particles to have different characteristics compared to those found in other turbid systems (e.g., shallow turbid lakes where turbidity is increased through sediment resuspension)10, we also investigate their organic carbon coating and particle size distribution. We hypothesized that glacial particles negatively affect the growth/survival of strict phagotrophic nanoflagellates due to their interference with bacteria, the dominant prey. We further anticipated that physical damage by ingestion of sub-micron minerogenic particles increases flagellate mortality and that these two mechanisms operate at different time scales (e.g., shorter in the case of physical damage). Therefore, we expected that addition of glacial ‘flour’ to a natural HNF assemblage from a clear lake, at
concentrations found in turbid glacial lakes, will cause a significant decrease in flagellate survival. Finally, we expected that mixotrophic flagellate species might cope with the interference of glacial particles because they are able to shift their nutritional strategy to a photosynthetic one.

**Results**

**Characterization of glacial particles.** Scanning electron microscopy (SEM) revealed that glacial particles were characterized by sharp edges (Fig. 1) and, in the youngest proglacial turbid lake (FAS1), were dominated by muscovite/illite (57%) followed by chlorite (31%) and quartz (12%). In older lakes in the deglaciation chronosequence, such as in Lake FAS3, particles were similarly dominated by muscovite/illite (55%), but also calcite (22%) was present. These clays are grey-white to silvery-white, which is also the typical hue of the water in new proglacial lakes (see Supplementary Fig. S1 online). Under the epifluorescence microscope, the particles fluoresced yellow (Supplementary Fig. S2 online). The average relative content of carbon and nitrogen in the glacial ‘flour’ was 0.47% and 0.060%, respectively, which is consistent with the low weight-loss-on-ignition value (0.81%). Most of the particles (98%) - analyzed using a Coulter Counter - were in the size range 0.7–4 μm and at 14 nephelometric turbidity units (NTU), their abundance represented 2.83 × 10⁶ ml⁻¹ (Supplementary Fig. S3 online). Laser diffractometry revealed that particles <2 μm represented a low percentage of the scattered volume (<3%), whereas 50% of the scattered volume was produced by particles <25 μm (Supplementary Fig. S3).

**Abundance of bacteria and HNF in clear and turbid FAS lakes.** The abundance of HNF in Lake FAS4, a system that is disconnected from the glacier and became clear (Supplementary Fig. S1, S2), ranged from 0.85 to 2.57 × 10³ cells ml⁻¹ and was higher in deep water layers than at the surface (Supplementary Table S1). In the turbid lakes, FAS1 and FAS3, HNF were not observed despite our having filtered large volumes of water. The addition of a HNF culture to those samples resulted in a positive detection. In contrast, the abundance of autotrophic nanoflagellates was higher in Lake FAS3, followed by FAS4 and FAS1 (Supplementary Table S1 online). Bacterial abundance was lowest in FAS1 (1.93 × 10⁵ cells ml⁻¹) and highest in FAS4 with the maximum (8.67 × 10⁵ cells ml⁻¹) observed in deep water layers (Supplementary Table S1).

**Effect of glacial ‘flour’ on a natural flagellate community.** To quantify the effect of glacial particles on a natural flagellate community, two different turbidity concentrations were tested on a sample collected from Lake FAS4. There was no significant difference in HNF abundance in the control, from the start to the end of the experiment (Holm-Sidak post-hoc test, p = 0.445) (Fig. 2a). However, in both treatments, the number of HNF dropped significantly after one week in comparison to the control (ANOVA F (3,8) = 13.62, p = 0.002, all pairwise multiple comparisons with Holm-Sidak significant to p < 0.01). The decrease in HNF cells was most evident in the 30 NTU treatment. Bacterial abundance in the control and in the 14 NTU and 30 NTU treatments increased significantly from the beginning to the end of the experiment (F (3,8) = 8.69, p = 0.007). However, after 1 week, bacterial abundance was not significantly different between the control and the two treatments (Holm-Sidak post-hoc test). As a consequence of the change in bacterial abundance, the bacteria (1–3 μm):particle ratio was higher at the end of the experiment in both treatments (Supplementary Fig. S4 online).

**Effect of glacial ‘flour’ on experimental model flagellate species.** The abundance of the strict heterotrophic *Spumella* sp. was significantly different among treatments and control (F (4,3) = 5.99, p = 0.01, Fig. 3). The negative effect of glacial ‘flour’ on growth was clearly concentration-dependent (30 NTU > 14 NTU > 7 NTU), though the difference between the control and the 7 NTU treatment was not significant (Holm-Sidak post-hoc test p = 0.096). In contrast, in experiments with the mixotrophic species *Dinobryon divergens* conducted under dark and light conditions, no
significant difference was found between its abundance in the control and the treatment (experiment with PAR: $F_{(4,1)} = 5.36, p < 0.05$). Thus, whereas the uptake rate (UR) in the control was $5.16$ FLB cell$^{-1}$ h$^{-1}$ corresponding to a clearance rate (CR) of $0.023$ nL cell$^{-1}$ h$^{-1}$, in the treatment, the UR was $1.14$ FLB cell$^{-1}$ h$^{-1}$ and the CR was $0.0051$ nL cell$^{-1}$ h$^{-1}$.

**Discussion**

Our results clearly demonstrate that the presence of glacial particles had a negative effect on the survival of a natural HNF community (Fig. 2a). Furthermore, the negative effect of the glacial ‘flour’ on the HNF community was concentration-dependent, i.e., the highest mortality was observed in the treatment with the highest turbidity. Overall, these results suggest that members of a natural HNF community from clear water lakes, such as that from Lake FAS4, will not survive under conditions of high mineral particle concentration and that glacial ‘flour’ hinders the establishment of HNF populations in glacial turbid lakes. Although we did not identify which HNF taxa were present in this natural community and, consequently, whether all species were affected in the same way, the large decrease in abundance suggests that the negative effect was widespread. There are also other potential factors which could decrease the chances of colonization of (pro)glacial lakes by HNF. One could be food limitation. This apparently occurs when bacterial numbers are $<10^6$ cells ml$^{-1}$ (Ref. 9). Thus, bacterial abundances of $10^5$ ml$^{-1}$ typically found in alpine lakes such as the FAS suggest that HNF could be at times food-limited. However, in alpine clear water lakes, HNF populations exist indicating that bacterial abundance is not relevant for establishing a community. In fact, the clear water Lake FAS4 had a bacterial abundance similar to that of Lake FAS3, but HNF were present. Nevertheless, the presence of high numbers of particles implies that search for and handling of a bacterium by a HNF in a diluted food medium takes more time and uses more energy.11

The results from the experiments with different flagellate species provided evidence for the mechanism behind the negative effect of glacial particles. The experiment with *Spumella* sp. (Fig. 3) clearly showed that growth was negatively affected by glacial ‘flour’ in a concentration-dependent manner and that HNF growth reduction is caused by a reduced grazing efficiency (Fig. 5). In the absence of glacial ‘flour’, the uptake and clearance rates were comparable to those reported for *Spumella* species with the same cell size range as

![Figure 3](image-url) | Growth of *Spumella* sp. in the control and in the presence of glacial ‘flour’ at 7, 14, and 30 NTU. Error bars represent ± 1SD.

![Figure 4](image-url) | Changes over time in the abundance of *Dinobryon divergens* when grown in the presence of light (A) or in darkness (B), and in the absence (control) and in the presence of glacial ‘flour’ (treatment). Error bars represent ± 1SD.

![Figure 5](image-url) | Changes over time in the average number of fluorescently labeled bacteria (FLB) ingested per *Spumella* cell in the absence (control) and in the presence of glacial ‘flour’ (treatment) corresponding to 14 NTU. Error bars represent ± 1SD.
the strain used in our experiments. In our experiments with Spumella sp., the bacteria:particle ratio was much higher than that found in proglacial lakes such as in Lake FAS1 (Supplementary Fig. S4). However, despite the potential higher contact rate between flagellates and bacteria, a negative effect was evident. A much stronger negative effect can be expected when the bacteria:particles ratio is low as found in lakes FAS1 or FAS3. This is feasible because the results from the size distribution analysis indicated that a significant fraction of the glacial particles (Supplementary Fig. S3) lies within the size range of bacteria preferentially consumed (1–3 μm) by phagotrophic flagellates. In this respect, our results resemble those obtained for Daphnia, for which survivorship in the presence of glacial ‘flour’ was dependent on the algal biomass available.

Another potential mechanism for the reduced HNF survival in the presence of glacial ‘flour’ can be physical damage. In fact, glacially- and glacially-derived suspended particles had sharp edges (Fig. 1) and were dominant in the presence of glacial ‘flour’ can be physical damage. In fact, glacially-derived suspended particles had sharp edges (Fig. 1) and were dominant (Fig. 1) and were dominant (Fig. 1) and were dominant (Fig. 1) and were dominant. In this respect, our results resemble those obtained for Daphnia, for which survivorship in the presence of glacial ‘flour’ was dependent on the algal biomass available.

Abundance of bacteria and flagellates in clear and turbid FAS lakes. To obtain reference values on the bacterial and flagellate abundance in the Faselfad lakes, water samples were collected from FAS1 (1 m depth), FAS3 (1 m, 6 m, 10 m, 15 m depth), and clear FAS4 (1 m, 6 m, 10 m, 13 m depth) during a summer field campaign. Water samples were collected using the same type of sampler, fixed in the field with formalin (2% final concentration), and transported by helicopter to the laboratory in Innsbruck in containers that were kept cool and dark.

For the analysis of bacterial abundance, subsamples of 5 to 15 mL were stained for 30 min with the fluorescent dye SYTO 9, which binds to DNA. After staining, the samples were filtered, and the number of bacteria was counted using epifluorescence microscopy (Zeiss AxioImager A1) at a magnification of 400x. The abundance of bacteria was determined by counting at least 1000 cells for each sample.

Effect of glacial ‘flour’ on a natural flagellate community from a clear high mountain lake. On 5 July 2011, a 5 L water sample was collected from Lake FAS4 at 13 m depth (typically the depth at which there was maximum HNF abundance) and transported to the laboratory in Innsbruck by helicopter in a plastic carboy that was kept in the dark and close to the in situ temperature. This sample was used to test the effect of two different particle concentrations (14 NTU, 57 mg L⁻¹ and 30 NTU, 113 mg L⁻¹) on the natural assemblage of flagellates. To exclude large organisms, the sample was screened through a zooplankton net (45 μm mesh size) and distributed into 2 ml glass bottles that were daily shaken to avoid sedimentation. The experiment was conducted in the darkness and at 15 °C using three replicates for the treatments and control (i.e., no particles added), respectively. Bottles were briefly opened every day to allow exchange of air. The abundance of bacteria and HNF were determined at the start of the experiment and after 1 week as described above.

Test organisms and culture conditions. Most experiments were done with the HNF species Spumella sp. strain JBM10 (diameter 4–7 μm), which is a common cryptomonad genus in freshwater communities. This species can be classified as an interception feeder that grazes on small- to medium-sized bacteria. For comparison with another phagotropic, but at the same time phototrophic (i.e., mixotrophic) species, we included the chrysophycean Dinobryon divergens (length 12–15 μm), which is a flagellate that often forms colonies and is commonly found in freshwaters and also in high mountain lakes. Spumella sp. and D. divergens were provided courtesy of Jens Boenigk. Spumella sp. was grown in inorganic basal medium (IBM) supplemented with 2.5 mg L⁻¹ of glucose and 40 mg L⁻¹ of peptone. D. divergens was cultured in WCg-medium. Cultures were kept in an environmental chamber with a light-dark photoperiod of 8 h:16 h in transparent 50 mL polystyrene tubes at 23 °C and were transferred to fresh medium every 2–3 weeks.

Effect of glacial ‘flour’ on flagellate growth. Two days prior to the experiments, 1 mL of culture and 9 mL of fresh medium were transferred into a 15 mL tube. At the start of the experiment, 100 μL of culture and 900 μL of fresh medium were transferred into 2 ml Eppendorf vials. The experiment was run using five replicates for the treatment and control (i.e., no glacial ‘flour’ added), respectively. Glacial particles were added to the experiment at a concentration of 14 NTU and at 7, 14, 30 NTU in the case of Spumella sp. These turbidity values are moderate and can be one order of magnitude higher in other turbid glacial meltwater lakes. The experiments were conducted at 15 °C in darkness, except for D. divergens for which additionally the pH was tested in the presence of light, with a light-dark photoperiod of 8:16 h at 23 °C. The pH-value in the treatments ‘flour’ was checked after addition of the glacial ‘flour’. This measurement revealed that addition of glacial ‘flour’ did not extensively alter the pH (control 7.09, treatment 7.05). To avoid sedimentation of the particles, all vials (i.e., also the controls) were placed in a rotator (VWR Tube Rotator) that has a fixed speed of 18 rpm. Rotation
mode was adjusted to 15 min (on) and 45 min (off) cycles to avoid potential shear stress on the flagellates. Changes in flagellate abundance over time were assessed by counting cells in drops of 0.5–2 μL under an Olympus BX 50 (brightfield, digital interference contrast) microscope at 40–200× magnification25. At least 200 cells were counted at time intervals of 24 h. Bacterial numbers were counted at the same time intervals as described above.

Short-term uptake experiment with fluorescently labeled bacteria. The grazing rate of Spumella sp. was determined by using fluorescently labeled bacteria (FLB) as surrogates26. Bacteria were stained with the yellow-green fluorescent dye, 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF). The bacteria used to prepare the FLB were those growing together with Spumella sp. and are a mixed culture. To remove the flagellates, the medium was filtered through a 2 μm pore size polycarbonate filter. To estimate the concentration of FLB in the suspension, cell abundance in a small sample (0.5 ml) was counted under the epifluorescence microscope at 1250× magnification. For the experiment, the Spumella sp. culture (10 ml) was first inoculated into a 500 ml glass bottle holding 390 ml IBM medium and, after 5 days, the dense flagellate culture was transferred to 50 ml tubes with three replicates for the treatment and control, respectively. To estimate the required volume of FLB to add (10–15% of the total bacteria abundance, ref. 28), a sample (2 ml) was fixed and stained and bacteria enumerated as described above. Control and treatment tubes were then supplemented with FLB and FLB plus glacial particles (57 mg L⁻¹ respectively). To estimate the required volume of FLB to add (10–15% of the total bacteria abundance, ref. 28), a sample (2 ml) was fixed and stained and bacteria enumerated as described above. Control and treatment tubes were then supplemented with FLB and FLB plus glacial particles (57 mg L⁻¹ respectively). The experiment was run in the darkness at 16 °C. Subsamples of 5 ml were taken at time 0 and after 2, 5, 10, and 15 min. The subsamples were immediately fixed with 0.25 μL alkaline Lugol’s solution (0.5%) and 0.25 ml buffered formalin (40%), and stored in the dark at 4 °C until processed within 24 h. Subsamples were then stained with DAPI and concentrated onto black polycarbonate filters (0.5 μm pore-size). Flagellates were examined under an epifluorescence microscope at a magnification of 1250×. The number of ingested FLB per individual was assessed by switching between the filter-set for UV (DAPI) and blue light excitation (DTAF). At least 60 individuals were checked on each filter. The filter- set for UV (DAPI) and blue light excitation (DAPI) and blue light excitation (DTAF). At least 60 individuals were checked on each filter. The filter- set for UV (DAPI) and blue light excitation (DTAF). At least 60 individuals were checked on each filter. The clearance rate (CR) in nL cell⁻¹ h⁻¹ was calculated by dividing the UR by the number of FLB used in the experiment29.

Statistics. Results of the experiments on the effect of glacial ‘foul’ on the short-term uptake experiment were analyzed by comparing the slopes of linear regressions between the control and treatments with a student t-test 29. The results from the uptake experiment were analyzed by comparing the slopes of linear regressions between the control and treatments with a student t-test 29. The results from the uptake experiment were analyzed by comparing the slopes of linear regressions between the control and treatments with a student t-test 29.

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Author contributions
R.S. designed the experiments and G.K. performed the experiments and field sampling. Both authors wrote the manuscript and prepared the figures.

Additional information
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