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# LIMNOLOGY and OCEANOGRAPHY



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# Mechanisms and fluid dynamics of foraging in heterotrophic nanoflagellates

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#### Abstract

Heterotrophic nanoflagellates are the main consumers of bacteria and picophytoplankton in the ocean. In their microscale world, viscosity impedes predator–prey contact, and the mechanisms that allow flagellates to daily clear a volume of water for prey corresponding to 10<sup>6</sup> times their own volume is unclear. It is also unclear what limits observed maximum ingestion rates of about 10<sup>4</sup> bacterial preys per day. We used high-speed video microscopy to describe feeding flows, flagellum kinematics, and prey searching, capture, and handling in four species with different foraging strategies. In three species, prey handling times limit ingestion rates and account well for their reported maximum values. Similarly, observed feeding flows match reported clearance rates. Simple point force models allowed us to estimate the forces required to generate the feeding flows, between 4 and 13 pN, and consistent with the force produced by the hairy (hispid) flagellum, as estimated using resistive force theory. Hispid flagella can produce a force that is much higher than the force produced by a naked (smooth) flagellum with similar kinematics, and the hairy flagellum is therefore key to foraging in most nanoflagellates. Our findings provide a mechanistic underpinning of observed functional responses of prey ingestion rates in nanoflagellates.

Heterotrophic nanoflagellates play a key role in microbial food webs in the oceans by feeding on phytoplankton and bacteria and by transferring primary production to higher trophic levels when grazed upon. Their top-down control shapes the structure and function of microbial communities and mediate essential biogeochemical cycles in the sea (Fenchel 1982a; Azam et al. 1983; Worden et al. 2015). Despite their importance, the mechanisms of prey capture and the processes limiting their ingestion rates are not fully understood (Boenigk and Arndt 2002; Weisse et al. 2016).

Flagellates live in a low Reynolds number world where viscosity impedes predator–prey contact (Jabbarzadeh and Fu 2018). Yet, nanoflagellates are capable of daily clearing a volume of water for prey that corresponds to about 1 million times their cell volume, which is equivalent to a significant fraction of their cell volume per flagellar beat period (Hansen

et al. 1997; Kiørboe and Hirst 2014). In the nutritionally dilute ocean, this is the clearance rate needed to sustain a viable population in the face of predation mortality (Kiørboe 2011). How the flagellates overcome the impeding effect of viscosity is unclear for many forms.

Most flagellates use their flagella to swim, to generate feeding currents, and to capture prev. Many studies have examined the fluid dynamics of flagellates from the perspective of swimming (Lauga 2020), but few have done so from the perspective of feeding on particulate food particles (Christensen-Dalsgaard and Fenchel 2003; Dölger et al. 2017; Nielsen et al. 2017), even though feeding is likely a more fundamental component of the fitness than propulsion per se. In a few cases, the flagellum forces have been estimated indirectly from swimming speeds (Christensen-Dalsgaard and Fenchel 2003) or from quantification of feeding flows (Roper et al. 2013; Nielsen et al. 2017). However, there is large variation in flagellar kinematics and arrangements between species that yields big differences in the strength and architecture of the feeding flows (Nielsen and Kiørboe 2021). In most cases, the forces generated by the flagellum and required to account for the necessary high clearance rates are unknown.

Direct observations of flagellate feeding were pioneered by Sleigh and Fenchel (Sleigh 1964; Fenchel 1982b), and followed by few additional studies (Ishigaki and Terazaki 1998; Boenigk and Arndt 2000a; Pfandl et al. 2004). These studies revealed a variety of prey acquisition and handling strategies. Prey is

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either intercepted by the cell body, a flagellum, or specialized structures, and then either rejected or transported to the spot on the cell surface where it is phagocytized. During capture and handling of prey, the feeding current may cease, and no further prey can be captured (Ishigaki and Terazaki 1998; Boenigk and Arndt 2000b). Handling time may therefore put an upper limit on prey ingestion rate. The maximum clearance rate governed by the feeding current, and the maximum ingestion rate, potentially governed by the prey handling time, together describe the functional response of the prey ingestion rate as function of prey concentration. This is the key function characterizing predator–prey interactions.

The aims of this study are twofold: (1) to understand what allows heterotrophic nanoflagellates to overcome the impeding effect of viscosity and (2) to provide a mechanistic underpinning of the functional response relations that have been obtained in incubation experiments (Hansen et al. 1997; Kiørboe and Hirst 2014). We build on and expand previous observational work on flagellate foraging, and we describe predator-prey encounters and prey handling in four species with characteristic predation modes. We portray the flagellar dynamics during the different grazing phases and quantify prey handling times to evaluate the potential for prey ingestion. We further quantify the feeding flow, estimate clearance rates from observed flow fields, and use simple fluid dynamic models to compute the forces needed to account for the observed flows as well as the forces that the flagellum produces.

#### Materials and methods

#### Study organisms, isolation, and culturing

The four selected marine flagellate species Pteridomonas danica, Paraphysomonas foraminifera, Cafeteria roenbergensis,



**Fig. 1.** Phase contrast micrographs and drawings of flagellates *P. foraminifera* (**a**, **e**), *P. danica* (**b**, **f**), *C. roenbergensis* (**c**, **g**), and *Pseudobodo* sp. (**d**, **h**). Abbreviations in drawings (**e**–**h**): C, cell; F, flagellum; SF, short flagellum; T, tentacles; PF, posterior flagellum; and S, surface. Scale bar = 5  $\mu$ m.

and *Pseudobodo* sp. are direct interception feeders (Fig. 1). They have a hairy (hispid) flagellum that drives the feeding current toward the cell, and in the opposite direction of the propagating flagellum wave. *C. roenbergensis* has been a key laboratory species, as its different feeding phases are easy to distinguish (Ishigaki and Terazaki 1998; Boenigk and Arndt 2000b). Christensen-Dalsgaard and Fenchel explored the fluid dynamics of *P. danica* and *Paraphysomonas vestita*; the latter sharing the genus with *P. foraminifera* (Fenchel 1982b; Christensen-Dalsgaard and Fenchel 2003). The feeding behavior of a close relative of *P. danica, Actinomonas* sp. has been studied (Sleigh 1964; Fenchel 1982b; Ishigaki and Terazaki 1998), and the predation mode of *Pseudobodo* sp. has been described briefly (Fenchel 1982b). Additionally, we made a few observations of *Ochromonas moestrupii* and *Chrysophyacea* sp.

All species were isolated from shallow (30 m) coastal waters (Øresund and Isefjord, Denmark). Cultures were maintained in the dark at 18°C in filtered and pasteurized North Sea water (salinity 30‰), using rice grains to feed the naturally occurring bacteria that served as prey. Species identification was verified using 18s rDNA molecular analysis except for *Pseudobodo* sp., which was morphologically matched with the description of *Pseudobodo tremulans* (Fenchel 1982b).

#### Microscopy and image analysis

A glass ring (16 mm inner diameter and 3 mm height) was fixed on a glass slide using stopcock grease, filled with 600  $\mu$ L of the culture, and covered with a glass coverslip. The sample was observed 5 min later to allow the flagellates to attach to the coverslip. The heating effects of light were reduced by having short periods of exposure (< 5 min) at moderate intensities during recordings. Room temperature was 16–20°C, and experiments did not last more than 1.5 h. Food particles consisted of naturally occurring bacteria and particulate organic matter. For cultures with low bacterial abundance, polystyrene microbeads (0.5  $\mu$ m in diameter and treated with Bovine Serum Albumine to avoid aggregation) were added (10<sup>-5</sup>%) to increase the rate of particle encounters.

Observations were carried out with an inverted microscope Olympus IX71, using an Olympus UPlanSApo oil immersion objective  $\times 100/1.40$  or an Olympus UPLanFL N oil immersion  $\times 100/1.30$  objective for phase contrast imaging. Recordings were carried out with a high-speed camera (Phantom Miro LAB 320). Prey captures were recorded at 500 frames per second (fps), and particle tracking was performed at 250 fps. Videos had a minimum resolution of  $512 \times 512$  pixels. The image analysis software ImageJ (Fiji) was used for detailed predator–prey interaction observations, morphometric measurements, and manual particle tracking (Schindelin et al. 2012; Rueden et al. 2017).

#### Predation behavior and time budget analysis

Predation was divided into five stages, largely following Montagnes et al. (2008): searching, contact, capture, and

ingestion or rejection. A rejection was defined as an active release of the prey, in contrast to a loss of prey. The handling time was defined as the duration of the period where the flagellum does not produce a feeding current and another prey cannot be encountered. Handling time does not necessarily commence upon prey contact, and it can end before or after the particle is fully ingested. Prey processing had three possible outcomes: ingestion, rejection, or loss. In total, 40 prey handling durations (20 ingestions and 20 rejections) were recorded for each species. In addition, 20 "lash rejections" by *Pseudobodo* sp. were analyzed (Supplementary Table S2).

#### Particle tracking and clearance rates

Flow fields were mapped by particle tracking. A particle was followed from a minimum distance of one cell length from the body, until it was either captured or it had gone well past the flagellate. We recorded 11-15 tracks per individual, studying two individuals per species. Most flagellates slightly shift their orientation while foraging (Supplementary Table S3), and the particle tracks are therefore shown relative to the observed flagellum coordinates. An imaginary, circular filtering area (clearance disc) for prey capture was assumed in front of the cell and perpendicular to the feeding flow. The size of the disc was defined by the tracks of particles that were captured or strongly interacted with the flagellate. A leastsquares five-point, centered finite difference scheme was applied to calculate the particle velocities from the measured particle positions. The average velocity component perpendicular to the clearance disc was used to calculate the clearance rate.

#### Model of the feeding flow

To describe the flow fields and estimate the flow-generating forces from the observed feeding currents we used a point force model (Blake 1971; Fenchel 1986; Rode et al. 2020). The low-Reynolds-number model describes the flow due to a point force above a plane no-slip surface. We examined two situations in which the force direction is either perpendicular (*P. danica, P. foraminifera*) or parallel to the surface (*Pseudobodo* sp., *C. roenbergensis*). In both cases, we use *F* to denote the magnitude of the force and *h* its height above the surface. In the perpendicular case, the flow has rotational symmetry, and the streamlines are the contour lines of the Stokes stream function:

$$\Psi(s,z) = \frac{Fs^2}{8\pi\mu} \left( \frac{1}{\sqrt{s^2 + (z-h)^2}} - \frac{1}{\sqrt{s^2 + (z+h)^2}} - \frac{2hz}{\left(s^2 + (z+h)^2\right)^{3/2}} \right)$$
(1)

where  $\mu$  denotes the dynamic viscosity, *s* the radial distance from the axis of symmetry, and *z* the height above the surface (Aderogba and Blake 1978; Blake and Otto 1996). Using the stream function, we can derive the clearance rate, *Q*, through a circular clearance disc centered on the axis of symmetry and oriented perpendicular to it:

$$Q = \frac{Fa^2}{4\mu} \left( \frac{1}{\sqrt{a^2 + (d-h)^2}} - \frac{1}{\sqrt{a^2 + (d+h)^2}} - \frac{2hd}{\left(a^2 + (d+h)^2\right)^{3/2}} \right)$$
(2)

where *a* denotes the radius of the clearance disc and *d* its height above the surface (Rode et al. 2020). The equation allows us to estimate the magnitude of the point force, *F*, using our clearance rate estimate obtained with particle tracking. In the parallel case, the flow does not have rotational symmetry and a Stokes stream function does not exist. We therefore integrate the velocity field numerically to obtain streamlines (Blake and Chwang 1974; Rode et al. 2020). To estimate the clearance rate through a circular clearance disc that is perpendicular to the direction of the point force and positioned the distance *h* above the surface in the symmetry plane of the flow, we assume that  $a \ll h$  and approximate the effect of the image system that ensures that the no-slip boundary condition is satisfied (Rode et al. 2020). We find the approximation:

$$Q \approx \frac{Fa^2}{4\mu} \left( \frac{1}{\sqrt{a^2 + l^2}} - \frac{l^2}{\left(4h^2 + l^2\right)^{3/2}} - \frac{12h^4}{\left(4h^2 + l^2\right)^{5/2}} \right)$$
(3)

where *l* denotes the distance between the position of the point force and the center of the clearance disc.

#### The force created by a hairy flagellum

To estimate the flow-generating force of *P. danica* directly from the observed motion of the flagellum, we used resistive force theory (Brennen 1976; Lauga and Powers 2009; Rodenborn et al. 2013). We assume that the flagellate is tethered, and that the motion of the flagellum is a traveling sine wave with wavelength  $\lambda$  and frequency *f* that is propagating in the positive *z*-direction:

$$x_f(z,t) = A\sin(kz - \omega t) \tag{4}$$

where  $x_f$  denotes the transversal deflection of the flagellum, A the amplitude,  $k = 2\pi/\lambda$  the wave number, and  $\omega = 2\pi f$  the angular frequency. Using resistive force theory, we obtain the component of the force on the flagellum in the *z*-direction:

$$F_{z} = -\mu \left(\xi_{\perp} - \xi_{\parallel}\right) \left(1 - \frac{1}{\sqrt{1 + k^{2} A^{2}}}\right) \frac{L\omega}{k}$$
(5)

where *L* denotes the length of the flagellum, and  $\xi_{\perp}$  and  $\xi_{\parallel}$  are the perpendicular and the parallel force coefficient, respectively (Gray and Hancock 1955; Brennen 1976). For a naked flagellum, we use the dimensionless force coefficients:

$$\xi_{\perp}^{f} = \frac{4\pi}{\ln(2\lambda/b) + 1/2}, \quad \xi_{\parallel}^{f} = \frac{2\pi}{\ln(2\lambda/b) - 1/2}$$
(6)

where *b* denotes the radius of the flagellum (Gray and Hancock 1955; Rodenborn et al. 2013). For a flagellum with two rows of stiff hairs that are in the beat plane and remain perpendicular to the flagellum during the beat, we use the dimensionless force coefficients:

$$\begin{aligned} \xi^{h}_{\perp} = & \frac{4\pi}{\ln(2\lambda/b) + 1/2} + \frac{2\pi\alpha/\chi}{\ln(\alpha/\beta) - 1/2}, \\ \xi^{h}_{\parallel} = & \frac{2\pi}{\ln(2\lambda/b) - 1/2} + \frac{4\pi\alpha/\chi}{\ln(\alpha/\beta) + 1/2} \end{aligned}$$
(7)

where  $\alpha$  denotes the total length of a pair of hairs,  $\beta$  their radius, and  $\chi$  the distance between neighboring pairs of hairs (Holwill and Sleigh 1967; Brennen 1976).

#### Results

#### Prey capture and handling

Supplementary Movies 1–4 illustrate the different behaviors described below; and morphometric data and flagellum properties can be found in Supplementary Table S1.

*P. foraminifera* attaches to the surface by a filamentous structure from the posterior end of the cell. Cells are located directly on the surface or at a distance. On the anterior side there are two flagella, with their base near the ingestion site. When searching for prey, the long flagellum continuously beats in a

curved fashion ( $46 \pm 6$  [mean  $\pm$  SD] Hz) and creates a feeding current toward the cell, while the second shorter flagellum is inactive. When a food particle enters the feeding current, it is pulled toward the flagellate (Fig. 2a). The flagellate responds to the prey before it establishes an observable contact with the flagellum (Fig. 2b). Most likely the first contact is with the invisible flagellar hairs. As also observed by Christensen-Dalsgaard and Fenchel, the presence of prev is followed by a series of changes in flagellar behavior (Christensen-Dalsgaard and Fenchel 2004). The end of the long flagellum hooks over into a fixed position while the wave amplitude and the beating frequency increases  $(67 \pm 8 \text{ Hz})$  and the short flagellum starts beating rapidly  $(104 \pm 15 \text{ Hz})$ . The particle is transported longitudinally until it is confined between the two flagella (Fig. 2c). Finally, the prey is positioned between the short flagellum and the body, ready for phagocytosis (Fig. 2d). During ingestion, three possible scenarios were observed. In the first case, the long flagellum returns to its original position and beating frequency; thus, a feeding current is generated immediately (Fig. 2e). Alternatively, the long flagellum returns to the searching position but with a reduced beating frequency ( $28 \pm 7$  Hz after 2 s); therefore, the flow rate is not restored until after more than 2 s. A third scenario involves an immobilized, stiff, and wavy long flagellum while the short flagellum continued beating until finally pausing. The flagellate remained inactive for a long period, which usually exceeded the recording capacity. Unrecorded observations confirmed that after these long breaks, P. foraminifera starts beating again to search for more prey. To reject a captured particle, the flagellate releases the prey by returning the long flagellum to the original beating pattern and position (Fig. 2f), and then continues creating a feeding current (Fig. 2g).

*O. moestrupii* and *Chrysophyacea* sp. have a similar feeding behavior as *P. foraminifera*. The prevailing difference is their straight, long, beating flagellum  $(52 \pm 9 \text{ and } 50 \pm 5 \text{ Hz},$ 



Fig. 2. Schematic representation of foraging by *P. foraminifera*. Prey handling steps: searching and capture (**a**–**c**); prey ingestion (**d**, **e**); and rejection (**f**, **g**). Figure objects: long and curved flagellum (solid line, red), short flagellum (solid line, blue), ingestion site (circle, green), feeding current (solid curved arrows), and object in motion (solid straight arrows).



**Fig. 3.** Handling times by *P. foraminifera*\*, *C. roenbergensis*, and *Pseudobodo* sp. Boxplots for the durations of ingestions (I), rejections (R), and lash rejections (LR); from prey capture to resuming the feeding current (median: dividing black line in the boxplot; mean: asterisk; error bars: dashed lines; outliers: empty circles). A one-way ANOVA test revealed that the three species of flagellates have statistically significant different mean handling times for ingestions ( $F_{2,49} = [52.58]$ ,  $p = 6.4 \times 10^{-13}$ ) and rejections ( $F_{2,52} = [17.44]$ ,  $p = 1.6 \times 10^{-6}$ ). The lash rejection of *Pseudobodo* sp. was excluded from this analysis. \*Not including the ingestion cases of *P. foraminifera* that were limited by the recording time.



**Fig. 4.** Schematic representation of foraging by *P. danica*. A single flagellum creates a feeding current and particles are captured on the crown of tentacles, and subsequently handled for ingestion or rejection.

respectively) in contrast to the curved flagellum that characterizes *P. foraminifera*. All three species attach posteriorly in the same manner, and contact and handle the prey with comparable flagellar behaviors for ingestions and rejections.

The handling time of *P. foraminifera* starts when the prey establishes contact with the flagellum. Rejected prey is handled more quickly than ingested prey (Fig. 3; Supplementary Table S2).

Handling times were independent of prey size over the range of encountered prey sizes (Supporting Information Fig. S1). When the flagellate stopped during an ingestion, the handling time ended when the flagellum reactivated, and the feeding current was restored. This frozen flagellum behavior was reported in 6 out of the 20 ingestions, with 5 exceeding the remaining recording time of 9–45 s.

*P. danica* is attached to the surface with a posterior stalk (Fig. 4). Its flagellum beats in a plane with constant frequency, creating a current toward the cell and perpendicular to the attachment surface as also described by Christensen-Dalsgaard and Fenchel (2004). Prey arriving in the flow is intercepted by the tentacle "crown." When food is captured by the tentacles, it is slowly transported toward the cell for phagocytosis. Some particles move outward and accumulate at the tentacle tips before drifting away. The beating pattern of the flagellate remains uniform throughout all prey encounters, behaving purely like a filtering predator. More than one food particle can be captured or handled simultaneously, and the handling time for *P. danica* is therefore zero.

When sessile, C. roenbergensis attaches to the surface with the tip of the posterior flagellum that flexes at irregular intervals. The anterior flagellum beats with constant frequency in a three-dimensional pattern with separate power and recovery phases to create a slightly erratic feeding current parallel to the attachment surface (Fig. 5a). As previously observed (Boenigk and Arndt 2000b), prey particles are intercepted by the cell, not the flagellum (Fig. 5b). Upon prey contact on the sensitive frontal side of the predator, the anterior flagellum stops beating and rapidly arches fully extended against the prey. Thus, food is physically retained between the flagellum and the cell, close to where phagocytosis takes place (Fig. 5c). If the prev establishes first contact elsewhere on the cell, the flagellum continues beating while the food is transported along the cell surface, upstream toward the frontal area. When the prey gets near the ingestion site, the flagellum stops beating to capture the prey and initiate phagocytosis (Fig. 5d). The anterior flagellum resumes its initial beating behavior while or after the prey is phagocytized (Fig. 5e). The flagellate can reject captured prey by returning the flagellum to its original position and releasing the food (Fig. 5f,g). Handling times for C. roenbergensis are shorter for rejected than for ingested prey (Fig. 3), and durations were uncorrelated with the prev size (Supporting Information Fig. S1).

When free-swimming, Pseudobodo sp. is pulled forward by the extended, beating anterior flagellum while the posterior flagellum inactively trails behind. Pseudobodo sp. attach to surfaces while feeding with the long anterior flagellum resembling a lasso loop (Fig. 6a). The flagellum beats  $(36 \pm 10 \text{ Hz})$ and creates a feeding flow through the loop as briefly described previously (Fenchel 1982b). The flow direction can vary from parallel to perpendicular to the surface. The distance between loop and cell (5.7  $\pm$  2.8  $\mu$ m) is variable during the searching mode. Food particles are intercepted by the anterior flagellum (Fig. 6b). Prey contact triggers an increase in beating frequency  $(63 \pm 15 \text{ Hz})$  at a reduced wave amplitude and a shorter helical pitch. The prey is retained between the cell and the flagellum and transported toward the body (Fig. 6c), either for ingestion (Fig. 6d,e) or rejection. Pseudobodo sp. has two ways to actively reject a particle: (1) the quick release and (2) the lash rejection. While the particle is



Fig. 5. Schematic representation of foraging by *C. roenbergensis*. Prey handling steps: searching and capture (**a**–**c**); prey ingestion (**d**, **e**); and rejection (**f**, **g**). Figure objects: frontal flagellum (solid line, red), posterior flagellum (solid line, blue), ingestion site (circle, green), feeding current (solid curved arrows), and object in motion (solid straight arrows).



Fig. 6. Schematic representation of foraging by *Pseudobodo* sp. Prey handling steps: searching and capture (**a**–**c**); prey ingestion (**d**, **e**); rejection (**f**, **g**); and "lash" rejection (**h**, **i**). Figure objects: "lasso" flagellum (solid line, red), posterior flagellum (solid line, blue), ingestion site (circle, green), feeding current (solid curved arrows), and object in motion (solid straight arrows).

captured between the flagellum and the body, it can be quickly released by reducing the beating frequency and returning the flagellum to its original position (Fig. 6f). Then, the feeding flow is rapidly restored (Fig. 6g). In the lash rejection, the flagellum stops beating for an instant before starting to "uncoil" from base to end, sometimes finalizing fully extended and straight (Fig. 6h). Then, it slowly starts beating  $(6 \pm 3 \text{ Hz})$  with a higher wavelength and amplitude  $(2.2 \pm 0.4 \ \mu\text{m})$ . In this rejection mode, the prey is physically pushed away by the flagellate after being captured. Once the prey is released, the flagellum slowly coils back and recovers the initial "loop" beating pattern (Fig. 6i). *Pseudobodo* sp. rejected particles with diameter smaller than 3  $\mu$ m with a quick release or a lash rejection, in contrast to particles with



**Fig. 7.** Observed feeding currents by particle tracking and theoretical flow fields generated with the point force model of the individuals *Pteridomonas* I (**a**, **b**), *Paraphysomonas* I (**c**, **d**), *Pseudobodo* I (**e**, **f**), and *Cafeteria* I (**g**, **h**). Clearance discs are in front of the flagellate, represented as a yellow solid line. Left-side panels: the cell body (gray circle) of the flagellate attaches (blue dotted line) to the surface (thick black line), and the beating flagellum (solid blue line) generates a feeding current. Green tracks are for captured particles, and red tracks are for uncaptured prey. Right-side panels: the point force (red arrow) dictates the direction of the feeding current described by the theoretical streamlines (blue solid lines).

diameter larger than 5  $\mu$ m that were only discriminated with the latter strategy (Supporting Information Fig. S1). The handling times of captured particles in *Pseudobodo* sp. were rather short and of similar duration for particles ingested or released quickly, while the lash rejections were of much longer duration (Fig. 3). For almost half of the ingested particles (8/20), the flagellate intercepted and processed the prey without modifying the flagellar beat, thus the handling time was zero. Similar to the other species, handling times were independent of prey size (Supporting Information Fig. S1).

#### Particle tracks and clearance rates

For all species, the particles followed hourglass-shaped paths, and only particles nearest the center line of the flow were captured by the cell (Fig. 7; Supporting Information Fig. S2). The sensitivity to the choice of clearance disc was found to be insignificant for clearance discs positioned sufficiently upstream of the beating flagellum (Supplementary Table S4). Estimated maximum clearance rates within species varied by a factor of about 2, and cell volume-specific clearance rates were all of the same order of magnitude and varied from 2 to  $18 \times 10^6 \text{ d}^{-1}$  (Table 1).

#### Estimation of force magnitudes and theoretical streamlines

The qualitative structures of the feeding flows are captured by the model (Fig. 7). The flow model ignores the cell body and assumes that the force acts at a point, while in reality the force production occurs along the flagellum. This means that the model can describe the flow quantitatively in the clearance disc regions but not in the immediate vicinity of the cell and the beating flagellum. A least-squares fit of the model to the full particle tracks would therefore not provide credible parameter estimation. Two previous studies of flows around sessile choanoflagellates and ciliates have excluded near-field data and made least-squares fits in the far-field (Roper et al. 2013; Pepper et al. 2021), but our information about the far-field is limited compared to the two studies. Instead, we manually positioned and oriented the point forces and applied Eqs. 2 and 3 directly using the detailed information contained in our particle tracks about the clearance rates and the clearance discs. The point force is positioned at the narrowest point of the hourglass-shaped track pattern where the particle speeds are highest. For P. danica, we used a point force located on the flagellum at <sup>3</sup>/<sub>4</sub> of its length from the cell body (Fig. 7a,b; Supporting Information Fig. S2a,b). For P. foraminifera, all particle tracks converged toward the curved distal segment of the flagellum (Fig. 7c,d; Supporting Information Fig. S2c,d), and therefore the point force was located in the middle of this section. Due to the complex and variable geometry of Pseudobodo sp., the point force was positioned in the middle of the "loop" halfway between the body and the tip of the helical flagellum (Fig. 7e,f; Supporting Information Fig. S2e,f). The point force in the cases of C. roenbergensis was set to be at half the projection length of the flagellum,

Table	<ol> <li>Morphological</li> </ol>	and kinematic parame	ters, and clearance rat	e and force estimat	tes for four species	of flagellates (P. f	oraminifera,
P. danic	a, Pseudobodo sp.	, and C. roenbergensis).	Particle tracking was	performed for two i	individuals (I and I	I) of each species	to estimate
the clea	rance rate and th	e force required to drive	e the flow.				

	P. foraminifera		P. danica		Pseudobodo sp.		C. roenbergensis	
	I	11	1	11	1	Ш	I	П
	3.9	3.5	4.6	4.4	3.5	5.5	4.0	3.9
Cell height (μm)	1.7	13.7	6.6	6.2	8.1	15.4	4.1	4.8
Flagellum projection length ( $\mu$ m)	14.0	10.8	8.4	6.7			4.6	5.2
Flagellum beat frequency, f (Hz)	52	44	30	33	21	33	38	47
Clearance disc radius, $a(\mu m)$	4.5	5.3	3.7	5.7	6.0	5.6	5.4	5.5
Clearance disc height, $d(\mu m)$	20.7	31.2	26.1	25.1				
Point force to disc distance, $I(\mu m)$					14.5	15.0	11.7	8.9
Average flow velocity, $U(\mu m s^{-1})$	78	52	44	30	19	18	17	16
Volume-specific clearance rate, $Q_{v}$ (10 <sup>6</sup> cell volume d <sup>-1</sup> )	13	18	3.3	6.2	8.1	1.7	4.2	4.1
Clearance rate, Q ( $10^3 \mu m^3 s^{-1}$ )	4.9	4.6	1.9	3.2	2.1	1.8	1.6	1.5
Point force height, $h(\mu m)$	13.7	21.7	17.5	15.6	9.9	18.2	6.1	6.8
Force, <i>F</i> (pN)	12.5	10.2	7.7	7.0	6.5	4.9	5.7	3.7

approximately where tracked particles reached maximum velocities (Fig. 7g,h; Supporting Information Fig. S2g,h). The resulting force estimates using Eqs. 2 and 3 were 3.7–12.5 pN with flows perpendicular to the surface (*P. danica* and *P. foraminifera*), requiring a slightly stronger force than the cases of parallel feeding currents (*Pseudobodo* sp. and *C. roenbergensis*; Table 1).

The force required to drive the observed flow can be compared with estimates of the force generated by the flagellum. P. danica beats its flagellum in a plane with a roughly sinusoidal beat pattern, allowing us to apply the resistive force theory expression in Eq. 5 to directly estimate the force produced by the flagellum. We have our observed parameters  $L = 11.5 \,\mu m_{e}$  $2A = 2.9 \,\mu\text{m}, \, \lambda = 5.3 \,\mu\text{m}, \text{ and } f = 33 \,\text{Hz}$  (Supplementary Table S1) and the parameters from the literature:  $b = 0.15 \,\mu m$ (Moestrup 1982),  $\alpha = 3 \mu m$ ,  $\beta = 0.01 \mu m$  (Fenchel 1982b; Patterson and Fenchel 1985), and  $\chi = 0.15 \,\mu m$  (Holwill and Sleigh 1967). With the parameters we would estimate  $F_z = -1.0$  pN using Eq. 6 if the flagellum were without hairs, that is, in opposite direction, and we find  $F_z = 15$  pN using the force coefficients in Eq. 7 for the flagellum with hairs. Similar estimates are not possible for the other species that have more complex three-dimensional beat patterns.

# Discussion

#### Attached vs. free-swimming

Our study complements earlier descriptions of the foraging behavior of heterotrophic nanoflagellates (Sleigh 1964; Fenchel 1982b; Boenigk and Arndt 2000b). We describe feeding only in flagellates attached to surfaces. Attached feeding appears to dominate for small forms, while larger forms, such as dinoflagellates, feed predominantly when freely swimming

(Boenigk and Arndt 2002; Nielsen and Kiørboe 2015). We observed P. danica capture prey while swimming, and Spumella sp., O. moestrupii, and loricated choanoflagellates are known to feed while swimming (Boenigk and Arndt 2000b; Pfandl et al. 2004; Nielsen et al. 2017). It has been argued that attachment enhances the feeding current of suspension feeders (Strickler 1982; Tiselius and Jonsson 1990; Christensen-Dalsgaard and Fenchel 2003), but fluid dynamical simulations and models suggest the opposite (Kirkegaard and Goldstein 2016; Andersen and Kiørboe 2020). The reason for attachment in bacterivorous nanoflagellates may therefore rather be favorable food conditions near surfaces, such as marine snow (Alldredge and Silver 1988; Simon et al. 2002). This is consistent with the observation that starving flagellates (Ochromonas sp., P. vestita, A. mirabilis) do not attach, while almost all cells experiencing high prey concentrations attach (Christensen-Dalsgaard and Fenchel 2003; Pfandl et al. 2004). Thus, swimming in heterotrophic nanoflagellates may for many species primarily serve the purpose of finding a nutrient-rich attachment surface. The probing behavior and different configuration of the flagellum of swimming and attached Pseudobodo sp. lend further support to this interpretation. Thus, stretching the flagellum in front of the cell allows faster swimming (Langlois et al. 2009). Some choanoflagellates similarly have an attached feeding stage with a short flagellum, and a free-swimming nonfeeding stage with a long flagellum and a smaller more streamlined cell body (Nguyen et al. 2019).

#### Handling time, clearance rate, and the functional response

Predator-prey interactions are often quantified by the prey ingestion rate as a function of the concentration of prey, typically described by a type II functional response (Holling 1959).

This equation has two parameters, the maximum clearance rate, that is, the volume of water cleared for particles per unit time at low prey concentration, and the prey handling time (= 1/maximum ingestion rate). Our behavioral observations allow us to estimate both parameters and to examine to what extent they underpin functional response relations estimated in incubation experiments.

In the species examined here, prey encounter is facilitated by the generation of a feeding current produced by the activity of one hairy flagellum that propels water toward the cell. We identified three different modes of prey encounter: prey particles arriving in the feeding current are perceived and captured by the flagellum, intercepted by the cell body, or by tentacles, and these represent the encounter mechanisms described for nanoflagellates. Prey is then handled by coordinated motions of one or two flagella, or, in the case of P. danica, by the tentacles. While the suspension feeding P. danica continues to generate a feeding current while handling prey, the feeding current ceases during prey handling in the other species. The prey handling time can be substantial, particularly in *P. foraminifera* that stops beating the flagellum for up to more than 1 min after the prey has been phagocytized. A similar "refractory period" has been reported for four species of nanoflagellates, including C. roenbergensis (Boenigk and Arndt 2000b), leading to handling times between 4 and 95 s per prey, similar to the range reported here. Eventually, ingestion rate may be limited by handling times, and the so estimated maximum ingestion rates vary by more than one order of magnitude between species, from 1000 to 20,000 preys per day. This corresponds largely to the range of speciesspecific maximum ingestion rates of bacteria in incubation experiments, 600–6000 bacteria  $d^{-1}$  (Fenchel 1982c; Boenigk and Arndt 2002). The match becomes better when considering that a varying but sometimes large fraction of captured bacteria may be rejected (Matz et al. 2002; Pfandl et al. 2004). Handling of rejected prey may further reduce time for searching. even though handling time is generally shorter for rejected than ingested prey (Boenigk and Arndt 2002).

Particle tracking allowed us to characterize the flow field generated by the feeding flagellates, to identify the extension of the prey capture zone, and to estimate maximum clearance rates. Our estimates of cell volume-specific maximum clearance rates varied between both individuals and species and ranged between  $10^6$  and  $10^7$  d<sup>-1</sup>. This magnitude is again similar to that obtained in incubation experiments, where estimates vary between species and range between  $10^5$  and  $10^7$  d<sup>-1</sup> (reviewed in Hansen et al. 1997; Kiørboe and Hirst 2014). Overall, functional responses measured in incubation experiments are mechanistically underpinned by behavioral observations.

#### Flow architecture and fluid dynamics

At the low Reynolds number at which nanoflagellates operate, viscosity impedes predator-prey contact, but the activity of the beating flagellum is obviously sufficient to overcome the effect of viscosity. The impeding effect of viscosity is somewhat relaxed in flagellates that contact prey by the flagellum or tentacles at some distance from the no-slip surface of the cell. However, even in *C. roenbergensis*, where first contact is on the cell surface, the feeding current is sufficiently strong to allow prey encounters.

By applying a point force model that describes the observed flow fields well, we estimated the flow-generating forces to be on the order of 4–13 pN for the four species. These estimates ignore the presence of the cell body, and the force produced by the flagellum has to be somewhat larger than the force required to produce the observed flow fields. Christensen-Dalsgaard and Fenchel used an alternative approach and measured the swimming speed of *P. vestita* towing a latex sphere and computed the flagellum force from the Stokes drag to be of similar magnitude, 7–13 pN (Christensen-Dalsgaard and Fenchel 2003). This approach neglects hydrodynamic interactions between flagellum, cell, and latex sphere, and the actual force is therefore larger than this estimate as well (Langlois et al. 2009).

How do these indirect estimates compare with direct estimates of the force generated by the beating flagella? The estimate derived for *P. danica* by applying resistive force theory is larger but of similar magnitude as the indirect estimate, 15 and 7–8 pN, respectively. The estimate ignores hydrodynamic interactions between adjacent sections of the flagellum (Holwill and Sleigh 1967; Brennen 1976), which is most likely not justified for flagella with closely spaced hairs (Rodenborn et al. 2013) and it is therefore speculative. The estimate suggests that the hairs reverse the direction of the force and increases its magnitude by an order of magnitude compared to a flagellum without hairs. This increase is similar to that estimated by comparing swimming speeds of flagellates with smooth and hispid flagella (Nielsen and Kiørboe 2021).

As noted above, most heterotrophic nanoflagellates have hispid flagella, and this seems to be optimal or even necessary for prev encounter for a number of reasons. First, the presence of hairs significantly increases the force production of the beating flagellum and thereby the clearance rate. Secondly, the presence of hairs makes prey scanning of the flagellum efficient, since prey intercepted by the hairs elicits a capture response. Thirdly, the dominant flow along a flagellum with hairs is outside the envelope of the beating flagellum (Jahn et al. 1964; Sleigh 1981, 1991), presumably allowing efficient prey transport toward the cell. Fourthly, the front-mounted flagellum increases the frequency of prey entrainment (Mathijssen et al. 2018). Finally, the reversal of the flow makes the streamlines come closer to the cell in the up-stream direction from where the prey arrives, and the transport of captured prey toward the cell body is facilitated by the flow.

#### Conclusions

Indirect and direct estimates of flagellum forces for one species are of similar magnitudes and consistent with the

observed feeding flow, and the estimates of maximum ingestion and clearance rates are similar to those obtained from previous incubation experiments. Thus, our observations and estimates suggest a mechanistic underpinning of functional responses in heterotrophic nanoflagellates. However, experimentally estimated specific clearance rates of flagellates vary by two orders of magnitude (Hansen et al. 1997; Kiørboe and Hirst 2014), and a significant fraction of this variation is accounted for by variation in flagellar arrangement and kinematics and consequent differences in flow architecture and predation risk: species with high clearance rates also disturb a large volume of water, attract flow-sensing predators from a further distance, and experience higher predation risk (Nielsen and Kiørboe 2021). A better mechanistic understanding of this foraging trade-off and the variation in clearance rates requires a better understanding of the fluid dynamics of hairy flagella. This in turn may be facilitated by accurate observations of the often complex three-dimensional beat patterns of the flagella (Christensen-Dalsgaard and Fenchel 2004) and the arrangement of hairs on the flagella in combination with computational fluid dynamics simulations and theoretical modeling.

#### Data availability statement

Data is available in the Dryad repository at https://doi.org/ 10.5061/dryad.bk3j9kdbs.

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#### **Conflict of Interest**

None declared.

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