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More than just slippery: the impact of biofilm on the attachment of non-sessile freshwater mayfly larvae

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While terrestrial insects can usually attach directly to a substrate, for aquatic insects the situation is more complicated owing to the presence of a biofilm on the primary substrates. This important fact has been neither the subject of investigation nor commonly taken into account in the interpretation of functional aspects of attachment in mobile freshwater animals. In this study, we investigate the impact of a biofilm on the attachment of living mayfly larvae. We performed *in vivo* attachment experiments in a flow channel using different substrates with defined surface roughness. Additionally, we measured friction forces directly generated by dissected tarsal claws on the same substrates. On substrates with smooth or slightly rough surfaces, which have little or no surface irregularities large enough for the claws to grasp, the presence of a biofilm significantly increases the friction force of claws. Consequently, larvae can endure higher flow velocities on these smooth substrates. The opposite effect takes place on rough substrates, where the friction force of claws decreases in the presence of a biofilm. Consequently, a biofilm is a critical ecological structure for these larvae, and other aquatic organisms, not only as a food source but also as a factor influencing attachment ability.

1. Introduction

When terrestrial insects attach to a substrate, they do so directly. However, in aquatic environments, there is a biofilm coating primary substrates, so contact is no longer direct. After just 2 h of exposure in an aquatic environment, organic material, bacteria and fungi form a primary biofilm on the surface of the substrate [1]. Subsequently, autotroph microorganisms and algae attach. The microorganisms secrete extracellular polymer substances (EPSs), which embed algae, bacteria, fungi and detritus particles in an organic sublayer [2]. Biofilms are complex highly hydrated structures, which show population heterogeneity in space and time [3].

Considering interactions of benthic animals with the biofilm, the relevance of the biofilm as a food source for grazers has been intensively investigated [4–10]. By contrast, its influence on animal attachment has not garnered much attention to date. While biofilms can vary greatly in composition and thickness [2], they are usually softer than the primary substrate, and change surface structure and chemistry. Such factors, substrate roughness, stiffness, wettability, chemistry and temperature, can have considerable effects on the adhesive strength of benthic animals [11].

Most of the literature dealing with the functional aspects of attachment of freshwater macroinvertebrates [12–19] does not investigate the possible influence of the biofilm surface. In some sessile freshwater animals, for example *Dreissena polymorpha*, the attachment ability is not influenced by a biofilm [20]. This is owing to the fact that *Dreissena* mussels replace the biofilm with their byssal threads and make direct contact with the substrate [21]. Nevertheless, in the presence of a biofilm, a higher number of individuals was observed during the first step of colonization [22]. By contrast, simuliid larvae show no difference in abundance during first

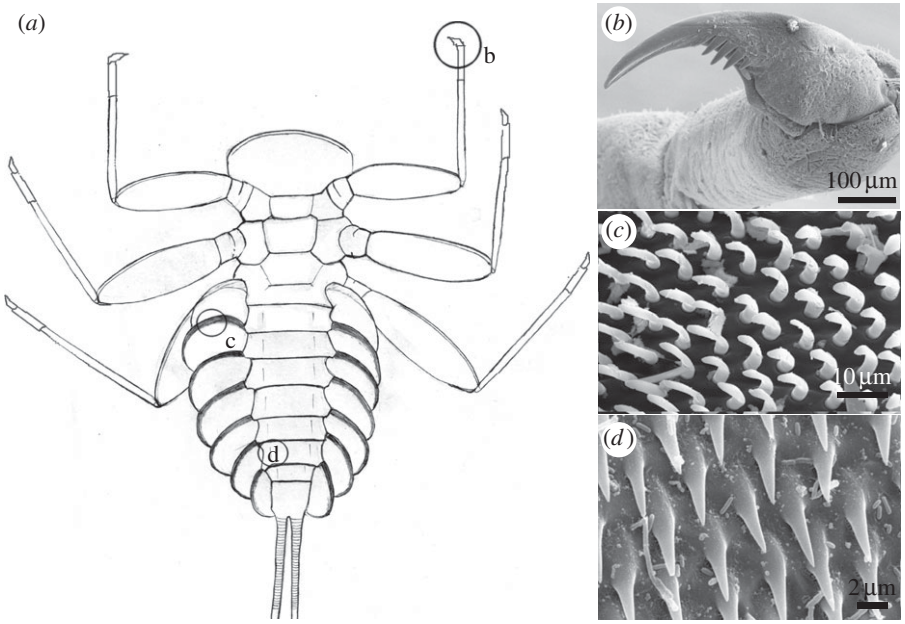

Figure 1. Attachment devices of *E. assimilis* larvae: (*a*) ventral view, (*b*) claw of the foreleg, (*c*) setae of the pads on the ventral side of the gill lamellae and (*d*) areas with spiky acanthae on the lateral parts of the abdominal sternits. (Adapted from [31].)

colonization, but it has been assumed that a biofilm has an effect on long-term settlement and attachment [23]. For many marine sessile animals, such as mussels, bryozoans, coelenterates or polychaetes, a biofilm can be either a settlement cue or inhibitor [23–29].

Sessile animals, however, are not alone in having to deal with a biofilm and, to our knowledge, the impact of biofilms on mobile insect larval attachment remains unexplored. Our recent investigations indicate that the presence of a biofilm considerably influences the ability of some aquatic insect larvae to attach to the substrate surface. For example, the reported ability of the running water mayfly *Epeorus* to attach to smooth surfaces [30,31] was no longer observed on smooth sterile substrates [32]. Besides a single tarsal claw, these mayfly larvae are equipped with additional attachment devices, for example friction pads (figure 1), which contribute to the general attachment force of the insect [31,33]. Surface roughness varies greatly in natural aquatic substrates and has a significant impact on the forces exerted by different attachment devices in *E. assimils* as well as in other insects [31,32,34]. However, the impact of a biofilm, especially in conjunction with surface roughness, has not been investigated.

The aim of this study is to understand the role of the biofilm in the attachment of mobile insect larvae, using the mayfly *Epeorus assimilis* as our model organism. We investigated the following questions. (i) Does the biofilm influence the ability of live *E. assimilis* larvae to attach to the substrate? (ii) Does the biofilm influence the friction forces generated by the tarsal claws? (iii) Is there any combined influence of biofilm and the surface roughness of the primary substrate on the mayfly's attachment ability?

2. Material and methods

2.1. Animals

The last instars of *E. assimilis* Eaton 1885 larvae were collected from streams located in the Taunus Forest close to Weilrod, Germany. The live larvae were packed on ice and were transported to the laboratory flume at the Federal Institute of Hydrology in Koblenz, Germany. The maximum transport time was approximately 1.5 h. A number of live specimens were fixed in 70% ethanol and were used to measure the friction force generated by the claws.

2.2. Substrates

In contrast to heterogeneous natural substrates, for example stone, artificial substrates can be selected according to which of their specific properties (stiffness, roughness, etc.) are of interest. Replication techniques allow the preparation of substrates with relatively homogeneous chemical composition but different, defined surface roughness.

For the experiments, we prepared substrates with four different surface roughnesses, chosen in accordance with former attachment studies focusing on the roughness impact without biofilm formation [31,32]. We used a two-step moulding process, using dental wax (President Light Body; Coltène Whaledent, Langenau, Germany) and epoxy resin-containing hardener (Epoxidharz L and Härter S; Conrad Electronics, Hirschau, Germany) in accordance with Koch *et al*. [35], to create replicate substrates. Glass (S1) replicas were used as a smooth reference. Moreover, replicas (size: 15 × 20 cm) were made of three different surface roughnesses: special polishing paper with a nominal asperity size of 1 µm (S2) and 12 µm (S3) (523 and 545; Buehler, Lake Bluff, IL, USA) and normal polishing paper 400 P (S4) (Tyage; Wolfcraft, Kempenich, Germany). These epoxy replicas were used both with and without a biofilm. To cultivate a biofilm of sufficient thickness, replicas of all substrate types were exposed in the laboratory flume for three weeks before the experiments started. During the daytime, substrates were exposed to artificial light. After the experiments, some biofilm samples were frozen and stored at −70°C for later examination (confocal laser scanning microscopy (CLSM), mechanical properties).

2.3. Confocal laser scanning microscopy

Thawed substrate samples were carefully transferred to water. In the case of the roughest substrate without a biofilm, air remained adhered to the surface, which was removed by briefly exposing the substrate to a vacuum and adding a few droplets of Triton X-100 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to the water. Later, the mixture of water and Triton X-100 was replaced by pure water. The surface topography of all substrates

was analysed by applying the confocal laser scanning microscope Zeiss LSM 700 (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with an upright microscope (Zeiss Axio Imager.M1m). For the visualization, a 25× objective (Zeiss Plan-Apochromat, multi-immersion (oil, glycerine and water) objective, numerical aperture = 0.8; directly immersed in the water above the substrates) was used. The substrates were exposed to light from a stable solid-state laser with a wavelength of 405 nm (5 mW at the fibre end, laser power = 1.5%), and the laser light reflected at the surface of the substrates was detected. Detector gain was manually adjusted prior to image stack collection in a way resulting in maximum signal intensity while simultaneously preventing oversaturation. Digital gain and digital offset were set to 1 and 0, respectively. We used the CLSM software Zeiss Efficient Navigation (ZEN) to automatically determine the optimal image size (limited by the CLSM system to a maximum of 2048 × 2048 pixels) necessary according to the Nyquist–Shannon sampling theorem. A pinhole size of 0.33 Airy units was applied. For each image stack, overlapping optical slices were visualized for the entire *z*-range of the surface. The distance between two consecutive focal planes was chosen in a way that the sampling rate was two times larger than necessary according to the Nyquist–Shannon sampling theorem. All image stacks were collected with a line average of 2. Scan rates resulting in a combination of both a very good signal-to-noise ratio and a reasonable collecting time were selected. ZEN software was used to create colour-coded height maps and to calculate the roughness parameters from the image stack data. For this purpose, no filter was applied, and the surfaces were fitted to a plane.

2.4. Mechanical properties of the biofilm

For testing, thawed biofilm samples on glass slides were fixed on the bottom of a Petri dish filled with tap water. Indentation measurements were performed under water using force tester Basalt-2 [36], a home-made microindentation set-up. It consists of a sample stage located on a six-axial nanopositioning system (Hexapod F206; PI, Karlsruhe, Germany), a flat glass spring (spring constant = 287 N m^{-1}) and a mini-interferometer SP-120 (SIOS Meßtechnik GmbH, Jena, Germany), fixed on a piezo stage (P611 nanocube stage; PI, Karlsruhe, Germany). An indenting sapphire sphere (radius = 1.5 mm, Goodfellow, UK) and a mirror were glued to the spring. The mirror displacement proportional to the force applied to the spring was monitored using an interferometer. The set-up was installed on a vibration isolation table (TS-150; Table Stable Ltd, Ammerbuch, Germany). The Hexapod nanopositioning system was controlled by means of the manufacturer's software. Load–displacement curves (figure 2) were acquired using a custom-made program written in LabVIEW (National Instruments, Austin, TX, USA).

As there was no adhesion observed in the biofilm indentation tests, the Oliver–Pharr model [37] was used to determine Young's modulus (E_r) and hardness (H). The following equations were derived from the model:

$$h_c = \delta_m - \frac{3F_m}{4S}, E_r = \frac{S}{2\sqrt{2Rh_c}}, H = \frac{F_m}{2\pi R h_c},$$

where F_m is the maximum applied force (figure 2), δ_m is the maximum indentation depth, h_c is the vertical distance along which contact is made (the contact depth), R is the radius of the indentation sphere and S is the slope of the force–distance curve at δ_m. The experimental data were analysed in Matlab (The MathWorks Inc.).

2.5. Estimation of insect attachment ability in a laboratory flume

Attachment experiments were carried out in an artificial stream flume (for details, see [33]). Substrates S1–S4 were placed in the flume and arranged so that each replicate experienced comparable flow conditions. For each experimental run, *E. assimilis* larvae were placed on a single substrate replica, when the flow velocity was zero. Then, the rotational speed of the paddle wheel was gradually increased until the animal left the substrate or the maximum speed of the flume (more than 40 cm s^{-1} bottom flow velocity) was reached. For each type of substrate, the experiment was repeated using 10 or 11 animals.

Experiments were recorded using a videoscope Iplex II camera (Olympus, Hamburg, Germany). Selected video sequences were analysed frame by frame using SIS Bildanalyse software EIS (Olympus, Münster, Germany). For calibration of distances and dimensions, a ruler was placed in the background during video recording. Using this scale, the bottom flow velocity was determined from the shutter speed of the video camera and from the length of the lines traced by single particles in single video frames.

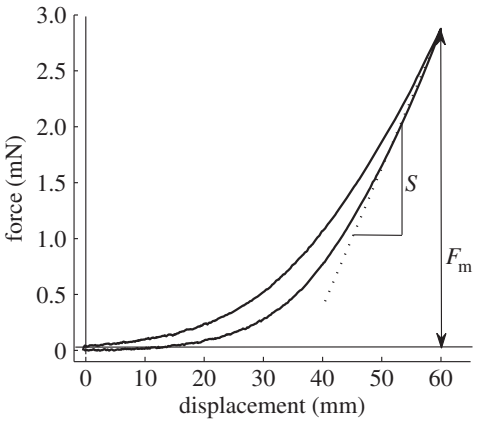

Figure 2. Example of a force–distance curve obtained in biofilm indentation experiments. F_m is the maximum applied force measured at the maximum indentation depth and S is the slope of the force–distance curve at F_m.

2.6. Friction measurements at claw tips of isolated insect legs

The right foreleg was dissected from larvae, fixed in 70% ethanol and cut between the trochanter and femur. A special clamp was used to clip single legs to the force sensor, oriented as naturally as possible: with the tibia parallel to the substrate and the claw in contact with the substrate. The force transducer was mounted on a motorized micromanipulator (MS314, M/W; Märzhäuser, Wetzlar, Germany), which was moved parallel to the substrate with a constant velocity of 100 µm s^{-1}. During movement, the friction force was monitored by load cell force transducers (10 and 25 g capacity; Biopac Systems Ltd, Santa Barbara, CA, USA). Normal forces applied by the claw to the substrate were measured and averaged 656 ± 311 µN for the entire series of experiments. Friction measurements were performed on all substrates with and without a biofilm.

3. Results

3.1. Surface properties of the substrates

The biofilm creates a secondary surface, on top of the primary substrate, which modifies the primary structure (figure 3). The most dramatic differences were observed on the smooth substrate (S1), where the height of the surface asperities increased considerably with the addition of a biofilm, from less than 1 µm to more than 100 µm. On the substrates with a

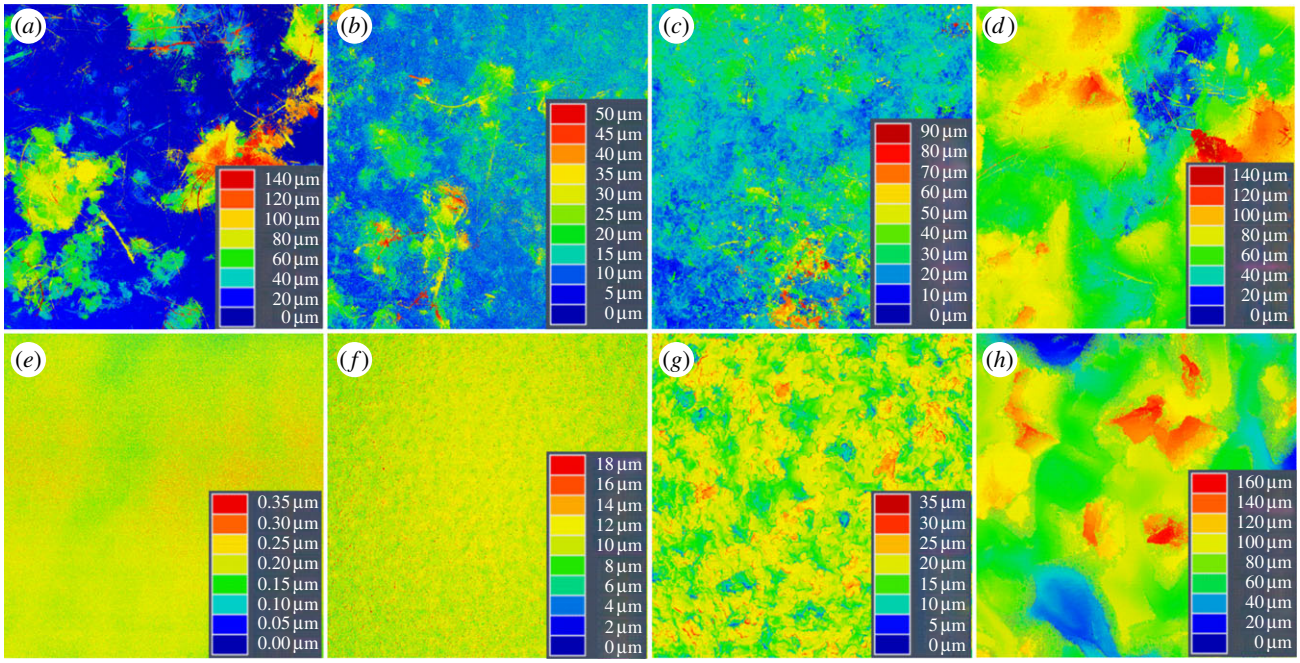

Figure 3. CLSM height maps showing the surface structure of the substrates: substrates with (*a–d*) and without (*e–h*) a layer of biofilm. On the smoother substrates, the presence of the biofilm layer resulted in an increased surface roughness compared with that of the primary surface (S1: *a*,*e*; S2: *b*,*f*; S3: *c*,*g*). On the substrates with the coarse initial surface roughness, the roughness remained almost the same after covering the surface with the biofilm (S4: *d*,*h*). S1, 100 × 100 µm; S2–S4, 512 × 512 µm. (Online version in colour.)

Table 1 Roughness parameters of the investigated substrates with and without biofilm, mean ± s.d. R_{max}, maximum roughness depth; R_a, roughness average.

	R_{max} (µm)		R_a (µm)	
	without biofilm	with biofilm	without biofilm	with biofilm
S1	0.50 ± 0.16	177.90 ± 19.47	0.036 ± 0.01	32.42 ± 20.06
S2	21.32 ± 2.14	66.28 ± 17.99	1.33 ± 0.11	5.13 ± 2.93
S3	36.52 ± 1.74	67.47 ± 19.16	3.19 ± 0.11	5.09 ± 1.65
S4	196.05 ± 44.01	153.26 ± 17.85	21.99 ± 2.6	18.17 ± 0.94

slight (S2) and medium (S3) roughness, the range of variation in height was more than two and a half times higher after the growth of a biofilm. By contrast, on the rough substrate (S4), the range of variation in height with biofilm was almost equal to the range of variation without. The described differences are reflected in the calculated roughness parameters (table 1). For example, the maximum roughness depth (R_{max}) shows a significant increase in surface roughness in the presence of a biofilm for the substrates S1–S3 (*t*-test: S1: $p < 0.005$, d.f. = 3, $t = 18.22$; S2: $p = 0.016$, d.f. = 3, $t = 4.97$; S3: $p < 0.049$, d.f. = 3, $t = 3.22$). By contrast, no significant difference was observed on substrate S4 (*t*-test: $p = 0.169$, d.f. = 3, $t = -1.80$).

The biofilm that developed on the test substrates was around 200 µm thick. The mechanical properties of the biofilm were considerably different from those of the primary substrate. On average, Young's modulus of the biofilm was 188 ± 112 kPa (mean ± s.e.m.). However, the material was strongly non-homogeneous, and Young's modulus varied from 0.812 to 2584 kPa (figure 4*a*). Hardness values were non-homogeneous as well and ranged from 0.079 to 14.55 kPa, with a mean value of 2.06 ± 0.41 kPa (figure 4*b*). Both Young's modulus and hardness values increased with indentation depth. One reason for this is a gradient of mechanical properties in a biofilm; nevertheless, the artefact caused by the stiff support cannot be excluded. Algae cells attaching to the support provide different mechanical properties from the gel-like EPS of the biofilm. Overall, the biofilm deformed elasto-plastically, and biofilm adhesion was not detectable. After load application, a pit appeared on the surface of the biofilm, which was seen as a successive shift of indentation curves (figure 4*c*).

3.2. The influence of the biofilm on the attachment of mobile mayfly larvae

On most substrates, attachment ability of live *E. assimilis* larvae changed in the presence of biofilm (figure 5; see electronic supplementary material). On substrates which provided no or marginal surface irregularities to grasp (S1–S3), the larvae were able to attach more strongly to substrates covered by biofilm than to bare substrates. The larvae were also able to stay on these substrates at higher flow velocities than on the bare substrates (Wilcoxon–Mann–Whitney: S1: $W = 62.5$, $p < 0.005$; S2: $W = 63.0$, $p < 0.005$; S3: $W = 62.5$, $p < 0.005$). By contrast, there were no differences found in the attachment ability on rough substrates (S4) with or without biofilm (Fisher's exact test:

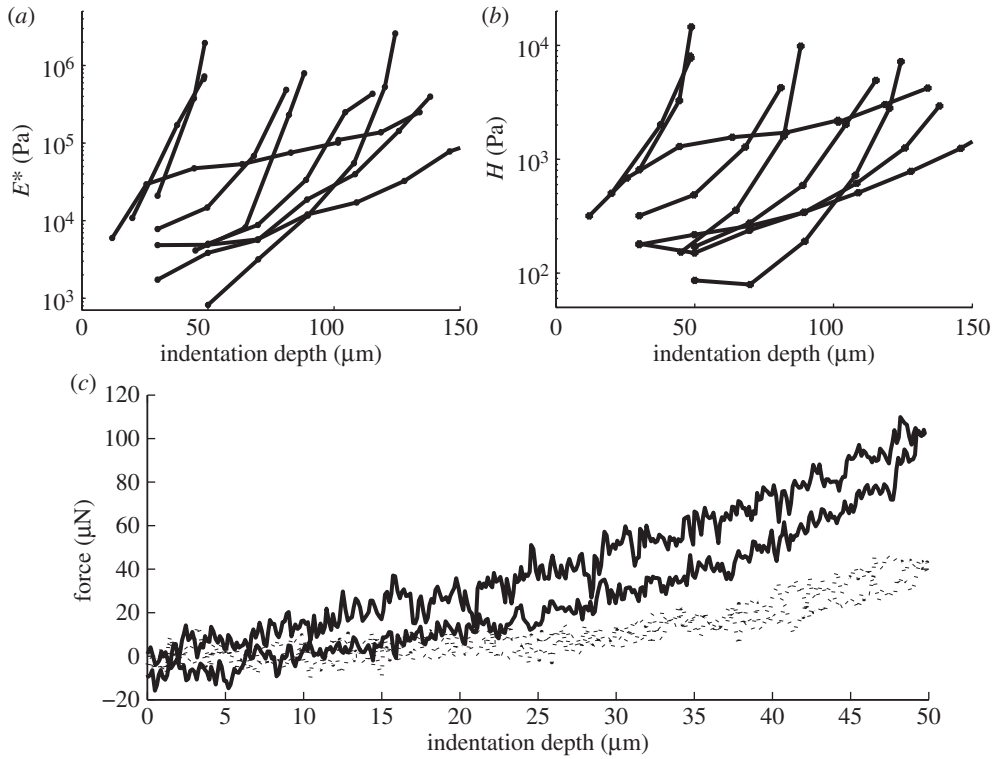

Figure 4. Young's modulus (*a*) and hardness (*b*) depend on indentation depth at different locations (different curves). Residual plastic deformation (*c*) is demonstrated as a shift of an indentation curve (dotted curve) after application of 2.13 kPa of average stress to the biofilm. Indentation curve, while applying stress of 2.13 kPa, is plotted as a solid line.

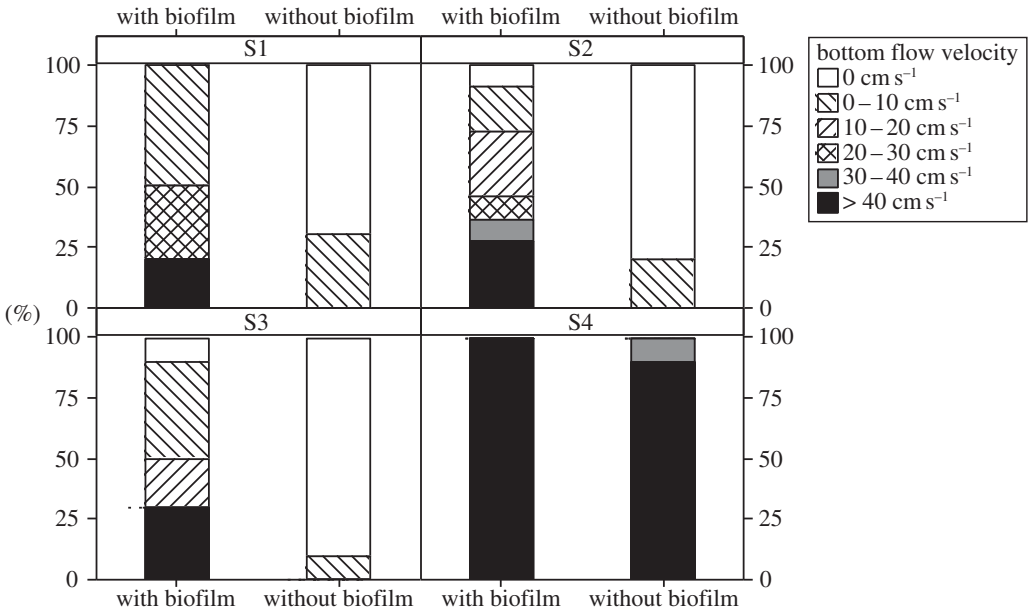

Figure 5. Attachment ability of *E. assimilis* larvae on substrates with different surface roughness. The same experiment was performed for the same substrates with and without biofilm. The maximal flow velocity at which the larvae remained on each substrate was determined. In the presence of the biofilm, more larvae stayed on substrates S1–S3 at higher flow velocities. Almost all larvae were able to withstand the maximum flow velocity with and without biofilm on the substrate S4. S1–S4, substrates of the types 1–4, respectively.

$p = 1$), and larvae stayed attached up to a bottom flow velocity of more than 40 cm s^{-1} on both. However, as this was the maximum flow velocity of the flume, we cannot rule out differences in attachment ability for the S4 substrate with and without biofilm at higher flow velocities. Video analysis showed that larvae primarily move a single leg, sometimes two, while moving forwards. In some cases, we observed that claws slowly slid backwards and the larvae had to re-adjust to maintain their position.

3.3. Claw-generated attachment force on the substrates with and without biofilm

We measured the forces generated by the tarsal claws of dissected legs parallel to the substrate to isolate the influence of the biofilm on the claws versus the influence on the other attachment structures of *Epeorus* larvae. The claw-generated attachment force increased significantly on smooth and slightly rough substrates in the presence of the biofilm (S1: Mann–Whitney, $W = 768$,

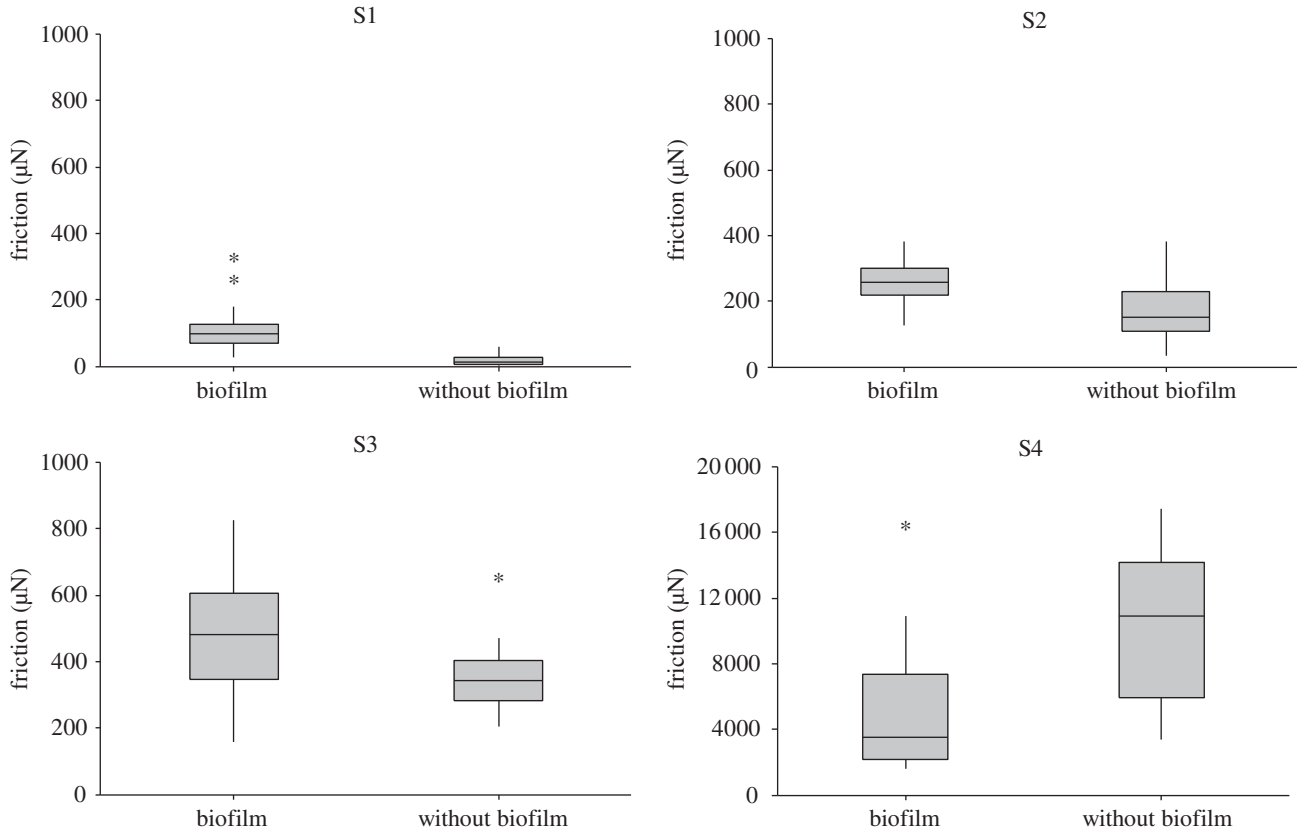

Figure 6. Attachment forces of claws measured on the substrates with and without biofilm while pulling laterally. On the smooth substrate (S1) and on the substrates with the slight and medium surface roughness (S2 and S3), forces generated by the claw were significantly higher in the presence of the biofilm. By contrast, on the coarse roughness (S4), the forces were lower in the presence of the biofilm. Graphs are box plots with median, upper and lower quartiles, interquartile range and outliner values. S1–S4, substrate types 1–4, respectively.

$N = 44$, $p < 0.001$; S2: two-sample t-test, $T = 4.88$; d.f. $= 51$, $p < 0.001$; S3: Mann–Whitney, $W = 721, N = 46, p < 0.05$). The opposite outcome was observed on the rough substrate (S4; figure 6; see electronic supplementary material), here the attachment force of the claws was lower for the substrate covered with the biofilm (S4: two-sample t-test, $T = 3.01$; d.f. $= 43$; $p < 0.005$).

4. Discussion

Anyone who has slipped on wet stones in a stream is familiar with the effects of biofilm cover. One might expect it to be more difficult for aquatic insect larvae to attach to a substrate covered with this biofilm. This study, however, demonstrates that on substrates with a smooth or slightly rough surface structure quite the opposite happens.

Depending on tarsal claw tip size, there is a species-specific minimum roughness past which claws cannot find sufficient surface irregularities to grasp onto primary substrates [32,38]. For these kinds of substrates (in this study: S1, S2 and S3), friction measurements demonstrate that the presence of biofilm leads to a significant increase in forces between the excised claws and the substrate. One possible explanation for this is that the biofilm, or the attached organisms that make the biofilm, increases the number and the amplitude of surface irregularities that tarsal claws can grasp. By contrast, on very rough substrates (in this study: S4), the particles and organisms of the biofilm can accumulate in the valleys of the substrate and decrease the overall change in amplitude. Nevertheless, even the roughness of the biofilm-covered substrate remains sufficient to generate high attachment forces. On these very rough surfaces, interlocking of the tarsal claws with surface irregularities plays an important role in attachment.

Moreover, the EPS secreted by organisms in the biofilm changes the material properties of the substrate that the insect larva is interacting with. Our measured Young's modulus (about 200 kPa) is in the range of values given in the literature for algae (100–500 kPa), bacteria (50–2000 kPa) and for the soft parts of a drinking water biofilm (less than 600 kPa) [39,40]. These low values for Young's modulus indicate the low stress needed to stretch or compress soft biofilm material. The increase in the Young's modulus close to the primary substrate surface is related to the primary substrate's effect on the indentation measurements [41], but also a gradient in the biofilm might contribute to this increase. Algal cells attaching to the substrate are expected to have higher values of elasticity modulus, like the gel-like EPS. We found that, unfortunately, there is no simple method for reconstructing a depth profile of either the elasticity modulus or hardness for such heterogeneous systems. Using the Oliver–Pharr method, it was found that the overestimation of both values, caused by the finite biofilm thickness, is about 30% at an indentation depth of around 0.75 [41]. This depth-dependent increase is negligible compared with the increase of that observed in the experiment for both hardness and elasticity. Therefore, the biofilm has an extremely strong depth gradient for both Young's modulus and hardness. Nevertheless, the average stress applied by the tip of a claw (according to the Hertz theory for 1 mN applied force and 3 μm radius of the claw's tip) is higher than the observed maximum biofilm hardness, even when measured at its deepest layers and close

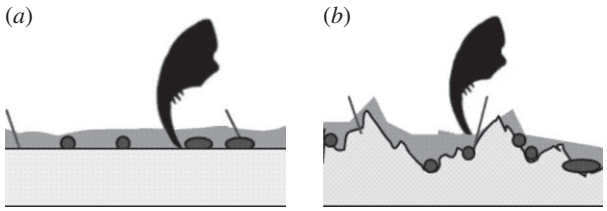

Figure 7. Diagram of the hypothetical interaction between claws and substrates covered with the biofilm. (*a*) The organisms of the biofilm and their EPSs increase the surface roughness of smooth substrates. (*b*) On rough substrates, surface roughness is not further increased. If the organisms of the biofilm preferably attach to the substrate valleys, the surface roughness might even be reduced. On smooth and rough substrates, claws might penetrate the EPSs.

to the substrate ($H \sim 15$ kPa) at Young's modulus higher than 0.7 kPa. Moreover, the hardness of the claws' chitin is orders of magnitude higher than the hardness of the biofilm. While the mean value of biofilm hardness in this study was 2.06 ± 0.41 kPa, in the literature there are hardness values reported for hydrated *Schistocerca* claws of 15 VHN (Vickers hardness numbers; 15 VHN is around 15 MPa) [42]. The tarsal claws used in this study are longer (length between the claw tip and the ventral–proximal edge of the claw is around 330 µm [32]) than the thickness of the biofilm (around 200 µm). Thus, the claws can freely pierce the gel-like biofilm and interlock with substrate irregularities caused by particles and algal cells attaching to the bare substrate (figure 7). Additionally, the biofilm surrounding the claw may increase resistance while claws are pulling parallel to the substrate owing to its high viscosity. On the other hand, the EPS of the biofilm between the claw tip and the substrate may also act as a lubricant and reduce friction.

It is important to remember that the composition and thickness of biofilms can strongly vary [2]. The thickness of the biofilm in our study is within the range of other river microbial biofilms, both those generated in a laboratory and those measured in the field [2,3,43]. Because of this, we can assume comparable thickness under most natural conditions. However, during winter, when there are fewer grazing invertebrates present, the thickness of the biofilm can increase up to a few mm [3]. This can be a considerably different situation for the remaining benthic invertebrates, as their claws may not be able to reach the primary substrate underneath. While biofilm structure and properties, such as elasticity, hardness and plasticity, may vary in different biofilms, increased surface roughness on smooth substrates and a layer of EPS can be expected for most types of biofilm. Therefore, the general trend in attachment forces for small-sized benthic animals on biofilm-covered substrates (of a comparable thickness) can be expected to be approximately the same. Thus, live larvae should be able to endure higher flow velocities on smooth substrates covered with a biofilm than without. This could also mean that drifting larvae may be better able to reattach, because reattachment begins with the tarsal claws [32,44]. On rough substrates, the attachment force of the claws decreased in the presence of biofilm, which may be the result of the lubricating effect of the biofilm EPS. However, this might not be biologically relevant for the live larvae, because their attachment force is about 8–40 times higher on the bare rough primary surfaces (S4) than on the smoother substrates with biofilm (S1–S3). In our experiments, live larvae showed no difference in their ability to attach to rough substrates with or without biofilm. Nevertheless, we cannot exclude the possibility that there might be differences at higher flow velocities.

In the resting position, all six legs of *E. assimilis* larvae are in contact with the substrate, and when they move, only one or two legs move at a time. Assuming that a minimum of four claws are grasping the substrate, we can estimate an overall larval attachment force of approximately 0.4 mN on S1, of approximately 1.2 mN on S2, of approximately 2 mN on S3 and of approximately 16 mN on S4. Applying C_w-values taken from the literature [45], we can estimate drag forces acting on the larvae parallel to the substrate according to the formula $F_d = 1/2\ C_d \rho S v^2$ (*Re* numbers were in the same range; F_d, drag forces; C_d, drag coefficient; ρ, water density; S, area in frontal projection; and v, flow velocity). At very high flow velocities of $2\ m\ s^{-1}$, which are typical for torrential streams [46], calculated drag forces reach approximately 20 mN, which is higher than our calculated attachment forces. At flow velocities up to $20\ cm\ s^{-1}$, we calculated drag forces of no more than approximately 0.2 mN, which is less than the attachment force generated by four claws on all substrates tested. At higher flow velocities, the drag forces are higher than the calculated attachment forces for S1 ($v = 0.3\ ms^{-1}$, $F_d = 0.5$ mN; $v = 0.4\ m\ s^{-1}$, $F_d = 0.9$ mN) or S2 ($v = 0.5\ m\ s^{-1}$, $F_d = 1.3$ mN). Despite this, 30% (S2, $v > 0.4\ m\ s^{-1}$) to 50% (S1, $v > 0.2\ m\ s^{-1}$) of the larvae were able to remain attached in these higher flow velocities. Possibly this disconnect is owing to variation in actual attachment forces corresponding to varying biofilm thickness, but other attachment mechanisms cannot be ruled out.

Our experiments were performed with the mayfly larva *E. assimilis*, and claws are not the only mechanism for attachment. These larvae also have special setose attachment pads on the ventral side of their gill lamellae, which are in close contact with the substrate when the larva rests in stronger currents. These attachment pads increase friction on sterile smooth substrates and might increase friction on the biofilm as well [31]. We hypothesize that both components together, the increased attachment force of the claws and the friction generated by attachment pads, can increase the friction force up to a value that enables the animals to inhabit smooth stones with a biofilm cover even while being exposed to higher flow forces.

Attachment is further complicated because larvae also experience lift forces acting perpendicular to the substrate, in addition to drag forces [47,48]. These lift forces can be even higher than the drag forces, but can vary greatly under similar flow velocities [45]. These lift forces might be the reason that live larvae were not able to remain on the substrates S2 and S3 without a biofilm, even if attachment forces of isolated claws (S2: 0.6 mN, S3: 1.4 mN for four claws) were higher than the calculated drag forces. The claws might not be able to grasp the substrate in a way that would withstand these tangential forces. On the other hand, the biofilm surrounding the claws can increase resistance, not only to drag but also to lift, owing to its viscosity, like placing the feet in honey.

We conclude that biofilm has a significant influence on the ability of *E. assimilis* larvae and other aquatic insects to attach to the substrate. Biofilm significantly increases friction forces on smooth and slightly rough substrates, where tarsal claws would not otherwise be able to find surface irregularities to grasp. Thus, larvae are able to stay on the same substrate, covered in biofilm, in higher flow velocities. Consequently, biofilm is of ecological relevance for the insect larvae not only as a food source but also as an important factor in their ability to remain

attached in stream habitats. Our results show the importance of taking into account the effects of biofilm when dealing with questions of aquatic attachment. Moreover, these findings can inform future work in other fields, such as antifouling research or the development of artificial attachment devices for underwater purposes.

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