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Working title: Beneath the surface: Application of transparent super absorbent polymer
substrates to track faunal activity within the sediment layer

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Summary

1. Tracking the movement of organisms is a fundamental goal of many ecological studies. A number of techniques exist in the study of terrestrial and aquatic fauna, however to date the ability to monitor aquatic fauna within the sediment layer efficiently and in multiple dimensions is lacking. Given the importance of subsurface sediments in supporting ecosystem functioning this inability to observe organism behaviour represents a fundamental gap in our knowledge and limits our capability to holistically characterise the response of freshwater systems to stressors.
2. Here we present an experimental study that employs novel transparent super absorbent polymer (SAP) substrates (ca. 8-12 mm in diameter) in combination with computer vision technology which enable, for the first time, real-time observation and tracking of organisms within the sediment layer under lotic flow conditions. Use of these substrates allowed the successful extraction of organism trajectories which enabled the velocity and body orientation of a freshwater amphipod (*Gammarus fossarum*) in the sediment layer to be calculated in response to a number of vertical hydrological exchange treatments (upwelling, downwelling and no vertical exchange).
3. Results indicate that under vertical hydrological exchange, a higher proportion of fast velocities (both horizontal and vertical) were recorded for *G. fossarum* in the sediment layer compared to no vertical exchange (control) conditions. This increase was most marked for upwelling flow exchange. We also observed a change in the body orientation of individuals in the sediment layer from a vertical alignment under no vertical exchange to a more horizontal one under downwelling and more notably upwelling flow exchange. This shift in body position was exacerbated under stronger vertical exchange rates.
4. We identified that following the flow transition of downwelling to upwelling conditions, there was an immediate shift (0-2 minutes) in both the orientation angle and activity level of individuals. This increased rate of activity was maintained for the individuals'

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3 53 velocity but not for their changing orientation angle. These trends were not apparent
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5 54 within the flow transition of no vertical exchange to downwelling flow.
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7 55 5. Our new methodological approach enables vital insights into the behaviour of
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9 56 organisms within the sediment layer. Use of SAP substrates allows real time multi-
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11 57 directional tracking of multiple organisms in parallel. We believe the method
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13 58 represents an innovative tool which can be employed to tackle a wide range of
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15 59 ecological questions and thereby improve our mechanistic understanding of
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17 60 ecological responses to biotic and abiotic processes / stressors.
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22 62 **1. Introduction**

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24 63 Quantifying the behavioural attributes and locomotion activity of biota is a fundamental
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26 64 element of many research questions in ecology (Sridhar et al., 2019). Within freshwater
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28 65 ecology a number of methods have been employed which seek to study the response of
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30 66 organisms to biotic and abiotic cues. These include the deployment of passive integrated
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32 67 transponder (PIT) tags and radio telemetry that enable monitoring of invertebrate and
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34 68 vertebrate movements (Barbin Zydlewski et al., 2001; Cooke et al., 2013); experimental
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36 69 observations of avoidance behaviours and locomotion levels to predation and competition
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38 70 (Alexander and Covich, 1991; Haddaway et al., 2014) and abiotic stresses (Wood et al.,
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40 71 2005; Conroy et al., 2018) and; flume studies to observe small scale movement patterns and
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42 72 responses to flow variations (Lancaster et al., 2005; Sidler et al., 2018). However, all of these
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44 73 methods have focussed on quantifying the activity of freshwater biota within the water
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46 74 column / surface sediments, whilst studies investigating activity within the sediment layer are
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48 75 rare.
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52 76 Interstitial pore space between gravel particles represents an important habitat for many
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54 77 organisms (Culp et al., 1983; Gee, 1982) and often provides a refuge from intra / inter
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56 78 specific predation (Mathers et al., 2019a), high shear stresses (Franken et al., 2006) in
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58 79 addition to extreme hydrological conditions such as floods or droughts (Dole-Olivier et al.,
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60 80 1997; Wood et al., 2010). Deeper subsurface (hyporheic) substrates are essential for

maintaining healthy ecosystem functioning and can be spatially extensive (Williams and Hynes, 1974; Stanford and Ward, 1988). Furthermore, for many river systems the hyporheic invertebrate production equals or exceeds that of the benthos (Smock et al., 1992; Dorff and Finn, 2020). Given the importance of subsurface sediments for supporting ecosystem functioning, the current limitations in quantifying behavioural attributes of fauna within the sediment layer is a considerable void in our knowledge.

Connectivity between surface and subsurface water is essential in permitting the exchange of water, dissolved oxygen (DO), nutrients, organic matter and organisms between ecosystem components (Jones and Holmes, 1996). Streambed permeability is an integral characteristic controlling hydrological exchange which results in the hydraulic conductivity of most streambeds typically being heterogeneous (Leek et al., 2009). Downwelling waters occur when surface water enters permeable river beds as a result of the hydraulic head being greater than that of the groundwater. This water passes into the groundwater or through the sediments before eventually emerging from the interstices as upwelling water (Brunke & Gonser, 1997). However, the connectivity and functioning of the hyporheic zone in many river systems is threatened by anthropogenic activities such as impoundment and excessive fine sedimentation which may modify substrate composition, infilling interstitial spaces, limiting vertical hydrological exchange and eventually disconnecting the hyporheic zone from the surface sediments (Sarriquet et al., 2007; Mathers et al., 2014; 2019). In contrast, in some rivers there may naturally be limited or no vertical hydrological exchange associated with impermeable substrates or reduced connectivity (Malcolm et al., 2003). Previous field (Pepin and Hauer, 2002; Davy-Bowker et al., 2006; Mathers et al., 2017) and laboratory (Mathers et al., 2014) studies have demonstrated that the direction of vertical hydrological exchange is important for determining the distribution and composition of macroinvertebrate communities, however this topic remains poorly understood (Davy-Bowker et al., 2006).

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3 107 Monitoring the real-time activity of fauna within the sediment layer is inherently difficult but is
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5 108 essential in order to gain a comprehensive and holistic understanding of how biotic and
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7 109 abiotic factors influence ecosystem functioning and in turn the threat that natural and / or
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9 110 anthropogenic stressors pose. Studies that have investigated the behaviour of organisms to
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11 111 environmental cues (e.g. vertical hydrological exchange, streambed drying events) within
12
13 112 subsurface sediments have focussed on the vertical distribution of biota by measuring their
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15 113 final position (Foy and Thistle, 1991; Stumpp and Hose, 2013; Mathers et al., 2014; 2019b;),
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17 114 by using a proxy such as leaf litter breakdown rates (Folegot et al., 2018), or by assuming
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19 115 use of subsurface sediments when individuals are not observed above surface substrates
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21 116 (Vadher et al., 2015). Stumpp and Hose (2017) employed transparent laboratory columns to
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23 117 assess the burrowing ability of a freshwater amphipod in redistributing fine sediment thereby
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25 118 creating migration networks. This method however only enables observations of activity at
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27 119 the edges of the columns due to the use of opaque natural substrates and continual real-time
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29 120 activity was not recorded as experiments were conducted over a year. To date we are aware
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31 121 of only two studies that have sought to quantify the real-time activity levels of fauna within
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33 122 subsurface sediments, but these are associated with limitations and did not employ lotic flow
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35 123 conditions. Vadher et al. (2017; 2018) utilised transparent acrylic particles to monitor the
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37 124 vertical migration of a freshwater amphipod in response to surface dewatering. In their
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39 125 experiments, the vertical distribution of amphipods were manually classified into 1 mm and 5
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41 126 cm intervals respectively, every 15 minutes prior to incremental water drawdown, providing a
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43 127 general vertical distribution profile over time. However, the use of transparent acrylic particles
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45 128 limits the horizontal field of observation as refraction still remains relatively high and therefore
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47 129 horizontal activity is difficult to quantify as substrate width increases. As such there remain
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49 130 clear gaps in our ability to simultaneously track multi-directional activity of multiple individuals
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51 131 using automated real-time observations under lotic flow conditions. Furthermore,
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53 132 parameterisation of faunal behaviour within substrates such as interstitial trajectories,
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55 133 velocities and body orientation have not been able to be quantified to date.
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To tackle the inherent problems associated with monitoring faunal behaviour and activity levels within subsurface sediments we present an experimental methodology that provides real-time tracking of fauna in multiple dimensions. Here, we detail the experimental setup and novel methodology which employs transparent super absorbent polymer (SAP) substrates in association with video tracking procedures to enable continuous tracking of multiple individuals for the first time in subsurface sediments under lotic flow conditions. To test the validity of our method we then employed it to determine horizontal and vertical velocities and the body orientation of a freshwater amphipod in the sediment layer in response to a number of vertical hydrological exchange treatments (no-vertical exchange, downwelling exchange and upwelling exchange; *sensu* Mathers et al., 2014). We selected the three flow treatments as vertical hydrological exchange has been shown to affect the vertical distribution of macroinvertebrates but to date monitoring the behavioural attributes of individuals continuously within the subsurface has not been possible. Flow treatments were run successively as we were interested in quantifying the transition period of individuals in acclimatising to new flow conditions and how their behaviours altered, for example via increased movement or changing their body orientation. We also hypothesized that the different flow treatments would result in different activity levels as individuals reacted to differing interstitial flow preferences. We also discuss the potential of SAP substrates in combination with a range of tracking procedures in the study of a number of ecological questions.

2. Methods

2.1 Experimental setup

Experiments were conducted in a custom-made recirculating acrylic glass flume (dimensions 179 cm long by 27.5 cm wide by 49.5 cm high; Figure S1; following Sidler et al., 2017; 2018). The sediment layer consisted of super absorbent polymer (SAP) spheres derived from polyacrylamide (PAM) with a diameter of 8-12 mm (M² Polymer Technologies), which were kept in place using an acrylic frame and butterfly netting (mesh size 8 mm). Use of SAP substrates enables tracking of organisms within the sediment layer due to the refractive index

162 of the spheres being very close to that of water and the material is not harmful to organisms.

163 Prior to experiments, SAP substrates were placed in a bucket of deionised water and left

164 overnight to swell to their maximum size (containing 90% water at this point and soft in texture).

165 All efforts were made to ensure that the experimental sediment layer was densely packed to

166 mitigate differences in vertical compression strength throughout the sediment layer. It should

167 be noted that once they have absorbed water, the spheres are almost neutrally buoyant and

168 therefore the spheres at the bottom of the sediment layer are not squeezed by the weight of

169 those above them. The experimental area was located in the middle section of the flume (120

170 cm long by 27.5 cm wide by 20 cm high) 40 cm from the header tank and therefore to obtain

171 uniform flow conditions, sections upstream and downstream of the SAP substrates (40 cm and

172 19 cm respectively) were filled with glass oval beads with a diameter of 17 mm (Figure S1).

173 The flume was filled with deionised water and water depths above the sediment bed were

174 maintained at ca. 16 cm via a perforated plate at the outlet of the flume. Flow velocities were

175 minimal to avoid additional confounding hydrological influences (depth-averaged horizontal

176 velocity of ca. 12 mm/s; Sidler et al. 2017). A valve with a flow meter regulated the discharge,

177 and uniform flow conditions were obtained via a combination of bricks, plastic honeycomb

178 panels and perforated plates at the inlet and outlet of the flume (Figure S1c). Downstream of

179 the flume, a thin metal mesh mounted in front of the outlet reservoir prevented individuals from

180 leaving the flume setup. Water temperatures were allowed to fluctuate under ambient air

181 temperatures ($20.2 \pm 0.3^{\circ}\text{C}$). This experimental setup has been used to successfully observe

182 the swimming behaviour of benthic copepods within the water column of a small volume (~1L)

183 of the flume (Sidler et al., 2017; 2018) but to date has not been tested to observe organisms

184 within deeper sediment layers. Here we also stitch together several camera views as described

185 below to observe fauna in the sediment layer of the whole experimental flume area.

186 The recording system consisted of three synchronised cameras (Mikrotron EoSens CL,

187 resolution of 1280 by 1024 pixels) equipped with Nikon 28 mm or 60 mm lens (set to f.16)

188 which recorded onto two arrays of solid-state disks (DVR Express Core 2, IO Industries). Two

189 halogen floodlights (400 W, 8545 lm, 2900 K) mounted above the flume provided

homogeneous illumination, and black acrylic sheeting was fixed to the flume wall opposite the cameras to provide a dark contrast against the organisms. It should be noted that Gammaridae display phototactic behaviour (migration away from the light source unless parasitized of which no individuals were from initial visual assessments; MacNeil et al., 1999), and as such results obtained from the artificial setup may differ from natural behaviour in opaque sediments and should therefore be applied with caution. Nonetheless for the purposes of quantifying behaviour between the interstitial flow treatments this effect is consistent throughout the experimental period. Alternative modes of observing individuals within SAP substrates without the use of artificial lighting are provided in the discussion. Cameras, all fixed at the same height, were mounted in planar configuration at the side of the flume and recorded at a frame rate of 50 Hz to enable continuous tracking of individuals' pathways within the experimental field of view which encompassed both the surface water and substrates (Figure S1a). The experimental facility can be used for any organism size with the camera lens and frame rate experiment specific to reflect the organism used and their mode of locomotion (see Sidler et al., 2017 for use with copepods and their intermittent behaviour).

2.2 Experimental design

To validate the experimental setup and apply the tracking methodology, we assessed the role of vertical hydrological exchange on the behaviour of a freshwater amphipod within subsurface sediments. By employing three synchronised cameras we were able to extract continuous trajectories of individuals during the recordings over a large section of the flume, which enabled the vertical and horizontal velocities and body orientation of each individual to be quantified. The diameter of SAP employed (8-12mm) provided an open framework with relatively large pore space therefore ensuring that movement was uninhibited and therefore had a negligible influence on an individual's orientation and interstitial velocity.

Three vertical hydrological exchange conditions were simulated: (a) no vertical exchange (control conditions; hereafter referred to as NVE for brevity); (b) downwelling exchange (DW); and (c) upwelling exchange (UW). Flow was delivered at a rate of 300 mL/s equivalent to a flow velocity of 3 mm/s within the interstitial pore space of the sediment. A second interstitial

flow rate was employed for recordings investigating the vertical orientation of individuals of 200 mL/s equivalent to a pore velocity of 2 mm/s. Both these interstitial flow conditions are representative of natural flow rates (Greig et al., 2007) and have been used in other laboratory studies measuring flow pathways in salmonid redds (Carling et al., 2006; Tonina and Buffington, 2009). On the base of the flume, a perforated ground plate covered with a thin plastic mesh enabled vertical hydrological exchange to be simulated whilst not permitting organisms to pass through. By pumping water into two chambers under the plate (Figure S1d), UW conditions were replicated, whilst pumping water out of the chamber created DW conditions. An external pump (Liverani Miniverter) connected to an electromagnetic flow sensor and transmitter (Siemens Sitrans MAG 1100) delivered flowing water to these chambers.

Gammarus fossarum was selected as the focal organism for the experiments. Gammaridae are commonly used in experimental studies including those examining faunal behaviour in the hyporheic zone (Folegot et al., 2018; Vadher et al., 2018, Little and Altermatt, 2018), and *G. fossarum* is a widespread and abundant model organism in Switzerland where the experiments were conducted (Altermatt et al., 2014). It is known to colonise benthic and hyporheic habitats, thereby enabling observation of a taxa that crosses habitat boundaries freely and, in many communities, dominates in terms of biomass (Robinson et al., 2003). All specimens were collected in the morning of each recording day from a local stream (unnamed tributary of Chatzenbach, Zurich, 47°40'97' N, 8°50'14' W) where they occurred in high abundances. Substrates were gently disturbed and drifting individuals captured with a pond net (mesh size 1 mm) thereby minimising damage and returned to the laboratory. Once in the laboratory, amphipods were allowed to acclimatise to laboratory conditions in holding containers for a minimum of 4 hours prior to the start of experiments.

Approximately 1,000 individuals of mixed size classes were released into the flume setup and allowed to acclimatise and distribute across the entire channel for 15-20 minutes prior to recording commencing. This period was determined to be sufficient for acclimatisation as active exploration and burrowing behaviour had ceased (*sensu* Gerhardt et al., 1994; Vadher

et al., 2015; 2017). Each hydrological exchange condition was filmed for 10 minutes to assess the behaviour attributes of individuals during the flow transition phase. It should be noted that the recording period is limited by storage capacity and as such experimental designs should be devised with this in mind. *G. fossarum* were first recorded for 10 minutes under no NVE, after this DW flow was simulated for 10 minutes and finally UW flow for a further 10 minutes. We conducted five replicates (with new organisms each time) with 28 mm lenses (field of view approx. 45 cm for each camera; Figure 1a) to record the velocity of individuals, providing a total of 150 minutes of recording (50 minutes for each camera) for each flow condition (NVE, UW at 3 mm/s and DW at 3 mm/s). To record the orientation of the individuals, 60 mm lenses (field of view approx. 20 cm for each camera; Figure 1b) were used with six replicates for the NVE condition and three replicates for each UW and DW flow rate (2 and 3 mm/s), again with new organisms each time. This provided a total of 180 minutes of recording (60 minutes for each camera) for the NVE condition and a total of 90 minutes of recording (30 minutes for each camera) for the UW and DW conditions at 2 and 3 mm/s.

2.3 Extraction of orientation angle, faunal trajectories and velocities

During the extraction process it became apparent that it was not possible to observe organisms deeper than ca. 150 mm below the surface of the sediment because of reflected light from the overhead floodlights onto the bottom acrylic glass plate of the main channel. This resulted in an overexposed area in the images that prevented the detection of *G. fossarum* and as such all analyses were conducted on sediment depths to 150 mm (and not the installed 200 mm depth). All image processing was conducted in MATLAB using standard functions from the image processing toolbox.

To calculate the orientation of individuals, immobile objects (e.g., debris or air bubbles trapped in the interstitial space) were first removed from the images by subtracting a background mask (Figures 1b,c) which consisted of an average of two thousand frames using the function *imsubtract*. Images were converted to black and white (*imbinarize*) based on a user defined threshold in the pixel intensity. Morphological operations of erosion, dilation and flood-fill operations (*imerode*, *imdilate*, *imfill*) were performed to improve isolation of bright objects

corresponding to individual organisms. These objects (i.e. individual organisms) were extracted from the images via connected-component labelling (blob extraction) which enables a subset of connected pixels in an image to be extracted. To determine the orientation of individuals, ellipses were fitted to the blobs (i.e. to the silhouettes of the organisms; Figure 1d). The body orientation was estimated as the angle θ between the horizontal axis of the image and the major axis of the ellipses and was only calculated for individuals in the sediment layer. To calculate the vertical and horizontal velocities of *G. fossarum* during the recording period trajectories were firstly calculated (Figure 2; Sidler et al., 2017). Trajectories were constructed using a two-dimensional implementation of the open-source three-dimensional particle tracking software OpenPTV (<https://www.openptv.net>). Organisms appeared in the images as bright blobs that were isolated from the darker background via high-pass filtering and simple user defined thresholds that allowed foreground pixels corresponding to the organisms to be separated from the background. The position of each organism in each image was determined using the grey-value weighted centre of gravity of its corresponding blob (Maas et al., 1993). Each individual organism was tracked in time by linking its successive positions in the images from one time step to the next. To predict the position of an organism in the next time step an algorithm was employed which calculated the prediction from an individual's velocity and acceleration in the previous time steps (with velocity or acceleration assumed to be constant). Within this algorithm the trajectory continues to be formed if a blob is found in the next image at the predicted position otherwise the trajectory stops. This approach allowed the tracking of a large number of organisms despite trajectories that often overlapped (Malik et al., 1993; Willneff and Gruen, 2002; Willneff, 2003). We discarded trajectories that were too short in space and time (less than 20 pixels in the images and less than one second in duration) as they generally resulted from wrong detections and tracking errors due to scratches on the front wall of the flume and / or the presence of bubbles and debris in the sediment. One set of trajectories were reconstructed for each flow condition (NVE, DW at 3 mm/s and UW at 3 mm/s) by combining all data from the replicates and cameras. Trajectories were subsequently smoothed with a second-order polynomial Savitzky-Golay filter with a span of 25 frames to

302 improve the precision of the data and reduce noise (Wolf et al., 2012). Data points at the
303 beginning and end of each trajectory, where the filter could not be fitted symmetrically, were
304 discarded to retain smoothed information only and reduce noise. From these trajectories the
305 horizontal and vertical components of the velocity vector for each organism at each time step
306 along the trajectories could be obtained.

307 Probability density functions were calculated on the datasets to account for the changing
308 abundances of individuals in the field of view over the course of the experimental recordings.
309 Probability density functions were calculated for the orientation angle and directional velocities
310 of individuals for 5-minute and 2-minute time intervals. Application of the finer 2-minute time
311 intervals enabled any temporal shifts in behaviour to be examined such as those immediately
312 following the flow transition period, whilst the 5-minute interval enabled any gross patterns in
313 behaviour to be visualised by flow exchange treatment. To specifically examine the response
314 of individuals to flow treatment independent of the previous flow treatment, the rate of change
315 in body orientation distribution was extracted. This was calculated by taking the probability
316 density function of one time interval and subtracting the previous one from it and dividing the
317 difference by the interval duration. For NVE no rate of change was able to be calculated for
318 the first time period (0-2) as no flow preceded this. By examining the rate of change in the first
319 two recording minutes of DW and UW flows, the reaction of *G. fossarum* in the flow transition
320 period could also be obtained for both orientation angle and velocities. Due to insufficient data
321 points in the water column statistically robust probability density functions could not be
322 obtained and we therefore restricted velocity analyses to data obtained in the sediment layer.

323 Differences in the mean orientation and mean absolute velocities were statistically tested via
324 Linear Mixed-Effects Models (LMMs) via the *lmer* function in the '*lme4*' package (Bates et al.,
325 2015) in the R environment (R Core Team, 2019). The model was fitted with the fixed effect of
326 treatment (five flow scenarios for orientation and three for velocities) with experimental
327 replicate fitted as a random effect to account for potential temporal autocorrelation. To assess
328 if there was any potential temporal dependence in the orientation measurements, the statistical
329 significance of the random effect was derived by employing a likelihood ratio test using the

anova function in the ‘*stats*’ package. Where significant differences occurred in flow treatment, post-hoc pairwise comparisons of groups were performed using least-square means and *p-values* were adjusted for multiple comparisons via Tukey tests within the ‘*lsmeans*’ package (Lenth, 2016). Standard error plots were constructed on the mean orientation data and velocities are presented as supporting information (Figures S2, S5).

3. Results

Application of the SAP substrates successfully enabled tracking of fauna within subsurface sediments. Orientation angles of individuals were calculated from a total of 300 Mio. data points and a total of 42 Mio. data points were used from the sediment layer to construct trajectories. Trajectory plots indicate that the mode of locomotion by *G. fossarum* differs substantially between the two habitat components (Figure 2). Within the open water, individuals demonstrate uninhibited swimming behaviour with long smooth trajectories. Within the porous sediment layer individuals displayed crawling behaviour around the particles, with a high number of directional changes and rounded trajectories. *G. fossarum* were found predominantly in the sediment layer during all recordings with only 562,530 data points extracted from the water column.

3.1 Orientation angle of fauna within the sediment layer under no, upwelling and downwelling vertical exchange

There were significant differences in the mean body orientation of *G. fossarum* associated with flow treatment ($F_{11,4} = 37.77$, $p = <0.001$; Figure S2). Under NVE conditions (control), *G. fossarum* displayed a body orientation which is predominantly aligned to the vertical axis of the image (body aligned vertically in the sediment) implying that individuals are often crawling up- or downwards. The distribution also shows a slight increase towards horizontal body alignment with a mean θ of $47.80^\circ (\pm 27.90^\circ)$; Figure 3a). DW flow conditions resulted in a change in the body orientation with *G. fossarum* demonstrating a shift towards the horizontal plane (mean θ of all treatments $46.00^\circ \pm 27.65^\circ$; Figure 3a). This change in body angle was more marked under upwelling flow where individuals demonstrated a greater tendency to align towards the

horizontal plane than the vertical one (mean θ of all treatments $42.28^\circ \pm 27.69^\circ$; Figure 3b). Pairwise comparisons indicated a significant difference in mean orientation between NVE and all other flow treatments (excluding DW 2 mm/s). Significant pairwise comparisons were also identified for DW 2 mm/s vs both UW treatments and DW 3 mm/s vs UW 3 mm/s (all $p < 0.05$; Table S1). There was no significant effect of experimental replicate on the orientation angle recorded indicating the orientation angle recorded was not a function of the previous flow treatment orientation angles (LR = 0.010, $p = 0.922$).

Despite the mean orientation differences being statistically insignificant between the two flow rates within the same treatment (Table S1), the strength of vertical exchange exhibited a minor effect on the body orientation with stronger exchange rates exerting stronger changes in body angles towards the horizontal plane. This effect was most marked under UW conditions but was also present under DW conditions (Figures 3, S2). When the rate of change of angle probability distributions were examined, differences were clearly evident between the flow treatment periods (Figure 4). Both UW treatments displayed greater rates of change particularly at the 0° angle, but also throughout the range of $^\circ$ suggesting that individuals repositioned themselves in response to the change of flow. When the period immediately following the flow transition was specifically examined it was apparent that this change in body orientation was instantaneous for UW treatments (Figure 5). Within DW conditions body orientation did display some immediate responses to the flow transitioning but these were minor compared to UW.

3.2 Vertical and horizontal velocities of fauna within the sediment layer, under no, upwelling and downwelling vertical exchange

Mean absolute vertical and horizontal velocities differed as a function of flow treatment ($F_{8,2} = 13.11$, $p = 0.003$ and $F_{8,2} = 10.77$, $p = 0.005$ respectively). *G. fossarum* displayed significantly greater horizontal and vertical velocities under UW vertical exchange conditions (mean absolute velocities of 1.2 ± 2.4 mm/s and 1.3 ± 2.2 mm/s) when compared to NVE (control; mean absolute velocities of 1.2 ± 2.2 mm/s and 1.2 ± 2.2 mm/s) and DW flow conditions (mean

absolute velocities of 1.2 ± 2.0 mm/s and 1.2 ± 2.3 mm/s; Figures 6, S3, S4; Table S3). This increase in velocities suggests more frequent and intense swimming. The change in activity was most notable for vertical velocities with exponential tails within the distribution plot under both UW and DW conditions indicating frequent bursts of fast velocities not seen in the no vertical exchange conditions (Figure S3a-c). There was little evidence to suggest any temporal variation in activity levels associated with the flow transition (Figure 6; Figure S3), however when rate of change was examined there was evidence that individuals behaved differently under UW flow conditions compared to NVE and DW. Under UW conditions, less individuals were stationary (lower probabilities of near zero velocities) whilst under NVE and DW more individuals became static (higher probabilities of near zero velocities; Figure S5). When the period immediately following the flow transition was specifically examined it was apparent that this change in activity was instantaneous for UW treatments (Figure S6) and remained elevated to a lesser degree throughout the treatment period.

4. Discussion

Application of innovative SAP transparent sediments in combination with computer vision techniques enabled the real-time tracking of multiple individuals in parallel in an artificial sediment layer more than a few cm's thick for the first time to our knowledge. To test the validity of our method, we successfully tracked *G. fossarum* individuals within a sediment layer 15 cm deep and 27 cm wide demonstrating the application of such a method to address ecological questions. The results from this study provide mechanistic insights into the response of freshwater fauna to vertical hydrological exchange albeit within an artificial experimental setting, with the direction and presence of such exchanges most likely being an important control in the activity levels and orientation of individuals within the sediment layer. Application of the artificial SAP substrates in combination with real-time tracking enabled two ecosystem compartments to be successfully crossed allowing detailed observations of how the behaviour of biota differed between the water column and within the interstitial pore space. As documented in the literature, we observed that the movement trajectories of *G.*

fossarum differed considerably between these two habitat types with uninhibited swimming in the water column and crawling behaviour between the interstitial pore spaces. Furthermore, we also observed that the majority of *G. fossarum* were located predominantly in the sediment layer during the experimental recordings (562,530 data points in water column compared to 41,946,791 in sediment layer). As *G. fossarum* is characterised predominately as a crawling species within fuzzy coded trait classifications (Tachet et al., 2010) our findings also successfully reflect this with locomotion preference with open water swimming being less dominant in our experiments. The successful characterisation of these widely documented locomotion modes by habitat component provides some validation of the methodology and experimental setup. Although we only extracted the basic characteristics of locomotion we believe that our method provides the ability to quantify the mechanics behind locomotion modes of biota and the role of body shape within the sediment layer (*sensu* Statzner and Holm, 1989 for surface flow conditions). Such efforts may aid and strengthen recent calls to incorporate functional and biological traits into ecological analyses and biomonitoring practices (Usseglio-Polatera et al., 2000; Desrosiers et al., 2019).

Typically, lotic animals often position their bodies to face the direction of flow (Statzner and Holm, 1989). Our results however demonstrate that this may not be the case within the sediment layer although the results should be applied with some caution due to the artificial nature of the experiments. We found that *G. fossarum* altered its body orientation under downwelling and upwelling flow conditions to be more aligned with the horizontal plane and not the vertical; this is most apparent under upwelling flows and with higher interstitial flow rates. It may be that as the flow velocities within the sediment layer are not as strong as those typically felt in the free-flowing water column (often between 0.15 and 1.5 m/s⁻¹) that organisms may not react as strongly to these flow cues. However, it may also be possible that *G. fossarum* align themselves under these weak interstitial currents such that they increase the oxygenated flowing water over the respiratory structures (and their brood if the females are carrying eggs). Under upwelling flows, by positioning themselves in a more horizontal structure (with the anterior side facing down) they increase the possibility of

oxygen diffusion through the gills due to their location under the abdomen. Under no vertical exchange conditions, it is likely their body position does not matter so much as they will have to create the current for respiration with their appendages. It is also likely, that as the dominant flow of water was laterally, albeit weak, that by facing vertically this may also aid the respiration process. By holding themselves in such body angles (vertically against lateral flows and horizontally against vertical flows) they also maximise flow velocities around themselves by creating the largest barrier to intragravel flows. This behaviour is likely to aid the respiration process in addition to reflecting their rheophilic (flowing water) preferences.

A third reason for these contrasting findings in orientation angles compared to open water flow conditions, may partially be a function of the artificial nature of the experimental setup. The phototactic behaviour of *G. fossarum* (MacNeil et al., 1999) may have resulted in some active orientation adjustments over the course of the experimental period such that individuals were not vertically facing the light source. However, this process is unlikely to explain completely the shift in body orientation as body orientation did not only vary associated with flow condition (which was run successively) but also demonstrated a linear trend associated with flow strength. As these differing interstitial flow velocities were not run successively within the same time frame, changes in the orientation angle cannot solely be explained by possible phototactic responses. We also observed contrasting patterns in the response of body orientation with upwelling treatments displaying strong responses particularly at the vertical 0° angle, but throughout the range of θ suggesting that individuals repositioned themselves in response to the change of flow. Most of the repositioning was instantaneous (0-2 minute time period), with changing orientations becoming stable and minimal (rate of change closer to 0) throughout the rest of the experimental period. This pattern was not matched for downwelling conditions suggesting that either this flow treatment did not require individuals to reposition or that the change in flow conditions (from no vertical exchange to downwelling) was not as severe as that from downwelling to upwelling which represented a complete reversal.

We recorded greater activity levels under the upwelling hydrological exchange conditions with more frequent bursts of fast vertical swimming not seen in the no exchange conditions. As noted above, *G. fossarum* is a rheophilic species and therefore it is likely that they are more active within their preferred habitat of flowing waters. Faster velocities were recorded for amphipods under upwelling flow conditions and it may be that these conditions could flush individuals into the water column, even with comparatively low intragravel velocities, and as such individuals may migrate more to find locations that are hydraulically stable; this change in activity levels was immediate. Under downwelling conditions, individuals may locate themselves in areas where local flow is enhanced therefore optimising the chances of food being transported to them from the surface in addition to providing oxygenated waters. We documented reduced activity levels immediately following the flow transition of no vertical exchange to downwelling flow conditions and this may reflect the more optimal conditions that downwelling waters potentially bring, however use of the hyporheic zone is patchy in nature (Dole- Oliver and Marmonier, 1992). Under extreme no flow conditions, particularly when fine sediment content is high, oxygen depletion is likely to be a significant threat and as such epigeal individuals (including *G. fossarum*) may move more to locate oxygenated waters, whilst hypogean individuals (e.g. *Niphargus* sp.) may reduce energy expenditure (Malard and Hervant; 1999; however oxygen was not an issue within our experiments being an open framework and trials were conducted under short durations). We call for further work to understand the mechanisms behind our experimental findings.

Use of the riverbed by biota is dependent on a number of factors and we propose that this innovative flume setup which employs SAP substrates can be employed to study a range of ecological processes and questions thereby enhancing our mechanistic knowledge of faunal interactions with the physical environment. Use of subsurface sediments is heavily dependent on its suitability with characteristics such as grain size and shape, in addition to the availability of pore space, altering the ability of fauna to access subsurface sediments (Xu et al., 2012; Vadher et al., 2017). Morphological (e.g. body shape and size) and behavioural traits (habitat affinity and locomotion strategy) of the individual may also alter the behaviour

of biota within the sediment layer (Omesová et al., 2008; Mathers et al., 2019b). There are also documented substrate preferences associated with the body size / life stage of an individual with intra and inter specific spatial segregation being observed in many amphipod species (McGrath et al., 2007; Clinton et al., 2018). Subsurface sediments may also act as a refuge from predation pressures of larger invertebrates and vertebrates (Starry et al., 1998; MacNeil et al. 2008; Mathers et al., 2019a). The diameter of SAP substrate is user defined and due to the refractive index being comparable to water, a mixture of sizes can be employed with little consequence for visibility. Besides using different grain sizes that are available commercially, it is possible to mimic a variety of grain textures using hydrogels manufactured in the laboratory using injection molding (Byron et al. 2013) or, for highly complex structures, stereo lithography (Arcaute et al. 2011). Accordingly, this allows the approach to be extended to a broad range of different organisms, much smaller or larger than those investigated here (please see Sidler et al., 2017, for application with cyclopoid copepods and their intermittent mode of locomotion using this experimental approach). Manipulation therefore of the above factors in combination with video tracking could enable enhanced understanding of how biota utilise this important habitat and therefore provide a strong foundation on which to model / predict the effect of natural and anthropogenic stressors.

One potential limitation of our study is the use of halogen lights which could have influenced *G. fossarum* behaviour to some degree. To overcome this it is possible to conduct measurements using near infrared illumination, in which case very strong light sources should be used to compensate for the generally lower sensitivity of standard camera sensors and for the strong absorption of IR light by water, which adds a financial constraint to conducting measurements. Alternatively, cameras featuring an excellent spectral response in the near infrared range can be used. However, the technology used in our measurements consists of standard machine vision cameras and computer vision techniques, and as such it can be applied widely without any specific expertise.

It has been hypothesised that the hyporheic zone may act as a nursery and refugium for benthic invertebrates during adverse surface conditions such as supra seasonal drought conditions (Boulton, 2003; Wood et al., 2010), ephemeral and intermittent streams (Datry, 2012; Loskotová, et al., 2019), and high flows (Dole-Oliver et al., 1997); however evidence is still equivocal. Recently the hyporheic zone has also been demonstrated to act as a thermal refuge from warming surface waters (Folegot et al., 2018) associated with the cooler temperatures often located in the deeper substrates. It is likely that subsurface sediments may also provide some protection from hydropeaking activities that instigate sharp thermal changes in the water temperature regime. The ability to make direct observations within the subsurface may make it possible to study these processes in detail.

The research presented here represents an interdisciplinary collaboration between hydraulic engineers and aquatic ecologists. Hyporheic zone research is challenging with many research questions laying at the interface of many disciplines and sub-disciplines. We still have many knowledge gaps that need to be addressed which have been identified by academics, environmental regulators and managers, some of which were first articulated over 25 years ago (Hakenkamp et al., 1993; Palmer, 1993). Tackling this interface requires interdisciplinary efforts and improved dialogues between disciplines to maximise scientific progress (Krause et al., 2011; Lewandowski et al., 2019).

Our new methodological approach outlined here, which combines innovative transparent SAP substrates with video tracking, has the potential to fill a large void in our understanding of how freshwater fauna organisms behave in subsurface habitats which are difficult to visually access due its very nature of being subsurface. To date, continuous tracking of individuals within subsurface sediments within running water has not been possible and thus successful extraction of movement and body orientation parameters represents a considerable advancement in freshwater ecology. This setup offers genuine potential in transforming our knowledge from descriptive patterns to mechanistic quantification of behaviours which underpin freshwater responses to physical processes and stressors. This

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2
3 548 is vital knowledge needed to halt the decline of freshwater diversity which at present far
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5 549 exceeds terrestrial and marine counterparts (Reid et al., 2019). We call for further
6
7 550 interdisciplinary research and methods which build on ours to address the behaviour and
8
9 551 response of organisms in a habitat which is out of sight but which shouldn't remain out of
10
11 552 mind.

13
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40 565 **Conflict of interest**

41
42 566 The authors declare no conflict of interest.

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45 567 **Authors' contributions**

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47 568 KM conceived the research idea and designed the methodology with FGM and MH, KM and
48
49 569 FGM ran the experiments, FGM led the data analyses with contributions from KM, KM led
50
51 570 the writing of the manuscript with contributions from FGM, MH and CW. All authors read and
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53 571 approved the final manuscript.

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55
56 572 **Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

List of Figures

Figure 1. (A) Image recorded with a 28 mm lens. Immobile objects (e.g., debris, frame holding the transparent beads) are removed during background subtraction. **(B)** Image recorded with a 60 mm lens **(C)** Image recorded with a 60 mm lens after background subtraction of immobile objects (e.g., debris, frame holding the transparent beads). Image corresponds to original shown in Figure 1b. **(D)** Process of fitting ellipses to organisms to retrieve the angle between the horizontal axis of the image and the major axis of the ellipsoid. Value shown is the orientation angle for that organism. Image corresponds to those shown in Figure 1b and 1c.

Figure 2. Subset of trajectories extracted from *Gammarus fossarum* activity in the water column and sediment layer. Shown are trajectories from one 5 minute recording taken from one camera.

Figure 3. Probability density function of the orientation angle (θ) of organisms over the first and second 5-minute periods of the recordings for **(A)** No vertical exchange (black and grey) and downwelling flow at 2 mm/s (dark and light red) and 3 mm/s (dark and light blue) and; **(B)** No vertical exchange and upwelling flow at 2 mm/s (dark and light red) and 3 mm/s (dark and light blue). $\theta = 90^\circ$ indicates vertical orientation.

Figure 4. Rate of change in orientation angle (θ) calculated from probability density functions for each flow treatment group. Note rate of change for minutes 0-2 are unable to be calculated for NVE. $\theta = 90^\circ$ indicates vertical orientation.

Figure 5. Rate of change in orientation angle (θ) calculated from probability density functions for the transition period to the new flow condition. Note rate of change unable to be calculated for NVE, but minutes 2-4 are provided as a comparison. $\theta = 90^\circ$ indicates vertical orientation.

Figure 6. Vertical (**A and B**) and horizontal (**C and D**) velocity (mm/s) of organisms within the sediment layer over the first and second 5-minute periods of the recordings for no vertical exchange (black and grey); (**A, C**) downwelling 3 mm/s and; (**B,D**) upwelling flow at 3 mm/s (light and dark red).

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Supporting information for “Beneath the surface: Application of transparent super absorbent polymer substrates to track faunal activity within the sediment layer”

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Figure S1. Photographs of the experimental setup.

Figure S2. Mean orientation angle (± 1 SE) of individuals for each flow treatment group. $= 90^\circ$ indicates vertical orientation.

Table S1. Summary of pairwise post-hoc tests on mean orientation angle by flow treatment. Significant ($p < 0.05$) values are emboldened.

Flow treatment	Downwelling 2 mm/s	Downwelling 3 mm/s	Upwelling 2 mm/s	Upwelling 3 mm/s
No vertical exchange	0.311	0.014	0.003	<0.001
Downwelling 2 mm/s		0.346	0.006	0.004
Downwelling 3 mm/s			0.090	0.003
Upwelling 2 mm/s				0.149

Figure S3. Vertical (A-C) and horizontal (D-F) velocity (mm/s) of individuals within the sediment layer for 2-minute intervals for the 10 minute recording under (A, D) no vertical exchange; (B, E) downwelling 3 mm/s and; (C, F) upwelling flow at 3 mm/s.

Figure S4. Mean ($\pm$ 1 SE) absolute (A) vertical and (B) horizontal velocities of individuals in the sediment layer for each flow treatment group.

Table S2. Summary of pairwise post-hoc tests on mean absolute vertical and horizontal velocities in the sediment layer by flow treatment. Significant ($p < 0.05$) values are emboldened. $N = 5$ and $df = 2$ for all flow treatments.

	Downwelling 3 mm/s	Upwelling 3 mm/s
<i>Vertical velocities</i>		
No vertical exchange	0.511	0.014
Downwelling 3 mm/s		0.003
<i>Horizontal velocities</i>		
No vertical exchange	0.146	0.083
Downwelling 3 mm/s		0.004

Figure S5. Rate of change in (A) vertical and (B) horizontal velocity (mm/s) of individuals calculated from probability density functions for each flow treatment group. Note rate of change for minutes 0-2 are unable to be calculated for NVE.

Figure S6. Rate of change in (A) vertical and (B) horizontal velocity (mm/s) of individuals calculated from probability density functions for the transition period for each flow treatment group. Note rate of change unable to be calculated for NVE, but minutes 2-4 are provided as a comparison.