

RESTOLINK
Joint sampling protocol
Version 2, October 17th, 2023

Content

1) Introduction.....	2
2) Selection of sites.....	2
3) Selection of habitats.....	3
4) General sampling procedure.....	4
5) WP 1 Hydromorphology.....	5
5.1 Reach-scale measurements.....	5
5.2 Hydraulic measurements at the spot scale.....	6
5.3 Morphological measurements at the patch scale.....	10
6) WP 2 Microbial and macrobial diversity.....	12
6.1 Parameters.....	12
6.2 Biofilm from green spots: chlorophyll-a and bacterial abundance.....	12
6.3 Laboratory analysis.....	13
6.4 Macroinvertebrates from green patches.....	14
7) WP 3 Ecosystem functioning.....	15
7.1 Stable isotope additions (¹³ C, ¹⁵ N).....	15
7.2 Organic matter decomposition.....	16
7.3 Biofilm from red spots for DNA and stable isotope uptake.....	17
7.4 Laboratory analysis DNA extraction.....	19
7.5 Food web complexity.....	20
7.6 Whole-stream metabolism.....	21
8) Literature cited.....	22
9) Appendix.....	25

1) Introduction

The following document contains the protocols for sampling, sample treatment and sample analysis of the RESTOLINK project. They represent the minimum requirements needed to determine the biodiversity of microbial and macrobial communities as well as selected ecosystem functions as defined in the proposal. The document is structured by work packages and a general section describing the site selection. Each regional partner is encouraged to undertake additional measurements in order to answer additional biome-specific questions. Please inform Mario Brauns about additional studies and measurements so that we can keep a record of these and distribute this information to the entire project team.

2) Selection of sites

Restolink adopts a biome approach and evaluates restoration success in the following regions:

Country	Biome	Köppen-Geiger climate
Brazil	Cerrado/Atlantic Rainforest	Cwa, Aw
Germany	Temperate broadleaf forest	Cfb
Spain	Mediterranean	Bsh
Sweden	Subarctic (Boreal)	Dfc

Within each biome (country), we will select three control-impact-restored pairs (triplets) in at least 3 streams. Streams should be permanently flowing, preferentially drain sub-mountain to mountain catchments, have 2nd to 3rd Strahler order and water depths > 10 cm. The discharge should ideally be < 300 L/s and NO₃-N concentrations should be < 2 mg/L. Candidate streams must be subjected to hydromorphological restoration measures at least five years before RESTOLINK has started. A hydromorphological restoration measure is defined as a measure that will produce a quantifiable effect on stream bed characteristics and/or stream hydraulics (see below for a list of hydromorphological parameters). If a given restoration measure has unclear hydromorphological effects, site selection will be discussed in the RESTOLINK consortium, and it will be jointly decided whether to include it or not. The type of restoration measures does not necessarily be replicated, i.e., it is possible to select 3 different measures within each biome to cover typical region-specific restoration measures.

It should be avoided that streams have other major human impacts that may affect restoration success, e.g., if a restored reach is located in an agricultural catchment. Each hydromorphologically restored site should be accompanied by a control and an impacted reach to form a control-restored-impact pair. The control reach should be located in a pristine catchment with native vegetation and no or minor human impact. The impacted reach should exhibit the hydromorphological degradation present in the restored reach before the restoration measure takes place. If other human stressors affecting the restored reach are inevitable, the control and impacted reach should be subjected to the same stressor.

The reach scale in RESTOLINK is defined by the length of the individual restoration measure. Control and impacted reaches should have a length comparable to the restored reach. However, reaches should be sufficiently long to include the entire affected area, e.g., impounded areas upstream of low-head dams. Reaches should be as homogeneous as possible and should lack tributaries, have homogenous canopy cover as well as comparable slope, width, geomorphic complexity and valley confinement.

3) Selection of habitats

Spatially, fluvial habitats are structured hierarchically from microhabitat ($\sim 10^{-2} - 10^{-1}$ m, hereafter **spot** for biofilms and **patch** for macroinvertebrates) and **mesohabitat** (100 m, hereafter habitat) to larger spatial scales like the **reach** scale ($\sim 10^1-10^2$ m or 10^{-12} x the bankfull width, Fig. 1) with processes emerging from both sides of the hierarchy (Frissell, Liss, Warren, & Hurley, 1986; Zavadil & Stewardson, 2013).

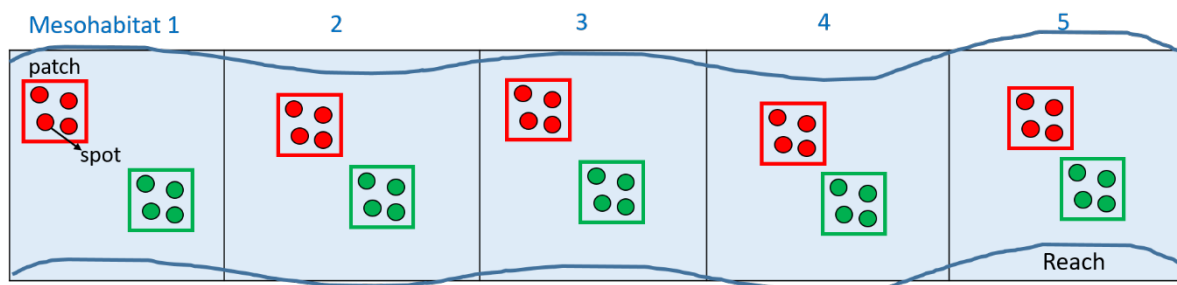


Figure 1. Schematic overview of the nested sampling design. The number of patches will be guided by the number of habitats available in the individual reach.

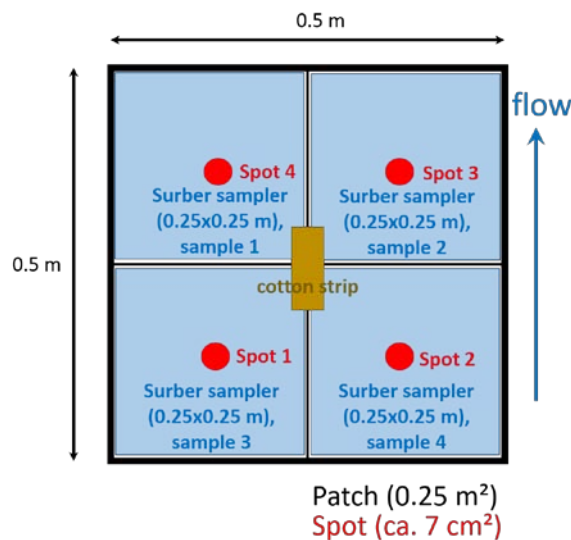


Figure 2. Details of a patch and its corresponding four spots.

RESTOLINK adopts a nested sampling design at four spatial scales (Fig. 1):

Spot: The smallest spatial scale pertains to the microscale, which is operationally defined by the size of the measuring volume of an acoustic Doppler velocimeter (3.64cm^2). The spot scale is where biofilms will be sampled.

Patch: Four neighboring spots delineate a patch that defines the area that is sampled for macroinvertebrates (0.5m²).

Mesohabitat: The mesohabitat is defined as an area of homogenous hydromorphological conditions (e.g., water depth, width, slope, surface flow type, grain size) and encompasses units such as pools, riffles, and glides. The mesoscale is delineated by the micro-scale measurements and samplings within the mesoscale. The number of patches within each mesohabitat is area-weighted. In case it is impossible to differentiate between habitats, use evenly spaced intervals for delineation of habitats

Reach: A reach is defined by the length of the restoration measure and is the largest spatial scale studied in RESTOLINK.

4) General sampling procedure

1. Sites selection including (1) "control", (2) "impact" and (3) "restored", in case all sites are at the same stream.
2. Delineation of reach for each site and mark start and end
3. Delineation of mesohabitats and patches: mesohabitats to be determined visually, e.g., distinguish between pool and riffle, 5 x 2 patches (distribution of patches within habitats depending on the areal extent of habitat – area weighted), always use a pair of two patches: a red patch, which is sampled after tracer addition and a green patch, which is sampled before tracer addition. Mark corners of patches with screws or flags.
4. Delineation of water stations (cross sections where water samples are taken): 6 water stations, i.e., one upstream, one downstream of the reach and the other 4 spread equidistantly in between
5. Expose loggers (e.g., light, temperature, stage, oxygen)
6. Measurements of hydraulics (40 spot measurements, 4 spots per patch) and morphology (10 patches) (see WP1)
7. Sample parameters associated with green patches (see WP 2)
8. Conduct tracer addition (15N-nitrate and 13C-acetate, NaCl/NaBr) addition (see WP3)
9. Sample parameters associated with red patches (see WP2 and WP3)
10. Expose cotton strips (see WP3)

Table 1. Overview of parameters to be sampled at different spatial scales.

Type	Parameter	Spot		Patch		Reach
		Green	Red	Green	Red	
Function	Biofilm DOC uptake		X			
Function	Biofilm NO ₃ -N uptake		X			
Function	Macroinvertebrate trophic niche width			X		
Function	Organic matter decomposition			X	X	
Function	PUC DOC uptake					X
Function	PUC NO ₃ -N uptake					X
Function	Whole-stream DOC cycling					X
Function	Whole-stream metabolism					X
Function	Whole-stream NO ₃ -N cycling					X
Structure	Bacterial abundance	X				
Structure	Biofilm biomass	X	X			
Structure	Biofilm chlorophyll <i>a</i>	X				
Structure	Macroinvertebrate biomass					X
Structure	Macroinvertebrate richness					X
Structure	Microbial richness		X			

5) WP 1 Hydromorphology

5.1 Reach-scale measurements

Parameters

- Mean water depth
- Stream width (wetted and optional: bankfull width)
- Water level and bed slope
- Mean flow velocity
- Discharge
- Grain size distribution

Equipment

- Ruler, tape measure, distance meter
- Level, or, if available, DGPS (global coordinates.), total station (local coordinate system)
- Conservative tracer addition (tracer, e.g., NaCl): salt, electric conductivity loggers, bucket, graduated pitcher.
- Protocols, writing material

Field instructions and post-processing

1. Using ruler, tape measure and level: mean water depths and width (wetted, bankfull if possible) of several cross-sections (approximately reach length divided by 10). Using DGPS/RTK-GPS (or total station with local coordinate system): coordinates in several cross-sections (distance spacing see above), incl. water edge and bankfull edge on both banks at each transect; longitudinal transect along thalweg of the reach for bed slope
2. Conservative tracer addition: please see WP 3 and Lamberti and Hauer (2017) for further instructions and calculations of discharge and mean velocities.

3. Pebble counts

Field instructions for pebble counts (Polvi, Nilsson, & Hasselquist, 2014)

1. Each reach is divided according to the water stations to connect pebble counts to the substrate mapping (see WP3)
2. Sampling is conducted by two people, one wading the stream and one taking notes
3. Reach is crossed-waded in a zigzag pattern
4. Sampling is conducted using a step-toe procedure: the person wading collects every two steps the first particle touched by his toe, measures the intermediate axis and communicates the particle length to the person taking notes on the shore
5. If particles cannot be removed due to size, weight, or other reasons, their size is measured underwater
6. Organic matter such as leaves and sticks are also picked up and measured
7. Particles smaller than 2 mm are classified as < 2 mm
8. 50 particles are collected in each section between 2 consecutive water stations except when:
9. the section is very short compared to the other sections (< 10 m), then only 25 particles are taken
10. if the reach is inaccessible (e.g., too deep), the number of samples can be reduced, but this should be noted

5.2 *Hydraulic measurements at the spot scale*

Measurements are done as close to the stream bed as possible since we want to characterize the benthic habitat. In particular, velocity fluctuations, which have been shown to be important for biofilms and macroinvertebrates (Biggs, Goring, & Nikora, 1998; Statzner, Gore, & Resh, 1988), are strongest in the vicinity of the streambed. The spot scale marks the sampling spot of flow measurements where biofilms are sampled later on.

Parameters

- Mean flow velocity
- Turbulent kinetic energy
- Mass transfer velocity
- Hydraulic diversity

Equipment list

See Appendix.

Field instructions (for ADVs)

1. Random positioning of tripods with attached ADV (e.g., Vectrino Profiler, Nortek AS, Norway; or other high-frequency devices) within a patch; watch out that tripod legs do not disturb flow in the vicinity of the ADV (Fig. 3)
2. Lowering ADV to the lowest position possible – recommended are 2cm above streambed when using acoustic devices because of interference with the streambed. When deploying the Vectrino Profiler, it is recommended to position the sweet spot of the device 2 cm above the stream bed (Brand, Noss, Dinkel, & Holzner, 2016; Koca, Noss, Anlanger, Brand, & Lorke, 2017 for position of the sweet spot.)
3. Set expected velocity range and start data collection, check the histogram for phase wrapping (in particular in fast-flowing areas), and adjust velocity range if necessary.

4. Start recording data for 10 min, which is sufficiently long for providing accurate characteristics of turbulence and facilitates spectral analysis of turbulent velocity fluctuations (Buffin-Bélanger & Roy, 2005).
5. Proposed settings (always depending on environmental conditions)
6. sampling frequency: 64 Hz
7. for Vectrino Profiler: max ping interval, except for very slow flowing areas (e.g., impoundments: min ping interval, or increase sampling volume with max sampling volume)
8. Mark the sampling location (e.g., with a little screw downstream of the sampling area) and take a macro picture with a laser pointer to highlight the precise location of the flow measurement on the bed. The photograph was used to identify the sampling location for later biofilm sampling (see Appendix and Fig. 4).



Figure 3. Tripod with attached Vectrino Profiler (Nortek AS, Norway) measuring high-frequency flow velocity at 2 cm above the stream bed.



Figure 4. Underwater photos of the sampling location marked with a metal nut (left) and a laser pointer at the precise sampling spot (right).

Post-processing

Matlab scripts for the processing of velocity time series measured with the Vectrino Profiler can be provided and adjusted for other ADVs by WP1. The processing follows Anlanger, Risse-Buhl, von Schiller, Noss, Weitere, and Lorke (2021). Briefly, raw data is filtered with a threshold of 15 dB for the signal-to-noise ratio and 70% for signal correlation. The filtered velocities are despiked using the method described by Goring and Nikora (2002), modified by Wahl (2003). Outliers are replaced by the nearest valid data points, and the resulting velocity vectors were rotated such that u , v , and w (m s^{-1}) denote the longitudinal, transversal, and vertical components of the mean flow velocity vector, respectively. Measurement noise was removed from the final velocities by low-pass filtering, while the cutoff frequency of the filter was estimated from the power spectra of each velocity time series and each velocity component. Noise was identified in the spectra as the breakpoint in spectral slope, where the spectra flattened at high frequencies.

Table 2. Overview of the components (α , β and γ diversity) of hydraulic and geomorphological diversity, which are expressed with data on flow velocity and streambed geomorphology.

Scale	Diversity	Flow velocity	Physical description	Streambed morphology	Physical description
Spot, Patch	α	Temporal flow diversity	Temporal variance of flow velocity $\overline{u'^2}$ normalized by the square of its temporal mean $\overline{u}^2 = (\text{twofold})$ ratio of turbulent kinetic energy TKE and squared mean flow velocity: $\alpha_{Flow} = \frac{\overline{u'^2}}{\overline{u}^2} = \frac{1/2 TKE}{\overline{u}^2}$	Streambed roughness	Spatial variance of water depths $\overline{h'^2}$ normalized by the square of the mean water depth $\overline{h}^2 = \text{squared relative streambed roughness}$: $\alpha_{Morpho.} = \frac{\overline{h'^2}}{\overline{h}^2}$
Meso, Reach	β	Spatial flow diversity	Spatial variance of time-averaged flow velocities $var(\overline{u})$ normalized by the square of their overall mean $\langle \overline{u} \rangle^2$: $\beta_{Flow} = \frac{var(\overline{u})}{\langle \overline{u} \rangle^2}$	(Mean) Water depth variability	Spatial variance of the mean water depths at the spot scale $var(\overline{h})$ normalized by the square of their overall mean $\langle \overline{h} \rangle^2$: $\beta_{Morpho.} = \frac{var(\overline{h})}{\langle \overline{h} \rangle^2}$
	γ	Overall flow diversity	Total temporal and spatial variance of flow velocity normalized by the square of their overall mean: $\gamma_{Flow} = \text{mean } \alpha_{Flow} + \beta_{Flow}$	Overall morphological diversity	Total spatial variance of water depths normalized by the square of their overall mean: $\gamma_{Morpho} = \text{mean } \alpha_{Morpho} + \beta_{Morpho}$

Data analysis

Mean flow velocity: The Reynolds averaged longitudinal flow velocity (u , m s^{-1}) was estimated as the arithmetic mean of all velocity post-processed measurements. Please note that v and w are zero after rotation.

Turbulent kinetic energy: The turbulent kinetic energy (TKE , $\text{m}^2 \text{s}^{-2}$) was estimated from turbulent velocity fluctuations (u , v , w ; m s^{-1}), which were obtained by subtracting the mean

values from measured (instantaneous) velocity components: $TKE = 0.5 (\overline{u'^2} + \overline{v'^2} + \overline{w'^2})$, where the overbar denotes temporal averaging.

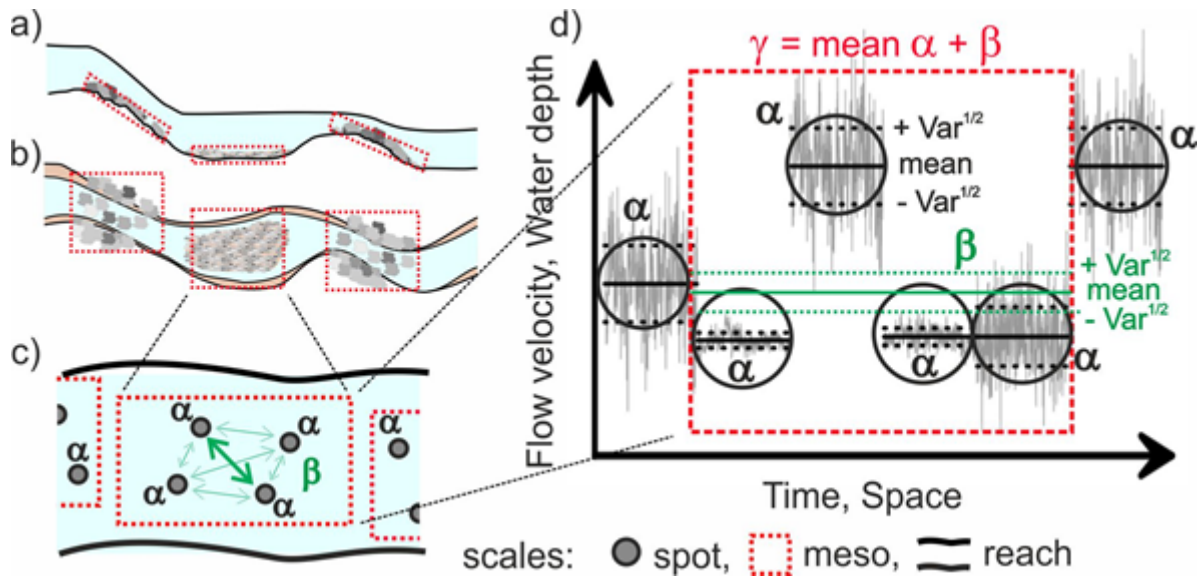


Figure 5. Conceptual framework for assessing the influence of hydromorphological variability on biodiversity and functioning in streams across scales. Larger scales in mountainous streams can be characterized by riffle and pool habitats or other distinct units (meso scale) or the reach scale (schematic longitudinal transect (a) and plan view (b)). Similar to biological diversities, hydraulic diversity can be calculated from individual spots (α diversity), between spots (β diversity, green arrows) and the overall diversity within a larger scale (γ diversity (c)). α diversity is the variance measured at the spot scale, and γ diversity is the total variance observed at larger scales (meso, reach). β diversity measures the difference in diversities between spots and, by using an additive approach, represents the variability of mean values among spots at larger scales. β and γ diversities are shown for the meso scale only; however, the diversities can also be calculated for the reach scale, with β diversity expressing the variation between meso habitats and the γ diversity expressing the overall diversity for the reach.

Mass transfer velocity: The mass transfer velocity (k , $m s^{-1}$) is estimated according to Katul and Liu (2017): $k = \alpha_1 (\epsilon v)^{0.25} Sc^{-0.5}$, where Sc is the Schmidt number, which is the ratio of the kinematic viscosity (ν , $m^2 s^{-1}$) depending on water temperature and the molecular diffusion coefficient of the compound under study (i.e., nitrate and dissolved organic carbon). α_1 is a dimensionless scaling factor and was chosen to be 0.4 (Lorke, Bodmer, Koca, & Noss, 2019). The dissipation rate of turbulent kinetic energy (ϵ , $W kg^{-1}$) was estimated from fitting power spectra of vertical velocity fluctuations to theoretical spectra within the inertial subrange (inertial dissipation technique; Bluteau, Jones, & Ivey, 2011).

Hydraulic diversity and extrapolation to larger scales: We will use a nested design to calculate alpha, beta and gamma diversity of flow and morphology at larger spatial scales. With this approach, we can account for temporal and spatial variability of flow and morphology, and we can directly relate it to biological diversity measures (e.g., α , β and γ diversities based on richness, Shannon diversity, Bray-Curtis etc.). Details for the expression of hydraulic diversity are described in the deliverable D7 and summarized in Fig. 5 and Table 2.

5.3 *Morphological measurements at the patch scale*

The smallest scale for morphology is the patch, which also defines the area sampled for macroinvertebrates (0.5 m²). Streambed topography and water depth are measured on this scale because they affect hydraulic habitat and mixing processes at the benthic interface (Lamarre & Roy, 2005; Noss & Lorke, 2016). Streambed topography is measured by a custom-made laser scanner similar to the systems described in Noss and Lorke (2016) and Noss, Wilkinson, and Lorke (2018). The measurements of riverbed elevation are based on the principle of laser scan triangulation and result in a topographic map with x, y, z coordinates (i.e., digital elevation model DEM). The streambed is illuminated with a laser line and observed by two cameras (Fig. 6). The bottom elevation along the laser line was reconstructed from the location of the laser line in the field of view of the cameras. Laser and cameras were mounted on a rack, which could be moved in a horizontal plane at an adjustable height above the bottom. The horizontal position is tracked by a third, emerged camera observing a simple LED attached to the vertical rod. The rack is adjusted for easy transport to all project partners.

Parameters

- Streambed roughness and water depth
- Morphological diversity

Equipment list

See Appendix

Field instructions

1. Mounting of cameras, laser and LED: take care that all camera angles and distances are adjusted for the local water depths still observing the laser line on the streambed. The emerged camera angle needs to observe the LED at all times.
2. Placing and leveling rack above patch
3. Start cameras, laser and LED and synchronize the cameras by a sudden movement that all cameras can see.
4. Adjust the vertical position of the laser and the two cameras so that they are submerged.
5. Carefully move the laser line along the horizontal direction of the frame. During laser deployment, use lightproof fabric to improve the visibility of the reflected laser line on the bed.
6. For each horizontal laser deployment, read the distance to the water surface from a fixed reference point on the laser holding vertical unit.
7. Replace the rack and repeat the scanning until the whole patch is scanned. In case the cameras are turned off during replacement, do not forget to synchronize them after restarting them.
8. For the conversion from pixel to metric coordinates, calibrate the laser line in a large bucket as outlined in Noss and Lorke (2016): observe the laser line on a calibration board at different heights ranging from min to max water depths (Fig. 4). Calibrate positioning of LED by at varying distances covering the whole horizontal movement

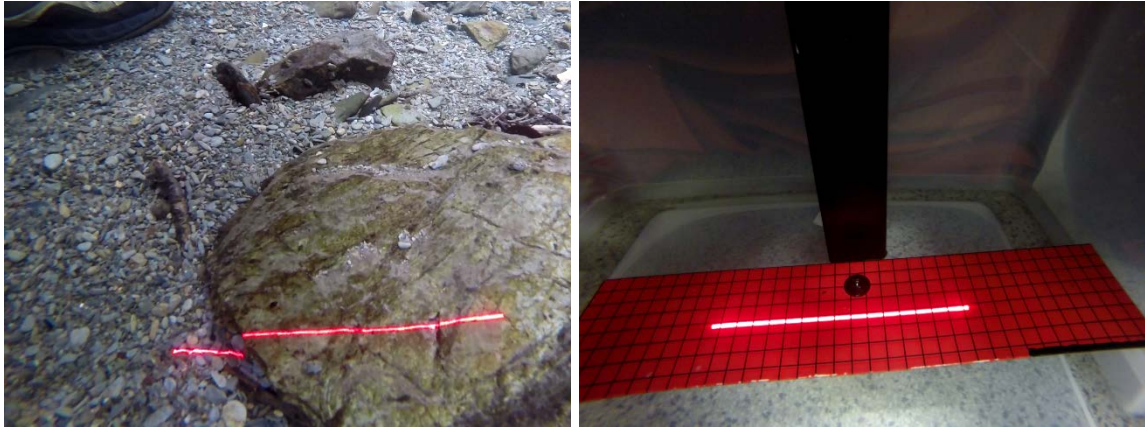


Figure 6. Streambed topography is measured by a laser line, which track is recorded by underwater cameras (left). The calibration procedure includes following the laser line on a calibration board at different heights ranging from min to max water depths (right).

Post-processing

Before processing the recorded data, synchronize videos from all three cameras by noting the time of the synchronization sign. A spreadsheet for this procedure is provided by WP1. In case the cameras were not turned off during replacing the rack while scanning a patch, cut all videos to the filming material necessary by using, e.g., AVIDEMUX free software. Finally, prepare snapshots from the calibration procedure for each position recorded. The snapshots are further used to transfer each pixel to metric coordinates.

MatLab scripts for calibration and further processing of videos are provided by WP1. Finally, the DEM is resampled on a regular grid using a final horizontal resolution of 0.2 cm (i.e., medium sand) to reduce data load but still represent the roughness of most common grain sizes.

Data analysis

Streambed roughness and water depth: Streambed roughness k is estimated as the standard deviation of the streambed elevation with respect to a planar surface, which is fitted to each DEM.

Geomorphological diversity and extrapolation to larger scales: same procedure as for hydraulic diversity except for geomorphological α diversity, which is calculated as the variance of water depth h normalized by the square of the mean depth at each patch (Table 1).

6) WP 2 Microbial and macrobial diversity

6.1 Parameters

- Macroinvertebrate communities, i.e., abundance, diversity and biomass
- Microbial communities, i.e., chlorophyll-a content, bacterial abundance, and microbial diversity

6.2 Biofilm from green spots: chlorophyll-a and bacterial abundance

Liftable stones

1. Gently lift the stone and place it in a tray without stream water.
2. Utilize reference photos to position the O-ring (with a 5 cm² diameter) on the target spot.
3. If macroinvertebrates are present, use tweezers to remove them
4. Rewet the biofilm at the target spot using an isosmotic solution.
5. Brush the stone as demonstrated here: <https://www.ohio.edu/voinovich-school/videos/biofilm-collection-streams> [at the 3.09-minute mark]. Transfer the biofilm slurry with a funnel into a 50 ml Falcon tube.
6. Adjust the final volume to 50 ml with isosmotic water
7. Sonicate the sample for 2 minutes at 125 W and 400 kHz.
8. Gently flip the sample three times and extract a 10 ml subsample for bacterial counting.
9. Transfer the 10 ml subsample into a 15 ml Falcon tube and add 1.2 ml of 37% formaldehyde to achieve a 4% v/v concentration. Store the Falcon tube in the fridge.
10. Make a note of the remaining volume.
11. Filter the remaining solution (40 ml or more) using a GFF filter Whatman 47 mm. Then, place the filter on a tissue, fold it and split it into two pieces using tweezers. Place the pieces into brown vials (kept in the dark) and press them to the bottom. Freeze at -20°C.
12. Note: If you have not collected sufficient material from the O-ring area, enlarge the sampled area and use aluminum foil to measure the sampled area.

Heavy (unliftable) stones

1. Sampling is done using a biofilm sampler.
2. Load a 60 ml syringe with the isosmotic solution.
3. Position the biofilm sampler over the target spot.
4. Rotate the handle three times to gently scratch the spot.
5. Lift the sampler with care.
6. Close the valve.
7. Reopen the valve and transfer the sample into the Falcon tube.
8. Proceed to follow the instructions from point 7 in the previous section

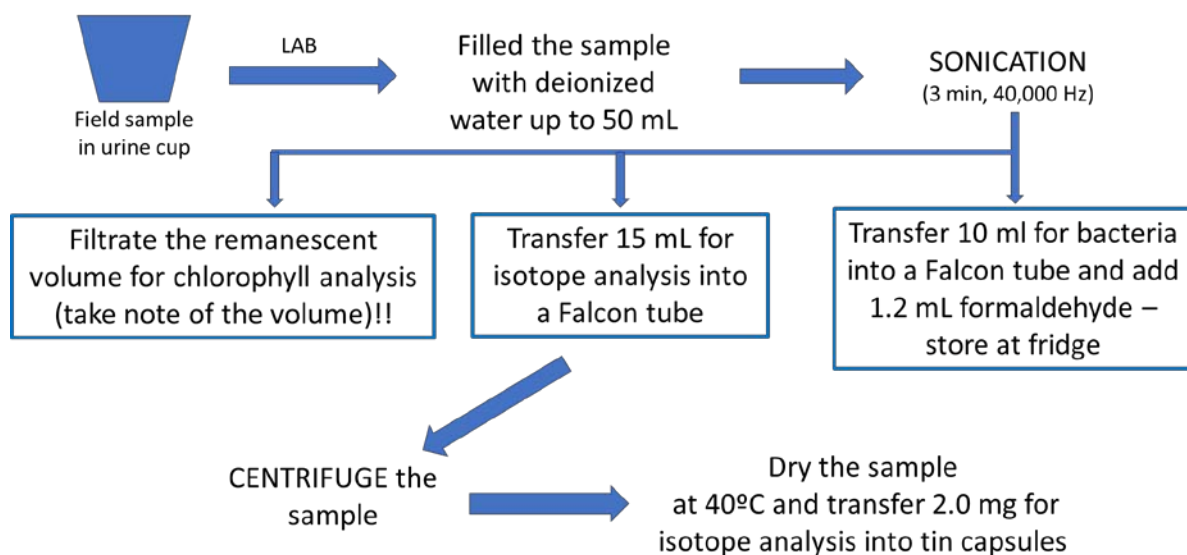
Stones covered with FPOM

1. Do not lift stones if they are FPOM-covered, as you will lose microbial communities.
2. Place the O ring on the designated sampling spot.
3. Utilize a 60 ml syringe to collect all the FPOM.
4. Follow point 6 in the section "Liftable stones"

Fine mineral substrate

1. Collect surface sediment from the top 1-2 cm using a small core (e.g., a 60-mL syringe with a cut tip or an inverted urine cup) along with the assistance of a thin plastic plate.
2. Place the sediment sample into a 50-mL Falcon tube.
3. Adjust the final volume to 50 ml by adding isosmotic water.
4. Sonicate the sample for 3 minutes.
5. In Spain only, use a 20-mL syringe to extract the supernatant.
6. Transfer the supernatant into a 50-mL Falcon tube.
7. In Spain only, adjust the final volume to 50 ml with osmotic water.
8. Gently flip the sample three times and draw a subsample of 10 ml for DAPI counting. Add 1.2 mL of formaldehyde.
9. Filter a known volume of the remaining solution using the GFF filter Whatman 47 mm for the chlorophyll-a subsample.
10. Store the DAPI subsample in the fridge at 4°C, while the chlorophyll-a filter goes into the freezer at -20°C.

BIOFILM PROTOCOL - GREEN PATCHES (FOR SAND AND GRAVEL SUBSTRATES)



6.3 *Laboratory analysis*

Bacteria extraction

Two different methods to extract and count the bacteria can be used.

- Flow cytometry as described in Perujo, Sanchez-Vila, Proia, and Romaní (2017)
- Counting of formalin-fixed samples after staining with acridine orange and counting using an epifluorescence microscope

Extraction procedure for flow cytometry

1. Vortex (5 seconds)
2. Sonicate with ice (2 cycles of 1 minute, 30 seconds rest in-between)
3. Vortex (8-10 seconds)
4. 1 ml of extract in 10 ml detaching solution in autoclaved glass vials
5. Shake in the dark at room temperature (30 minutes at 150-200 rpm)

6. Cool at 4 Celsius degrees for 10 minutes
7. Vortex (5 seconds)
8. 1 ml of extract in sterile Eppendorf
9. 1 ml optiprep density gradient in the bottom of the Eppendorf (using a needle)
10. Centrifuge 90 minutes at 14000 rpm at 4 Celsius degrees
11. Transfer the liquid (2 ml, without the pellet) and place it in a new sterile Eppendorf
12. Centrifuge again 30 minutes at 14000 rpm at 4 Celsius degrees/
13. Transfer the liquid (2 ml, without the pellet) and place it in a new sterile Eppendorf
14. Vortex
15. 400 µl of sample in a cytometer tube
16. Add 4 µl de SYTO 13 (FISHER, 5µM)
17. Incubate in the dark for 15-30 minutes
18. Add 10 µl of beads (solution 10⁶ beads/ml, FISHER, 1.0 µm), sonicate previously for 1 minute + vortex (5 seconds)
19. Read the sample in the cytometer.
20. Write down M1 and M2 values and the volume of each cytometer reading.

Chlorophyll-a analysis:

The biofilm suspension is filtered on GFF (Whatman®, Glass fiber filter) and analyzed photometrically (DIN 38409-60:2019-12)

6.4 Macroinvertebrates from green patches

From each patch, 4 replicated samples are collected with a Surber sampler (500µm mesh) and pooled to form a composite sample. The Surber replicates within a patch need to match with the area defined by hydraulic measurements (delineated with flags or similar). Samples should be elutriated after sampling to remove organic matter and pebbles. Samples are preserved in 75% ethanol and processed in the laboratory by counting and identifying them to the lowest taxonomic level feasible (usually genus level), except Diptera (family), Chironomidae (subfamily), Oligochaeta (order level) and Coleoptera larvae (family). From each taxon, at least 10 individuals should be measure for body length or head capsule width to determine dry weight by length-mass relationships.

7) WP 3 Ecosystem functioning

7.1 Stable isotope additions (^{13}C , ^{15}N)

Design

- 12 h addition of combined nitrate/acetate/NaCl solution dissolved in distilled water. NaCl slug additions before and after the isotope additions in streams with EC > 300 $\mu\text{S}/\text{cm}$ to avoid excessive salt concentrations.
- Individual additions for each reach unless high ambient nitrate or DOC concentration, in such cases
 - Conduct a nitrate or a acetate addition only
 - Conduct a single addition that encompasses control and restored reach, if they are located in close proximity
- Addition should encompass half night and half day
- Injection of solution with a metered peristaltic pump at least 25x stream width upstream of water station 1
- Injection should increase background bioavailable ^{13}C -DOC (10% of total DOC) and ^{15}N - NO_3 by a factor of 1.5 and 3, respectively
- Injection should double stream water EC
- Injection preferentially into a turbulence structure (end of a riffle, cascade) upstream of the start of the reach to ensure complete mixing, use a diffusor if possible
- Bromide can be used as conservative tracer together with the isotopic tracers

Equipment list

- Peristaltic field pump assembly
- Conductivity loggers (to monitor injection at the start and end of the reach)
- Pre-weighed ^{13}C -acetate, ^{15}N - NO_3 , and NaCl, considering field concentrations and Q
- Bucket/barrel for the solution, stick to mix
- Equipment and containers for biofilm sampling (5 patches x 4 spots = 20 50-mL flasks per reach and substrate, if more than one substrate (stones, sand ...) per spot)
- Flasks for stream water sampling (at least 6 stations [+1 upstream station] x 3 replicates = 21 flasks); flask volumes according to analytical methods used.

Sampling procedure and storage

- Biofilm sampling according to WP2 both before (background SI) and after 12 h of SI addition (addition still running), one extra sampling spot for SI analysis if biomass is low, transfer biofilm+water into flasks, store on ice, transport to the lab
- Sample all available primary uptake compartments around each patch following LINX2 protocols¹.
- Stream water sampling (Lagrangian) with injection still running, 6 water stations + 1 upstream station sampled in triplicate, distance between stations according to stream size (v, w, Q) and reactivity, sample filtration in the field (Whatman GFF)
- ^{15}N - NO_3 samples should be filtered over 0.2 μm and stored on ice or frozen immediately
- DOC samples should be filtered over 0.2 μm following Wilson, Munizzi, and Erhardt (2020), but rinse filter 2-3 times with stream water before filtering
- DO NOT preserve the samples with HgCl because no lab will analyze them

¹ <https://lter.kbs.msu.edu/research/areas-of-research/lotic-intersite-nitrogen-experiment-linx/>

- DOC samples can be stored in the fridge and should be analyzed within ~6wks

Lab instructions

- Biofilm: freeze-dry (no filtering; alternative: filter on GFF, dry, carefully remove OM from the filter), weigh ~2 mg of dry material (to 0.001 mg) in tin capsules (2 replicates per spot), transfer to well plates
- Each partner organizes SI analyses (particulate material, ^{13}C -DOC, and ^{15}N - NO_3) and processes samples accordingly
- Measure stream water background concentrations (DOC, NO_3 , chloride) and basic physicochemical characterization of the stream reaches (pH, EC, DO, Alkalinity, TP, SRP, NH_4 , TN).

7.2 *Organic matter decomposition*

Design

- Cotton strips and method description according to Tiegs, Clapcott, Griffiths, and Boulton (2013)
- 10 strips per reach, 1 in each patch, 3 control strips (same material, no incubation)
- In the lab, separate the strip threads 0.5 cm from one edge with a forceps to create a small opening. Push a cable binder through the hole (do not close).
- Restrict handling of each strip to a 1-cm wide area at the very end of each strip. Work as sterile as possible and wear gloves.
- After inserting the cable binders in each strip, place them into a plastic bag for transport.
- In the field, attach the binder to a loop tied to a thin piece of nylon rope (0.5 m). The end without a loop will be attached to a stake hammered into the stream sediment. Tie the end of the rope that does not have a cotton strip to the base of the stake close to the stream water/sediment interface. A small flat rock is placed on the rope immediately upstream from the cotton strip to prevent the cotton strip from flapping about in the current.
- Three control strips are used to estimate the initial tensile strength of the strips. Return these to the lab and keep them in the plastic bags.
- Strips will be incubated in the field for ~30 days (+/- 7 days). In warm (~25 °C) or cold streams (~12 °C), the incubation period should be shortened or lengthened by a week. Target: 50% tensile strength loss.

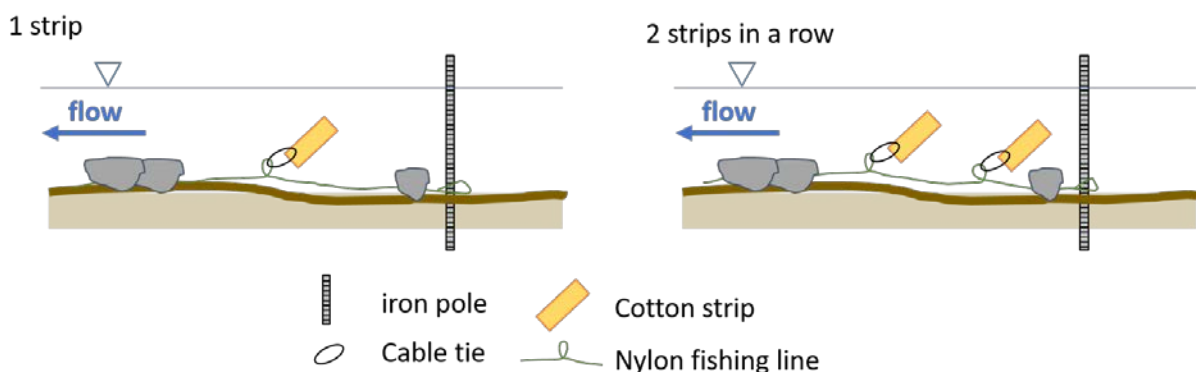


Figure 7. Details on how to install the cotton strips.

Equipment list

Incubation

- 10 cotton strips with cable binders inserted (and 3 control strips not incubated in the field) per reach
- Nylon cord
- 10 wooden stakes/metal bars and a hammer

Retrieval

- Scissors
- Labels
- Ethanol (approx. 500 ml)
- Shallow tray
- Aluminum foil cut into 10 10 cm x 10 cm squares (1 for each strip).
- Transport container
- Latex gloves

Sampling procedure and storage

- Cut the rope from each stake, cut the cable binder and detach it from the strip. Handle the strips gently and hold them only on the very end.
- Still in the field, after taking the strips from the stream, place each strip in a small tray containing a centimeter of ethanol (70% or greater, treat control strips the same way). Using a finger, gently brush the strip for 10 seconds on each side to remove adhering sediment and biofilm.
- Place each cotton strip individually onto the center of a sheet of aluminum foil and fold the sides of the foil over the strip, making an envelope. Keep the strip flat, do not fold or crumple. Label each envelope.
- Transport the strips to the lab in a rigid container.

Lab instructions

- Slightly unfold each foil envelope. Place the strips in an oven at 40 °C to dry for 24 h
- Once dry, close each foil envelope and store envelopes in a zip-lock plastic bag with some desiccant (instant rice, silica gel).
- Pack and ship the strips in a rigid container or padded envelope to the lab that will perform tensiometer measurements.
- Tensiometer measurements with a pulling rate of 2 cm/min, ~2–200 kgF measurement range

7.3 Biofilm from red spots for DNA and stable isotope uptake

Sampling

After each sample, ensure thorough cleaning of the biofilm collector and funnel with Milli-Q water. Do not use alcohol to not interfere with the $\delta^{13}\text{C}$.

Liftable stones

1. Follow steps 1-5 in the corresponding section of WP 2
2. Utilize a scale with precision to one decimal place and record the weight of both the Urine cup and the sample.
3. Ensure thorough mixing of the sample by attaching a new plastic tip to the pipette, aspirating 5 ml, and then dispensing the withdrawn volume back into the sample.

Repeat this process three times to ensure proper suspension and mixing of the material.

4. Use the same plastic tip used for mixing to draw 5 ml from the total volume and place it into a 15 ml sterile Falcon tube. Note: For DNA analysis, 0.5 mg dry mass is required. If you suspect that 5 ml may not provide enough material, increase the volume and make a note of it.
5. After taking the sample, weigh the urine cup with the sample. (For the reason behind this, refer to the next section.)
6. If possible, freeze both samples to -20°C while still in the field. Otherwise, keep them cool, and upon reaching the laboratory, freeze the small Falcon tubes containing the DNA sample to -80°C, and the Urine cups to -20°C. Note: To reduce freeze-drying time, allow the material to settle at the container's bottom and remove as much water as possible.

Heavy (unliftable) stones

1. Follow steps 1-5 in the corresponding section of WP 2
2. Proceed by following instructions from step 6 in the previous section

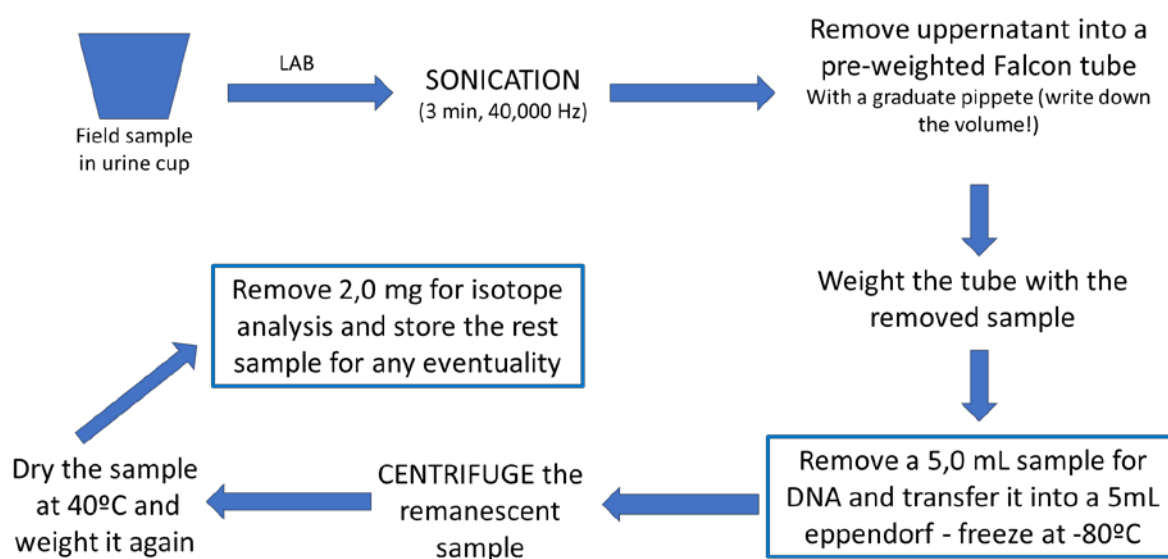
Stones covered by FPOM

1. Follow steps 1-3 in the corresponding section of WP 2
2. Proceed by following instructions from step 6 in the previous section

Fine mineral substrate

1. Pre-weigh a urine cup and record its weight.
2. Collect surface sediment from the top 1-2 cm using a small corer (e.g., a 60-ml syringe with a cut tip or an inverted urine cup) with the assistance of a thin plastic plate.
3. Place the sediment sample into a 50-ml Falcon tube.
4. Adjust the final volume to 50 ml by adding osmotic water.
5. Sonicate the sample for 3 minutes.
6. In Spain only, extract the supernatant using a 20-ml syringe.
7. Transfer the supernatant into the pre-weighed urine cup.
8. In Spain only, adjust the final volume to 40 ml with osmotic water.
9. Record the weight of the urine cup and the sample.
10. Thoroughly mix the sample by attaching a new plastic tip to the pipette, drawing 5 ml, and then returning the withdrawn volume to the sample. Repeat this process three times to ensure proper suspension and mixing of the material.
11. Using the same plastic tip used for mixing, draw 5 ml from the entire volume and place it into a 15-ml sterile Falcon tube. Note: For DNA analysis, 0.5 mg dry mass is required. If you believe that 5 ml may not contain enough material, increase the volume and make a note of it.
12. Record the weight of the urine cup minus the 5-ml sample.
13. Place the DNA sample and the remaining urine cup sample into the freezer.

BIOFILM PROTOCOL - RED PATCHES (FOR SAND AND GRAVEL SUBSTRATES)



7.4 Laboratory analysis DNA extraction

Equipment

- Qiagen DNeasy Blood & Tissue Kit.
- Protocol used for extraction: Purification or Total DNA from Animal tissues (Spin-column protocol)

Procedure

1. Firstly, the biofilm is transferred to a sample vessel in which 20 ceramic beads (1.4 mm, <https://de.vwr.com/store/product?keyword=432-0356>, Number:432-0356) are placed. Then add 180 ml of ATL buffer and 20 ml of Proteinase - K. After this is done, these vessels are placed in a tissue lyser (<https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/instruments-equipment/tissuelyser-lt>) for two times 20 seconds at 50 1/s.
2. Add 20 µl Proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample or place in a thermomixer, shaking water bath or on a rocking platform. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h or, for rodent tails, for 8 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely. After incubation, the lysate may appear viscous but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, see the "Troubleshooting Guide", page 52, for recommendations.
3. Vortex for 15 s. Add 200 µl buffer AL to the sample and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%) and mix again thoroughly by vortexing. It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. A white precipitate may form on the addition of Buffer AL and ethanol. This precipitate does not interfere with the

DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after the addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at $\geq 6000 \times g$ (8000 rpm) for 1 min. Discard flow-through and collection tube.
5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), and add 500 μ l Buffer AW2 and centrifuge for 3 min at 20,000 $\times g$ (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. It is important to dry the membrane of the DNeasy Mini spin column since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through since this will result in a carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 $\times g$ (14,000rpm).
6. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml micro-centrifuge tube (not provided), and pipet 200 μ l Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at $\geq 6000 \times g$ (8000 rpm) to elute.
7. Elution with 100 μ l (instead of 200 μ l) increases the final DNA concentration in the eluate but also decreases the overall DNA yield (see Figure 2, page 23).
8. Recommended: For maximum DNA yield, repeat elution once as described in step 7. This step leads to increased overall DNA yield. A new micro-centrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the micro-centrifuge tube from step 7 can be reused for the second elution step. Note: Do not elute more than 200 μ l into a 1.5 ml micro-centrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

7.5 *Food web complexity*

Sampling Design

Macroinvertebrate consumers are sampled from green patches as described in WP 2.

Sampling procedure and storage

- Life sort individuals of each abundant taxon (e.g., macroinvertebrates having a dry weight > 500 μ g)
- Remove guts from all omnivorous or predator species and store guts on alcohol for later gut content analysis.
- Identify macroinvertebrates to the lowest taxonomic level possible in the field. If this is not possible, freeze each consumer sample.

Lab instructions

- Remove tissue from shells (mollusks). Use boiling water to kill the bugs (if samples are not frozen) and remove tissue with two forceps.
- Dry (oven drying at 45C is fine) and grind the individuals of each taxon and replicate separately.
- Weigh 0.5-1 mg of dry material in tin capsules and transfer individual samples to well plates.

- Each partner is responsible for SIA analyses but should report lab and international standards.

7.6 *Whole-stream metabolism*

Design

- 2- station upstream-downstream DO change method (cross-calibrated DO and temperature loggers at the start and end of each reach) if reach lengths are 0.4 to 1.0 v/k (Demars, Thompson, & Manson, 2015)
- 1-station method (one DO logger at the end of the reach) if reach length is >3 v/k (or stream conditions are homogenous over >3 v/k) (Reichert, Uehlinger, & Acuna, 2009)
- Measurements for at least 3 days during the cotton strip incubation
- Reaeration according to methods available (propane/SF6 additions, empirical equations)
- Parallel measurements of w, d, v, Q, groundwater and lateral dilution, groundwater DO concentration (piezometers, only applicable in sites with large groundwater inputs), once at the start and end of DO measurements
- Atmospheric pressure and PAR records above the stream channel, continuous

Equipment list

- Calibrated DO and T logger, 2 cross-calibrated loggers for the 2-station method
- Pressure and PAR loggers
- Propane and injection/sampling equipment (if applicable)
- Piezometer for groundwater DO measurement (if applicable)
- Equipment for NaCl addition (slug, better constant rate) and conductance loggers to estimate Q, v, and dilution
- Tape measure/laser device and meter stick to measure wetted channel width and depth

Sampling procedure and storage

- Obtain an estimate of gas transfer (k) and average current velocity for each reach from previous studies, field measurements, and/or empirical equations (Raymond et al., 2012)
- Choose adequate method and reach length based on $L \sim 0.7 (0.4 \text{ to } 1.0) v/k \rightarrow 2\text{-station} / L > 3 v/k \rightarrow 1\text{-station}$
- Calibrate DO/T probes directly before measurements according to manual
- Cross-calibrate probes (2-station method) by placing them in the same position side-by-side in the thalweg of the stream and measuring DO for one day. The obtained data will be used to adjust measured data later.
- Place one probe in the thalweg, middle of the water column at the end of the reach, and another (2-station method only) at the start of the reach. Avoid waterfalls and unusual turbulence directly upstream of probes, and make sure that the measured reach is homogenous and does not include non-target sections (impacted sections in the natural reach, etc.) or tributary streams.
- Place a PAR logger at a position representing typical average light conditions.
- Place an atmospheric pressure logger somewhere in the riparian zone.
- Measure DO, T, PAR, and atm. pressure for 2 weeks at 5 min intervals (for the 2-station method, 1 min works better) during the cotton-strip incubation.

- Retrieve DO/T probes after 2 weeks. Cross-calibrate probes (2-station method) again, as described before.
- Before the measurement and directly after, estimate k , Q , v , d , and w , and ideally, groundwater input and DO concentration into the reach with available methods. See e.g., Hall and Hotchkiss (2017)

Lab instructions

- Download data from all loggers and synchronize time series for all variables in a single spreadsheet.
- 2-station method: run regressions between the two DO and T cross-calibration time series of the two probes for both the before and after cross-calibration (before-after corrections usually only necessary if you do not use optical sensors). Use regression equations to adjust data along the sampling interval (if needed) and between probes.
- Metabolism estimates according to one of the available methods. Suggested: streamMetabolizer (Appling, Hall Jr., Yackulic, & Arroita, 2018) for 1-station method, Bayesian R script by Hall, Tank, Baker, Rosi-Marshall, and Hotchkiss (2016) Hall et al. 2016 for 2-station method.

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9) Appendix

Restolink WP 1:

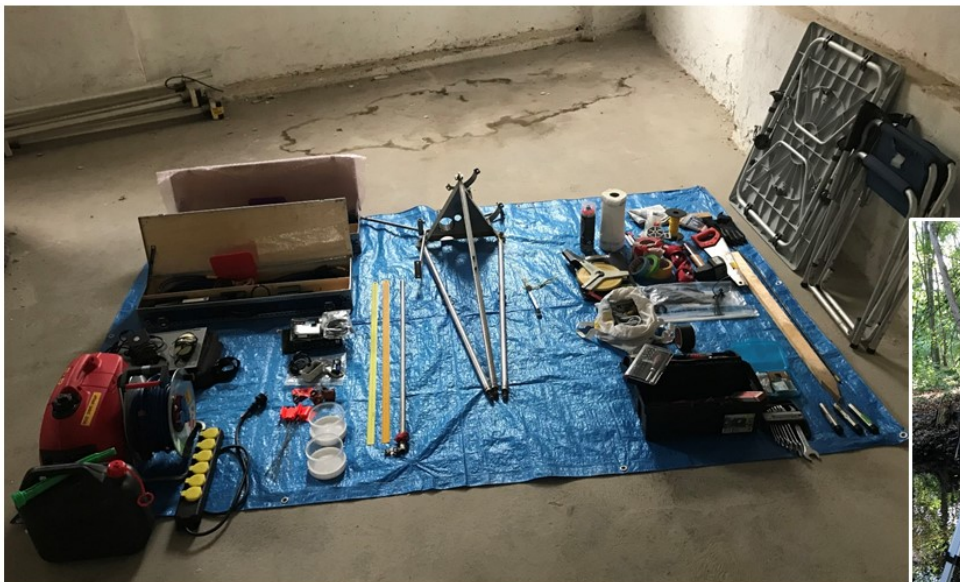
Measurements of:

- Flow
- Streambed roughness

Equipment list



Overview



Flow measurements



Details



High frequency flow measurement devices
Here: 2 Nortek AS, Vectrino Profiler incl. 20m cable, plugs, etc.

Flow measurements



Table
Chair(s)

3

Details



Holding frame "Eddy"

Holding frame "Sven"
consisting of

- 1 commercially available tripod: important are the screws!
- 1.50m Alu rod, full-tube
- Inlet – custom made – details see next slide

Flow measurements



4

Details

Holding frame "Sven", consisting of

- a commercially available tripod,
- a custom made holding inlet, and
- an Alu rod in two pieces (total length: 1.5 m)



construction details can be obtained from WP1 leaders

Flow measurements



Details



Computer to run the data collection software



Flow measurements

Electricity (230V) to run the Vectrinos and the computer

Here:

- generator,
- cable,
- fuel,
- socket strip (water proof)

Alternatively: car battery and voltage transformer (1 Vectrino: max. 4 Watt, Computer: 20 Watt?)
6

Details

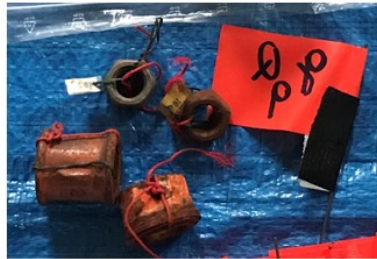


Examples of how to mark the position of spot – see Appendix

Flow measurements

Helpful things to find and mark the position of flow measurement:

- Camera (water proof) (BRA ?, ESP ?)
- Laserpointer (water proof) (BRA, ESP)
- Flags (in GER: to find patches, but could also be used to find spots..), (BRA ?, ESP ?)
- Metal pieces (screws of different size depending on the streambed: for rough, stable streambed with high flow I used bigger, heavier ones; for smooth, slow flowing areas I used smaller ones. For dynamic sand-bed streams we maybe should think about something else because the screws might cause local scour – flags might work better?)
- Plastic containers used as aquascopes 😊 (BRA, ESP)



7

Details

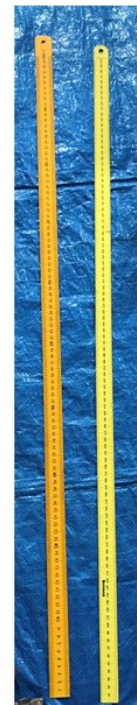


Useful things:

Necessary are:

- Tape
- Screwdrivers
- Open-end wrench
- Ropes and plane (for rain events)
- Cable ties

Ruler for measuring water depths. Those metal rulers (either 50 or 100 cm) work nicely.



8

Overview

Streambed roughness



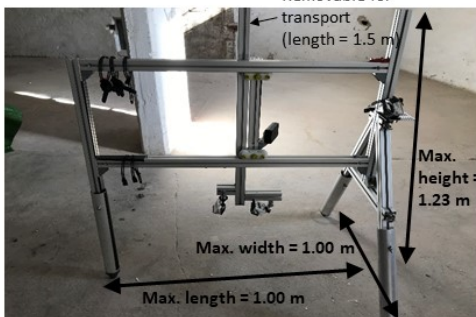
Carboy of tracer addition is also used for calibration of the laser scan



9

Details

Streambed roughness



Holding frame out of 4 x 4 cm alu profiles
Legs extendable

Holding frame disassembled:



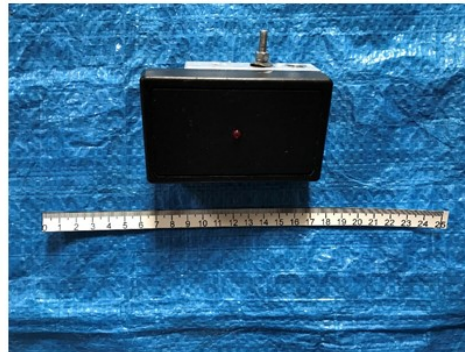
10

Details



Streambed roughness

- 3 GoPros and accessories (water proof housing, batteries, mounting, charging stuff)
- Red line laser in water proof housing
- LED in splash proof housing



11

Details



Streambed roughness

Opaque fabric for darkening to increase contrast with laser light on streambed
Umbrella, stick, etc. (things to span the fabrics)

Tank for calibration (alternatively, calibration is also possible in stream at place with high water depth)



Calibration tank:
Optimal height = 0.6 m
Minimum length (or diameter) = 0.4 m

12