PROTECTING URBAN AQUATIC ECOSYSTEMS TO PROMOTE ONE HEALTH

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OneAquaHealth Field Sampling Protocols for Urban Stream Ecosystems

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Contents

1 Introduction 3
2 Sampling site characterization 3
2.2 Catchment area and land cover information 4
2.3 Water samples 4
2.4 Hydromorphology 4
3 Ecosystem Health Indicators sampling 5
3.1 Benthic macroinvertebrates 5
3.2 Fish 6
3.3 Diatoms 6
3.4 Macrophytes 7
3.5 Riparian vegetation 7
4 Biological Indicators of Health risk sampling 8
4.1 Epilithic Microbiomes 8
4.2 Adult Diptera 9
4.3 Birds 9
5 References: 11
Annex I 12

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1 Introduction

This document provides standardized field sampling protocols developed under the European project OneAquaHealth with the aim of assessing urban aquatic ecosystems under a One Health perspective.

The protocols describe harmonized procedures for sampling site characterization**,** ecosystem health indicators and biological indicators of health risks. They are designed to ensure methodological consistency enabling comparable datasets and integrated analyses across cities and countries. These protocols were used to obtain the data presented at the City Dashboards, in predictive models developed under OneAquaHealth project and in the project publications.

Sampling site characterization includes geolocation, altitude, air and water physicochemical parameters (temperature, dissolved oxygen, pH, conductivity, total dissolved solids), stream morphology (width, depth, flow velocity), hydromorphology, and catchment land cover characterization (via GIS).

Ecosystem health indicators covered in this protocol include benthic macroinvertebrates, fish, diatoms, macrophytes and riparian vegetation, as well as birds in riparian corridors. Descriptions include sample collection, with field and laboratory recommendations aligned with European standards where applicable.

Biological indicators of health risks are also addressed, including protocols for sampling epilithic biofilms, water microbiomes and disease vectors such as mosquitoes and other Diptera in the riparian zone. A standardized field sampling form is provided to support consistent data recording across sites.

These protocols support integrated monitoring of biodiversity, ecosystem functioning and potential human and wildlife health risks in urban stream environments, contributing to evidence-based management and nature-based solutions in European cities.

2 Sampling site characterization

2.1 Geolocation, altitude and multiple parameters

Aim: To record the geographical location, altitude, and in situ physicochemical parameters of the sampling site.

Material:

- Field form (**Annex I**)
- GPS device
- Multi-parameter water quality meter
- Measuring tape
- Current velocity meter
- Datalogger for temperature

Procedure:

Fill the field sampling form (**Annex I**) taking all the site characterization information required:

1) Write down the GPS coordinates and altitude;
2) Measure air temperature and take the date and time of sampling;

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3) Measure water temperature, dissolved O_2, pH, conductivity and total dissolved solids in the water using the multiparameter probe;
4) Measure water width, depth and velocity using measuring tape and current velocity meter, respectively.

2.2 Catchment area and land cover information

Aim: To characterize land use variables within the catchment and surrounding buffer area.

Land use characterization (e.g., % impervious areas, % natural area, % agriculture) in the catchment area and a buffer area (500 meters) around the site will be surveyed through GIS later.

2.3 Water samples

Aim: To obtain water samples for the analysis of physicochemical parameters, including nutrients, organic matter, suspended solids, and pharmaceutical compounds.

Material:

- 3 water sampling bottles, PET, sterile or acid washed (2 x 1000 mL, 1 x 500 ml per stream)
- 2 Falcon tubes (1 x 15mL, 1 x 50 mL per stream)
- Freezer (-20ºC)
- Formic acid (80%) (optional)

Procedure:

1) Rinse the collection bottles with water *in situ* before making the collection.
2) Fill 1000 mL bottle with flowing water for pharmaceuticals. Freeze the samples and adjust pH to 3 with 80% formic acid solution (about 0.5 mL for each 500 mL sample) before freezing (-20ºC).
3) For nutrients determination (total P, total N, phosphates, sulphates, ammonia, nitrates, nitrites, and other cations and anions fill a 50 mL Falcon. Freeze the samples (-20 ºC).
4) Collect water in the 15 mL Falcon (for TOC, TON). Freeze the samples (-20 ºC).
5) Fill 500 mL PET bottle for TSS and keep cold (do not freeze).

2.4 Hydromorphology

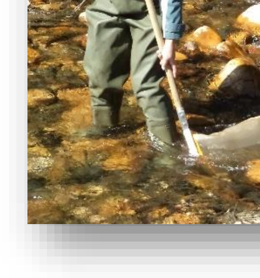
Aim: To assess the hydromorphological conditions of the stream reach, including channel structure, substrate composition, flow diversity, and connectivity alterations.

The main hydromorphological characteristics of the stream (100 meters reach) are characterized regarding the presence, abundance and/or diversity of flow types, substrate types in the channel and banks, and presence of barriers to longitudinal connectivity, among others.

Procedure: Fill the field sampling protocol (**Annex I**).

3 Ecosystem Health Indicators sampling

3.1 Benthic macroinvertebrates



Aim: To use benthic macroinvertebrates as an ecological indicator/biodiversity measure.

Aquatic macroinvertebrate sampling is based on European standards of sampling and treatment (European Committee for Standardization, 2003, 2004, 2006).

Material:

- Hand-net (0.5-mm mesh size, 0.25 × 0.25 m opening)
- Waders
- Plastic containers (wide-mouth, 1-2 L) or large ziplock-bags or similar.
- Permanent markers, pencil and labels
- 10% formalin or 96% ethanol

Procedure:

1) Select a stream section of 50 meters, including different types of substrate and flow velocity (e.g., riffle zones, pools, etc), if available.
2) Perform 6 sub-samples distributed by the existing habitats (in proportion to their existence). Each sub-sample covers 1 m long and ca. 25 cm width (corresponding to the net opening).
3) The net is placed in the bottom of the stream facing upstream (against the current) and the sampling is performed by kicking the substrate and stir the bottom and sweep along 1 m upstream.
4) Transfer the sample from the hand net to the plastic bottle container or ziplock-bag, previously labeled (use always an internal label and an external one with: the name of the stream and code, date and project name). Samples should be transported/maintained in a cooler until preserved with 10% formalin or using ethanol 96% (note: remove as much of the supernatant water as possible from the sample before using ethanol). (If the invertebrates are sorted alive just use ethanol after sorting – see 5)).
5) In the lab (or in the field before preserving the samples), to facilitate the work of finding invertebrates, wash the sample over 3 different sieves of 0.5mm, 1mm and 2mm. Separate each one for a tray and distribute the sample homogenously in the tray. Sort all the invertebrates from both 2mm and 1mm fractions and from the smaller (0.5mm) perform sub-samplings if needed (e.g., 1/3 of the sample or ¼) and take note of that in the bottles and label.
6) Then identify all individuals to the lowest possible level (at least family).

3.2 Fish

Aim: To use fish as an ecological indicator/biodiversity measure.

Fish sampling is based in the European standard for electrofishing in wadable rivers (European Committee for Standardization, 2003b).

Material:

- Electrofishing gear (backpack electrofisher and corresponding accessories)
- Waders
- Bucket 10L
- Fishing net with handle
- Cooler/container 30L
- Notebook, pencil

Procedure:

1) Select a stream wadable section of 100 meters, including different types of substrate and flow velocity (e.g., riffle zones, pools, etc) containing at least one riffle zone if available.
2) Walk *in stream* along the selected section for 30 minutes, using the electrofisher device and collecting the stunned fish with the fishing net to the bucket filled with stream water.
3) If the number of individuals becomes too high in the bucket during the sampling period, transfer them to the bigger cooler/container with local water in the margin, until finishing sample.
4) Identify and count each captured species and after return them to the stream with the exceptions of the exotic species (not be returned to the stream).

3.3 Diatoms

Aim: To use diatoms as an ecological indicator/biodiversity measure.

Diatom sampling is based in European standards of sampling and treatment (European Committee for Standardization, 2003c, 2004b).

Material:

- Waders
- Soft brush (e.g. toothbrush)
- Falcon tubes 50 mL (or similar to urine cups with screw cap 60 mL)
- Tray/container
- Permanent markers, pencil and labels
- 96% ethanol
- Distilled water

Procedure:

1) The upper surfaces of five submerged stones (an area of ~100 cm2) will be scrubbed with a toothbrush and washed with running water or distilled water in the tray/container.
2) The scrapped material (with the minimum amount of water) will be transferred to a Falcon tube/sampling container and preserved in 75% ethanol (fill the tubes). Close the container very well, covering the lid with parafilm.
3) The diatoms are later identified in the laboratory under a microscope and counted (ca. 400 valves), according to standardized protocols.

3.4 Macrophytes

Aim: To obtain a simple structural classification of the different types of macrophytes found in the urban streams.

Material:

- Notebook, pencil
- Field Form (**Annex I**)

Procedure:

1) Select a stream section of 100 m, including different types of substrate and flow velocity (e.g., riffle zones, pools, etc) if available.
2) Record in the field form (Annex I) the different *in* channel vegetation types (e.g., filamentous algae, submerged broad-leaved, free-floating, emergent reeds, etc…) along the river section using the presence and indicating if it is extensive (≥33% area) and native/non/native.
3) Take pictures!

3.5 Riparian vegetation

Aim: To characterize the structure of the riparian vegetation in the margins of urban streams.

Material:

- Field sampling form (**Annex I**)
- Notebook, pencil

Procedure:

1) Select a stream section of 100 m, including the 50 m used for the remaining sampling procedures.
2) Fill the field sampling form (**Annex I**) for riparian site characterization information required.
3) A census is conducted on the vegetation coverage percentage in the study area, that includes 10 m in each margin. Vegetation is divided in 3 different categories:
    a. Herbaceous (height < 1.5m)
    b. Shrubby (height 1.5 - 3m)

c. Arboreal (height > 3m)

To each of these categories is attributed a score from 1 to 5 which represent the cover percentage ranging from: 0-20%, 21-40%, 41-60%, 61-80% and 81-100%. Plants in different categories may be overlapping in the area, such that is possible that different categories have high cover percentages, and as such, its sum will not be related the total cover percentage of the area.

4) Additionally, information was also collected on the dominant shrubby and arboreal species present in the riverbed in which their coverage represents 10% or more alongside the 100m transept, as they may influence biological communities in these sites by providing shade and shelter over the water line.

4 Biological Indicators of Health risk sampling

4.1 Epilithic Microbiomes

Aim: To obtain information on bacteria and micro-eukaryotic communities through molecular identification.

Material:

- Gloves,
- Scalpel/knife/spatula
- 2 mL Eppendorf
- Distilled water
- Ethanol
- Cooler/freezer (at least -20ºC)/dry ice

Procedure:

1) Select a stone/stones or a solid surface (concrete) with biofilm (water depth of 15 / 30 cm). Note the material you sampled.
2) Scratch biofilm material with a clean scalpel/knife/spatula (cleaned with ethanol and then distilled water) into a labelled 2 mL Eppendorf. There should be at least 0.5 mL of biofilms.
3) Freeze the samples as soon as possible (preferably in dry ice).

Important: wear gloves and avoid touching the (scratched) biofilm.

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4.2 Adult Diptera

Aim: To collect flying Diptera communities, analyze the presence of disease vectors, and analyse their pathogens (causing e.g., Dengue, Zika, West Nile Virus, Usutu and Chikungunya).

Material:

- Mosquito traps (e.g. Biogents BG Pro; https://research-shop.biogents.com/collections/all-articles/products/bg-pro-set-of-4; 2 traps per stream site)
- Powerbanks (min. 10000mA)
- Tubes for CO_2 (like aquarium tubes)
- CO_2 from dry ice (ca 1 kg per night of sampling/ stream)
- 50mL tubes to store samples/or Petri dishes

Procedure:

1) Select a place to install the traps near the water (approximately at eye level) at dusk (2h before sunset); traps device placement and assembly explained in: https://eu.biogents.com/bg-pro/; https://youtu.be/bzMgeCx1WGo. Take care that the 2 traps are apart at least ca. 5 m from each other (ca. 25m are suggested) and avoid their exposure to direct sunlight when sun rises.
2) Place the dry ice in the thermal insulated bag (included in the trap), please use the "hats" of the trap which concentrate the CO_2 and protects from the rain, and connect the thermal bag to the area bellow the trap hat with the CO_2 tube;
3) Turn on the fan by connecting to the powerbank; be sure the powerbank is not close to the dry ice to avoid frosting (the pocket in the thermal box did not work for us).
4) Leave the trap in the field until 2h after sunrise; be sure to collect samples before it gets too warm for the mosquitoes);
5) Retrieve the section of the trap containing the diptera (net bag) and take it to the laboratory. There, freeze the mosquitoes and transfer them into the 50mL tubes/ Petri dishes. Keep them frozen, preferably at -80ºC;

Additional: Note the meteorological conditions of the sampling (e.g. use a thermohigrometer)

4.3 Birds

Option 1 – Mist net bird catches:

Aim: To identify the bird species using the streams and riparian zones, use them as a biological indicator of stream quality, as vectors or reservoirs of pathogens, and as biological controls (e.g., mosquitos and other Diptera).

Material:

- Mist nets
- Paper bags (ideally with baking paper in the bottom and metallic grid for faeces collection for microplastic analysis, according to doi: 10.1007/s00248-018-1182-4).
- White or light color fabric bags.

- poles and strings
- 150mm calliper
- Wing ruller
- 50g and 100g Pesola
- 1.5mL microtubes
- Fine pointed tweezers
- 27G and 25G needles
- Heparinised microcapillary tubes
- Slides
- Slide boxes
- Microtube boxes
- Cool box
- Cotton
- Metanol
- Serology filter paper
- Ethanol 70%

Procedure 1:

1) Install the nets (60 meters) before dawn and during 5h, in the shade and in days with no wind or rain.
2) Place an open 1.5mL tube in the ringing table in vertical position as control for microplastic contamination
3) Visit the nets regularly (every 40min) and retrieve birds from the nets.
4) Leave birds in the paper bags closed with a clothes' peg for 10min, inside the fabric bag, in the shade for them to defecate
5) Collect any faeces from the bags into 1.5mL microtubes (use clean tweezers between individuals) - keep refrigerated and freeze at the laboratory for microplastic analysis
6) Identify the bird, its age and sex (when possible)
7) Collect biometrics (wing length, tarsus length, body mass)
8) Collect blood by puncturing the brachial vein and collection with the heparinized tubes. Stop the blood with cotton. Do 2 thin blood smears, air dry (at the end of the day fix the smears by submerging 7min in methanol). Keep at room temperature. Place two thick drops of blood in serology filter paper (air dry and keep at room temperature in separate paper envelopes per individual (for WNV antibody analysis). Eject any remaining blood into a 1.5mL tube containing 96% ethanol (for malaria analysis).
9) Collect any ticks parasitizing the bird (look carefully around the eyes, beak and ears by blowing the feathers apart) with tweezers and place them in a 1.5mL microtube. Add 0.5mL RNAlater or freeze at -80ºC - if to screen for viral RNA. For zoonotic bacteria ethanol 70% may be used as preservative.

Option 2 – Acoustic Identification of Birds:

Aim: To use bird species presence and bird community traits as an ecological indicator/biodiversity measure.

Material:

- Smartphone with bird identification app (recommended: BirdNET or Merlin Bird ID).
- Field notebook or digital recording sheet.

Procedure 2:

1) Conduct the survey during the breeding season, when bird vocal activity is highest. Surveys should take place in the early morning, between the 2nd and 5th hour after sunrise (E.g.: If sunrise occurs at 06am, surveys should be conducted between 07am and 10am. Choose spots that characterize well the habitat surrounding the stream and away from strong noise sources.
2) Upon arrival at the sampling point, remain quiet and still to avoid disturbing birds and to improve sound detection.
3) Use the acoustic identification app such as BirdNET or Merlin Bird ID to identify bird species based on their vocalizations. The app should be used continuously during the survey period. When possible, ensure that the app records and stores the sound clips. E.g.: Merlin saves the sound clip and in case of uncertainty of ID, you should flag those records and then send only those to an expert.
4) At each sampling point, register all the different species of birds by their calls during a 10 minutes period.

5 References:

European Committee for Standardization (CEN). (2003). Water quality — Sampling of macro-invertebrates with hand net (EN 27828). Brussels: CEN.

European Committee for Standardization (CEN). (2003b). Water quality — Sampling of fish with electricity (EN 14011). Brussels: CEN.

European Committee for Standardization (CEN). (2003c). Water quality — Guidance standard for the routine sampling and preparation of benthic diatoms from rivers and lakes (EN 13946). Brussels: CEN.

European Committee for Standardization (CEN). (2004). Water quality — Guidance on the selection of sampling methods for benthic macroinvertebrates in fresh waters (EN 25667-3). Brussels: CEN.

European Committee for Standardization (CEN). (2004b). Water quality — Guidance standard for the identification, enumeration and interpretation of benthic diatom samples from running waters (EN 14407). Brussels: CEN.

European Committee for Standardization (CEN). (2006). Water quality — Guidance standard for the sampling of benthic macro-invertebrates from wadable rivers (EN 16150). Brussels: CEN.

Annex I

FIELD SAMPLING FORM

1. Sampling site identification (river section of **50 meters**, performed during Spring)

Site number:	Site Name:	Stream Name:
Sampling date:		Time:
Coordinates (GPS; Lat, Long):		
Altitude (m)		

2. Environmental parameters measurement (if possible in a run zone)

Air Temperature (ºC): _______________

Water Temperature (ºC): _______________

Water Dissolved Oxygen: _______________ (mg/L) / _______________ (%)

Conductivity (µS/cm): ________________

Total Dissolved Solids (TDS, mg/L): _______________

pH: ______________

Flow Current Velocity* (m/s): i. ______________ ii. _______________ iii. ______________

Water Column Depth* (cm): i. ______________ ii. _______________ iii. ______________

Water width* (m): i. ______________ ii. _______________ iii. ______________

*please take 3 measurements within your sampling section: i, ii, iii

3. Hydromorphological characterization (in the 50 m stretch)

Hydromorphological parameter	**Measurement**
Flow types (RU: no waves/runs; RI: unbroken standing waves/riffles; PO: pools; NP: no perceptible flow; D: Dry areas/Intermittent flow) *(use P for Present; E for Extensive – more than 33% of the channel; A for absent; note the number of riffles and pools)*	RU = RI = PO = D =

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Substrate types present in the channel (BE: Bedrock; BO: Boulders; ST: Stones/Cobbles; G: Gravel; SA: Sand; MU: Mud; OM: Organic Matter Deposits; AR: Artificial) *(use P for Present; E for Extensive – more than 33% of the channel; A for absent)*	BE = BO = ST= G= SA= MU= OM= AR=
Substrate in the banks (EA: Earth/soil; ST: Layed Stones (natural stones not compacted by concrete); GA: Gabion (non-natural stones or from other areas, not compacted by concrete, inside a metal mesh); CC: concrete or similar artificial impervious materials; Other: *specify*) *(use P for Present; E for Extensive – more than 33% of the banks; A for absent and analyse each margin separately)*	**Right margin** EA = ST = GA CC Other= **Left margin** EA = ST = GA CC Other =
Barriers to longitudinal connectivity (number of weirs/dams)	
Outflows (number of pipes or similar discharging pluvial waters to the stream)	
Intakes (number of pipes or similar abstracting water from the stream)	
Bridges (number of bridges pedestrian or for car traffic crossing the stream)	Pedestrian = For cars =
Presence of **other artificial structures** occupying the stream banks, or margins – up to 10m after the stream bank (specify, e.g. banks, street lights, sidewalks, bike paths, roads, houses, industrial facilities)	

4. Riverine and riparian vegetation characterization

a. Macrophytes – Channel vegetation types

Indicate in the table below if these types of aquatic plants are absent (**A**), present (**P**), Extensive (**E**; i.e., occupying >33% of the channel). Take pictures and make a video.

Indicate also, for each type, if there are native or non-native species (use the following symbols: **?**, if you don't know; **0**, if there are none; **1** if you see **1** non-native species; **>1** if you have several non-native species for that vegetation type; add the name of the species, if you know them)

Channel vegetation type	**Abundance** (A/P/E)	**Non-native species** (?, 1, >1; species names)
Liveworts/mosses/lichens		
Emergent broad-leaved herbs		
Floating-leaved (rooted)		
Free-floating		
Amphibious (occupy the transition between land and water)		
Submerged broad-leaved		
Submerged linear-leaved		
Filamentous algae		

b. Riparian vegetation

Vegetation type	**Cover category** Select one: 1 - 0-20%, 2 - 21-40% 3 - 41-60% 4 - 61-80% 5 - 81-100%	**Non-native species** (?, 1, >1; species names)
Trees (height >3m)		
Bushes (height 1.5-3m)		
Herbaceous (height <1.5m)		