# Assessing Metropolitan Biodiversity Using Aquatic Environmental DNA Metabarcoding

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## 1. Abstract

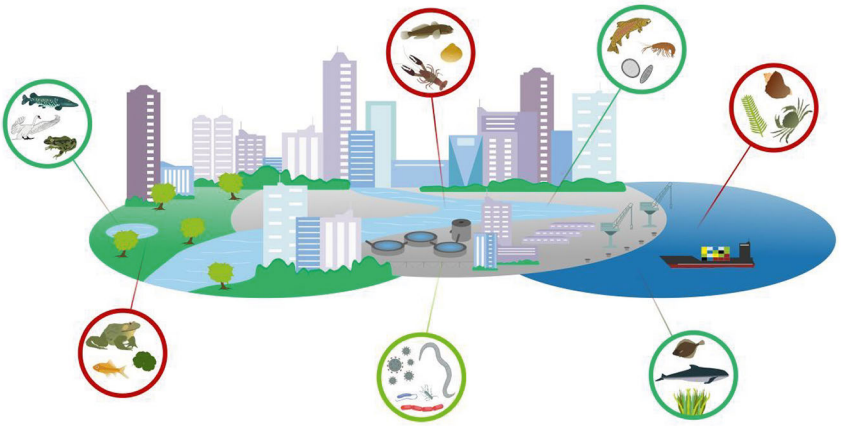
In recent years, metropolitan areas are expanding faster than ever, largely affecting neighboring biodiversity and even forming an ecosystem of its own inhabited by often peculiar fauna and flora. Ongoing urbanization is known to affect the metropolitan biodiversity by altering the available habitats, causing biotic homogenization and introducing alien, often invasive species. Urban freshwater ecosystems are particularly vulnerable, and since all cities heavily rely on healthy aquatic ecosystems, further understanding and recognition of metropolitan freshwater biodiversity is key for sustainable planning and management of freshwater ecosystem services. Thus, we here showcase the potential of using DNA-based methods, in particular environmental DNA (eDNA) metabarcoding, i.e. a technique for biodiversity assessment from DNA traces in the environment, for assessing the metropolitan diversity and evaluating potential threats to healthy aquatic ecosystems. We present the advantages as well as the shortcomings of eDNA metabarcoding and by evaluating several studies, we discuss pathways for its future application in routine biomonitoring of metropolitan freshwaters (fig. 1) while at the same time also engaging city inhabitants. With that, we show that environmental DNA is a very capable tracer of environmental change in aquatic ecosystems and can be a promising solution for future sustainable development of metropolitan ecosystems.

## 2. Introduction

### 2.1 Impact of Urbanization on Metropolitan Biodiversity

Cities are arguably the fastest developing ecosystems in the world. Over half of humanity is currently living in metropolitan areas, and it is predicted that by 2050 the number of city inhabitants will exceed two thirds of the global population (United Nations 2019). This trend is particularly striking in Europe, where already about 70% of the population

*Fig. 1: City of the future – utilizing eDNA-based biomonitoring tools to assess metropolitan aquatic ecosystems, including monitoring species of interest (examples presented in green circles), invasive species (red circles) and pathogens (light green circle)*



lives in cities, which cover approximately 4% of the continent (Koceva et al. 2016). Given that the physical extent of metropolitan areas grows even faster than the metropolitan population, it has major implications for metropolitan biodiversity resulting in habitat loss, biotic homogenization and the introduction of alien species (McKinney 2006; Elmqvist et al. 2013). On the other hand, metropolitan areas host a unique and often exceptionally high biodiversity and with that, they are often considered as hotspots of peculiar, urban biodiversity (McKinney 2008; Dearborn/Kark 2010). Nonetheless, since it is predicted that urbanization will in particular affect natural or near-natural areas recognized as key biodiversity hotspots (Seto et al. 2012), the trend poses a serious conservation challenge (McKinney 2002). Thus, a better understanding of the composition and dynamics of metropolitan biodiversity seems essential for the sustainable planning and management of metropolitan ecosystems and their services.

Recently, public interest in biodiversity in cities and metropolitan areas is growing with an increasing number of studies focusing on the impact of urbanization on the diversity and distribution of metropolitan biodiversity. This also translates into an increased awareness of metropolitan society (e.g., via citizen science projects) and policies of local decision-makers to protect metropolitan ecosystems, e.g., through various restoration efforts (McKinney 2002). However, to plan and implement conservation efforts reasonably and sustainably in metropolitan ecosystems, vast and global knowledge on the impact of urbanization on city-dwelling species is needed from all ecosystems encompassed within metropolitan areas (Rebele 1994). Yet, up to now the majority of the global surveys regarding metropolitan biodiversity focus mostly on terrestrial organisms (e.g., Aronson et al. 2014), leaving metropolitan aquatic ecosystems relatively poorly studied.

## 2.2 Metropolitan Freshwater Environments – Biodiversity as a Proxy for Healthy Ecosystems

*Fig. 2: Example of a metropolitan stream: the river Emscher located in the Ruhr region (Germany)*

Every city depends on various freshwater environments to provide inhabitants with clean water for direct use and recreational purposes, but also for transport, industry and agriculture (Elmqvist et al. 2013; Higgins et al. 2019). Thus, having healthy aquatic ecosystems is of paramount importance and is recognized as one of the top priorities in municipal management (Palmer et al. 2004; Walsh et al. 2005). On the other hand, urbanization heavily alters the land cover, which then impacts metropolitan water resources in terms of both quality and quantity (McDonald et al. 2011a; Elmqvist et al. 2013). Facing constantly increasing anthropogenic pressure results in increased loads of pollutants, altered geomorphology and finally, loss of native biodiversity with the establishment of alien, often invasive species in the resulting heavily modified water bodies (Paul/Meyer 2001; Walsh et al. 2005). On top of this, global climate change leads to drastic changes in the availability of suitable water as a resource and habitat (Tonkin et al. 2019). In effect, these factors pose a great challenge for sustainable management and distribution of freshwaters in metropolitan environments worldwide (McDonald et al. 2011a; 2011b).

Recognizing cities as ecosystems marked a gradual paradigm shift in conservation policy for metropolitan environments (Rebele 1994; McKinney 2002; Hobbs et al. 2006). Putting stronger emphasis on metropolitan ecology and ecosystem functioning corresponds with the increased interest in the composition and structure of metropolitan biodiversity (Rebele 1994; Grimm et al. 2000; Savard et al. 2000). Similar trends have been observed in particular in metropolitan freshwater biomes (Hering et al. 2013; Oertli/Parris 2019). Treating freshwater biodiversity as a proxy for a healthy aquatic ecosystem has been a focal point of most biomonitoring and ecological restoration initiatives worldwide (Geist/Hawkins 2016) and is a building block of the concept under-

lying the EU Water Framework Directive (WFD; 2000/60/EC). For metropolitan freshwaters (fig. 2), the reference conditions set by the WFD refer to near-natural waters. Obviously, such conditions can never be met in urban environments. Therefore, these urban water bodies have to be assessed independently. However, to date there is still no urban-specific framework for assessing and monitoring freshwater biodiversity present in metropolitan streams. Implementing systematic scanning of freshwater biodiversity in metropolitan management policies could be beneficial for planning reasonable and effective strategies for protecting and developing healthy metropolitan aquatic ecosystems.

## 2.3 DNA-Based Research on Metropolitan Biodiversity

**Box 1: From Single Specimens to Whole Communities: A Brief History of DNA-Based Research**
In the early 2000s a new method to identify species based on DNA sequences was introduced: DNA barcoding (Hebert et al. 2003). Here, the DNA from a single specimen of a given species is extracted and sequenced to generate a genetic barcode. These genetic barcodes are in most cases unique in their DNA sequence composition for most species and thus were proposed as a straightforward way of species identification. The individual barcodes are stored in publicly available databases, for example GenBank (NCBI), Barcode of Life Data Systems (BOLD) or the UNITE database, and whenever the barcode of a new specimen is sequenced, its identity can be revealed by cross-matching its barcode to a reference database. Even though at the moment not all described species have been genetically barcoded, the taxonomic coverage of many ecosystems is already high and still rising, with new initiatives in place working towards completion of DNA barcode reference databases in the foreseeable future (e.g., BIOSCAN, eBioAtlas).

Then, in the early 2010s the advancements in sequencing technology allowed researchers to take the next step: Suddenly many million sequences could be sequenced in a single experimental run. Now, instead of just sequencing a single barcode of a single specimen, whole collections of specimens from different species (bulk samples) could be analyzed at once. The rise of this so-called DNA metabarcoding has led to a drastic increase in DNA-based bioassessment studies during the last decade (Taberlet et al. 2012). Even though it rapidly increased the throughput and outcome of the DNA-based species assignment, it became apparent that for many organismal groups, in particular large vertebrates such as fish, it is not feasible to collect these in large quantities, i.e. as bulk samples. On the other hand, it was recognized that all species release genetic traces, such as cells, hair, feces, skin, and mucus, into their environment. So, the idea of simply collecting an environmental sample (e.g., water, soil, air) and sequencing the genetic traces in the sample, led to the development of the so-called environmental DNA (eDNA) metabarcoding (Deiner et al. 2017), which allowed for rapid and non-invasive studies of entire communities of organisms. Today, researchers work towards application and validation of eDNA metabarcoding in routine biomonitoring studies by conducting eDNA-based research all over the world and across various taxonomic groups, from freshwater macroinvertebrates, via tropical fish to arctic microbes.

DNA as a molecular fingerprint has been used for decades in multiple disciplines. The uniqueness of DNA allows comparing individuals, e.g., in order to investigate paternity or suspects of crimes. DNA-based analyses also became a frequently applied methodology in taxonomy. Books had to be rewritten after DNA investigations revealed unexpected evolutionary relationships. Depending on the questions asked (e.g., relationship among members of a population, phylogenetic relationship among taxonomic groups), the choice of the investigated DNA section differs. Highly conserved and slowly evolving sections can be used to address phylogenetic questions among species or higher taxa (genus, family, order, etc.). Variable, fast evolving gene sections can be used to differentiate individuals within a single species. In a biodiversity context, we differentiate between the analysis of DNA for single species or individuals (single-specimen DNA barcoding) and the analysis of entire communities with multiple species at once (DNA metabarcoding; see Box 1). Both approaches were successfully applied to study biodiversity in a variety of different habitats across the globe (Janzen et al. 2009; Bucklin et al. 2011; Valentini et al. 2016; Schütz/Tollrian/Schweinsberg 2020).

In recent years, it was recognized that all organisms constantly shed DNA to the environment they inhabit. This can be hair, fur, skin, feathers, scales, saliva or excretions. Traces of DNA are everywhere. Those DNA traces are collectively referred to as environmental DNA (eDNA), which allows the passive and non-invasive detection of species. Environmental DNA can be extracted and analyzed from a variety of different media such as soil, sediment, water and air. Since all life depends on water in some form, it can be seen as a sink for DNA that was released by life within and surrounding the catchment. Cities, metropolises in particular, have almost always been built near freshwater habitats, ranging from metropolitan rivers, to harbor coastlines, park lakes, canals or small ponds in citizens' backyards. Therefore, assessing biodiversity through eDNA collected from these waters is a rather straightforward task. Since the collection of the samples is simple, it can be performed by citizens and can increase the interest of communities in science, raising awareness of biodiversity and the value of metropolitan wildlife. Such eDNA-based approaches have already been applied to metropolitan environments (Francis/Chadwick 2015; Stoeckle et al. 2017; Bagley et al. 2019), in particular aquatic catchments, delivering invaluable data on communities, which would otherwise be very difficult to obtain. The results of eDNA metabarcoding, when used routinely, would offer important data on species distribution, the state and health of the environment and the success of restoration measures right around us. Scientists as well as officials can implement this data for a sustainable city development that meets the needs of the citizens, while also offering suitable habitats for metropolitan wildlife and with that increasing the quality of life.

## 2.4 Environmental DNA Metabarcoding – A Promising Tool for Estimating Metropolitan Aquatic Biodiversity

As mentioned in the beginning of the chapter, biodiversity assessment in metropolitan areas is still a highly undervalued, yet very important topic. The diversity of novel species and the complexity of their interactions in particular with native ones is drastically higher than in natural habitats, despite habitats often being artificial. This holds true in

particular for metropolitan freshwater ecosystems that act as recreational ecosystems, as drinking water resources, as entry points of surface runoff water or as sinks for purified water from sewage plants. Biodiversity traces from land and water can be captured using environmental DNA comparable to the forensic DNA fingerprinting, offering unparalleled insights into the biodiversity associated with these freshwater ecosystems. Here we want to i) highlight the potential of environmental DNA metabarcoding methods to assess metropolitan biodiversity much more holistically than traditional assessment methods, ii) provide an overview of contemporary approaches to capturing biodiversity with eDNA metabarcoding and iii) outline perspectives to implement eDNA metabarcoding as a simple analytical tool for metropolitan biodiversity monitoring.

## 3. Environmental DNA Methodology – A Holistic Approach for Biomonitoring

The choice of eDNA methodology strongly depends on the type of ecosystem and taxonomic group that are to be investigated. Despite a generally similar workflow, the methodologies can drastically differ by sample type, habitat and target organism. We will here outline general principles for collecting, analyzing and interpreting eDNA data.

### 3.1 Environmental Sample Types

First, eDNA can be collected from different environmental sample types (sediment, water, ice, etc.), which can give insights into different communities and on varying time scales. With the collection of water samples, mostly the active and present fauna and flora of the water body and the surrounding area is detected, depending on the persistence and stability of DNA traces as well as the character of the water body (Harrison et al. 2019). On the other hand, the collection of sediment samples can give insights into the benthic (i.e. living at the bottom of a water body) and terrestrial fauna and can also date back years to millennia in time when collecting sediment from greater depths, permafrost or even ice cores. However, this chapter focuses on the application of the aquatic eDNA and therefore, the following parts will be oriented around the potentials of using DNA information gathered from water samples.

### 3.2 Freshwater Habitats

Freshwater habitats can generally be divided into flowing water bodies (lotic), such as streams and rivers, standing water bodies (lentic), such as ponds and lakes, and groundwater habitats. The sample collection differs between these habitats. Lotic ecosystems are sinks in their environment and act as conveyor belts, transporting eDNA downstream for many kilometers. Thus, water samples from lotic environments usually have a greater spatial inference, representing the community of even up to (or more than) 12 km upstream of the sampling site (Deiner/Altermatt 2014; Deiner et al. 2016). The eDNA detection range, however, can be strongly influenced by DNA degradation and retention

as well as resuspension and dilution. Therefore, typically already within a few hundred meters or less the eDNA community can substantially differ (Harrison et al. 2019). For flowing water bodies, rather small volumes (1–2 l) are often collected in biomonitoring campaigns and already allow the detection of a great portion of the present community. However, to depict the whole community, usually larger volumes have to be collected (up to 100 l; Cantera et al. 2019).

In standing waters, on the other hand, the water column of lentic ecosystems is usually highly stratified with little transportation of water in vertical or horizontal directions, which makes eDNA signals very local. During the collection of water samples from ponds and lakes, this is accounted for by sub-sampling from different locations along the shore and from the middle of the water body and additionally from various depths. Usually, greater water sample volumes from different spots are required to depict the whole community. Several guidelines to optimize the sampling strategy of ponds and lakes have been proposed (Beentjes et al. 2019; Harper et al. 2019).

Groundwater bodies are a highly important source of drinking water in metropolitan areas and encompass an aquatic habitat particularly difficult to access and assess. Nevertheless, the cold temperature and absence of light offer optimal conditions for eDNA preservation. However, only a few studies have investigated groundwater using eDNA (Niemiller et al. 2018), collecting only limited water volumes. While being mostly unexplored to this day, groundwaters should be sampled similarly to surface standing waters to account for stratification effects.

## 3.3 eDNA Sampling Techniques

*Fig. 3: Water samples for eDNA analyses are collected in sterile bottles*

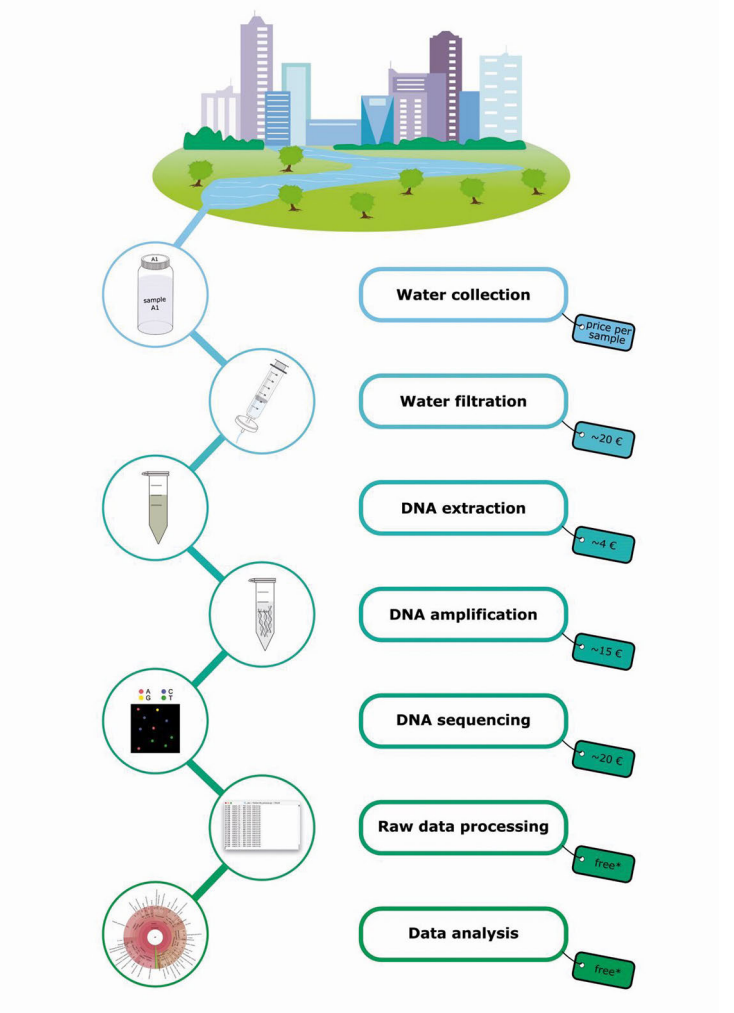
When collecting environmental samples (fig. 3), contamination with other DNA traces should be avoided. Thus, the usage of sterile gloves and sterile field equipment is of extreme importance. Not only can contamination between samples occur (cross-contamination), but also human DNA traces originating from the sample handler can

potentially enter the sample. The starting point of all water-based eDNA methodologies is the collection of a water sample in sterile 1–2 l bottles or bags that are placed in the water body. Alternatively, eDNA samples can be directly taken from the water using sterile pipes and specialized pumps (Thomas et al. 2018). The volume of water and the number of samples depends largely on the type of water body (flowing/standing), its depth and number of available microhabitats present.

*Fig. 4: The collected water is pumped through a special encapsulated filter to isolate the eDNA from the water*

After the collection of the water samples, the eDNA needs to be isolated from the water and collected on a dedicated filter (fig. 4). Finally, after the water is filtered, the sample consisting of a filter with collected eDNA requires preservation. Depending on the preservation method chosen (e.g., in highly concentrated ethanol), the collected eDNA sample can be stored at cool temperatures (4°C or -20°C) or even at room temperature. In conclusion, the required sample replication and filter volume depends on the choice of target organism, the research question and the water body. Depending on the approach chosen, including the number of replicates and types of filters used, cost estimates per sample can vary between 20 and 100 Euros, excluding personnel expenses (fig. 5). That being said, when planning for eDNA research, one has to bear in mind that sometimes a trade-off between sampling sufficient amounts of water and cost and time efficiency has to be made (Macher et al. 2021).

*Fig. 5: Metropolitan freshwater biodiversity can be assessed quickly and on a large scale using eDNA metabarcoding, while retaining comparably low costs. However, the costs per sample scale with the total number of samples/replicates that are sequenced simultaneously (see Buchner/Macher/Beermann/Werner/Leese 2021). (*) Most bioinformatic tools to process and analyze the eDNA metabarcoding data are free of charge but can require the acquisition of computing power*




## 3.4 Molecular Laboratory

The laboratory processing of eDNA samples (fig. 6) with a focus on animal and plant (and not microbial) species usually implies working with a low concentration of target DNA.

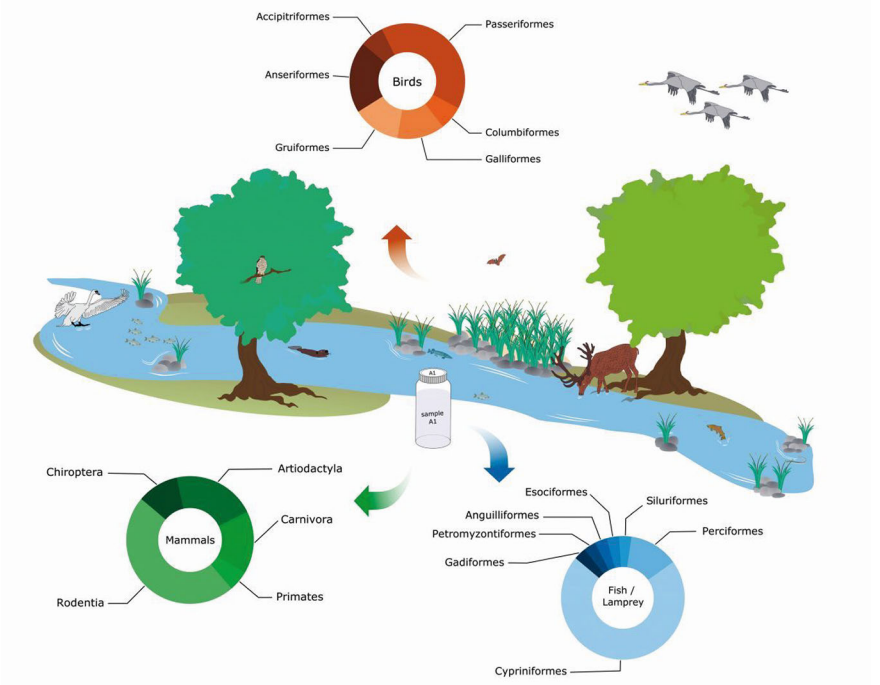
*Fig. 6: After the fieldwork, the eDNA is extracted from the filters and prepared for sequencing in a sterile laboratory*

Thus, significant precaution measures have to be taken to prevent contamination of the samples. Usually, eDNA samples should preferably be processed in a dedicated sterile lab with a unidirectional workflow. Here, the working places for the two major steps of DNA extraction (low amounts of DNA) and DNA amplification (high amounts of DNA) are spatially separated and samples only proceed forward. This mitigates potential contamination from already PCR-amplified eDNA samples. Furthermore, working places have to be decontaminated either with UV light or bleach after each working step. To prevent contamination with human DNA, the usage of a single-use, sterile overall suit and long gloves is an established good practice.

The initial laboratory step is the DNA extraction. Here, many different laboratory protocols and various commercial kits are available, but all follow a similar principle. First, the captured eDNA has to be separated from the filter. Open filters can usually be removed from the housing and are then ground or partitioned either with beads in a bead mill or with sterile scissors. Encapsulated filters, on the other hand, are filled with a buffer that is removed from the capsule and used for the DNA extraction. The next step is the lysis, where the DNA is isolated from cells in the sample. A lysis can either be performed enzymatically, chemically or mechanically. Then, the DNA is extracted from the sample, which comprises the removal of all organic and inorganic components except DNA. The following step is the amplification of the target DNA with a polymerase chain reaction (PCR). Here, the amount of target DNA copies is exponentially increased. The choice of target fragment, however, largely depends on the research question and the target species. For the assessment of whole communities, universal primers that can target DNA fragments ('DNA barcodes') characteristic for certain taxonomic groups, e.g., fish or invertebrates, are amplified in PCR reactions. Depending on the questions, various other organismal groups can be targeted, e.g., fungal, algal and bacterial communities. Interestingly, the eDNA information collected from water samples can also inform about the surrounding diversity as DNA traces shed by terrestrial organisms in

the water bodies can be successfully detected (e.g., Macher et al. 2021; fig. 7). To ensure a high reliability of obtained results, the implementation of multiple extraction and/or PCR replicates is strongly recommended. Afterwards, the eDNA samples have to be prepared for high-throughput sequencing (HTS) and are subsequently converted into millions of sequence reads. The HTS is performed on the machines that are capable of transforming the PCR-amplified products into the letter codes (ACGT) using fluorescent chemistry, which then form the end DNA sequences. The raw data then obtained from the HTS machine is basically a large text file with millions of lines that represent the letter code of the sequences ('reads') as well as ASCII codes that denote the quality of the sequence.

*Fig. 7: Overview of a freshwater associated vertebrate community including some of the detected species. The OTU (operational taxonomic unit) richness among the classes of birds, mammals and fish/lamprey found in this study are illustrated in pie charts (Source: Macher et al. 2021)*




## 3.5 Raw Data Processing

After sequencing, the multitude of DNA sequences obtained (usually millions of reads per sequencing run) have to be processed bioinformatically prior to scientific analyses. Initially, all reads of a sequencing run are stored in a single file and since they usually contain information from multiple sampling sites, require a demultiplexing step. Thus, the reads are divided into single files according to their sample-specific tagging se-

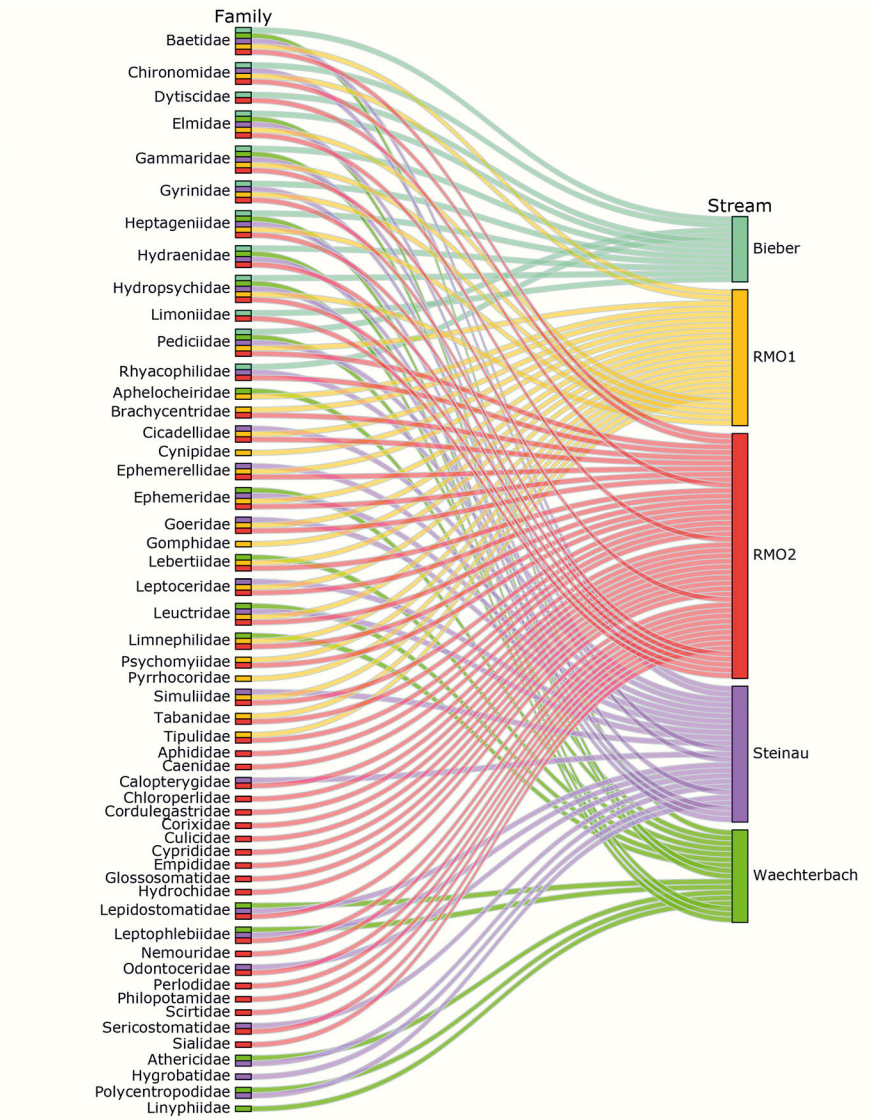
quence. This allows the simultaneous sequencing of dozens and even hundreds of samples on a single sequencing run, which drastically reduces costs. Nowadays, most DNA metabarcoding studies apply paired-end sequencing runs where the DNA sequences are read from both directions. This increases the quality of the reads and allows the sequencing of longer reads. After demultiplexing, the forward and reverse reads are merged and their quality is subsequently evaluated. When all high-quality reads are assigned to their respective samples, they can then be joined into biological entities. Here, two main approaches can be distinguished: clustering and denoising approaches. Clustering groups together all sequences within a certain threshold into a single entity (i.e. the operational taxonomic unit – OTU). Denoising approaches, on the other hand, aim to remove sequencing errors from the dataset and retain all correct biological sequences, without clustering them. These entities are referred to by various abbreviations, such as ASVs (amplicon sequence variants), ESVs (exact sequence variants) or zOTUs (zero-radius OTUs). Both approaches have their specific strengths and fields of application and can be used in parallel: OTUs are a proxy for species and should preferably be used for diversity measures (e.g., local or regional species diversity [alpha and beta diversity]), ecological indices and biogeography, while denoised sequences should rather be used for population genetics, connectivity and haplotype indices (Antich et al. 2021).

The final step of the raw data processing workflow involves taxonomic assignment (identification of a single sequence to a taxonomic, preferably species level). Therefore, the sequences are matched against a reference database. The choice of database depends on the target marker used in the study. Various online databases exist that host reference sequences of different markers and organisms, including NCBI GenBank, Barcode of Life Data Systems (BOLD), UNITE or R-Syst. In most cases, due to incompleteness of reference databases, not all reads can be reliably assigned to a reference species and the obtained taxonomy table requires additional filtering steps. Furthermore, it is recommended to manually check the assigned taxonomy for plausibility and potential errors (replicate consistency, negative controls).

## 3.6 Data Analysis

The final product after data processing is a taxon list with species names and associated read numbers per site. This table is the basis for downstream analyses. With the final taxon table, one can perform statistical analyses to investigate patterns of observed diversity and use multiple tools to visualize the data (fig. 8). Different software tools exist to perform statistical and ecological analyses, such as the R package vegan, the web-based tool MicrobiomeAnalyst, or the graphical-user interface-based software TaxonTableTools that was specifically developed for the processing and analysis of DNA metabarcoding data (Macher/Beermann/Leese 2021). The choice of analysis strongly depends on the design of the study and researchers' preferences. In most cases, the occurrence over space and time will be the most relevant dependent variables, as well as analyses that link the occurrence of species or taxa with ecological traits to, e.g., assess ecological status.

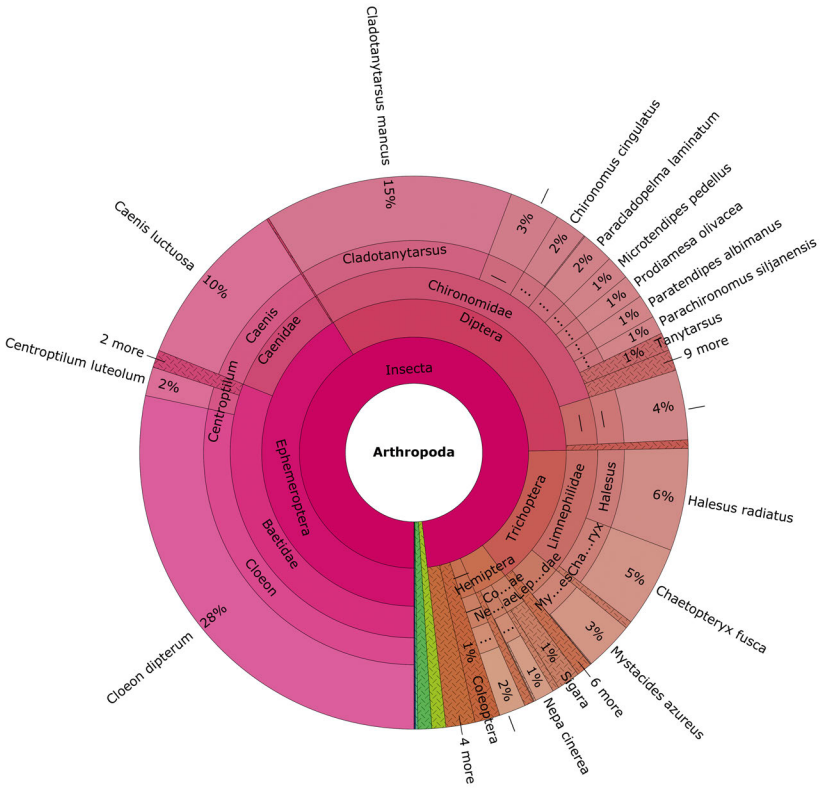
*Fig. 8 a): Exemplary eDNA metabarcoding data analysis results: Parallel category plots are a comprehensive method to visualize biodiversity of a dataset and illustrate differences in taxonomic composition between samples.*




## 3.7 Shortcomings of eDNA Metabarcoding

Undoubtedly, eDNA metabarcoding data hold many strengths. However, the interpretation of eDNA-derived data always needs to be taken with caution because, contrary to traditionally derived species lists, taxa that are found in eDNA datasets were not nec-

*Fig. 8 b): Exemplary eDNA metabarcoding data analysis results: Krona charts are hierarchical, interactive pie charts to explore the detected taxa inventory of a dataset on different taxonomic levels.*




essarily present at the site at the time of sampling. Particularly in anthropogenically influenced ecosystems, such as metropolitan areas, wastewater effluents can introduce signals of marine food fish, while in agricultural areas many terrestrial domestic animals can be found. This poses a challenge of false positive (species reported as present while it is not) and false negative (species reported as absent while it is present) DNA signals. Although false positives might occur due to the transportation of the DNA signal in lotic ecosystems or its persistence, still the most common cause of false positive DNA signals is contamination. False negative results are of paramount importance for detecting species of interest, particularly rare, threatened or invasive taxa, where their absence in the data could be deriving from lack of their DNA rather than their actual absence in the ecosystem. False negative results often derive from insufficient water sample volume to retrieve the DNA signal, often from rare, underrepresented species. While its implementation is beneficial for producing reliable and comprehensive presence and absence data, up to now eDNA metabarcoding does not offer reliable informa-

tion about species abundance or the population structure (e.g., sex or age distribution). Although in some cases, quantitative interpretations of read counts and biomass have been reported, e.g., for fish (Hänfling et al. 2016; Ushio et al. 2018), they still should be treated with caution. A final major concern is the incompleteness and reliability of reference databases. As described previously, reference taxonomic information is crucial to translate the sequencing data into a species list. The gaps in public DNA reference databases are unequally distributed among taxa groups and regions worldwide. However, with the ongoing efforts towards curating those databases and the addition of substantial amounts of new molecular data, the databases continue to improve constantly. Until the goal of having curated fairly complete DNA reference databases is achieved, we advise that the taxa lists should be verified by a specialist of the respective taxonomic group to further limit the shortcomings of reference material.

The shortcomings described above have to be taken into consideration when working with eDNA data. However, as also highlighted, there are several approaches that currently exist to reduce the impact of most of them. With the eDNA field rapidly improving, it is likely that most of those issues will be of a lesser concern in the foreseeable future. Overall, eDNA metabarcoding has been proven to be a reliable, comprehensive and reproducible approach for species bioassessment and would prove a promising solution also for metropolitan aquatic ecosystems.

## 4. Potential Applications of eDNA in Metropolitan Research

Environmental DNA-based methods are non-invasive and time-efficient ways of studying aquatic biodiversity, providing information about the presence of single species or even entire communities inhabiting aquatic ecosystems. In recent years the application of eDNA-based biodiversity assessment has expanded beyond simply assessing species composition towards more targeted and specific approaches. From monitoring invasive or elusive species to evaluating the success of restoration initiatives, eDNA metabarcoding has shown great promise for detecting aquatic life. Various studies have successfully applied eDNA-based biodiversity assessments in metropolitan research, for example, by initiating citizen science projects, the monitoring of reintroduced species or assessing pathogenic activity. Here, we present examples of eDNA-based approaches already tested in various environments that could readily be adapted to metropolitan aquatic research.

### 4.1 Monitoring Species of Interest

Metropolitan ecosystems, even though seemingly hostile for native fauna and flora, often harbor unexpected taxa often recognized as rare and threatened (Dearborn/Kark 2010; e.g., in the Ruhr region: Rhine sculpin, fire salamander, midwife toads). On the other hand, mainly due to anthropogenic activity, many invasive species have been thriving in city environments. Here, eDNA traces have been successfully used to detect rare, threatened taxa as well as invasive alien species (e.g., in the Ruhr region: signal crayfish, invasive gobiids, American bullfrog; see, e.g., Jerde et al. 2011; Thomsen

et al. 2012; Thomas et al. 2020). This species detection approach has considerable advantages over traditional assessment methods, mainly because of its non-invasiveness and easy scalable application. No need for disturbance of the target organism to prove its presence is of paramount importance, particularly for threatened taxa. Contrary to traditional monitoring methods, eDNA-based surveys can avoid the handling of specimens (e.g., electrofishing, preservation in ethanol), but are still in agreement with the conventional approaches. Moreover, in some cases species were found in sites where they were not previously detected with traditional methods, which further supports eDNA-based approaches as a highly sensitive method of species detection. The sensitivity of eDNA methodology, along with time and cost efficiency, was the reason why it was proposed for application in routine biomonitoring of threatened aquatic species of high importance like the Eastern hellbender, the European weather loach or the great crested newt. In all of the cases mentioned, eDNA-based identification outperformed traditional detection, revealing targeted species' presence even at low eDNA molecule concentrations. Further development and validation of eDNA protocols has been conducted in recent years to further enhance the detection reliability of endangered taxa, additionally aiming to also provide information on quantities and abundances (Thomsen et al. 2012; Harper et al. 2018; Kusanke et al. 2020). The further development of eDNA methodology has led to the implementation of eDNA-based biomonitoring of endangered taxa in standard national legislation, similarly to routine monitoring of great crested newts in the United Kingdom. The detection of aquatic invasive species via eDNA has also proven highly efficient. Studies on several candidate vertebrate invasives, like the American bullfrog, the Bluegill sunfish and the Asian carp as well as smaller organisms like invertebrates proves the high efficiency of eDNA-based species detection even at very low abundances of target organisms. However, with some freshwater arthropods, eDNA-based detectability was difficult, arguably due to low abundances and presence of chitin exoskeleton likely hindering the DNA release (Thomsen et al. 2012; Tréguier et al. 2014).

Keeping in mind the shortcomings of the method, eDNA-based approaches have certain advantages that could be useful in monitoring species of interest in metropolitan environments. It could certainly help planning reasonable conservation actions towards protecting those species and provide a cost-efficient and non-invasive way of informing local authorities about species' presence and dynamics. For example, eDNA metabarcoding may be a desirable solution for informing both city conservation agencies as well as the citizens about highly endangered species living in their vicinity like axolotl populations within Mexico City or rare leeches living in the metropolitan ecosystems of Warsaw (Koperski 2010; Recuero et al. 2010). The eDNA methodology could be equally useful for controlling the dispersal of invasive species thriving in human-altered metropolitan environments. It has recently been proposed as an effective tool aiding in estimating the presence and impact of invasive mollusks in metropolitan areas in Spain (Clusa et al. 2017).

## 4.2 Assessing Pathogens in Metropolitan Waters

Water security is one of the top priorities of metropolitan ecosystem services. Among the main threats to clean water access is water pollution, caused mainly by sewage discharge, stormwater runoff or animal fecal input, resulting in the presence of pathogens affecting the well-being of the city inhabitants. Health risk deriving from pathogenic exposure was confirmed in several studies from metropolitan recreational waters as well as sources of drinking water (Craun et al. 2005; Wullings/van der Kooij 2006; Sterk et al. 2015). In the field of microbiology, eDNA metabarcoding has been a well-established method for estimating diversity of various pathogenic organisms. An eDNA-based detection has also been implemented for successfully detecting dangerous animal pathogens, such as *Batrachochytrium salamandrivorans* or *Ranavirus,* threatening endangered amphibian species (Miaud et al. 2019; Sieber et al. 2020; Spitzen-van der Sluijs et al. 2020). Data collected from eDNA traces have also been used for extensive research on improving the detectability of the fungus *Aphanomyces astaci* responsible for the crayfish plague decimating threatened native crayfish species throughout Europe (Wittwer et al. 2018b; 2018a; Strand et al. 2019).

Genetic information retrieved from aquatic environmental samples has recently been proposed as a very promising solution for detecting human pathogens. It has proven particularly efficient in detecting and monitoring local outbreaks of SARS-CoV-2. Here, environmental viral RNA signals were identified both from air samples as well as from wastewater from multiple locations (Ahmed et al. 2020; Hart/Halden 2020; Lednicky et al. 2020; Street et al. 2020). Detection of SARS-CoV-2 virus from wastewater has already been thoroughly evaluated, in some cases detecting a higher rate of infections compared to the number of confirmed clinical cases (Wu et al. 2020; Farrell et al. 2021). Using aquatic eRNA for detecting SARS-CoV-2 virus has also been tested in a metropolitan setting in the region of Valencia in Spain. Here, the viral signal from the urban wastewater was detected even before the number of reported cases started to indicate a local outbreak, highlighting that eRNA-based detection could serve as an early warning system for monitoring pathogens in metropolitan areas (Randazzo et al. 2020).

Even though eDNA metabarcoding has been recognized as a promising tool that could be used in stormwater and wastewater management, it has not yet been widely implemented in urban environments. Thanks to increased sensitivity, eDNA could provide a valuable tool for reliable estimation of the microbial community, which could lead to a better understanding of the potential pathogenic risks present in metropolitan waters. By using eDNA/eRNA-derived data, one could improve the monitoring of drinking water sources or sewer discharges with increased possibility of tracing potential pollution sources. An eDNA-based approach has already shown great promise for tracking fecal contamination in metropolitan recreational areas (Staley et al. 2018). It could also largely facilitate understanding harmful cyanobacterial blooms taking place in urban recreational waters by better understanding the diversity and dynamics of communities responsible (Y. Jiang et al. 2020). Establishing eDNA-based research in cities could also be of paramount importance to metropolitan freshwater diversity by analyzing the

pathogens affecting both local diversity, e.g., fish in local aquacultures and threatened amphibians as well as the citizens.

## 4.3 Evaluating Metropolitan Restoration Initiatives

To counteract continuous degradation of freshwater ecosystems and slow down, halt or even reverse ongoing biodiversity loss, various restoration initiatives have been proposed for riverine ecosystems worldwide (Bernhardt et al. 2005; Pander/Geist 2013; Muhar et al. 2016). Restoration activities typically comprise various modifications to river course and adjacent riparian zones as well as the habitats within, sharing the common aim of improving the hydrologic and ecological status of a degraded riverine ecosystem, which could then increase community, biological and utilitarian values. Gradually, the need for river restoration has been recognized by policy- and decision-makers, which resulted in its implementation in the legislative acts on local as well as international levels (e.g., EU Water Framework Directive, US Clean Water Act). Although there are certain benefits from restoration projects, one of the main issues discussed is the lack of standardized evaluation of the restoration success. Although several approaches to evaluating the success of restoration initiatives have been proposed (Palmer et al. 2005; Woolsey et al. 2007; Jähnig et al. 2011), there is still a lack of standardization and robustness. The potential of using eDNA-based approaches in restoration ecology has been thoroughly discussed and proven to be a promising solution for a future way of assessing biodiversity in restored sites. Similarly, eDNA metabarcoding has been acknowledged as a highly promising tool for biodiversity assessment, also in the context of ecological restoration (Williams et al. 2014; Ruppert et al. 2019). However, to date very few studies have applied eDNA-based evaluation of restoration success and only from a single-species perspective. In metropolitan environments, freshwater ecosystems are subject to substantial degradation and deterioration of their ecosystem functions due to gradual urbanization. Thus, a multitude of actions were proposed to initiate stream restoration initiatives within urbanized areas. To evaluate the success of many metropolitan stream restoration projects, the composition of macroinvertebrate communities is investigated (Purcell et al. 2002; Selvakumar et al. 2010; Violin et al. 2011). However, since all of those assessments are based on morphological determination, some of the organisms can only be assigned to family level, which could possibly hamper interpreting the richness of observed diversity. Here, eDNA metabarcoding could provide a higher taxonomic resolution and thus enhance the reliability of the biological evaluation of metropolitan stream restoration initiatives.

## 4.4 Engaging Metropolitan Society

Recently, the number of research projects involving the engagement of the public is constantly growing, with more and more benefits of so-called 'citizen science' being acknowledged. Acquisition of large volumes of data collected voluntarily along with high coverage, otherwise difficult to achieve, have been primary advantages of communities' involvement behind a plethora of environmental monitoring initiatives. Public activity seems to be a particularly effective solution for biomonitoring in residential ecosystems

like metropolitan areas. Citizen science has already proven useful for obtaining environmental data from city ecosystems, e.g., regarding urban biodiversity (Wang Wei et al. 2016; Anton et al. 2018; Mason/Arathi 2019). It has also been proposed and tested as a promising solution for measuring metropolitan environmental pollution (Q. Jiang et al. 2016; Longo et al. 2020). In metropolitan aquatic ecosystems, citizen science has been validated to deliver valuable environmental data, mostly concerning water quality, which are comparable to those obtained by trained professionals. However, engaging the public has not yet been implemented in studying metropolitan freshwater biodiversity, even though some solutions have already been proposed (Rae et al. 2019). On the other hand, there are more and more initiatives engaging the public by studying biodiversity using eDNA metabarcoding. eDNA-based citizen science research was proposed, e.g., to enhance the knowledge about diversity of amphibians living in residential ponds in Austria ("Frosch im Wassertropfen" project; http://www.uibk.ac.at/350-jahre/veranstaltungen/frosch-im-wassertropfen/), to assess the freshwater macroinvertebrate biodiversity of selected watersheds in Canada (STREAM initiative; http://www.stream-dna.com), to assess biodiversity in local BioBlitzes (public event focusing on collecting and identifying as many species as possible in a specific area over a short period of time) in the USA (CALeDNA; http://www.ucedna.com) or even to enhance nationwide biomonitoring of the endangered great crested newt in the United Kingdom. Citizen science has also become an important part of the outreach activities undertaken by scientific institutions and commercial companies focusing on eDNA analyses like NatureMetrics in the UK or EnviroDNA in Australia, offering easy-to-use kits that can be readily applied by non-scientists for straightforward eDNA sampling. The value of eDNA-based biodiversity records gathered through local community-derived initiatives are also considered as one of the crucial input data underlying new global initiatives like eBioAtlas by the IUCN and NatureMetrics (www.ebioatlas.org). There is a growing concern that eDNA studies will disattach the public from nature by translating it into numbers and letter codes. However, as shown above, the eDNA approach provides a promising straightforward solution for raising interest in nature and life in the immediate vicinity. Thus, implementing eDNA-based research in city freshwater ecosystems engaging the citizens would not only provide a valuable method for monitoring species of interest, assessing pathogens or evaluating local restoration initiatives, but might possibly also have a profound educational value in increasing scientific literacy and biodiversity awareness of metropolitan citizens (Box 2).

**Box 2: Urban BioBlitz Using Aquatic eDNA – Assessing Biodiversity and Educating Citizens**
Since freshwater biodiversity is in peril with the number of freshwater species being in decline, it seems of high importance to better engage researchers, decision-makers and the public on the way to protect aquatic organisms. This seems particularly true for biodiversity of metropolitan freshwater ecosystems, which on the one hand is heavily impacted by urban land use and on the other is poorly known. To create awareness and educate the citizens about local freshwater biodiversity, a rapid survey ('BioBlitz') using environmental DNA is proposed (Hupało et al. 2021). In only two days of sampling per-

formed by two people at 15 sites in the city of Trondheim in Norway, 435 taxa, representing at least 265 putative species, were detected. The results of this study demonstrate the usefulness of eDNA metabarcoding for rapid biodiversity surveys and its value for educational purposes. The authors also point out the relative ease and cost effectiveness of generating and analyzing a large biodiversity dataset. When combined with openly available services and software, eDNA information can be a powerful educational tool for expanding scientific literacy, increasing citizen inclusiveness and raising awareness about the importance of the diversity living in the close surroundings.

## 5. Conclusions and Outlook

To assess and monitor metropolitan biodiversity is an important task in times of biodiversity loss, invasive species spread and the emergence of pathogens. Environmental DNA is a very suitable tracer that allows an assessment of animal and plant but also unicellular bacterial and viral diversity from water samples. The collection of eDNA samples is simple, straightforward and analyses are cost-efficient. It is important that the collected data are analyzed appropriately and made accessible to managers, researchers, but also to the interested public. Therefore, the next urgent steps are less about method development but rather about the installation of common platforms for data access and visualization (similar, e.g., to GBIF – Global Biodiversity Information Facility, http://www.gbif.org) as well as the implementation of quality control and quality assurance routines to improve the reliability of new data for global biodiversity monitoring. Dedicated regulations, standardized protocols as well as unified personnel training with a high degree of automation have to follow to ensure the reliability and reproducibility of eDNA-based metropolitan biomonitoring. With that in place, novel solutions can be implemented in the cities of the future including, e.g., screening for invasive species detection in ballast waters in city harbors, early warning detection of harmful pathogens in recreational waters as well as drinking water sources or routine DNA-based biomonitoring of restoration initiatives. Those solutions based on environmental DNA support a pathway for future sustainable development of urban areas including metropolitan biodiversity.

## 6. Works Cited

Ahmed, Warish, et al. "First Confirmed Detection of SARS-CoV-2 in Untreated Wastewater in Australia: A Proof of Concept for the Wastewater Surveillance of COVID-19 in the Community." Science of the Total Environment 728 (2020): 138764.

Antich, Adrià, et al. "To Denoise or to Cluster, that is not the Question: Optimizing Pipelines for COI Metabarcoding and Metaphylogeography." BMC Bioinformatics 22.1 (2021): 1–24.

Anton, Victor, Stephen Hartley, Andre Geldenhuis, Heiko U. Wittmer. "Monitoring the Mammalian Fauna of Urban Areas Using Remote Cameras and Citizen Science." Journal of Urban Ecology 4.1 (2018): doi.org/10.1093/jue/juy002

Aronson, Myla F. J., et al. "A Global Analysis of the Impacts of Urbanization on Bird and Plant Diversity Reveals Key Anthropogenic Drivers." Proceedings of the Royal Society B: Biological Sciences 281.1780 (2014): 20133330.

Bagley, Mark, et al. "High-Throughput Environmental DNA Analysis Informs a Biological Assessment of an Urban Stream." Ecological Indicators 104 (2019): 378–389.

Beentjes, Kevin K., et al. "The Effects of Spatial and Temporal Replicate Sampling on eDNA Metabarcoding." PeerJ 7 (2019): e7335.

Bernhardt, Emily S., et al. "Synthesizing US River Restoration Efforts." Science 308.5722 (2005): 636–637.

Buchner, Dominik, Till-Hendrik Macher, Arne J. Beermann, Marie-Thérése Werner, Florian Leese. "Standardized High-throughput Biomonitoring Using DNA Metabarcoding: Strategies for the Adoption of Automated Liquid Handlers." Environmental Science and Ecotechnology 8 (2021): 100122.

Bucklin, Ann, Dirk Steinke, Leocadio Blanco-Bercial. "DNA Barcoding of Marine Metazoa." Annual Review of Marine Science 3 (2011): 471–508.

Cantera, Isabel, et al. "Optimizing Environmental DNA Sampling Effort for Fish Inventories in Tropical Streams and Rivers." Scientific Reports 9.1 (2019): 3085. http://doi.org/10.1038/s41598-019-39399-5

Clusa, Laura, et al. "eDNA for Detection of Five Highly Invasive Molluscs: A Case Study in Urban Rivers from the Iberian Peninsula." PloS One 12.11 (2017): e0188126.

Craun, Gunther F., Rebecca L. Calderon, Michael F. Craun. "Outbreaks Associated with Recreational Water in the United States." International Journal of Environmental Health Research 15.4 (2005): 243–262.

Dearborn, Donald C., Salit Kark. "Motivations for Conserving Urban Biodiversity." Conservation Biology 24.2 (2010): 432–440.

Deiner, Kristy, Florian Altermatt. "Transport Distance of Invertebrate Environmental DNA in a Natural River." PloS One 9.2 (2014): e88786.

Deiner, Kristy, et al. "Environmental DNA Reveals that Rivers Are Conveyer Belts of Biodiversity Information." Nature Communications 7.1 (2016): 12544. http://doi.org/10.1038/ncomms12544

Deiner, Kristy, et al. "Environmental DNA Metabarcoding: Transforming How We Survey Animal and Plant Communities." Molecular Ecology 26.21 (2017): 5872–5895.

Elmqvist, Thomas, et al. Urbanization, Biodiversity and Ecosystem Services: Challenges and Opportunities; A Global Assessment. Dordrecht: Springer Nature, 2013.

Farrell, Jessica A., Liam Whitmore, David J. Duffy. "The Promise and Pitfalls of Environmental DNA and RNA Approaches for the Monitoring of Human and Animal Pathogens from Aquatic Sources." BioScience 71.6 (2021): 609–625.

Francis, Robert A., Michael A. Chadwick. "Urban Invasions: Non-Native and Invasive Species in Cities." Geography 100.3 (2015): 144–151.

Geist, Juergen, Stephen J. Hawkins. "Habitat Recovery and Restoration in Aquatic Ecosystems: Current Progress and Future Challenges." Aquatic Conservation: Marine and Freshwater Ecosystems 26.5 (2016): 942–962.

Grimm, Nancy B., J. Morgan Grove, Steward T. A. Pickett, Charles L. Redman. "Integrated Approaches to Long-Term Studies of Urban Ecological Systems: Urban Ecological Systems Present Multiple Challenges to Ecologists – Pervasive Human Impact and Extreme Heterogeneity of Cities, and the Need to Integrate Social and Ecological Approaches, Concepts, and Theory." BioScience 50.7 (2000): 571–584.

Hänfling, Bernd, et al. "Environmental DNA Metabarcoding of Lake Fish Communities Reflects Long-Term Data from Established Survey Methods." Molecular Ecology 25.13 (2016): 3101–3119.

Harper, Lynsey R., et al. "Needle in a Haystack? A Comparison of eDNA Metabarcoding and Targeted qPCR for Detection of the Great Crested Newt (Triturus Cristatus)." Ecology and Evolution 8.12 (2018): 6330–6341.

Harper, Lynsey R., et al. "Environmental DNA (eDNA) Metabarcoding of Pond Water as a Tool to Survey Conservation and Management Priority Mammals." Biological Conservation 238 (2019): 108225.

Harrison, Jori B., Jennifer M. Sunday, Sean M. Rogers. "Predicting the Fate of eDNA in the Environment and Implications for Studying Biodiversity." Proceedings of the Royal Society B: Biological Sciences 286.1915 (2019). http://doi.org/10.1098/rspb.2019.1409

Hart, Olga E., Rolf U. Halden. "Computational Analysis of SARS-CoV-2/COVID-19 Surveillance by Wastewater-Based Epidemiology Locally and Globally: Feasibility, Economy, Opportunities and Challenges." Science of the Total Environment 730 (2020): 138875.

Hebert, Paul D.N., Alina Cywinska, Shelley L. Ball, Jeremy R. deWaard. "Biological Identifications through DNA Barcodes." Proceedings of the Royal Society of London. Series B: Biological Sciences 270.1512 (2003): 313–321.

Hering, Janet G. et al. "A Changing Framework for Urban Water Systems." Environmental Science & Technology 47.19 (2013): 10721–10726.

Higgins, Sahran L. et al. "Urban Freshwaters, Biodiversity, and Human Health and Well-Being: Setting an Interdisciplinary Research Agenda." Wiley Interdisciplinary Reviews: Water 6.2 (2019): e1339.

Hobbs, Richard J. et al. "Novel Ecosystems: Theoretical and Management Aspects of the New Ecological World Order." Global Ecology and Biogeography 15.1 (2006): 1–7.

Hupało, Kamil et al. "An Urban Blitz with a Twist: Rapid Biodiversity Assessment Using Aquatic Environmental DNA." Environmental DNA 3.1 (2021): 200–213.

Jähnig, S.C. et al. "River Restoration Success: A Question of Perception." Ecological Applications 21.6 (2011): 2007–2015.

Janzen, Daniel H. et al. "Integration of DNA Barcoding into an Ongoing Inventory of Complex Tropical Biodiversity." Molecular Ecology Resources 9 (2009): 1–26.

Jerde, Christopher L., Andrew R. Mahon, W. Lindsay Chadderton, David M. Lodge. "'Sight-Unseen' Detection of Rare Aquatic Species Using Environmental DNA." Conservation Letters 4.2 (2011): 150–157.

Jiang, Qijun et al. "Citizen Sensing for Improved Urban Environmental Monitoring." Journal of Sensors 2016 (2016): n.p.

Jiang, Yongguang et al. "Revealing Cryptic Changes of Cyanobacterial Community Structure in Two Eutrophic Lakes Using eDNA Sequencing." International Journal of Environmental Research and Public Health 17.17 (2020): 6356.

Koceva, Mariana M. et al. (eds.). Urban Europe: Statistics on Cities, Towns and Suburbs. Luxembourg: Publications Office of the European Union, 2016.

Koperski, Paweł. "Urban Environments as Habitats for Rare Aquatic Species: The Case of Leeches (Euhirudinea, Clitellata) in Warsaw Freshwaters." Limnologica – Ecology and Management of Inland Waters 40.3 (2010): 233–240.

Kusanke, Lena Maureen et al. "Detection of the Endangered European Weather Loach (Misgurnus fossilis) via Water and Sediment Samples: Testing Multiple eDNA Workflows." Ecology and Evolution 10.15 (2020): 8331–8344.

Lednicky, John A. et al. "Viable SARS-CoV-2 in the Air of a Hospital Room with COVID-19 Patients." International Journal of Infectious Diseases 100 (2020): 476–482.

Longo, Antonella, Marco Zappatore, Mario A. Bochicchio. "Apollon: Towards a Citizen Science Methodology for Urban Environmental Monitoring." Future Generation Computer Systems 112 (2020): 899–912.

Macher, Till-Hendrik, Arne J. Beermann, Florian Leese. "TaxonTableTools: A Comprehensive, Platform-Independent Graphical User Interface Software to Explore and Visualise DNA Metabarcoding Data." Molecular Ecology Resources 21.5 (2021): 1705–1714.

Macher, Till-Hendrik, Robin Schütz, Jens Arle, Arne J. Beermann, Jan Koschorreck, Florian Leese. "Beyond Fish eDNA Metabarcoding: Field Replicates Disproportionately Improve the Detection of Stream Associated Vertebrate Species." Metabarcoding and Metagenomics 5 (2021): e66557. http://doi.org/10.3897/mbmg.5.66557

Mason, Lisa, H. S. Arathi. "Assessing the Efficacy of Citizen Scientists Monitoring Native Bees in Urban Areas." Global Ecology and Conservation 17 (2019): e00561.

McDonald, Robert I. et al. "Global Urban Growth and the Geography of Water Availability, Quality, and Delivery." Ambio 40.5 (2011a): 437–446.

McDonald, Robert I. et al. "Urban Growth, Climate Change, and Freshwater Availability." PNAS: Proceedings of the National Academy of Sciences 108.15 (2011b): 6312–6317.

McKinney, Michael L. "Urbanization, Biodiversity, and Conservation: The Impacts of Urbanization on Native Species are Poorly Studied, but Educating a Highly Urbanized Human Population about these Impacts can Greatly Improve Species Conservation in all Ecosystems." BioScience 52.10 (2002): 883–890.

McKinney, Michael L. "Urbanization as a Major Cause of Biotic Homogenization." Biological Conservation 127.3 (2006): 247–260.

McKinney, Michael L. "Effects of Urbanization on Species Richness: A Review of Plants and Animals." Urban Ecosystems 11.2 (2008): 161–176.

Miaud, Claude et al. "eDNA Increases the Detectability of Ranavirus Infection in an Alpine Amphibian Population." Viruses 11.6 (2019): 526. DOI: 10.3390/v11060526.

Muhar, S. et al. "Evaluating Good-Practice Cases for River Restoration across Europe: Context, Methodological Framework, Selected Results and Recommendations." Hydrobiologia 769.1 (2016): 3–19.

Niemiller, Matthew L. et al. "Evaluation of eDNA for Groundwater Invertebrate Detection and Monitoring: A Case Study with Endangered Stygobromus (Amphipoda: Crangonyctidae)." Conservation Genetics Resources 10.2 (2018): 247–257.

Oertli, Beat, Kirsten M. Parris. "Toward Management of Urban Ponds for Freshwater Biodiversity." Ecosphere 10.7 (2019): e02810.

Palmer, Margaret A. et al. "Ecology for a Crowded Planet." Science 304.5675 (2004): 1251–1252.

Palmer, Margaret A. et al. "Standards for Ecologically Successful River Restoration." Journal of Applied Ecology 42.2 (2005): 208–217.

Pander, Joachim, Juergen Geist. "Ecological Indicators for Stream Restoration Success." Ecological Indicators 30 (2013): 106–118.

Paul, Michael J., Judy L. Meyer. "Streams in the Urban Landscape." Annual Review of Ecology and Systematics 32.1 (2001): 333–365.

Purcell, Alison H., Carla Friedrich, Vincent H. Resh. "An Assessment of a Small Urban Stream Restoration Project in Northern California." Restoration Ecology 10.4 (2002): 685–694.

Rae, Marcia et al. "Evaluating the Validity of a Simple Citizen Science Index for Assessing the Ecological Status of Urban Drainage Ponds." Ecological Indicators 98 (2019): 1–8.

Randazzo, Walter et al. "Metropolitan Wastewater Analysis for COVID-19 Epidemiological Surveillance." International Journal of Hygiene and Environmental Health 230 (2020): 113621.

Rebele, Franz. "Urban Ecology and Special Features of Urban Ecosystems." Global Ecology and Biogeography Letters 4.6 (1994): 173–187.

Recuero, Ernesto, Juan Cruzado-Cortes, Gabriela Parra-Olea, Kelly R. Zamudio. "Urban Aquatic Habitats and Conservation of Highly Endangered Species: The Case of Ambystoma mexicanum (Caudata, Ambystomatidae)." Annales Zoologici Fennici 47.4 (2010): 223–238.

Ruppert, Krista M., Richard J. Kline, Saydur Rahman. "Past, Present, and Future Perspectives of Environmental DNA (eDNA) Metabarcoding: A Systematic Review in Methods, Monitoring, and Applications of Global eDNA." Global Ecology and Conservation 17 (2019): e00547.

Savard, Jean-Pierre L., Philippe Clergeau, Gwenaelle Mennechez. "Biodiversity Concepts and Urban Ecosystems." Landscape and Urban Planning 48.3–4 (2000): 131–142.

Schütz, Robin, Ralph Tollrian, Maximilian Schweinsberg. "A Novel Environmental DNA Detection Approach for the Wading Birds Platalea Leucorodia, Recurvirostra Avosetta and Tringa Totanus." Conservation Genetics Resources 12.4 (2020): 529–531.

Selvakumar, Ariamalar, Thomas P. O'Connor, Scott D. Struck. "Role of Stream Restoration on Improving Benthic Macroinvertebrates and In-Stream Water Quality in an Urban Watershed: Case Study." Journal of Environmental Engineering 136.1 (2010): 127–139.

Seto, Karen C., Burak Güneralp, Lucy R. Hutyra. "Global Forecasts of Urban Expansion to 2030 and Direct Impacts on Biodiversity and Carbon Pools." Proceedings of the National Academy of Sciences 109.40 (2012): 16083–16088.

Sieber, Natalie, Hanna Hartikainen, Christoph Vorburger. "Validation of an eDNA-Based Method for the Detection of Wildlife Pathogens in Water." Diseases of Aquatic Organisms 141 (2020): 171–184.

Spitzen-van der Sluijs, Annemarieke et al. "Using Environmental DNA for Detection of Batrachochytrium salamandrivorans in Natural Water." Environmental DNA 2.4 (2020): 565–571.

Staley, Zachery R. et al. "Fecal Source Tracking and eDNA Profiling in an Urban Creek Following an Extreme Rain Event." Scientific Reports 8.1 (2018): 1–12.

Sterk, Ankie et al. "Effect of Climate Change on the Concentration and Associated Risks of Vibrio spp. in Dutch Recreational Waters." Risk Analysis 35.9 (2015): 1717–1729.

Stoeckle, Mark Y., Lyubov Soboleva, Zachary Charlop-Powers. "Aquatic Environmental DNA Detects Seasonal Fish Abundance and Habitat Preference in an Urban Estuary." PloS One 12.4 (2017): e0175186.

Strand, David A. et al. "Monitoring a Norwegian Freshwater Crayfish Tragedy: eDNA Snapshots of Invasion, Infection and Extinction." Journal of Applied Ecology 56.7 (2019): 1661–1673.

Street, Renée, Shirley Malema, Nomfundo Mahlangeni, Angela Mathee. "Wastewater Surveillance for Covid-19: An African Perspective." Science of the Total Environment 743 (2020): 140719.

Taberlet, Pierre, Eric Coissac, Mehrdad Hajibabaei, Loren H. Rieseberg. "Environmental DNA." Molecular Ecology 21.8 (2012): 1789–1793.

Thomas, Austen C. et al. "eDNA Sampler: A Fully Integrated Environmental DNA Sampling System." Methods in Ecology and Evolution 9.6 (2018): 1379–1385.

Thomas, Austen C. et al. "A System for Rapid eDNA Detection of Aquatic Invasive Species." Environmental DNA 2.3 (2020): 261–270.

Thomsen, Philip Francis, et al. "Monitoring Endangered Freshwater Biodiversity Using Environmental DNA." Molecular Ecology 21.11 (2012): 2565–2573.

Tonkin, Jonathan D. et al. "Prepare River Ecosystems for an Uncertain Future." Nature 570.7761 (2019): 301–303.

Tréguier, Anne et al. "Environmental DNA Surveillance for Invertebrate Species: Advantages and Technical Limitations to Detect Invasive Crayfish Procambarus clarkii in Freshwater Ponds." Journal of Applied Ecology 51.4 (2014): 871–879.

United Nations. World Urbanization Prospects: The 2018 Revision. New York, 2019.

Ushio, Masayuki et al. "Demonstration of the Potential of Environmental DNA as a Tool for the Detection of Avian Species." Scientific Reports 8.1 (2018): 4493. http://doi.org/10.1038/s41598-018-22817-5

Valentini, Alice et al. "Next-Generation Monitoring of Aquatic Biodiversity Using Environmental DNA Metabarcoding." Molecular Ecology 25.4 (2016): 929–942.

Violin, Christy R. et al. "Effects of Urbanization and Urban Stream Restoration on the Physical and Biological Structure of Stream Ecosystems." Ecological Applications 21.6 (2011): 1932–1949.

Walsh, Christopher J. et al. "The Urban Stream Syndrome: Current Knowledge and the Search for a Cure." Journal of the North American Benthological Society 24.3 (2005): 706–723.

Wang Wei, James, Benjamin P.Y.H. Lee, Low Bing Wen. "Citizen Science and the Urban Ecology of Birds and Butterflies: A Systematic Review." PloS One 11.6 (2016): e0156425.

Williams, Anna V., Paul G. Nevill, Siegfried L. Krauss. "Next Generation Restoration Genetics: Applications and Opportunities." Trends in Plant Science 19.8 (2014): 529–537.

Wittwer, Claudia et al. "Comparison of Two Water Sampling Approaches for eDNA-Based Crayfish Plague Detection." Limnologica – Ecology and Management of Inland Waters 70 (2018a): 1–9.

Wittwer, Claudia et al. "eDNA-Based Crayfish Plague Monitoring is Superior to Conventional Trap-Based Assessments in Year-Round Detection Probability." Hydrobiologia 807.1 (2018b): 87–97.

Woolsey, Sharon et al. "A Strategy to Assess River Restoration Success." Freshwater Biology 52.4 (2007): 752–769.

Wu, Fuqing et al. "SARS-CoV-2 Titers in Wastewater Are Higher than Expected from Clinically Confirmed Cases." mSystems 5.4 (2020): e00614-20.

Wullings, Bart A., Dick van der Kooij. "Occurrence and Genetic Diversity of Uncultured Legionella spp. in Drinking Water Treated at Temperatures Below 15 C." Applied and Environmental Microbiology 72.1 (2006): 157–166.