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From molecules to monitoring: integrating genetic tools into freshwater quality assessments

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CHAPTER 1

General introduction and literature review

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Parts of this text and its illustrations have been adapted to
STOWA Deltafact: DNA-technieken voor waterbeheerders.
www.stowa.nl/deltafacts/waterkwaliteit/diversen/dna-technieken-voor-waterbeheerders

1.1 THE STATE OF FRESHWATER AND ITS INHABITANTS

The continuing anthropogenic decline of the Earth's biodiversity (Barnosky et al. 2011) is one of the most serious threats of the 21st century, with no outlook on significant reduction in the rate of biodiversity loss (Butchart et al. 2010). Human influences have created extinction rates that go beyond those of pre-human periods, current estimates being a thousand-fold the expected background rate, in what has been dubbed "the sixth extinction wave" (Pimm et al. 2014, Dirzo et al. 2014).

Freshwater species appear to be at a greater threat than terrestrial and marine species. Freshwater ecosystems contain a rich diversity of both taxa and habitats, despite the fact that they cover less than one percent of the Earth's surface. Of all water, 2.5% is freshwater, with only 1.2% of freshwater being surface water (Gleick 1993). The 2014 Living Planet Report presented an average decline in size of monitored populations of 76% in freshwater, against 39% for both terrestrial and marine biomes (WWF 2014). The main drivers that threaten freshwater species are habitat loss or degradation, pollution of water, over-exploitation, flow modification and invasive species, the first being the most prevalent by far (Dudgeon et al. 2006, Collen et al. 2014).

Freshwater habitats are, in essence, islands within a sea of dry land or salt water, creating barriers that are unbridgeable for many species living in these ecosystems. This physical isolation makes for limited dispersal opportunities across these islands. The insular nature of freshwater ecosystems has led to the evolution of species with small geographic ranges, and resulted in biotas with high rates of endemism and turnover (Strayer 2006). This fragmentation and relatively high proportion of endemism greatly reduce the ability of freshwater taxa to respond to environmental change, as they limit the ability to freely disperse and re-establish local populations that have been extirpated. This makes those freshwater species that do not have large geographic ranges especially sensitive to human impacts (Strayer & Dudgeon 2010).

Freshwater invertebrates form a phylogenetically diverse group, which are usually not well studied in terms of conservation biology. Hence, they often receive different or less protection than their vertebrate co-occupants of freshwater habitats. Invertebrates live in most freshwater sources, save for the most polluted waters. Densities of all freshwater invertebrates together range between 10^5 and 10^6 individuals per cubic meter (Wetzel 2001), and although the inventories of freshwater invertebrates, even macroinvertebrates, are often incomplete, local faunas may contain hundreds, if not thousands of species (Strayer 2006). The distribution, species richness, and threatened-species richness data for vertebrate taxa show little congruence with those of invertebrate taxa in freshwater (Collen et al. 2014), making

the well-studied fish and amphibians imperfect indicators of macroinvertebrate communities. Conservation statuses for freshwater vertebrates may therefore not be suitable proxies for those of invertebrate taxa in the same habitats (Dudgeon et al. 2006). Data on the geographical distribution ranges and relative extinction risks are limited, but it is expected that the small ranges that many aquatic invertebrates exhibit will be even more dissimilar from the few large-bodied groups that have been studied (amphibians, fish, mammals, reptiles, and crustaceans) (Collen et al. 2014).

Freshwater is not only important for the life that it contains, but also for most other organisms living on our world, including human beings. Our species already uses over half of the accessible global freshwater runoff, with demand steadily increasing (Jackson et al. 2001). Rapid changes in the use of freshwater are causing dramatic changes in patterns of water stress, and we are close to overstepping the limits set in the planetary boundaries for global sustainability (Alcamo et al. 2008, Rockström et al. 2009). Hence, managing water quality is not only important for the aquatic flora and fauna, but also for the ecosystem services that are essential to the well-being and health of mankind (Corvalan et al. 2005).

1.2 THE MONITORING OF BIOLOGICAL QUALITY

Because of its importance, in the past decades a range of monitoring methods have been developed to assess the “health” of freshwater, ranging from abiotic properties to multimetric biological indices. The concept of health within an ecological context has seen much debate in the last decade of the 20th century, with critics insisting that it is not an observable ecological property, and merely a property of the organisms within an ecosystem, or that “preferred ecosystem states” cannot be well-defined (Suter 1993, Scrimgeour & Wicklum 1996). The discussion was complicated by the involvement of societal values, where people argued that health is dependent on human values, and that efforts to protect the health of ecosystems should consider the “human uses and amenities derived from the system” (Rapport 1989, Regier 1993). Supporters of the concept of health in an ecosystem setting have often looked for more objective and scientifically relevant arguments, such as a system’s primary productivity, species diversity and connectivity, and resiliency to stress, as well as the interactions between such variables (Costanza 1992). Others have argued that the heavy reliance on ecological theories without any form of validation in the real world might lead to inadequate public environmental policies, and in essence, mislead society (Karr 1999). To illustrate his case, Karr provides examples of situations in which a tropical forest may be classified as more healthy than a spruce-fir forest,

based solely on the fact that it is more diverse and has a higher primary production, or where a community of oligochaete worms at a wastewater treatment outflow may be classified as healthy because of their resiliency to disturbances.

Most parties involved in freshwater quality monitoring and management can agree with the fact that health is an important aspect of systems, especially in those that are of importance to human health, such as freshwater bodies. Directive 2000/60/EC of the European Parliament and of the Council established "a framework for [European] Community action in the field of water policy". In this EU Water Framework Directive (WFD), the European Parliament states that water is "not a commercial product like any other but, rather, a heritage which must be protected, defended and treated as such". The WFD reiterates the declaration of the 1991 Ministerial Seminar on groundwater, that argued for a need for "action to avoid long-term deterioration of freshwater quality and quantity" and called for a program of actions aiming at sustainable management to be implemented by the year 2000 (European Union 2000). The WFD emphasizes the importance of freshwater organisms, as the composition of their communities is now used to determine the condition of water bodies, and therefore defines the need for restoration efforts and investments. The annexes of the WFD provide normative definitions of ecological status classifications, which include quality elements from hydromorphology (e.g. hydrological regime, river continuity), physico-chemistry (e.g. specific synthetic or non-synthetic pollutants) and biology (e.g. phytoplankton, benthic invertebrate fauna, fish fauna). Each quality element can be categorized as high, good, moderate, poor or bad, but all are defined in comparison to totally, or almost totally, undisturbed conditions. The comparison to undisturbed, ideal communities as a reference, however, also makes that this approach provides a general valuation of the biodiversity itself, and not just evaluates the classical response of indicator species (Schmidt-Kloiber & Hering 2015). Almost 300 different assessment methods for biological quality are in use in Europe alone (Birk et al. 2012), many focusing on invertebrate surveys to calculate Ecological Quality Ratios (EQRs). In the Netherlands, the measures set forth in the WFD are implemented in the Kader Richtlijn Water (KRW). The KRW assesses quality on a scale of 0 to 1, subdivided into five quality classes: "bad"(EQR <0.2), "poor" (0.2–0.4), "moderate" (0.4–0.6), "good" (0.6–0.8) and "high" (0.8–1.0) (Evers et al. 2012, van der Molen et al. 2016) (Figure 1.1).

While some common methods will employ the use of physical and chemical properties of the water, such as levels of dissolved oxygen, acidity or turbidity, these parameters only offer a snapshot of the actual conditions, and fail to provide a more integrative measure of the overall condition of a water body. It may therefore be

insufficient to recognize impaired waters (Kenney et al. 2009). Instead, biological indicators—or bio-criteria—are better capable of offering a more integrated assessment of the health of water bodies (Karr 1999). These bio-criteria use measures of biological communities, spanning multiple trophic levels. Policies such as the EU WFD have already adopted the use of different trophic levels, as witnessed by the inclusion of phytoplankton, macrophytes and phytobenthos, macroalgae, benthic invertebrate fauna, and fish fauna as biological quality elements for the different water types defined in the annexes of its establishing directive. Barbour et al. (1999) summarized the advantages of using biological communities for monitoring, or bio-surveys, as reflecting overall ecological integrity and integrating stressors and stresses

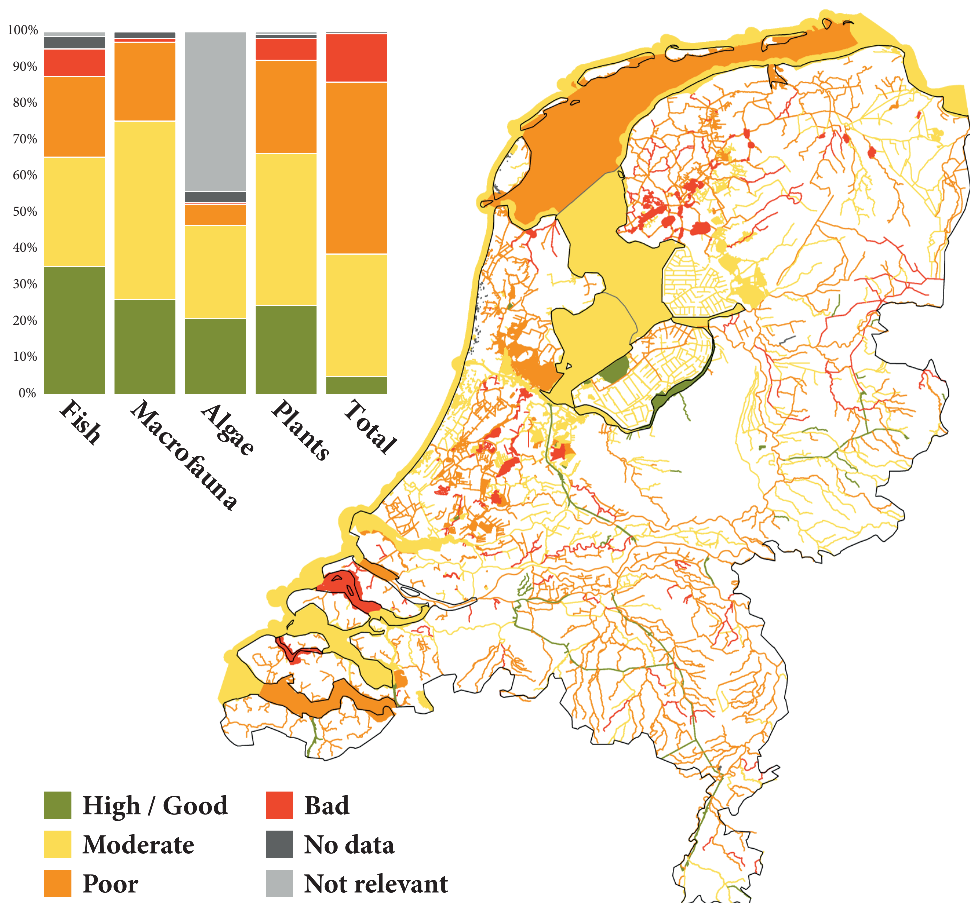

FIGURE 1.1. Ecological quality ratio (EQR) scoring of Dutch water systems in 2015, based on fish, macrofauna, algae, and plants. Map represents the total EQR score of all four elements combined. Data and map obtained from Planbureau voor de Leefomgeving (www.clo.nl/nl142003).

over time to provide a measure of fluctuating environmental conditions. In addition, the routine monitoring of biological communities is argued to be relatively expensive when compared to the assessment of toxic pollutants with chemical tests.

The use of benthic macroinvertebrates for biomonitoring has several advantages. Macroinvertebrate communities are abundant, and reflect localized conditions due to the limited migration patterns of many taxa. This makes them suitable for the assessment of site-specific impacts, such as those measured in upstream-downstream studies. The invertebrate communities are made up of species that represent a broad range of trophic levels, ecological functions and tolerances to stressors. Experienced identifiers can easily recognize most taxa, including the taxa that are most sensitive to changing conditions, allowing for even cursory examinations to yield insights into water quality conditions. In comparison to fish monitoring, the sampling of benthic macroinvertebrates is relatively easy and cheap, with minimal effect of the sampling on the resident biota (Barbour et al. 1999, Kenney et al. 2009).

The advantages already highlight one of the major disadvantages of the use of benthic macroinvertebrates, or any taxonomic group in that respect, as it calls for experienced identifiers. The sheer amount of species in the macroinvertebrate assessments, spread over a huge range of taxonomic groups, requires multiple specialists that divide the workload among the different taxa, or identifiers who are familiar with at least hundreds of species. Such expertise is rare and decreasing: for example, an inquiry among British taxonomists revealed a continuing decline both professional and amateur taxonomists (Hopkins & Freckleton 2002). This taxonomic impediment is furthermore seen in the decreasing number of taxonomic courses offered at universities, and the difficulties encountered by when applying for funding for taxonomic activities by researchers (Drew 2011).

1.3 THE QUALITY OF BIOLOGICAL MONITORING

Variations observed in the macroinvertebrate community—or any biological community—can have several origins. First of all, there are the effects of pollution or other environmental stressors, which are usually the variations that water quality assessments attempt to detect and quantify. Second, there is a natural variation in time, caused by other factors than stress or pollution. Seasonality is the main cause behind this temporal variation. An assessment of macroinvertebrate communities during a one-year period revealed that the best results are obtained by sampling twice a year, in early spring and in late autumn, whereas sampling in summer and winter months is discouraged due to strong seasonal influences and logistical reasons,

respectively (Šporka et al. 2006). Finally, there is variation that occurs during the assessment itself. These have been classified as (1) variations in sampling or sampling methods, (2) sample processing errors, and (3) sample identification errors (Clarke & Hering 2006). The last two steps seem to be the source of most inconsistencies: regular quality control of sample processing is lacking in most laboratories, and only appears to be implemented in the United Kingdom (Haase et al. 2010). Both are labor-intensive, making them susceptible to human errors. Other stages of the water quality assessments at risk of human error include site selection, data entry, and interpretation of the data (Clarke & Hering 2006). Studies have found a significant amount of human errors in the sorting and identification processes, which impacted most of the functional metrics used in water quality assessments (Haase et al. 2006).

During a national survey of streams in the United States, a detailed evaluation of 74 benthic macroinvertebrate samples revealed a taxonomic disagreement of 21% between primary analyst and auditor. This percentage decreased in a second round of evaluations, after primary analyst and auditor communicated and corrective actions were implemented (Stribling et al. 2008). Similar conclusions were drawn in Germany, where an audit on the water quality monitoring program of German streams and rivers was performed. In this audit, 50 out of 414 macroinvertebrate samples were scrutinized on sorting level, identification level, and the combination of both levels. Samples were collected by different commercial laboratories using EU WFD protocols. The human errors were substantial, with 29% of all specimens overlooked during the sorting process by the primary analysts, which led to one in five species being excluded from further analysis. The identification audit revealed that roughly one third of the taxa were different between the primary analyst and the auditor. One of the surprising results was that the error rate was not higher in taxa considered difficult to identify, as compared to those considered easy to recognize. It is postulated that this is caused by the fact that identifiers unconsciously paid less attention to “easy taxa”. In the end, about a sixth of all samples was placed into a different ecological assessment compared to the original assessment (Haase et al. 2010).

Several studies identify similar taxonomic groups that are difficult to identify, such as Baetidae, Chironomidae and Hydropsychidae, which are dependent on freshwater during their larval stages. This indicates that the underlying problem is not just a lack of expertise in the audited studies, but that these groups may pose a challenge in general. Inventories of streams and rivers in the United States indicate that there may be several hundreds, or even over a thousand species that live in monitored sections of water, with most of them only identifiable using adult male specimens or relatively late juvenile stages (Jackson et al. 2014). As a considerable amount of

the collected material consists of those stages hardest to identify to species level on morphological grounds, the results of the few published audits do not come as a surprise. This susceptibility to errors in traditional assessments urges the monitoring efforts of freshwater macroinvertebrates to find alternatives for the identification of collected material, or better yet, skip the time-consuming collecting and sample handling completely.

1.4 INTEGRATION OF MOLECULAR TOOLS

Identifying specimens from freshwater samples to species level based on morphology alone remains a challenge. The taxonomic knowledge about various groups of organisms is often rudimentary, and dichotomous keys are of limited use due to the variation in morphology within benthic macroinvertebrate species. In addition, most of these taxa are small, have few accessible morphological characters, often have closely related species, and often need to be reared to adulthood since pre-adult stages are usually not covered in identification keys (Jones 2008). Several studies have looked into the costs of morphological identifications, and the general conclusion is that the difference in cost between order- or family-level on the one hand and genus- or species-level identifications on the other is considerable, whereas the additional costs to increase the resolution from genus- to species-level are relatively modest (Marshall et al. 2006).

Jackson et al. (2014) state that most studies that assign macroinvertebrates to the lowest possible taxonomic level, generally leave around half of the individuals identified at genus level or higher. They argue that the use of molecular methods will enable assessments to take full advantage of all collected specimens, and in turn may even lead to new species-specific insights on ecology and regulations. Species designations and delineations based on DNA barcodes seem to be in good agreement with those based on morphology, ecology or even behavior. DNA barcoding, the technique of using short fragments of molecular data to identify species, has been around for decades. The use of DNA barcodes for species identification grew tremendously after the introduction of the roughly 650 base pair long mitochondrial COI barcode in the early 2000s (Hebert et al. 2003). While there was some initial doubt about the acceptance of DNA barcoding (DeSalle et al. 2005), the continued growth of the Barcode of Life Database (Ratnasingham & Hebert 2007), together with the sheer number of citations of the original publication from 2003 (well over 11,000 at the time of writing), can be seen as proof of the effectiveness and acceptance by the scientific community. The technique has become embedded in the daily work of

many biologists, and many papers about DNA barcoding are still published each year (Figure 1.2). DNA barcoding has enabled the use of improved taxonomic resolutions, reduced costs and a reduction in the human error in identifications (Pauls et al. 2014). Pauls et al. summarized the benefits of the application of molecular tools in freshwater science as (1) the ability to characterize spatial patterns in diversity on a broader range of taxa, with much greater resolution, (2) the ability to assess functional genetic variation and responses to environmental changes, and (3) increased speed and taxonomic resolution in assessing current status of freshwater.

In the case of Sweeney et al. (2011), for example, the use of DNA barcodes allowed for the identification of many more taxa than with morphology alone. When comparing DNA barcode generated taxon data to expert level inventories on genus and species level, they found a 125% and 70% increase, respectively. When comparing to amateur level identifications they even found a 475% (124 taxa) increase. Using barcodes also revealed additional species that were not described in larval keys, as well as coexisting congeners that may well have been missed due to morphological similarity. Increases in species richness in taxonomic inventories were reported by others as well, such as Jackson et al. (2014). They recovered 104 more species based on DNA barcoding, which amounted to a 108% increase in species richness estimations. Results were best for some of those groups which have been described as "difficult",

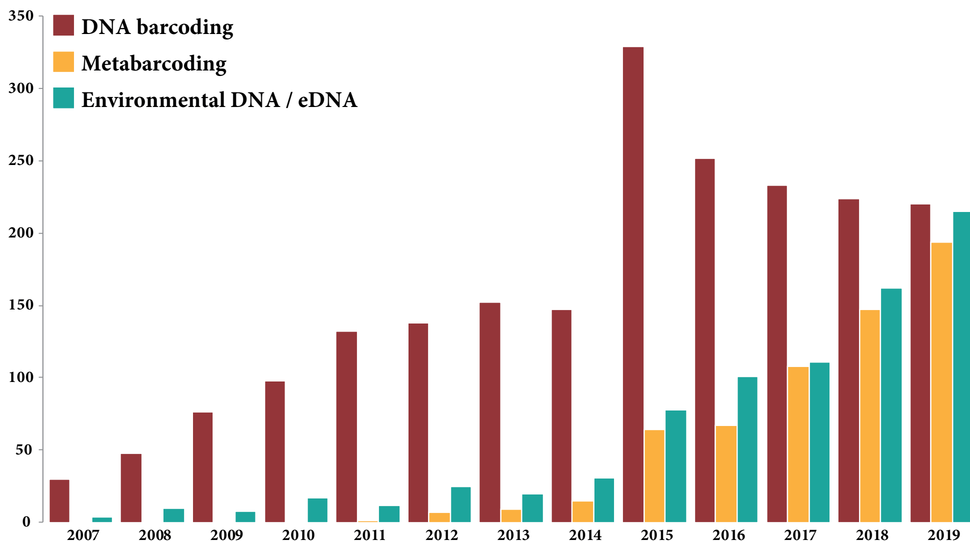

FIGURE 1.2. The number of indexed papers published from 2007 to 2019 on DNA barcoding, metabarcoding, and environmental DNA. Data was retrieved from Web of Science (https://www.webofknowledge.com), based on papers with titles containing "DNA barcoding", "metabarcoding", and "environmental DNA" or "eDNA", respectively.

such as the Chironomidae (194% increase) and Ephemeroptera (77% increase), but also Acari (200% increase). Species identified with DNA were often species known to be uncommon or usually only found in small numbers. Similarly, when creating species lists for alpine lakes, Deiner et al. (2013) found that in about 75% of the cases where young or damaged individuals could not be identified using morphological characters, DNA barcodes allowed for identification up to species or genus level.

Being able to generate species-level taxon lists for freshwater communities also enables the use of species-level ecological characteristics and traits. Even though these may not yet exist for all taxa, as the term “species traits” is often used to refer to genus- or family-level characteristics, their use would greatly improve the ability to reliably identify subtle changes in community structure, and therefore in water quality (Jackson et al. 2014). It has already been shown that even without binomial taxonomic names, DNA barcoding can distinguish between putative species that show differing responses to environmental stressors. DNA barcoding of mayflies in New Zealand stream sites revealed up to twelve different clades or cryptic species, which had contrasting tolerances to common environmental stressors (Macher et al. 2016). Similarly, sequencing of chironomids from a mesocosm experiment showed different response patterns for different biological entities, even though the majority of these operational taxonomic units (OTUs) could not be identified due to lacking references (Beermann et al. 2018). These studies indicate that even with an incomplete reference library or unresolved cryptic species complexes, DNA barcodes provide higher-resolution taxonomic information that can be used for assessments.

1.4.1 High-Throughput Sequencing

Methodological advances in the past decade have led to a situation in which research can focus more on the merger of molecular biology and ecology, and less on the design of studies around technical restrictions. As shown before, the use of DNA barcodes can provide easier and more reliable (at least more standardized) identifications of macroinvertebrates, especially where it concerns pre-adult life stages. To increase the applicability of molecular identification techniques further, DNA barcodes, or any marker for that matter, can be used for simultaneous identification of multiple taxa in complex samples, via DNA metabarcoding (Taberlet et al. 2012a). At the base of this lies what is often called next-generation sequencing (NGS), even though such techniques are nowadays common use, and better refered to as high-throughput sequencing (HTS). Limitations in sequencing platforms at the time, however, made the full COI barcode region unsuitable for use, as its length exceeded the maximum of most platforms. This again spurred some debate as to whether COI was the right

marker for molecular approaches, as it may not contain many suitable conserved regions for broad-spectrum taxonomic coverage (Deagle et al. 2014). Alternatives, such as ribosomal RNA were offered in place of COI, mainly due to the possibility to obtain shorter amplicons. However, the benefit of the COI barcode library, which has much better taxonomic coverage than any other gene for metazoan diversity, has made that COI is still the marker of choice in many studies (Elbrecht et al. 2016, Andújar et al. 2018b), with the exception of fish, where ribosomal markers (such as 12S or 16S) are used, as they allow for better primer design (e.g. Valentini et al. 2015, Fujii et al. 2019).

While COI reference libraries are far from complete, they are sufficiently populated with most of the commonly observed freshwater macroinvertebrates

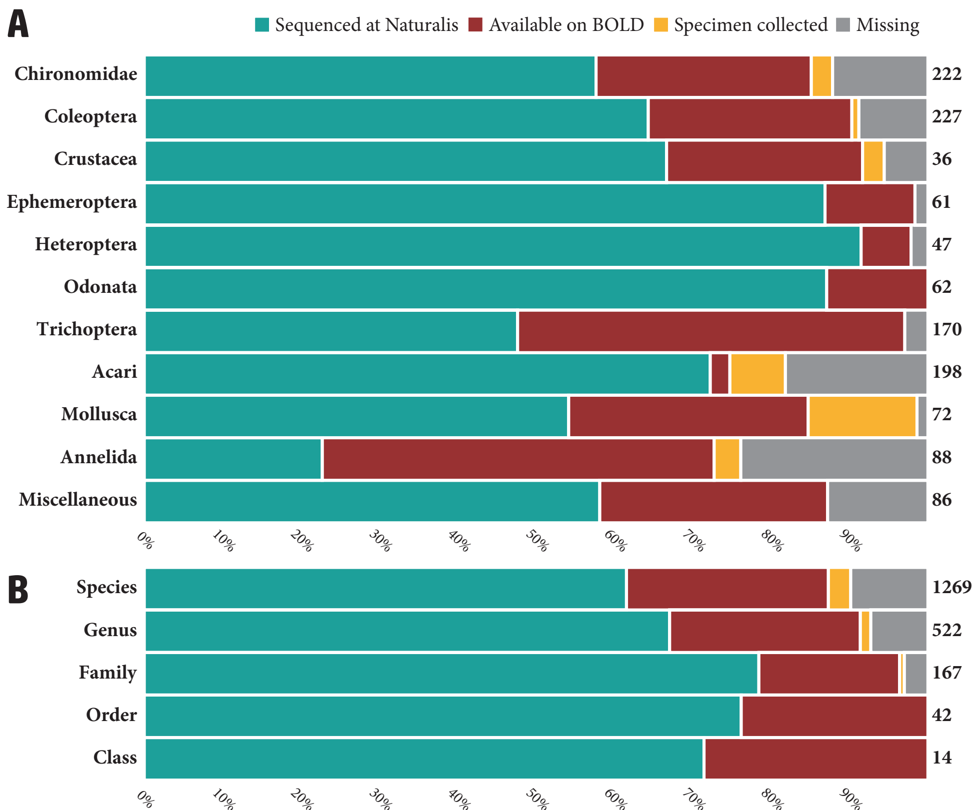

FIGURE 1.3. Percentages of taxa from the Dutch WFD list covered by DNA reference libraries, from the Dutch barcoding campaign at Naturalis Biodiversity Center (as of May 2020), and public data available on the Barcode of Life Database (http://boldsystems.org/). Data is shown for (A) the most important groups of taxa and (B) five taxonomic levels of identification. Indicated to the right of each bar is the number of taxa for each category.

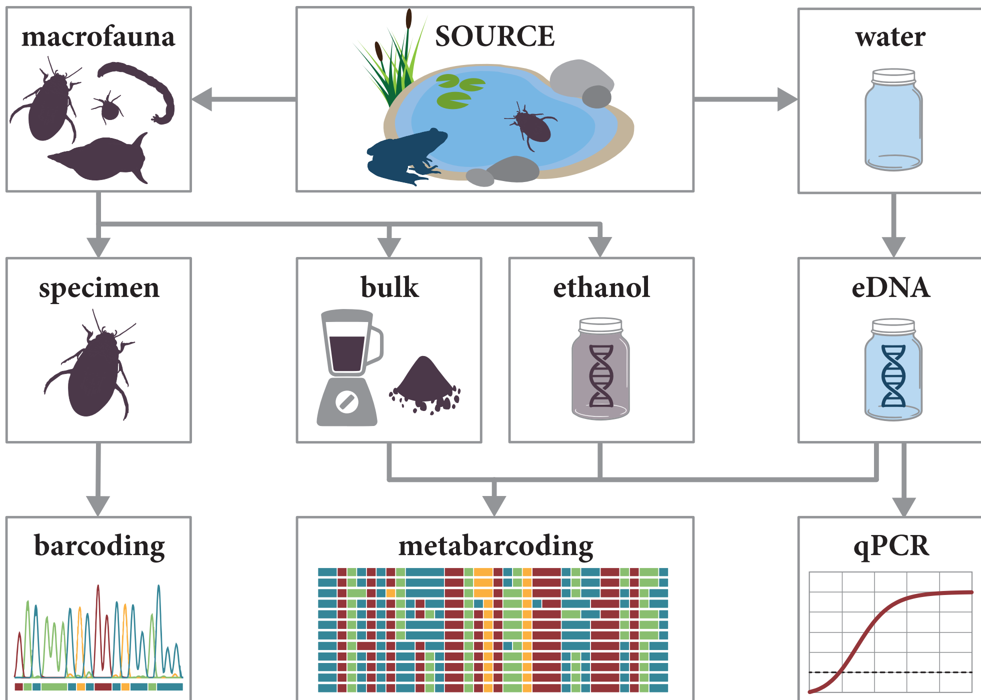

FIGURE 1.4. A schematic overview of the main techniques used in water quality monitoring, based on the type of samples. Individual specimens collected using traditional techniques are essential for the creation of a reliable reference database using single-species DNA barcoding. Bulk, ethanol from bulk, and directly collected environmental DNA can be used for DNA metabarcoding. Species-specific PCR detection is best performed on eDNA samples. Original illustration.

to allow for the use of COI in routine monitoring applications (Curry et al. 2018) (figure 1.3). Shorter fragments of COI can still be used to separate closely-related taxa (Meusnier et al. 2008), and several primer sets have been developed on shorter amplicon lengths and have proven to be successful in capturing relevant groups for biodiversity monitoring, such as marine metazoa (Leray et al. 2013) and freshwater macroinvertebrates (Elbrecht & Leese 2017). DNA metabarcoding techniques have been used for identifications of specimens in bulk samples, simply by homogenizing the samples and performing DNA extractions on the resulting "slurry" (Hajibabaei et al. 2011, Gibson et al. 2015) (figure 1.4). In addition to the bulk samples obtained from biodiversity monitoring programs, the use of DNA metabarcoding has also proven its worth in diet studies, where the original bulk data is impractical to use for morphological identification, due to the degraded state of most tissue found in gut contents or fecal matter (Pompanon et al. 2012, Gibson et al. 2014, Corse et al. 2017).

Non-destructive DNA metabarcoding has also been performed on storage ethanol of bulk samples, leaving the specimens intact and available for further study (figure 1.4). In 2010, it was shown specimens stored in ethanol “leak” DNA into the preservative, when DNA barcodes were obtained from both freshly stored and archival specimens (Shokralla et al. 2010). Metabarcoding studies on preservative ethanol show promise, in some cases obtaining more species than bulk metabarcoding, although there are still differences with traditional morphological assessments, especially where it considers species that represent low proportions of the total biomass of a sample (Hajibabaei et al. 2012, Erdozain et al. 2019).

1.4.2 Environmental DNA

While DNA metabarcoding can potentially replace morphological identifications, and the sampling of preservative ethanol is a non-destructive approach, both still require traditional sampling of specimens. However, in the last decade a new method has become popular in the field of molecular biomonitoring of multicellular organisms: environmental DNA (eDNA) (figure 1.4). Inspired by studies that retrieved ancient DNA from sediment or ice cores (e.g. Willerslev et al. 2003), Ficetola et al. (2008) showed they were able to detect the presence of the invasive American bullfrog (*Lithobates catesbeianus*) in both controlled aquarium setups and natural ponds in France by sampling water and precipitating organic material contained therein. Since then, the use of environmental DNA for the detection of species diversity has increased rapidly (Taberlet et al. 2012b, Thomsen & Willerslev 2015), which is reflected in the number of papers growing steadily each year (Figure 1.2).

Many early papers dealing with eDNA in freshwater and the marine environment focused on single-species detection using specific primer/probe sets. These allowed for the amplification of only target DNA in real-time PCR, resulting in a “yes” or “no” (and quantitative indication) without the need for sequencing any DNA. A fair number of these studies used invasive species as a model organism, as they are relevant for ecosystem management. In these cases, eDNA could provide an “early warning” insight system, in which it would theoretically be possible to detect presence of invasive species in early stages without intensively sampling systems using traditional methods. The majority of the papers employing eDNA for such detections focused on amphibians (e.g. Dejean et al. 2012, Smart et al. 2015) or fish (e.g. Jerde et al. 2011, Takahara et al. 2013), organisms known to shed relatively large amounts of DNA into the water column, in comparison to many hard-bodied macroinvertebrates, although there have been several studies that showed eDNA is also usable to detect invasive crayfish (Tréguier et al. 2014, Agersnap et al. 2017). In

a similar fashion, species-specific PCR assays have also been used for the detection of rare, endangered or policy-relevant species. This effectively allows monitoring agencies to cover more terrain by simply sampling water at any location of interest and foregoing the invasive and labor-intensive traditional assessment methods, and has been coined "conservation in a cup of water" (Lodge et al. 2012). As with the invasive species, most of the target organisms in these eDNA studies are vertebrates, ranging from amphibians (Goldberg et al. 2011, Spear et al. 2015) to fish (Sigsgaard et al. 2015) and cetaceans (Stewart et al. 2017), although several inquiries have been made into invertebrates as well (Thomsen et al. 2012b, Mächler et al. 2014), and even aquatic plants (Matsuhashi et al. 2016). The use of eDNA for the detection of single species has been shown to be less labor-intensive and more cost-effective than traditional monitoring, but require well-planned sampling strategies adapted to the target organisms (Smart et al. 2016, Evans et al. 2017a, Lugg et al. 2018).

Important with species-specific assays is the specificity and sensitivity of the primer/probe sets used for detection, especially when there are closely-related species that might provide false positive signal (Wilcox et al. 2013). Thomsen et al. (2012) also showed, however, that high-throughput sequencing was also possible for species detection of fish and amphibians, which makes that primers may not necessarily have to be species-specific. On the contrary, HTS allows for the simultaneous sequencing of multiple organisms, so in theory it is best used in combination with primers that are not species-specific. Environmental DNA metabarcoding has become more and more mainstream, and forms the final stage in the transition from traditional monitoring to molecular biomonitoring: (1) replace taxon identification by DNA barcoding, (2) replace specimen handling by DNA metabarcoding, and (3) replace traditional sampling by environmental DNA. Several primer sets have been developed for the monitoring of fish (and other vertebrates), both freshwater and marine (Thomsen et al. 2012a, Miya et al. 2015, Valentini et al. 2015, Andruszkiewicz et al. 2017), which often show congruence with traditional inventories (e.g. Hänfling et al. 2016). In addition to fish, group-specific assays have been developed for several other relevant organism groups for biomonitoring, such as indicator chironomids (Bista et al. 2017), invasive molluscs (Klymus et al. 2017), and mosquitoes that act as disease vectors (Schneider et al. 2016, Krol et al. 2019). For good comparison with traditional monitoring, such group-specific primers would be optimal, as general primers often amplify a wide range of non-target taxa.

1.5 NEXT-GENERATION BIOMONITORING

The developments in sequencing and alternative DNA sources also brought with them an increased resolution of information to be obtained from natural systems. While many European systems that assess macroinvertebrates rely on species-level identifications for the resulting EQR (at least on paper), there are still others in which only higher-level identifications are used, as well as many taxonomic groups for which species-level identifications are not always possible. However, some challenges still need to be overcome to allow for full incorporation into standard monitoring practice, depending on the taxonomic groups assessed (Hering et al. 2018). Whereas the use of DNA barcoding may not be cheaper than morphological analysis, costs will be driven down by adopting metabarcoding as method for taxon identification, especially when compared to species-level identifications using morphology (Jones 2008, Stein et al. 2014, Aylagas et al. 2018). Newly emerging techniques for high-throughput sequencing will make sequencing even cheaper in the near future. It also opens up possibilities for groups that are now largely ignored due to their difficulty with identifications, such as planktonic taxa, to be included in routine monitoring or impact studies. While the inclusion of any such groups needs a completely new method of assessment, the tools are already largely available to start working towards this “biomonitoring 2.0” (Baird & Hajibabaei 2012, Pawlowski et al. 2018). It has already been shown in several studies that genetic assessments of biodiversity can yield significantly different results compared to morphological assessments, although both genetic and traditional surveys can complement each other and present a more complete view of the ecosystem (Shaw et al. 2016, Kelly et al. 2017).

Genetic assessments have already been performed and compared to traditional monitoring in several studies. For marine monitoring, a genetics-based version of the AZTI’s Marine Biotic Index (gAMBI) has been proposed and compared to the traditional AMBI index, in which there was a moderately strong correlation between the two (Aylagas et al. 2016). The best performing DNA-based method, using the 313 bp fragment by Leray et al. (2013), resulted in a 62.4% match between the morphological and molecular taxa list, and 76.3% when only looking at species level. A similar study by the same authors only found about half the taxa in metabarcoding compared to traditional identifications (54.4%), but metabarcoding and traditional data led to similar assessments, comparable to the earlier study. Additionally, the metabarcoding was calculated to be around 55% less costly and 72% less time consuming (Aylagas et al. 2018). A study comparing morphological and metabarcoding-based stream assessment in Finnish monitoring sites, on the other hand, found twice as many

taxa with DNA as with morphology, although that conclusion is somewhat inflated due to the fact that some species-rich groups like chironomids were only identified up to family level in the morphological analysis. The EQR and other assessment metrics were significantly correlated (Elbrecht et al. 2017a), indicating that DNA metabarcoding-based approaches certainly have potential to replace traditional monitoring with the necessary recalibration of metrics involved.

Micro-organisms, such as planktonic taxa or bacteria, are an ideal target group for the use of molecular tools. The sampling methods for the collection of samples are relatively easy, compared to the traditional collection methods employed for macroinvertebrates, and the identification requires microscopic inspection by specialists. These groups are often also more diverse than most macroinvertebrate groups, meaning they might provide a better insight into the ecosystem due to the increased resolution they provide in comparison to relatively species-poor vertebrate and macro-invertebrate groups used in traditional surveys. Diatom indices inferred from metabarcoding data have already been shown to be significantly correlated to morphological assessments, demonstrating the feasibility of applying metabarcoding in such surveys (Visco et al. 2015). The relatively high diversity can, however, also be a limiting factor, since it is likely that taxon groups such as plankton may hide a large proportion of undescribed or understudied taxa, which could cause issues inferring quality assessments. There is still ample opportunity to incorporate these unknown taxa into biomonitoring, using so-called “taxonomy-free” methods. A paper on diatoms showed that three-quarters of the examined sites could be assessed correctly using molecular operational taxonomic units (MOTUs), rather than classic taxonomic assignments (Apothéloz-Perret-Gentil et al. 2017). Unassigned MOTUs were also used to infer assessments based on benthic foraminifera via machine learning techniques, again leading to a similar ecological status as the traditional monitoring using macroinvertebrates (Cordier et al. 2017). The same machine learning techniques were later used to show that different markers (both prokaryote and eukaryote) could accurately predict environmental impact of marine aquaculture, and all outperformed the assessment based on traditional methods (Cordier et al. 2018).

Such impact assessments are a prime target for the use of (environmental) DNA metabarcoding. Not only have these molecular tools the potential to provide much higher resolution views on an ecosystem, impact assessment studies are not necessarily bound to traditional survey methods like the ecological quality assessments dictated by the EU WFD. Several studies have already been performed using metabarcoding for impact assessments in the marine realm, dealing with impacts from fish farms (Pochon et al. 2015, Stoeck et al. 2018, Cordier et al. 2018) and offshore oil and gas

drilling (Laroche et al. 2018). Metabarcoding has also been successfully applied in various impact studies in freshwater in recent years, ranging from metabarcoding of bulk macroinvertebrates samples to investigate pesticide spills (Andújar et al. 2018a) to the collection of planktonic organisms to study the effects of urbanization and wastewater (Chonova et al. 2019, Hanashiro et al. 2019). Environmental DNA obtained from water samples contained few metazoans, making comparison to traditional methods difficult, but nonetheless the MOTUs could still be used to reveal impaired sites (Bagley et al. 2019), demonstrating again that eDNA is a powerful tool in water quality assessment and management.

While DNA techniques have proven useful in various analyses performed for water management, from the detection of invasive species, to the assessment of stressor impacts, there is still some way to go before molecular tools can be integrated into water quality monitoring across the board. Many assessments, especially those performed under the WFD, are still performed using traditional methods, simply because that is, from a legal perspective, the golden standard. The current implementation of the WFD runs until 2027, meaning that there is still time before DNA methods can be legally entered into the standard practices. This leaves room for much needed standardization in some approaches, which would arguably make it easier for policy makers and stakeholders to accept DNA techniques as actual "standards". Some of the discussions about any such standardization are currently taking place in DNAqua-Net, a COST Action network aimed to "nucleate a group of researchers across disciplines with the task to identify gold-standard genomic tools and novel eco- genomic indices for routine application in biodiversity assessments of European fresh- and marine water bodies" (Leese et al. 2016).

There are as many different protocols for DNA metabarcoding as there are labs, and probably even more. The idea that one protocol will arise as "the golden standard" and consequently be used by all players in the field of water quality management sounds utopian. Different nations will likely have their own interpretations of any WFD protocol, as is already the case with the traditional monitoring today. Nonetheless, it is reasonable to have some standards that allow for cross-comparison of results, and to implement some basic guidelines which improve reliability and reproducibility of results. Several papers have already been published that try to further this agenda, by suggesting recommendations for sampling and analysis, and minimum recommended reporting guidelines for eDNA studies (Goldberg et al. 2016, Nicholson et al. 2020), or highlighting the need to take the necessary controls in each step of the eDNA metabarcoding process (Zinger et al. 2019). In addition to the standardization of techniques for successful integration into quality assessments,

it is imperative that end-users become familiar with new technologies and their terminology, and are also shown the shortcomings of current methodology (Bush et al. 2019). The terminology that molecular tools bring with them may be unfamiliar for practitioners and policy makers, especially regarding NGS technology, which can hinder the successful implementation of these tools into water quality assessments. For instance, reporting "presence/absence" when using eDNA may be incorrect, and terms like "detected/not detected" would be better suited (Roussel et al. 2015), since false positives and negatives are still commonplace in eDNA analyses (Ficetola et al. 2016).

1.6 THE ECOLOGY OF EDNA

Besides terminology and the need for better reporting, there are several key challenges in the use of environmental DNA and DNA metabarcoding. For eDNA, some of the main challenges deal with the ecology of DNA within the environment, in particular the origin, state, transport, and fate (Figure 1.5), which have significant impact on the sampling design and DNA extraction methods. Where it concerns DNA metabarcoding, one of the key discussion topics surrounds the inference of abundance from molecular data; another deals with the use of MOTU data and genetic variation between taxonomic units, and both have considerable impact on the subsequent (ecological) analyses and assessments. Since the first papers emerged that demonstrated the potential of eDNA for the detection of aquatic organisms, there has been an increasing number of publications delving into the behavior of environmental DNA (see also Figure 1.2). Central questions in the "ecology of eDNA" (figure 1.5) deal with the state and fate of eDNA. Factors that play a role in this ecology of eDNA, such as transportation and degradation of eDNA, as well as spatial and temporal distribution patterns are key to the interpretation of eDNA results.

There are many potential sources of environmental DNA. Living organisms release DNA into the environment via shedding and extraction. The slimy coating that fish and amphibians use as a form of self-defense seems to be a decent source of eDNA, considering the many papers that use eDNA for the detection of these organism groups (e.g. Ficetola et al. 2008, Jerde et al. 2011). Other organisms that are known for shedding relatively high quantities of eDNA are bivalves, which not only continuously filter water, but also have periods of spawning that introduce large quantities of genetic material into the water column (Sansom & Sassoubre 2017). Aquatic insects that have larval stages in the water column introduce eDNA by molting and pupating, such as the larvae and pupae of Chironomidae (Bista et al. 2017). Feces are also a source of eDNA, from both prey and predator species, as are any dead

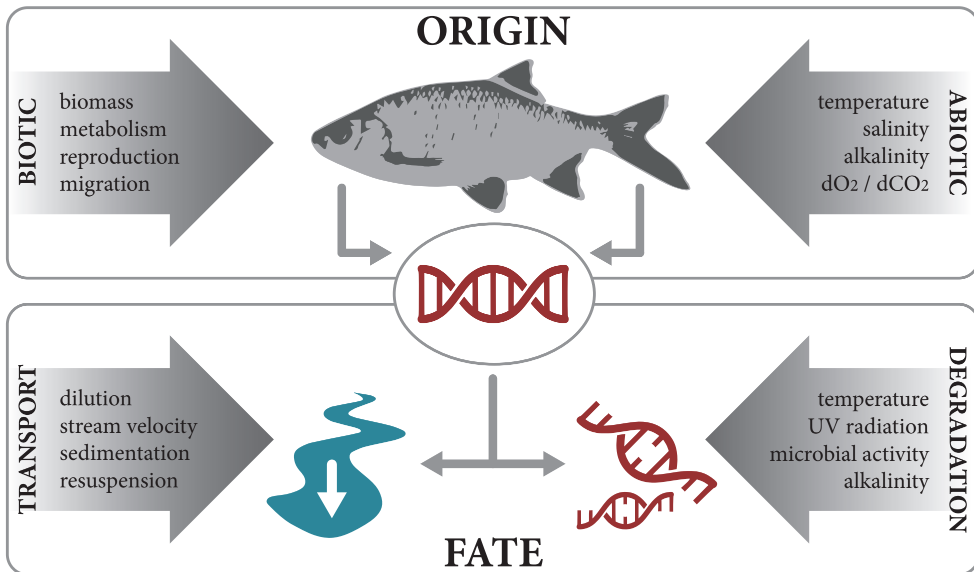

FIGURE 1.5. The main components in the cycle of eDNA in the water column: the origin of eDNA and the fate of eDNA through transportation and degradation, as well as some of the most important biotic and abiotic factors that influence the ecology of eDNA. Original illustration, based on Barnes & Turner (2015) and Stewart (2019).

organisms (Merkes et al. 2014). Shedding rates of living organisms vary throughout the seasons, and are influenced by different environmental factors, such as increases in water temperature (Jo et al. 2019). A study using bighead and silver carp also showed that feeding patterns of fish had an influence on the amount of eDNA shed into the water, probably due to increased excretion of sloughed gut cells with higher food intakes (Klymus et al. 2015). Other biotic factors, such as metabolism, age, stress, reproductive state, and migration, as well as a wide variety of abiotic factors like temperature, salinity, alkalinity, and levels of dissolved O_2 and CO_2 have been linked to variations in eDNA release and shedding from its source organism (Stewart 2019) (Figure 1.5). Environmental DNA sourced from dead animals can in some cases provide difficulties, for example in case of monitoring of invasive species from ballast water. Ballast water from transport ships is often treated to kill any remaining organisms, which are potential invaders. Environmental DNA might not be sufficient to separate signals from living invasive species and dead species dumped with ballast water, and some limited work has been done to circumvent these difficulties by using the more instable RNA as environmental signal (Pochon et al. 2017, Cristescu 2019).

The source of eDNA also influences its state. Whilst eDNA is often thought of as

DNA strands that exist freely in the water column, a large part of the environmental DNA is in fact still bound in cellular remains and adsorbed on particulate matter. Extracellular eDNA is relatively unstable, and exposed to the elements that break down DNA. Conflicting reports are found in the literature regarding the particle size distributions of eDNA. Fractioned filtration of water samples with filters with decreasing pore sizes revealed that fish eDNA was most abundant in fractions that would not pass through 1.0 μm pores (Turner et al. 2014a, Wilcox et al. 2015). These findings are consistent with hypotheses that a large proportion of eDNA is still bound in cellular remains or to particles, and also explains the heterogeneity observed in water samples. Turner et al. (2014a), however, also showed that total eDNA yields were highest using 0.2 μm pore size, as did a paper looking at particle size distribution in water fleas (Moushomi et al. 2019), which would suggest that eDNA is predominantly subcellular. Many studies, however, do not distinguish between intra- and extracellular DNA when assessing the processes within the ecology of eDNA, such as the factors playing a role in the persistence (Barnes et al. 2014). Often such studies use model organisms that are removed from an aquarium setup at a certain point in time to evaluate the persistence of DNA under various conditions, but use eDNA extraction methods that do not allow for distinctions between intra- and extracellular DNA (e.g. Dejean et al. 2011). This is not necessarily an issue, although it does highlight the continuing discussion around the use of the term “environmental DNA”. Many papers use “eDNA” in a way that covers both intra- and extracellular DNA, but sometimes bulk-collected specimens are also treated as “environmental DNA”. Fortunately, most studies around the ecology of eDNA use vertebrate or macroinvertebrate model organisms, in combination with collection methods that are not aimed at collecting bulk specimens. Generally, persistence of eDNA in the water column is relatively low, with signals becoming undetectable within days or weeks after removal of the source organisms (Dejean et al. 2011). Environments that have lower temperatures, higher pH, and are more protected from UV radiation have been found to allow a longer persistence time than water bodies with higher temperatures, lower pH, and more exposure to UV radiation (Strickler et al. 2015, Goldberg et al. 2018), all processes that either directly or indirectly (via increased microbial activity) influence the degradation of eDNA.

Transport of eDNA is another factor that plays an important role in the analysis of eDNA results, and something that should be considered during the sampling strategy design. Transport of eDNA is most obvious in lotic systems, in which eDNA has been shown to be transported from a point source with the flow of the water. DNA of two species living in a lake in Switzerland was observed up to almost 10

kilometers downstream in an outflowing river (Deiner & Altermatt 2014). Similar studies with different organisms found different detection ranges, from several kilometers for fish in a river in France (Civade et al. 2016), down to only five meters for amphibians in a stream in the United States (Pilliod et al. 2014). Several factors explain these differences, such as those involved in the persistence, but also density of organisms at the source, as well as stream velocity and turbulence, and their effect on sedimentation rates of particulate matter with adsorbed DNA. The influence of such factors were also postulated by Jane et al. (2015) from an experiment with caged trout in two headwater streams. In these experiments, they found that low stream velocities resulted in high eDNA concentrations near the cage, with concentrations quickly dropping further downstream, whereas high stream velocities resulted in low eDNA concentrations both near the cage and downstream. A study by Pont et al. (2018) combined observations from literature and their own field data into a model that showed that eDNA in lotic waters behaves much like fine particulate organic matter, and reported detection distances of up to a hundred kilometers. In a follow-up study in the catchment system in Switzerland, Deiner et al. (2016) concluded that eDNA was better able to provide information of a catchment area than kicknet sampling, with eDNA samples provided higher richness estimates in samples further downstream (and thus representing larger catchment area), as well as lower community dissimilarities compared to kicknet sampling.

The number of publications on the transport of eDNA in lentic systems is limited, although various studies have looked at the spatial distribution patterns of eDNA in freshwater bodies. The general conclusions from these studies is that the distribution of eDNA in lentic systems is quite heterogeneous, with very local signals of eDNA representing local presence of aquatic organisms. Optimal sampling requires spatial replicates to improve detection of organisms (Thomsen et al. 2012b, Harper et al. 2019b) or increase the number of taxa detected with NGS (Evans et al. 2017b, Grey et al. 2018, Lawson Handley et al. 2019). This suggests that the transport of eDNA in lakes and non-flowing ditches based on diffusion is limited. Similar findings of spatial dissimilarities have been reported for marine sampling, which is not surprising seeing how study sites there are larger than most freshwater systems (Guardiola et al. 2016, O'Donnell et al. 2017, Stat et al. 2019).

In addition to spatial distribution and transportation of eDNA, seasonal differences in eDNA have been studied in more detail the last few years. Some work has been done to highlight the need for spatial and temporal replicate sampling macroinvertebrate communities, although seasonal differences are mainly attributed to phenological patterns that have already been observed in morphological monitoring (Šporka et al.

2006), especially for those groups that are aquatic only in the larval stages (Bista et al. 2017). Most papers that study seasonal differences in aquatic eDNA, however, focus on fish (Stoeckle et al. 2017, Sigsgaard et al. 2017) or amphibians (Rees et al. 2017, Buxton et al. 2018). Environmental DNA seems to be more abundant in warmer seasons of the year, requiring fewer spatial replicates for successful detection of organisms (De Souza et al. 2016). Differences between winter and summer have been attributed to different factors, most of all the increased activity of many organisms, including reproductive activity (Figure 1.5). Other factors that are involved in seasonal differences of eDNA detectability are abiotic factors that influence persistence of eDNA, or physical processes like stratification and admixture in lakes (Lawson Handley et al. 2019).

In addition to the influence of the ecology of eDNA on DNA-based biodiversity monitoring, there are numerous practical and technical considerations, from the handling of samples in the lab to the interpretation of data during the analyses, which will be reflected upon in this thesis. The increasing number of studies published in the field of eDNA, metabarcoding (Figure 1.2), and their applications in biomonitoring, however, is a sign that the research field is making progress.

1.7 OUTLINE OF THIS THESIS

With this thesis I aim to shed some light on a few important considerations when dealing with molecular data, but also show the potential of these techniques. Since the DNA barcode reference libraries for Dutch aquatic macrofauna are relatively complete (Figure 1.3), using Dutch freshwaters as a study focus was an obvious choice. The Netherlands also has a long history of standardized WFD monitoring, which allowed us to make use of historic data and samples in the research presented in this thesis.

Chapter 2

One of the major obstacles in using data generated by DNA metabarcoding is the unreliable abundance data obtained from next-generation sequencing reads. Abundance is an important factor for WFD quality assessments under the current benchmarks. Developing methods that provide accurate abundance information from metabarcoding data is difficult, if not impossible, and problems are probably even greater when dealing with eDNA. So, if DNA metabarcoding or eDNA metabarcoding is ever to play a role in WFD monitoring and the calculations of EQRs, it would be necessary to make a transition to an EQR scoring system independent of abundance

data. In Chapter 2, titled "The influence of macroinvertebrate abundance on the assessment of freshwater quality in the Netherlands", I used historical monitoring data from several water authorities in the Netherlands (which were based on abundance data), and transformed these into presence/absence data. By directly comparing EQR scores calculated on both abundance and presence/absence data, I concluded that for macroinvertebrates, the importance of abundance data was only limited, and, perhaps surprisingly, removing abundances had little impact on the resulting EQR scores.

Chapter 3

The next step was to start comparing molecular tools for the identification of macroinvertebrates with traditional analyses of WFD samples. Current practices for WFD monitoring rely on the cumbersome and time-consuming visual identification of all the specimens collected at the monitoring sites. In addition to their time-consuming nature, morphological assessments are reliant on the expertise of individual assessors, and thus prone to error (see also paragraph 1.3). While DNA barcodes may not be able to distinguish all of the >1000 Dutch aquatic macroinvertebrate species, especially with the gaps in the reference libraries as they are, DNA-based methods have the benefit of not relying on individual taxonomic expertise.

In Chapter 3, titled "Increased performance of DNA metabarcoding of macroinvertebrates by taxonomic sorting", I tried to limit the influence of preferential amplification by using taxonomically sorted samples. Historical WFD samples were obtained, and kept the specimens separated according to the taxonomic categories used during the morphological analysis; Annelida, Crustacea, Heteroptera/Coleoptera, Mollusca, Trichoptera/Odonata/Ephemeroptera and "rest" (predominantly Chironomidae and other Diptera). Sorting specimens into these six basic groups before DNA extraction and amplification improved taxon recovery by 46.5%. When comparing the species lists obtained with DNA metabarcoding to those from the morphological assessment, there were considerable differences, although the numbers of taxa detected were similar. With an average overlap of 56.8% between the two, the impact on the EQR calculations was severe. However, for a few taxonomic groups the use of DNA barcodes resulted in much more detailed information, especially in those groups that are considered difficult to identify based on morphology.

Chapter 4

While using a blender and DNA metabarcoding WFD samples in bulk seems to be successful, when certain steps are taken to overcome the worst of the preferential

amplification, such methods would still require the specimens to be collected. While not as time-consuming as the identification process, the collecting of specimens still imposes considerable effort on the part of the monitoring agency. Sampling of eDNA would alleviate this. However, the approach is still under heavy scrutiny, especially where it concerns the sampling design. In Chapter 4, titled "The effects of spatial and temporal replicate sampling on eDNA metabarcoding", I investigated the effects of replication at three different levels during collecting and processing of eDNA samples: spatial replicate sampling, temporal replicate sampling, and PCR replicates. Two undisturbed natural lakes in the dunes of Wassenaar were sampled over 20 consecutive weeks, and eDNA was analyzed using the same general COI primer set used in the analysis of bulk samples. While our initial observation was that these general primers were not optimal for the detection of macroinvertebrates in eDNA samples due to the amplification of many non-target taxa, the replicate patterns were clear. Temporal differences over intervals larger than two weeks were larger than differences in spatial replicates, suggesting that turnover effects might be more important for the dynamics of eDNA than its heterogeneity within a study site. PCR replicates also showed dissimilarities, although not as profound as the replicate sampling.

Chapter 5

The effects of temporal replication were also witnessed in the study presented in Chapter 5 of this thesis, titled "Environmental DNA metabarcoding reveals comparable responses to agricultural stressors on different trophic levels of a freshwater community". In this study, I put eDNA to the test as a tool for monitoring impact of agricultural stressors on aquatic ecosystems. Because previous research had already shown that general macroinvertebrate primers for COI showed a lot of "bycatch", I opted to have an in-depth look into the ecosystem on three different trophic levels. Bacteria represented the decomposers, phytoplankton acted as a representative for the primary producers, and Chironomidae represented the macrofaunal community. Chironomidae are a key indicator group for water quality, and relatively well-represented in the custom reference databases created over the duration of the PhD project. All three groups are often understudied in water quality assessments, mostly due to the difficulties with morphological identifications.

To get a good grip on the individual and combined effects of two major agricultural stressors, the neonicotinoid insecticide thiacloprid, and fertilizer influx, this study was performed in the "Living Lab" facility of the University of Leiden. This unique setup strikes a balance between controlled laboratory mesocosms, and a natural field

situation. Replication of single and combined treatments, and undisturbed control situations, allowed us to disentangle the stressor effects, as well as differences caused by turnover through time. All three groups under assessment showed significant impact from both agricultural stressors, where even bacteria and phytoplankton communities were influenced by insecticides at concentrations regularly observed in surface waters in the Netherlands. Concurrently with the eDNA research, the Living Lab samples were also analyzed using traditional morphology, allowing us to directly compare results. The impact patterns seen with traditional assessments were comparable to those observed with eDNA, but at a much lower resolution (i.e., fewer taxa), and more replicates were needed to come to the same conclusions. The use of three understudied groups also allowed to us to uncover potential new bioindicators for freshwater stressors, although except for the Chironomidae, these usually were unidentified MOTUs. Large parts of the freshwater biodiversity, especially microorganisms, are still underrepresented in DNA reference databases.

Chapter 6

In the final chapter of this thesis, Chapter 6, I combine the insights into the applicability of DNA-based methods in freshwater monitoring obtained within my PhD project with results and conclusions from research in this field from the past decade. I discuss the potential and pitfalls of the use of environmental DNA for different assessments into the quality of freshwater, especially where it concerns technical considerations. Additionally, I give directions for future research to increase the understanding of eDNA, and how this knowledge should be integrated into methods that are suitable for direct application in the field.