

# Identification of invasive invertebrates in freshwater using eDNA

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Is there a correlation between the spatial distribution of the invasive freshwater mussel species *Dreissena polymorpha* and the invasive amphipod crustacean *Dikerogammarus villosus*, as measured by the amount of eDNA of both species detected by qPCR from four sampling sites distributed along the river Limmat and the Lake of Zurich?

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## 1. ABSTRACT

With the intensification of climate change, the invasion of Swiss freshwater bodies by alien species will become a rising phenomenon. Therefore, adequate, time- and cost-efficient monitoring of the concerned organisms and their dynamic interactions with others will be crucial to anticipate and milden their impact on fluvial biodiversity.

Since the invasion of the zebra mussel *Dreissena polymorpha* and the killer shrimp *Dikerogammarus villosus* in the Lake of Zurich and the Limmat in the last decades, their spatial distribution has been primarily investigated using kick-sampling, which is harmful to the biome and inefficient in terms of time and replicate numbers.

This Maturitätsarbeit aims to evaluate if the detection of environmental DNA by real-time quantitative PCR is a promising method to detect a potential correlation between the presence of both invasive species in the Lake of Zurich and the Limmat.

The main steps of the experimental design consisted of freshwater sampling on the four sites along the Lake of Zurich and the Limmat, filtering to immobilize eDNA on microporous membranes, isolation, and species-specific detection by qPCR. eDNA yield and qPCR amplification cycle values ( $C_q$  values, indicator of relative eDNA abundance of each species) were acquired from multiple sampling sites and replicates.

Following this experimental pipeline, eDNA from *D. polymorpha* and *D. villosus* was reliably detected on three sampling sites, confirming their co-occurrence in the freshwater bodies investigated. However, although previous observations suggested that both species could interact in the biome for micronutrient supply and habitat preferences, no significant positive correlation between their eDNA levels was found, suggesting no quantitative association between both species at present.

Lastly, concerns about fluvial biodiversity are emitted using a case study on how the co-occurrence of *D. villosus* and *D. polymorpha* could, *inter alia*, negatively influence the dynamic relationship of algae and cyanobacteria in the water.



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### 3. FOREWORD

Every organism on earth has a singular genome made of DNA and is a product of *nature's* evolutionary bricolage. Since Watson's, Crick's, and Franklin's discovery of the double-helical structure of DNA in 1953 that is often considered to represent the birth of modern molecular biology,<sup>1</sup> geneticists have been longing to find fast and cost-efficient possibilities to sequence hereditary material for diverse applications in the fields of biotechnology, forensics, and environmental biology.

The Human Genome Project (1990-2003) is considered<sup>2</sup> one of the most significant cooperative international research projects. Its main goal<sup>3</sup> was identifying and mapping all DNA bases that make up the 23 human chromosome pairs. Another, more controversial objective was to patent genes in the human genome having the potential for making recombinant proteins and then to charge potential payers in various fields, especially in the medical milieu.<sup>4</sup> Using the Sanger sequencing method based on electrophoresis, the project cost was about 3 billion US dollars, involved 20 universities, and lasted for 13 years.<sup>5</sup> By 2003, most of the human genome was characterized, although it lasted until May 2022 to publish the last DNA sequences.<sup>6</sup>

In contrast, next-generation sequencing technologies that were developed in these last years have allowed sequencing for only a few thousand dollars. Moreover, they require less manpower for sample preparation. More recently, an even more revolutionary technique developed by Oxford Nanopore Technology, called "third-generation sequencing", allows sequencing on the field with a simple laptop and a portable sequencing device.<sup>7</sup>

This short introduction shows how the perception of DNA has changed over the last decades.

An up-and-coming field is currently developing around environmental DNA (eDNA). "eDNA refers to the total pool of DNA isolated from the environment and is composed of both organismal (whole individuals that were probably alive at the time of sampling) and extraorganismal DNA (material shed from organisms, or biologically active propagules)."<sup>8</sup> For example, *Nature* recently published a study using eDNA to uncover the Arctic biological community from two million years ago that consisted of open boreal forests, reindeer, and geese, all of which were previously not detected by macrofossil analysis.<sup>9</sup>

In this Maturitätsarbeit paper, I immersed myself in the world of biotechnology by extracting eDNA to characterize biome interactions in my local environment. The availability of technologies around eDNA will rise exponentially in the following years, allowing cost-efficient experiments to characterize with non-invasive methods water ecosystems. In this paper, I aimed to familiarize myself with currently available eDNA technologies in the field. This Maturitätsarbeit is considered a case study to prove that a high-school student can nowadays manipulate and experiment with eDNA with minimal technical support.

## 4. EXPLANATION/JUSTIFICATION OF EXPERIMENTAL DESIGN

This paper used three different eDNA extraction methods and two different qPCR experimental designs (including a preliminary experiment) to answer its research question. The filtering step was inherent to both methods.

The purpose of having these different methods was to allow their respective comparison afterward in terms of cost-efficiency, accuracy, and time. Furthermore, the research question was answered using several positive controls.

## 5. DESIGN

### 5.1. Aim of the investigation and research question

On a global level, the geographical invasion and the impact of both invasive populations of *D. villosus* and *D. polymorpha* in freshwater ecosystems have been primarily investigated on their respective prey communities. Therefore, one motivation for this Maturitätsarbeit was to investigate the relationship between both invasive populations. Indeed, in the future, with the intensification of climate change and the increase of global water temperatures, Swiss freshwater bodies will become more and more suitable and exposed to foreign and exotics species; their invasion by alien species will become more frequent. It is hence of great importance to investigate the impact of such populations on each other: Does their presence cause a mutual population increase or decrease? How threatening could their increased invasion be to the ecosystem biodiversity?

*D. polymorpha* colonized the Lake of Constance in the mid-1960s, while *D. villosus* macroinvertebrates were first recorded in 2002 there.<sup>10</sup> In 2009, and hence with the advanced invasion of the lake by both alien species, the physical attachment of *D. polymorpha* and *D. villosus* there was observed in the research paper “*Dreissena polymorpha* in Lake Constance: An example of a keystone engineer?”<sup>11</sup> (2009) by R. Gergs.

The physical attachment of both species was also observed in the paper “Bathymetric expansion of an invasive gammarid (*Dikerogammarus villosus*, Crustacea, Amphipoda) in Lake Léman.”<sup>12</sup> (2006) by B. Lods-Crozet in Lake Léman.

*D. polymorpha* colonized the Lake of Zurich and the river Limmat in 1969,<sup>13</sup> while *D. villosus* macroinvertebrates were first recorded in 2006 there.<sup>14</sup> This invasion time lag of about seven years compared to the Lake of Constance could reflect the geographical distribution evolution of both species that entered the Zurich area through shipping activity on the Rhine and the Limmat. By 2007, no statistically significant correlation was found between both species in the Limmat or the Lake of Zurich, as seen in the research paper “*Dikerogammarus*- Monitoring im Zürichsee und in der Limmat/Bestandesmonitoring 2007”<sup>15</sup> (2007) performed by the *Kanton Zürich Baudirektion Amt für Abfall, Wasser, Energie und Luft (AWEL)* using direct sampling of the

organisms to count them. This may have been due to the uncompleted establishment and adaptation of the species in the lake at that time.

In this Maturitätsarbeit, the author proposes to use eDNA as an alternative readout to assess this correlation. This sampling mode is less invasive for the environment and offers the opportunity to sample at more extensive times and locations. Fifteen years after the mentioned statistically insignificant study by AWEL, this Maturitätsarbeit aimed to investigate whether, in 2022, a correlation can be established between the spatial distribution of the invasive alien species *Dreissena polymorpha* and *Dikerogammarus villosus* in the Limmat and the Lake of Zurich. Hence the following research question was chosen:

Is there a correlation between the spatial distribution of the invasive freshwater mussel species *Dreissena polymorpha* and the invasive amphipod crustacean *Dikerogammarus villosus*, as measured by the amount of eDNA of both species detected by qPCR from four sampling sites distributed along the river Limmat and the Lake of Zurich?

## 5.2. Background information

### 5.2.1. *Dikerogammarus villosus* (see Figure 1)<sup>16</sup>

The following paragraph refers to the paper “The killer shrimp *Dikerogammarus villosus* (Crustacea, Amphipoda) invades Lithuanian waters, South-Eastern Baltic Sea.”<sup>17</sup> (2016).



**Figure 1:** Picture of a *D. villosus* organism

*Dikerogammarus villosus*, the so-called *killer shrimp*, is “an amphipod originating from the Ponto-Caspian region.” The crustacean began its geographical expansion “in the 20<sup>th</sup> century after the re-opening of the Rhine-Main-Danube canal.” Known as an alien species, *D. villosus* has become invasive to most rivers in Europe, disturbing or even destroying local ecosystems; In recent years,

the amphipod has thus become a severe threat to fluvial biodiversity. The extent of the killer shrimp’s impact on Western ecosystems can be explained by numerous factors, such as “its large body, high fecundity, and wide environmental tolerance.”

The following paragraph refers to the paper “The profile of a ‘perfect’ invader – the case of killer shrimp”<sup>18</sup> (2014) by T. Rewicz.

Owning the same trophic level as a fish, this crustacean is an omnivorous species, feeding on many organisms such as aquatic bugs, fish eggs, and microalgae. This ability of *D. villosus* to feed on numerous nutritional sources is an evolutionary advantage allowing better survival chances than other macroinvertebrates. The extinction of the mentioned species is catalyzed by the fact that *D. villosus* does not necessarily consume its prey after killing them. Additionally, as the prey communities are about the same size as *D. villosus*, they cannot penetrate and hide safely in small refugia to escape predators.



5.2.2. *Dreissena polymorpha* (see Figure 2)<sup>19</sup>



Figure 2: Picture of two *Dreissina polymorpha* organisms showing different shell appearances

The following paragraph refers to the paper “*Dreissena polymorpha* (zebra mussel)”<sup>20</sup> (2008) by CABI International.

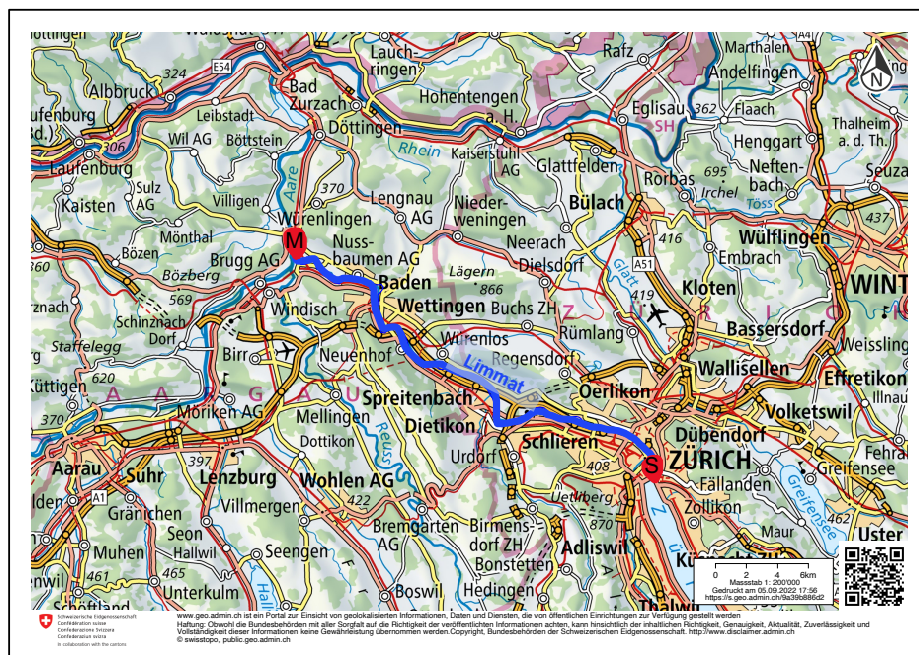
*Dreissena polymorpha*, also known as the “zebra mussel”, is a freshwater invader which is native to the Ponto-Caspian drainage basin. The word *polymorpha* refers to the wide range in appearance of the mussel, as seen in Figure 2. Its geographical

expansion and invasion of Western fluvial ecosystems began 200 years ago and is still ongoing. The extent of the zebra mussel’s impact on Western ecosystems can be explained by numerous factors, such as its tolerance to starvation (and hence oligotrophic/eutrophic systems), high range in fluvial temperature, and broad environmental conditions. The mussel feeds on microscopic plankton organisms and biomass particles. Additionally, needing a substrate to attach to, numerous other mussel species have become threatened as they lose their ability to breathe.

Additionally to my motivations in 5.1, both species were chosen as they are abundant invertebrates in the Limmat and Lake of Zurich, allowing their optimal detectability by qPCR.<sup>21</sup>

5.2.3. Limmat and Lake of Zurich

For this research paper, the Lake of Zurich and the river Limmat (Map 1),<sup>22</sup> located near Zurich, the largest Swiss economic and population center, were chosen as a case study.



Map 1: Area around the Swiss city of Zurich with the Limmat transect in blue with its river mouth (M) and source (S) labelled in red

The source (S) of the Limmat is situated at the Lake of Zurich, its mouth (M) at the confluence with the river Aare near Brugg AG.

### 5.3. Hypothesis & Explanation

The study hypothesis is that there is a positive correlation between the spatial distribution of *D. polymorpha* and *D. villosus*. The killer shrimp feeds on the feces from *Dreissena polymorpha*, and, additionally, colonies of *D. polymorpha* accumulate biomass, the food base of the chironomid larvae, a highly calorific organism that promotes *D. villosus* growth.<sup>23</sup> It is therefore assumed that a high concentration of *Dreissena polymorpha* eDNA leads to a high *Dikerogammarus villosus* eDNA concentration.

### 5.4. Measurements

qPCR, or **quantitative polymerase chain reaction**, is a technique for the “real-time detection of PCR products, enabled by the inclusion of a fluorescent reporter molecule in each reaction well that yields increased fluorescence with an increasing amount of product DNA.”<sup>24</sup>

Of note, “the  $C_q$  value is the cycle number at which the sample’s reaction curve [of fluorescence] intersects the threshold line and is detected, whereas the threshold line is the point where samples reach a fluorescence above the background level.”<sup>25</sup> If little target DNA concentration is present in a sample, it will need more amplification cycles to be detected by qPCR and have a higher  $C_q$  value. Hence the number of amplification cycles needed for the eDNA of the organisms to be detected with qPCR is a quantitative approach to the original eDNA yield in the samples.

- **$C_q$  value** determined for *Dreissena polymorpha* eDNA by quantitative PCR (qPCR) at four Limmat/Lake of Zurich sites [uncertainty of measurement: +/- 0.01]
- **$C_q$  value** determined for *Dikerogammarus villosus* eDNA by qPCR at four Limmat/Lake of Zurich sites [uncertainty of measurement: +/- 0.01]

### 5.5. Controls

#### 5.5.1. Sampling sites, method, and replicates<sup>26</sup>

A homogenized and systematic collection scheme was established at each site, and three repeated measurements were operated to obtain reliable primary data. During water sampling, gentle steps were made not to whirl the sediments deposited on the bed as they would have blocked the filters. Additionally, sampling was performed in the counter-direction of the stream for the sampler (and his/her DNA) not to contact the water sampled.

#### 5.5.2. Sterility during sampling, filtration, extraction, dilution, and qPCRs

Sterile material was used during the experiments. To reduce DNA and bacterial contamination risk, all samples were handled with sterile forceps. Mask and gloves were changed frequently to prevent human DNA from skin or saliva from mixing with sample eDNA. Scalpels and pipette tips were changed after each manipulation to avoid sample cross-contamination.

#### 5.5.3. Homogeneity in temperature, humidity, pressure, and material (size)

All experiments were performed in the same laboratory to ensure homogeneity in the controlled variables temperature, humidity, and pressure. The experimental design used the same

centrifuge, vortex, Thermomixer, and TissueLyser II machines to assure homogeneity. The chemical solutions originated from ordered kits (except for the ethanol and the ultrapure water that came from the laboratory) to assure chemical purity and data reliability.

#### 5.5.4. Water filtering, eDNA extraction, and qPCRs

Filter membranes were cut in four to be used in independent experiments. It was assumed that there was an equal amount of eDNA on each filter quarter. Although DNA distribution could not be homogeneous on the filter (meaning that some species might have been more abundant in some fourths but not others), it was assumed that the random assignments of all 48 technical replicates to each extraction method averaged it, assuring data consistency. Three different extraction methods (two commercial kits and an adaptation of a commercial kit) were used on the water samples to compare the results afterward. To increase data reliability, four additional replicates per filter fourth were performed for qPCR.

#### 5.5.5. Positive and negative controls

Negative controls were included for each primer and consisted of ultrapure water. Melting curves during qPCRs were analyzed to ensure that the eDNA detected did not result from primer self-amplification.<sup>27</sup> In addition, ultrapure water underwent the entire procedure on two independent filters to assert the absence of eDNA in the filters, tubes, and solutions. Positive controls for the presence of eDNA were performed with *E. coli* as it is one of the most common species in freshwater systems<sup>28</sup> and was expected to be present in most samples.

## 6. METHOD: WATER SAMPLING

### 6.1. Sampling conditions



Figure 3: Weather conditions on collection day

Primary data collection was performed on the 11<sup>th</sup> of September 2022 from 2 pm to 4 pm on a sunny day (20.2 to 22.0°C)<sup>29</sup> with no precipitation (see **Figure 3**).<sup>30</sup> The water samples were directly brought from the field to the laboratory for filtering. The short sampling time of two hours allowed for minimizing the contamination risk of the samples.

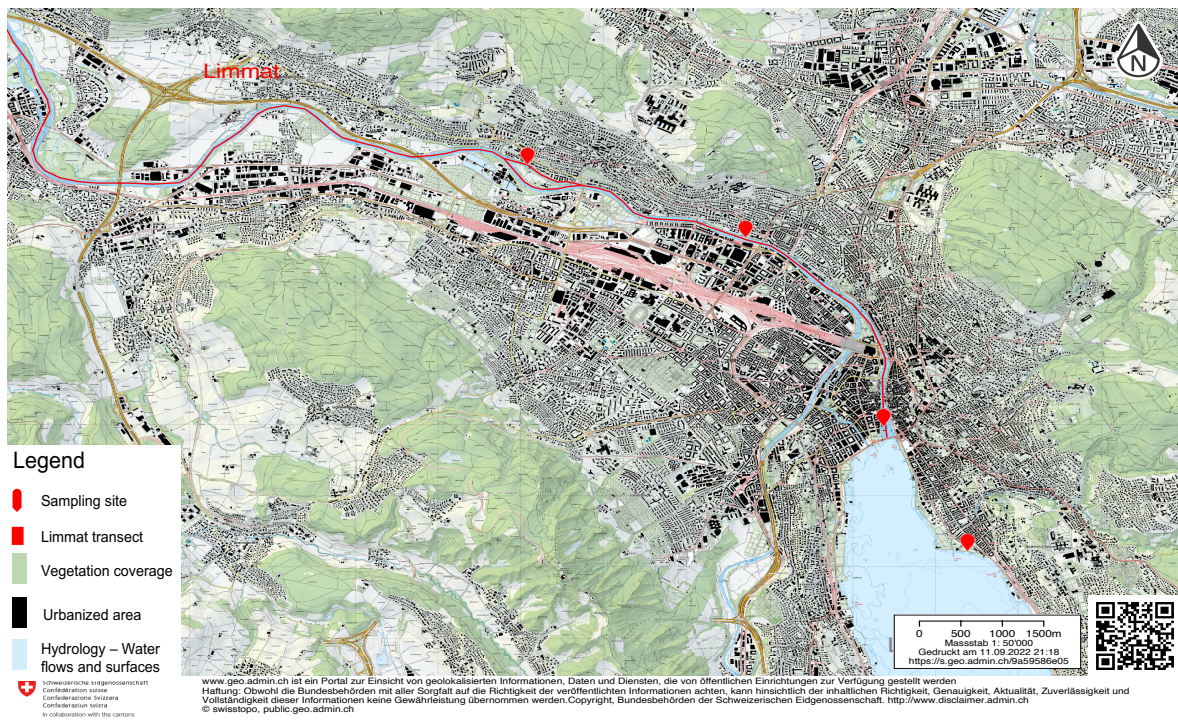
### 6.2. Sampling sites and replicates

Three sites along the Limmat and one on the Lake of Zurich were selected to collect primary data. The sampling sites were located near or in public bathing areas. The reason for this hydrologic site characteristic is that the high discharge of the Limmat does not allow systematic sampling along its banks; hence, convenience sampling was favored for safety and accessibility reasons.

### 6.3. Site locations

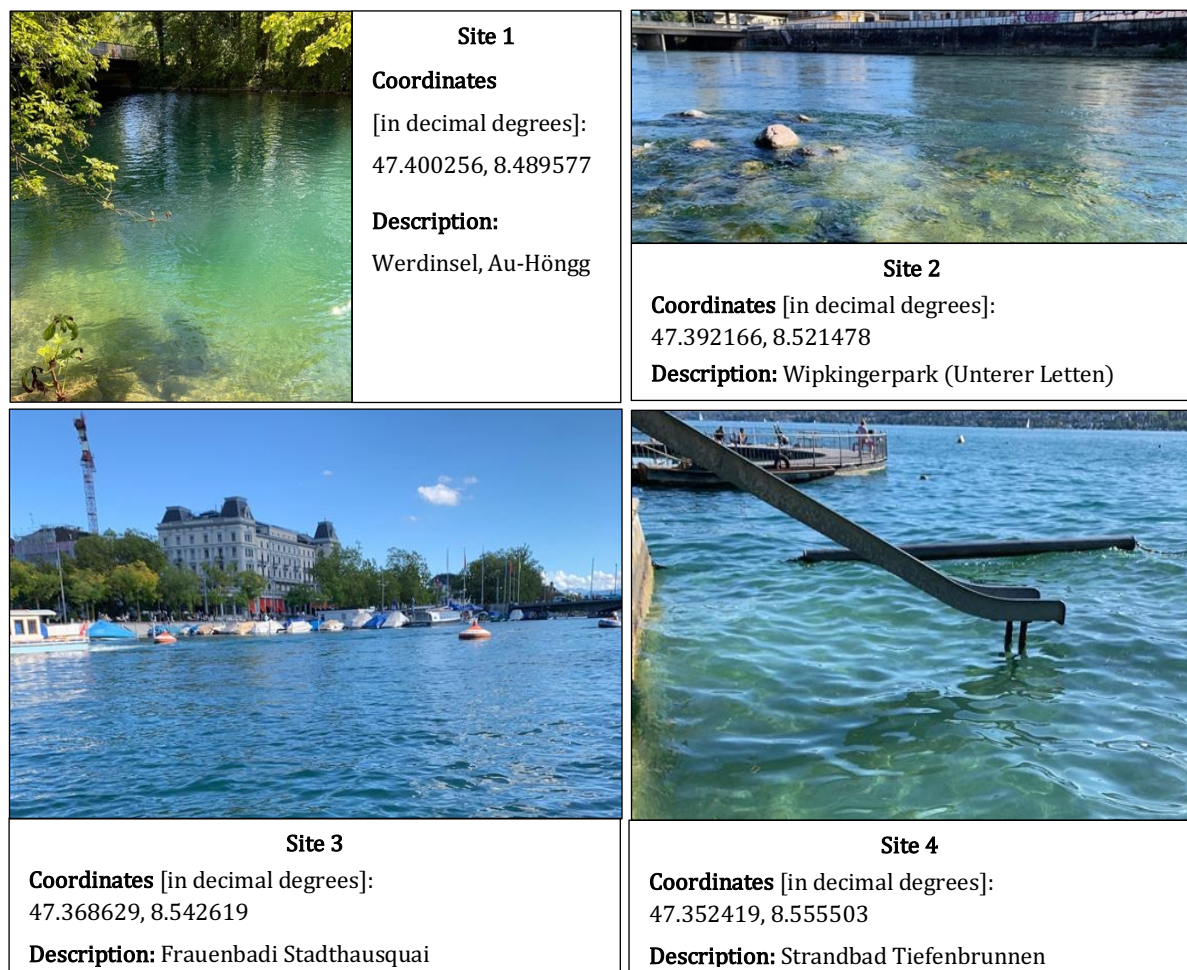
The sampling sites selected are shown on **Map 2**<sup>31</sup> with red dots. The Limmat transect is highlighted in red. Although convenience sampling was used, sites were about equidistant.





**Map 2:** Limmat transect highlighted in red with four associated sampling sites labeled in red

Figure 4<sup>32</sup> summarizes the site characteristics.



**Figure 4:** Sampling site photographs with associated coordinates and location descriptions



## 6.4. Materials

- 1 waterproof pencil
  - 1 refrigerated bag (40-l capacity)
  - 14 disposable scalpels (Swann-Morton®)
  - 14 radio-sterilized Stericup® Quick Release (Millipore Express® PLUS 0.22µm, 500-ml)
  - 2000ml of ultrapure water (Thermo Scientific)
  - 1 test tube rack (5x16, for 2-ml tubes)
- 56 DNA 2ml LoBind Tube (Eppendorf)
  - 1 box of nitrile gloves
  - 1 lab coat
  - 1 box of face masks
  - 1 write-on label tape
  - 1 vacuum pump
  - 1 SHARPSAFE container
  - 14 glass bottles (1000-ml capacity)

## 6.5. Method: Field sampling (see Figure 5)<sup>33</sup>

1. Put on a pair of nitrile gloves and locate the sampling site. Fill a glass bottle with 1000ml of water in a stream-upward direction.
2. Place the cap on the bottle top and label it with a piece of tape and pencil (site, replicate).
3. Redo steps 1. to 3. for the second and third replicates. Put all bottles in a refrigerated bag.
4. Move on to the second, third, and fourth sites and redo steps 1. to 3.



Figure 5: Bottles of fluvial water in refrigerated bag

### 6.5.1. Negative control

1. Fill two glass bottles with 1000ml of ultrapure water. Place the caps on top of the bottles and label them as “negative control 1” and “negative control 2” with tape and a waterproof pencil.

## 6.6. Method: Vacuum Filtering<sup>34</sup>

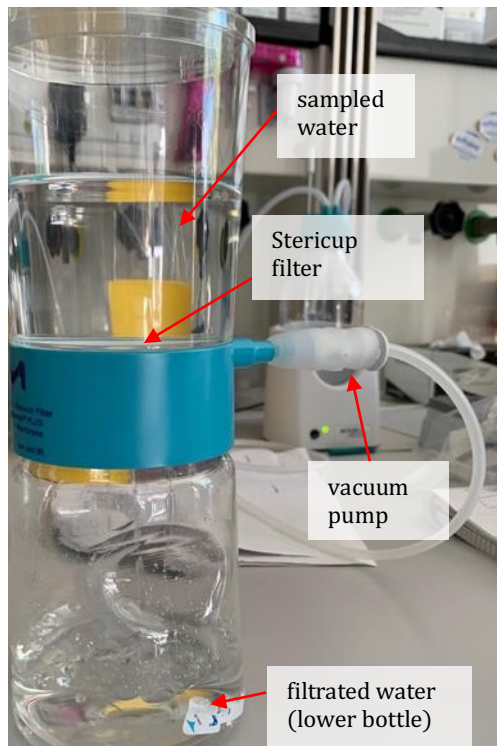
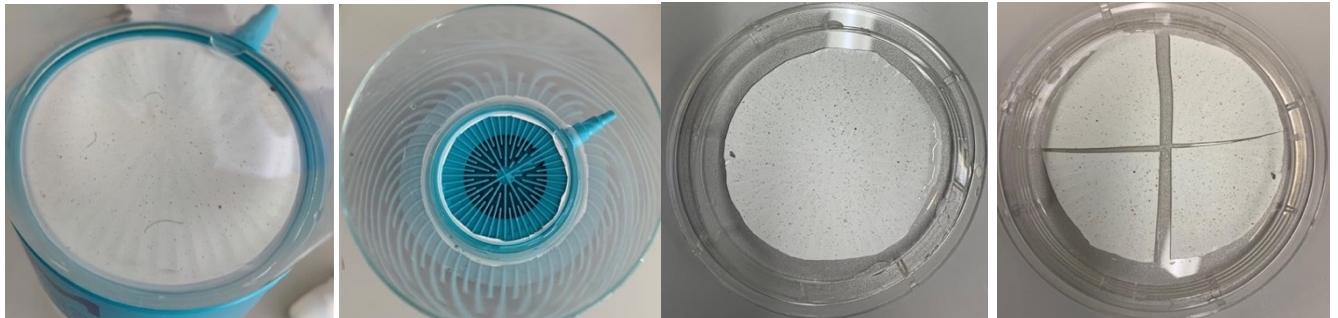


Figure 6: Photograph of filtration system set-up

1. To limit sample contamination, put on nitrile gloves, a lab coat, and a face mask.
2. Take a Stericup® Quick Release with the gloves and remove the cap. Fill the Stericup® reservoir with 500ml of the water bottle labeled “site 1, replicate 1”.
3. Put the cap on the reservoir. Next, attach the vacuum pump to Stericup® and activate it (Figure 6).<sup>35</sup>
4. Stop the vacuum pump when the water level is above the filter. Discard the lower water from the lower bottle in a sink.
5. Refill the reservoir with the rest of the water bottle and redo steps 2. to 4.
6. Stop the vacuum pump a few seconds after all water is filtered through (when the filter paper changes color and becomes dry).

7. Remove the Stericup® cap and use a scalpel to cut out the filter (see **Figure 7**).<sup>36</sup>
8. Put the filter on the Stericup® cap. Then, use a scalpel to cut the filter into four (see **Figure 8**).<sup>37</sup>



**Figure 7:** Attached filter to the Stericup® (left) that was cut-off with a scalpel (right)      **Figure 8:** Cut-off filter paper on the Stericup® cap (left) that was cut in four with a scalpel (right)

9. Using a pair of tweezers and the scalpel, fold each of the four paper pieces with the upper surface on the outside to ease contact with the liquid during DNA extraction.
10. Put all papers in individual DNA LoBind Tubes and close them.
11. Label the DNA LoBind Tubes accordingly with the following coding system (site)-(replicate)-(technical replicate) (see **Figure 9**).<sup>38</sup>
12. Put the DNA LoBind Tubes on the tube rack. Place the rack in a freezer at a temperature of -20°C.
13. Discard the scalpel in the SHARPSAFE container and take a new one.
14. Put the pair of tweezers in an autoclaving machine for disinfection.
15. Redo steps 2. to 14. twice for the second and third replicate of site 1.



**Figure 9:** Photographs of the DNA LoBind Tubes containing the filter papers (left) on the tube rack (right)

16. Redo steps 1. to 15. three times for Sites 2, 3, and 4 and each associated technical replicate.

### 6.6.1. Negative control

1. Redo **6.6** (without step 16) twice for both negative control bottles and each associated technical replicate.
2. Label all 8 DNA negative control LoBind Tubes as NC 1, NC 2, ... NC 8 (=Negative Control).



## 7. METHOD: eDNA EXTRACTION AND QUANTIFICATION

The extraction kit for Method 1 was chosen accordingly to the one used in the research paper “Freshwater monitoring by nanopore sequencing”<sup>39</sup> (2021) by L. Urban *et al.* After online research, the extraction kit for Method 2 was chosen for its high sensitivity to low DNA concentrations.<sup>40</sup>

### 7.1. Method 1: Materials

- |   |  |
|---|--|
| <ul style="list-style-type: none"> <li>- 1 DNeasy® Powerwater® Kit (Qiagen)</li> <li>- 2 pairs of tweezers</li> <li>- 1 centrifuge machine for Eppendorf tubes (Eppendorf, 2-ml tube capacity)</li> <li>- 1 Vortex Genie 2™ machine (INOTECH)</li> <li>- 100 pipette tips (1000-µl capacity)</li> </ul> | <ul style="list-style-type: none"> <li>- 1 pipette (Eppendorf, 100 to 1000-µl capacity, +/- 1 µl)</li> <li>- 1 tube rack (2-ml tube capacity)</li> <li>- 1 box of nitrile gloves</li> <li>- 1 lab coat</li> <li>- 1 pair of safety goggles</li> <li>- 1 waterproof pencil</li> </ul> |
|---|--|

Of note, the DNeasy® Powerwater® Kit contains the following:<sup>41</sup>

- |   |  |
|---|--|
| <ul style="list-style-type: none"> <li>- MB Spin Columns (50)</li> <li>- PowerWater Bead Pro Tubes (50)</li> <li>- Solution PW1 (55ml)</li> <li>- Solution IRS (15ml)</li> <li>- Solution PW3 (2x18ml)</li> </ul> | <ul style="list-style-type: none"> <li>- Solution PW4 (2x24ml)</li> <li>- Ethanol (2x30ml)</li> <li>- Solution EB (9ml)</li> <li>- Collection Tubes (2-ml capacity, 5x50)</li> </ul> |
|---|--|

### 7.2. Method 1: DNeasy® Powerwater® Kit

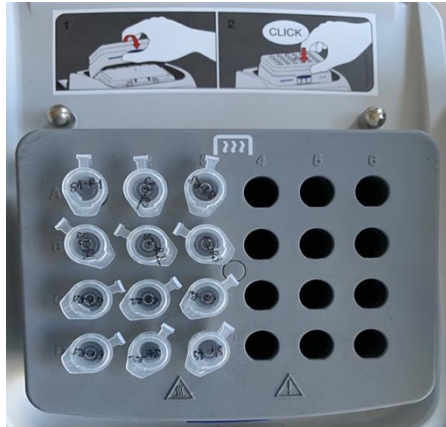
This method follows the manufacturer’s instructions from the DNeasy® Powerwater® Kit. This protocol can be found on the website under **Resources, Kit Handbooks**:

<https://www.qiagen.com/ko-ch/products/discoveryand-translationalresearch/dnarnapurification/dna-purification/microbial-dna/dneasy-powerwater-kit/?catno=14900-50-NF>

The following points were considered before each DNA extraction:

- For this method, only one technical replicate from each site replicate is needed (total: 12 samples, labeled 1-1-1, 1-2-1, 1-3-1, 2-1-1, 2-2-1, 2-3-1, 3-1-1, ...)
  - For each manipulation with the pipette, change the pipette tip to avoid sample contamination. (Important: Always hold the pipette tip towards the ground.)
  - Label them accordingly (S1, R1/S1, R2) for collection tube transfer to prevent mix-ups.
  - After centrifuging steps, do not wiggle the samples so the pellet remains on the bottom.
  - During centrifuging steps, mind distributing the samples evenly across the template.
1. Plug in the Thermomixer machine and put it to 4°C.
  2. Take the DNA LoBind Tubes (including two negative controls (NC 1; NC 5)) out of the freezer.
  3. Use two tweezers to unfold the filter paper partially and expose more filter surface. Then, put the filter in a collection tube.
  4. Follow the protocol above. When finished, store the samples in the freezer at –20°C.

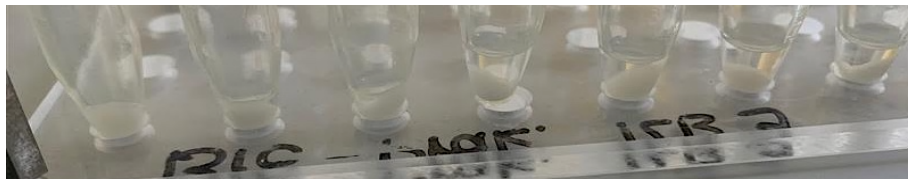
**Figures**<sup>42</sup> 10, 11, and 12 illustrate the different steps of the Experienced User Protocol:



**Figure 10:** Step 11: Incubation of the samples in the Thermomixer machine



**Figure 11:** Step 22: Centrifuge



**Figure 12:** Step 13: Leftover pellet after supernatant removal

### 7.3. Method 1 : DNA quantification using a fluorometer<sup>43</sup>

- |   |   |
|---|---|
| <ul style="list-style-type: none"> <li>- 1 Qubit™ dsDNA HS Assay Kit (Thermo Scientific)</li> <li>- 1 Qubit™ Fluorometer machine (Thermo Scientific)</li> <li>- 1 Vortex Genie 2™ machine (INOTECH)</li> <li>- 30 Qubit™ Assay Tubes (2-ml capacity)</li> <li>- 15 pipette tips (500-<math>\mu</math>l capacity)</li> <li>- 15 pipette tips (100-<math>\mu</math>l capacity)</li> <li>- 1 pipette (Eppendorf, 5 to 100-<math>\mu</math>l capacity, +/- 0.1<math>\mu</math>l)</li> </ul> | <ul style="list-style-type: none"> <li>- 1 pipette (Eppendorf, 100 to 1000-<math>\mu</math>l capacity, +/- 1 <math>\mu</math>l)</li> <li>- 5<math>\mu</math>l of Solution EB (Qiagen, elution buffer) from 7.1 for negative control</li> <li>- 1 tube rack (2-ml tube capacity)</li> <li>- 1 box of nitrile gloves</li> <li>- 1 lab coat</li> <li>- 1 pair of safety goggles</li> </ul> |
|---|---|

Of note, the Qubit™ dsDNA HS Assay Kit contains the following:<sup>44</sup>

- |   |  |
|---|--|
| <ul style="list-style-type: none"> <li>- Qubit ssDNA Reagent (250 <math>\mu</math>l)</li> <li>- Qubit ssDNA Buffer (50 ml)</li> </ul> | <ul style="list-style-type: none"> <li>- Qubit ssDNA Standard #1 (1 ml)</li> <li>- Qubit ssDNA Standard #1 (2 ml)</li> </ul> |
|---|--|

1. Take the samples from 7.2 out of the refrigerator.
2. With the help of the 5 to 100- $\mu$ l pipette, transfer 5 $\mu$ l of the samples into new Qubit™ Assay Tubes. Then, put the samples back in the freezer.
3. With the help of the 5 to 100- $\mu$ l pipette, transfer 5 $\mu$ l of the NC1/NC5 into a new Qubit™ Assay Tube for negative control. From then on, follow the protocol as seen below.

Of note, this method follows the manufactural steps of the protocol associated with the Qubit™ dsDNA HS Assay Kit using fluorescence to quantify eDNA. This protocol can be found on the website under resources, Kit Handbooks:

[https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FELSG%2Fmanuals%2FQubit\\_dsDNA\\_HS\\_Assay\\_UG.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FELSG%2Fmanuals%2FQubit_dsDNA_HS_Assay_UG.pdf)

## 7.4. Method 2: Materials

- |  |   |
|--|---|
| <ul style="list-style-type: none"> <li>- 1 ZymoBIOMICS™ DNA/RNA Miniprep Kit (Zymo)</li> <li>- 2 pairs of tweezers</li> <li>- 1 centrifuge machine for Eppendorf tubes (Eppendorf, 2-ml tube capacity)</li> <li>- 1 Vortex Genie 2™ machine (INOTECH)</li> <li>- 200 pipette tips (1000-µl capacity)</li> <li>- 1 pipette (Eppendorf, 100 to 1000-µl capacity, +/- 1µl)</li> </ul> | <ul style="list-style-type: none"> <li>- 200ml of ethanol (100%)</li> <li>- 100 2ml DNA LoBind Tube (Eppendorf)</li> <li>- 1 TissueLyser II machine (Retsch)</li> <li>- 2 tube rack (2-ml tube capacity)</li> <li>- 1 box of nitrile gloves</li> <li>- 1 lab coat</li> <li>- 1 pair of safety goggles</li> <li>- 1 waterproof pencil</li> </ul> |
|--|---|

Of note, the ZymoBIOMICS™ DNA/RNA Miniprep Kit contains the following:<sup>45</sup>

- |   |  |
|---|--|
| <ul style="list-style-type: none"> <li>- ZR BashingBead Lysis Tubes (50)</li> <li>- DNA/RNA Shield (50ml)</li> <li>- DNA/RNA Lysis Buffer (50ml)</li> <li>- DNA/RNA Prep Buffer (50ml)</li> <li>- DNA/RNA Wash Buffer (24x2ml)</li> <li>- ZymoBIOMICS HRC Prep Solution (30x3ml)</li> </ul> | <ul style="list-style-type: none"> <li>- ZymoBIOMICS HRC DNase/RNase-Free Water (30ml)</li> <li>- Zymo-Spin III-HRC Filters (100)</li> <li>- Zymo-Spin IIICG Columns (50)</li> <li>- Spin-Away Filters (50)</li> <li>- Collection Tubes (300)</li> </ul> |
|---|--|

## 7.5. Method 2: Zymobiomics™ DNA/RNA Miniprep Kit<sup>46</sup>

1. Take the filter membrane samples (inclusive negative control membranes NC2/NC6) out of the freezer at -20°C.
2. With the help of the tweezers, gently pull out the filter samples from their respective collection tube and transfer each of them directly into one ZR BashingBead Lysis Tube.
3. From then on, follow the protocol as seen below.

This method follows the manufactural steps of the protocol associated with the ZymoBIOMICS™ DNA/RNA Miniprep Kit. This protocol can be found on the website under resources, Kit Handbooks:

[https://files.zymoresearch.com/protocols/ r2002\\_zymobiomics\\_dna-rna\\_miniprep\\_kit.pdf](https://files.zymoresearch.com/protocols/ r2002_zymobiomics_dna-rna_miniprep_kit.pdf)

## 7.6. Method 2: DNA quantification using a fluorometer

Follow the protocol in 7.3.

## 7.7. Method 3: Modified DNA Easy® PowerWater® Kit

For the third extraction method, the protocol from the first extraction method was followed; however, using a TissueLyser II machine (Retsch) after incubating the filter paper in the first solution for DNA lysis instead of vortexing. Lysis tubes were taken from the ZymoBIOMICS™ DNA/RNA Miniprep Kit. Indeed, seeing that more DNA was extracted with Method 2 than 1, although many more tube transfers and elutions took place, potentially leading to eDNA loss, using the TissueLyser II machine was considered the decisive step for increased lysis of the samples and increased DNA extraction (see **Table 15**). The same modification was made in the paper “Freshwater monitoring by nanopore sequencing”<sup>47</sup> (2021) by L. Urban *et al.*

## 7.8. Method 3: Materials

- |  |  |
|--|--|
| <ul style="list-style-type: none"> <li>- 1 DNeasy® Powerwater® Kit (Qiagen)</li> <li>- 14 ZR BashingBead Lysis Tubes (Zymo)</li> <li>- 2 pairs of tweezers</li> <li>- 1 centrifuge machine for Eppendorf tubes (Eppendorf, 2-ml tube capacity)</li> <li>- 1 Thermomixer machine (Eppendorf, 2-ml tube capacity)</li> <li>- 1 Vortex Genie 2™ machine (INOTECH)</li> <li>- 1 TissueLyser II machine (Retsch)</li> </ul> | <ul style="list-style-type: none"> <li>- 100 pipette tips (100-µl capacity)</li> <li>- 1 eppendorf pipette (100 to 1000-µl capacity, +/- 1µl)</li> <li>- 1 tube rack (2-ml tube capacity)</li> <li>- 1 box of nitrile gloves</li> <li>- 1 lab coat</li> <li>- 1 pair of safety goggles</li> <li>- 1 waterproof pencil</li> </ul> |
|--|--|

1. Take the filter membrane samples (inclusive negative controls NC3/NC7) out of the refrigerator.
2. With the help of the tweezers, gently pull out the filter samples from their respective collection tube, unfold them, cut them into little pieces for increased liquid exposure, and transfer each of them directly into a ZR BashingBead Lysis Tube.
3. With the help of the pipette, transfer 1 ml of the solution PW1 to each tube.
4. Put the tubes in the TissueLyser II machine for 5 minutes.
5. Follow the manufacturer instructions from step 8 on from the Experienced User Protocol:

<https://www.qiagen.com/ko-ch/products/discoveryand-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerwater-kit/?catno=14900-50-NF>

## 7.9. Method 3: DNA quantification using a fluorometer

Follow the protocol in 7.3.

## 8. METHOD: qPCR

The samples were diluted to the same eDNA concentration before performing the qPCRs.<sup>48</sup>

### 8.1. Dilution (see Table 1)<sup>49</sup>

#### 8.1.1. Materials

- |   |  |
|---|--|
| <ul style="list-style-type: none"> <li>- 100ml of ultrapure water (Thermo Scientific)</li> <li>- 1 PCR strip tube plate (12x8 for 0.2 ml tubes)</li> <li>- 1 PCR tube rack (12x8 for 0.2 ml tubes)</li> <li>- 1 aluminum PCR sealing foil</li> <li>- 1 Vortex Genie 2™ machine (INOTECH)</li> <li>- 50 pipette tips (20-µl capacity)</li> </ul> | <ul style="list-style-type: none"> <li>- 1 pipette (Eppendorf, 2 to 20-µl capacity, +/- 0.5µl)</li> <li>- 1 box of nitrile gloves</li> <li>- 1 lab coat</li> <li>- 1 pair of safety goggles</li> </ul> |
|---|--|

#### 8.1.2. Method

All concentrations (see 7.3, 7.6, and 7.9) were diluted to the lowest concentration present (7.8 ng/ml, Extraction Method 1). However, a compromise was found not to dilute too much the more concentrated samples and compromise qPCR accuracy. Hence samples from Method 1 were not used and were kept as technical replicates for further procedure. The lowest eDNA concentration in Extractions 2 and 3 was 15.2ng/ml. The following formula was used:<sup>50</sup>

$$C_1 \cdot V_1 = C_2 \cdot V_2$$

$C_1$ , or Concentration 1, is the DNA concentration recorded by the Qubit® 2.0 Fluorometer. Mind that the unit [ng/ml] is equal to [pg/μl] (see 7.3, 7.6, and 7.9 and Tables 12, 13, and 14).

$C_2$ , or Concentration 2, is the wanted DNA concentration for the qPCR chain reaction. The value of 15.0ng/μl was chosen accordingly to the lowest  $C_1$  present.

$V_2$ , or Volume 2, is the required DNA sample volume for performing all qPCRs assays. It was chosen at 30.0μl. Considering that the qPCRs were performed with 2μl sample solution and that a single sample was tested for three primers four times (technical qPCR replicates),  $2\mu\text{l} \cdot 3 \cdot 4 = 24\mu\text{l}$  of the solution was needed, which was guaranteed by this initial volume of 30.0μl.

$V_1$ , highlighted in violet, or Volume 1, is the calculated sample volume needed from the DNA sample resulting from the extraction step. Of note, the DNA fixed on the *Spin Columns* was eluted using 100μl of the *buffer*. However, 5μl was needed for each sample for fluorescent analysis. Hence  $100\mu\text{l} - 5\mu\text{l} = 95\mu\text{l}$  of the sample solution was left over. The obtained  $V_1$ -values (range: 10.6 to 29.0μl) seemed appropriate as they represented  $\frac{1}{8}$  to  $\frac{1}{3}$  of the 95μl, and hence enough remained in case of experimental errors.

$V_3$ , highlighted in red, or Volume 3, represents the calculated volume of ultrapure water added to the samples for equivalent dilution.

**Table 1:** Summary of sample dilutions using ultrapure water performed for qPCRs

Site/Replicate and Extraction Method		initial DNA sample concentration	diluted DNA concentration needed for qPCR	final sample volume needed for qPCR	calculated volume of sample DNA for qPCR	calculated volume of ultrapure water for dilution
		$C_1$ [+/- 0.1pg/μl]	$C_2$ [+/- 0.1pg/μl]	$V_2$ [+/- 0.1μl]	$V_1 = \frac{C_2 \cdot V_2}{C_1}$ [+/- 0.1μl]	$V_3 = V_2 - V_1$ [+/- 0.1μl]
Extraction Method 2	S1, R1	16.1	15.0	30.0	28.0	2.0
	S1, R2	19.5	15.0	30.0	23.1	6.9
	S1, R3	15.2	15.0	30.0	29.6	0.4
	S2, R1	18.1	15.0	30.0	24.9	5.1
	S2, R2	23.9	15.0	30.0	18.8	11.2
	S2, R3	18.5	15.0	30.0	24.3	5.7
	S3, R1	20.8	15.0	30.0	21.6	8.4
	S3, R2	32.3	15.0	30.0	13.9	16.1
	S3, R3	27.2	15.0	30.0	16.5	13.5
	S4, R1	26.0	15.0	30.0	17.3	12.7
	S4, R2	19.1	15.0	30.0	23.6	6.4
	S4, R3	22.9	15.0	30.0	19.7	10.3
Extraction Method 3	S1, R1	18.2	15.0	30.0	24.7	5.3
	S1, R2	19.5	15.0	30.0	23.1	6.9
	S1, R3	16.9	15.0	30.0	26.6	3.4
	S2, R1	15.5	15.0	30.0	29.0	1.0
	S2, R2	16.6	15.0	30.0	27.1	2.9
	S2, R3	19.5	15.0	30.0	23.1	6.9
	S3, R1	32.3	15.0	30.0	13.9	16.1
	S3, R2	35.6	15.0	30.0	12.6	17.4
	S3, R3	42.4	15.0	30.0	10.6	19.4
	S4, R1	40.0	15.0	30.0	11.3	18.8
	S4, R2	32.5	15.0	30.0	13.8	16.2
	S4, R3	28.4	15.0	30.0	15.8	14.2

1. Take the sample tubes from Extraction Method 2 and 3 from the refrigerator and let them for 20 minutes at room temperature, then vortex each tube for 5 seconds.
2. Put the PCR strip tube plate on the PCR tube rack.

- With the help of the pipette, transfer the ultrapure water volumes (according to each site and replicate) highlighted in red in **Table 1** in the PCR strip tube plate according to the PCR well plan in **Table 2**.<sup>51</sup>

**Table 2:** PCR wells plan for diluted eDNA samples for qPCR

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3
B	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3
C	NC 2	NC 6	UW	UW	NC 3	NC 7	UW	UW	UW	UW	UW	UW
D												
E												
F												
G												
H												

- Add 30 µl of ultrapure water in all cells in **Table 2** labeled UW (=ultrapure water).
- Add 14.2 µl of ultrapure water in all cells in **Table 2** labeled NC x (=negative controls).
- With the help of the pipette, transfer the DNA sample volumes (according to each site and replicate) highlighted in purple in **Table 1** in the PCR strip tube plate according to the PCR well plan in **Table 2** (Do not add any DNA in the cells labeled NC x!)
- Add 15.8 µl of each NC x sample volume in his according well in **Table 2**.
- Cover the PCR strip tube plate with aluminum foil and press on it for the wells to be completely closed.

## 8.2. Oligonucleotides resuspension<sup>52</sup>

The following section for oligonucleotide resuspension refers to the manufacturer information that can be found on the website <https://www.microsynth.com/hints-and-tips.html>.

The specific DNA oligonucleotides were ordered from Microsynth AG via the UZH laboratory. The specific primer base sequences and reasons for their selection are shown in the **Appendix**. As they were ordered in dried form, they had to be dissolved and resuspended.

### 8.2.1. Materials

- |   |  |
|---|--|
| <ul style="list-style-type: none"> <li>- forward and reverse primer for <i>D. polymorpha</i></li> <li>- forward and reverse primer for <i>D. villosus</i></li> <li>- forward and reverse primer for <i>E. coli</i> (positive control)</li> <li>- 100ml of ultrapure water</li> <li>- 1 Vortex Genie 2™ machine (INOTECH)</li> </ul> | <ul style="list-style-type: none"> <li>- 1 Thermomixer machine (Eppendorf, 2-ml tube capacity)</li> <li>- 1 centrifuge machine for Eppendorf tubes (Eppendorf, 2-ml tube capacity)</li> <li>- 3 pipette tips (100-µl capacity)</li> <li>- 1 pipette (Eppendorf, 100 to 1000-µl capacity, +/- 1µl)</li> </ul> |
|---|--|

### 8.2.2. Method

- Take the first forward primer for *D. polymorpha*. Put it into the centrifuge at 13'000 rpm for 1 minute.
- With the help of the pipette, transfer the amount of ultrapure water indicated on the sheet in the Appendix (for 100µM final concentration) to the primer tube.
- Put it in the Thermomixer machine at 65°C for 5 minutes. Vortex shortly.
- Repeat steps 1 to 3 for the reverse primer for *D. polymorpha* and the rest of the primers.



### 8.3. qPCR: Preliminary experiment

Of note, when performing this preliminary experiment for the first time, this author was guided and supervised during the procedure in the laboratory as needed to become more familiar with the experimental setup and procedure (for example, how to create an optimal PCR well plan). Furthermore, the supervisor went through each procedure step beforehand with the author and explained how the reagent volumes and concentrations were calculated. Nevertheless, for the other qPCRs, the author altered the parameters, recalculated all the concentrations and volumes without external help and supervision, and performed the procedure alone.

This section follows the manufacturer's instructions (SYBR Green qPCR Master Mix) on the following website:

[https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018825\\_PowerTrackSYBRGreenMasterMix\\_UG.pdf](https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018825_PowerTrackSYBRGreenMasterMix_UG.pdf)

In the UZH laboratory, this author was advised to use an adjusted protocol with increased volume and higher concentrations of DNA templates and primers, the same Master Mix volume, and, followingly, less water. The following reagent concentrations and volumes resulted (**Table 3**):<sup>53</sup>

**Table 3:** Indicated reagent concentrations and volumes for qPCR

Reagents	Volumes [+/- 0.1µl]
SYBR Master Mix (2x)	5.0
PCR Forward Primer [3µM]	0.5
PCR Reverse Primer [3µM]	0.5
DNA	2.0
ddH <sub>2</sub> O	2.0
Total	10.0

The primers were present in 100µM concentration (see **Appendix**) and hence had to be diluted to 3µM as seen in **Table 3**, using the same formula as in **8.1.2**. First, 15 µL of each forward/reverse primer per species were mixed to get a 50µM concentration and a sample volume of 30 µL. The ultrapure water volume needed for further dilution is displayed in **Table 4**.<sup>54</sup>

**Table 4:** Ultrapure water volume needed for dilution of the specific primers for qPCR

primer name	specific primer volume [+/- 0.1µl]	initial primer concentration [+/- 0.1µM]	final primer concentration needed for qPCR [+/- 0.1µM]	calculated volume of primer solution for qPCR $V_2 = \frac{C_1 \cdot V_1}{C_2}$ [+/- 0.1µl]	calculated volume of ultrapure water needed for dilution $V_3 = V_2 - V_1$ [+/- 0.1µl]
DbuCOI3F/R	30.0	50.0	3.0	500.0	470.0
DvillosiusF1/R1	30.0	50.0	3.0	500.0	470.0
coli_ybbW_F/R	30.0	50.0	3.0	500.0	470.0

#### 8.3.1. Materials

- forward and reverse primer for *D. polymorpha*
- forward and reverse primer for *D. villosus*
- forward and reverse primer for *E. coli* (positive control)
- 100ml of ultrapure water (Thermo Scientific)
- 2.5ml of SYBR Green qPCR Master Mix (Thermo Scientific)
- 1 Vortex Genie 2™ machine (INOTECH)
- 1 bucket with ice
- 6 DNA 2ml LoBind Tube (Eppendorf)

- |  |  |
|--|--|
| <ul style="list-style-type: none"> <li>- 1 centrifuge machine for Eppendorf tubes (Eppendorf, 2-ml tube capacity)</li> <li>- 100 pipette tips (20-<math>\mu</math>l capacity)</li> <li>- 1 multi-channel pipette (Eppendorf, 12, 20-<math>\mu</math>l capacity)</li> <li>- 1 pipette (0.2 to 20-<math>\mu</math>l capacity, +/- 0.1<math>\mu</math>l)</li> </ul> | <ul style="list-style-type: none"> <li>- 1 pipette (50 to 100-<math>\mu</math>l capacity, +/- 1<math>\mu</math>l)</li> <li>- 20 pipette tips (100-<math>\mu</math>l capacity)</li> <li>- 100 pipette tips (20-<math>\mu</math>l capacity)</li> <li>- 1 aluminum PCR sealing foil</li> <li>- 2 qPCR machines (LightCycler 480 Instrument II (Roche))</li> </ul> |
|--|--|

### 8.3.2. Method

As seen in **Table 2**, 36 wells must be tested for each specific primer. If doing four technical replicates per sample, 144 wells are needed, and hence 8 $\mu$ L of Master Mix (volume Master Mix + primers + water) for each of them ( $144 \cdot 8\mu\text{L} = 1152\mu\text{L}$ ). Reagents were prepared for 162 wells in case of an experimental error ( $162 \cdot 8\mu\text{L} = 1296\mu\text{L}$ ).

As reagent ratios must remain the same, the following formula was used (see also **Table 3**):

$$5x \text{ (Master Mix)} + 1x \text{ (primer)} + 2x \text{ (water)} = 1296\mu\text{L}$$

$$8x = 1296\mu\text{L}$$

Simplifying, the following equation results:

$$x = 162\mu\text{L}$$

Hence the following volumes must be used for the final Master Mix preparation (**Table 5**):<sup>55</sup>

**Table 5:** Reagent concentrations and volumes for final Master Mix

Reagents	Volumes [+/- 0.1 $\mu$ l]
SYBR Master Mix (2x)	$5x = 5 \cdot 162.0 = 810.0$
PCR Primers [3 $\mu$ M]	$x = 162.0 = 162.0$
ddH <sub>2</sub> O	$2x = 2 \cdot 162.0 = 324.0$
Total	1296.0
per well	8.0

1. Vortex and resuspended primers for 5 seconds. Transfer 15 $\mu$ L of the forward and 15 $\mu$ L of the reverse primer for *D. polymorpha* in a 2ml-DNA LoBind Tube. Vortex shortly.
2. Add 470 $\mu$ L of ultrapure water (see **Table 4**) to the tube. Vortex shortly.
3. Fill a bucket with ice and put the Master Mix on an on-ice rack.
4. In a 2ml-DNA LoBind Tube, mix (with according pipette) 324 $\mu$ L of ultrapure water with 810 $\mu$ L of Master Mix, and 162 $\mu$ L of the primer solution from steps 1 and 2. Vortex quickly.
5. Considering the PCR plate organization in **Table 6**,<sup>56</sup> transfer 104 $\mu$ L of the solution from step 4 in each orange well marked with a pipette (4 $\mu$ L will remain for easier pipetting). This volume was chosen in this way so that when later pipetting 8 $\mu$ L twelve times out of each well, there is a rest volume of 8 $\mu$ L for easier pipetting ( $104\mu\text{L} - 8\mu\text{L} \cdot 12 = 8\mu\text{L}$ ).

**Table 6:** Plan of qPCR Master Mix on 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
1 A	MMDV	MMDV	MMDV	MMDV	MMDV	MMDV	MMDV	MMDV	MMDV	MMDV	MMDV	MMDV
2 B												
3 C	MMDP	MMDP	MMDP	MMDP	MMDP	MMDP	MMDP	MMDP	MMDP	MMDP	MMDP	MMDP
4 D												
5 E	MMEC	MMEC	MMEC	MMEC	MMEC	MMEC	MMEC	MMEC	MMEC	MMEC	MMEC	MMEC
6 F												
7 G												
8 H												

6. Redo steps 1 to 5 for *D.villosus* and *E.coli*. For step 5, transfer the *D. villosus* Master Mix in the green and the *E. coli* Master Mix in the black highlighted wells on **Table 6**.
7. With the help of the multi-channel pipette, transfer 8µL each well (orange, A1 to A12) in **Table 6** into all wells in **Table 7**<sup>57</sup> circled in orange. To do so, fill the pipettes, and fill the wells A1, A3, A5 ....., and A23 in one go, then the wells A2, A4, A6 ....., and A24 in one go.
8. Repeat this procedure for rows B, C, D, E, and F.
9. Repeat steps 7 and 8 for *D. polymorpha* (in green) and *E. coli* (in black), whereby the *E.coli* wells must be located on a second qPCR plate (see **Table 8**).<sup>58</sup>
10. Take the qPCR plate with the DNA samples (**Table 2**) out of the refrigerator and let it warm up at room temperature for 10 minutes.
11. Using the multi-channel pipette, transfer 8µL the blue-highlighted wells from **Table 2** to the blue-highlighted wells in **Tables 7 and 8** (same principle as in step 7). Redo this step for the pink and green highlighted wells.
12. Cover the PCR strip tube plate with aluminum foil and press on it for the wells to be closed.
13. Place both plates in a centrifuge for 30 seconds at 20 rpm.

**Table 7:** Plan for first plate for qPCR (blue: Extraction Method 2, green: Extraction Method 3, pink: controls)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	A	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
2	B	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
3	C	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
4	D	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
5	E	NC2	NC2	NC6	NC6	UW	UW	UW	UW	NC3	NC3	NC7	NC7	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW
6	F	NC2	NC2	NC6	NC6	UW	UW	UW	UW	NC3	NC3	NC7	NC7	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW
7	G	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
8	H	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
9	I	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
10	J	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
11	K	NC2	NC2	NC6	NC6	UW	UW	UW	UW	NC3	NC3	NC7	NC7	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW
12	L	NC2	NC2	NC6	NC6	UW	UW	UW	UW	NC3	NC3	NC7	NC7	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW
13	M																								
14	N																								
15	O																								
16	P																								

*D. villosus*

*D. polymorpha*

**Table 8:** Plan for the second plate for qPCR (blue: Extraction Method 2, green: Extraction Method 3, pink: controls)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	A	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
2	B	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
3	C	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
4	D	S1, R1	S1, R1	S1, R2	S1, R2	S1, R3	S1, R3	S2, R1	S2, R1	S2, R2	S2, R2	S2, R3	S2, R3	S3, R1	S3, R1	S3, R2	S3, R2	S3, R3	S3, R3	S4, R1	S4, R1	S4, R2	S4, R2	S4, R3	S4, R3
5	E	NC2	NC2	NC6	NC6	UW	UW	UW	UW	NC3	NC3	NC7	NC7	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW
6	F	NC2	NC2	NC6	NC6	UW	UW	UW	UW	NC3	NC3	NC7	NC7	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW	UW
7	G																								
8	H																								
9	I																								
10	J																								
11	K																								
12	L																								
13	M																								
14	N																								
15	O																								
16	P																								

*E.coli*

14. Place the qPCR plates in the qPCR machines. Then, turn on the computers and start the qPCR program (LightCycler 480 Software) following the temperature targets in the protocol.

## 8.4. qPCR: Experiment

The experiment was performed with another qPCR mix: PowerTrack SYBR Green Master Mix, chosen as it was more sensitive, allowing the detection of lower concentrations of target DNA. Indeed, as discussed in 10.3, the mix used in the preliminary experiment did not detect investigated DNA reliably, most likely because of its low abundance. Therefore, this section follows the manufacturer's instructions on the following website:

[https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FSLG%2Fmanuals%2FMAN0018826\\_PowerTrackSYBRGreenMasterMix\\_QR.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FSLG%2Fmanuals%2FMAN0018826_PowerTrackSYBRGreenMasterMix_QR.pdf)

The yellow sample buffer was not used. Given the low DNA sample concentrations, 2 $\mu$ L of eDNA was used per well (and not 1 $\mu$ L as stated in the protocol). The ultrapure water volume was adapted to achieve the 10 $\mu$ L volume concentration at the end, given the DNA surplus and the buffer's absence. The following reagent concentrations and volumes resulted (Table 9):<sup>59</sup>

**Table 9:** Reagent concentrations and volumes for qPCR

Reagents	Volumes for one reaction [+/-0.1 $\mu$ l]
DNA	2.0
Yellow Sample Buffer	-
SYBR PowerTrack Mix (2x)	5.0
PCR Forward/Reverse Primer [8 $\mu$ M]	0.5
ddH <sub>2</sub> O	2.5
total volume	10.0

Initial primer stocks were prepared at a concentration of 100 $\mu$ M concentration (see Appendix) and hence had to be diluted to 8 $\mu$ M using ultrapure water, as seen in Table 9, using the same formula as in 8.1.2. First, 15  $\mu$ L of each forward/reverse primer per species was mixed to get a 50 $\mu$ M concentration. Then, this solution was further diluted using the water volume in Table 10.<sup>60</sup>

**Table 10:** Ultrapure water volume needed for dilution of the specific primers for qPCR

	primer volume	initial primer concentration	final primer concentration needed for qPCR	calculated volume of primer solution for qPCR	calculated volume of ultrapure water needed for dilution
primer name	$V_1$ [+/-0.1 $\mu$ l]	$C_1$ [+/- 0.1 $\mu$ M]	$C_2$ [+/- 0.1 $\mu$ M]	$V_2 = \frac{C_1 \cdot V_1}{C_2}$ [+/-0.1 $\mu$ l]	$V_3 = V_2 - V_1$ [+/-0.1 $\mu$ l]
DbuCOI3F/R	30.0	50.0	8.0	187.5	157.5
DvillosiusF1/R1	30.0	50.0	8.0	187.5	157.5
coli_ybbW_F/R	30.0	50.0	8.0	187.5	157.5

### 8.4.1. Materials

- |  |   |
|--|---|
| <ul style="list-style-type: none"> <li>- forward and reverse primer for <i>D. polymorpha</i></li> <li>- forward and reverse primer for <i>D. villosus</i></li> <li>- forward and reverse primer for <i>E. coli</i></li> <li>- 100ml of ultrapure water</li> <li>- 2.5ml of SYBR Powertrack SYBR Green Master Mix (Qiagen)</li> <li>- 1 Vortex Genie 2TM machine (INOTECH)</li> <li>- 1 bucket with ice</li> <li>- 6 DNA 2ml LoBind Tube (Eppendorf)</li> <li>- 1 centrifuge machine for Eppendorf tubes (Eppendorf, 2-ml tube capacity)</li> </ul> | <ul style="list-style-type: none"> <li>- 100 pipette tips (20-<math>\mu</math>l capacity)</li> <li>- 1 multi-channel pipette (12, 20-<math>\mu</math>l capacity)</li> <li>- 1 pipette (0.2 to 20-<math>\mu</math>l capacity, +/- 0.1<math>\mu</math>l)</li> <li>- 1 pipette (50 to 100-<math>\mu</math>l capacity, +/- 1<math>\mu</math>l)</li> <li>- 20 pipette tips (100-<math>\mu</math>l capacity)</li> <li>- 100 pipette tips (20-<math>\mu</math>l capacity)</li> <li>- 1 aluminum PCR sealing foil</li> <li>- 2 qPCR machines (LightCycler 480 Instrument II (Roche))</li> </ul> |
|--|---|

### 8.4.2. Method

When considering one replicate for each of the 12 wells,  $12 \cdot 8\mu\text{L} = 96\mu\text{L}$  of qPCR Mix is needed (see Table without DNA volume). To allow easier pipetting,  $110\mu\text{L}$  was planned for this step. Hence, for planning four replicates for each well and considering the 12 wells for Extraction Method 1, Extraction Method 2, and the negative/positive controls,  $110 \cdot 3 \cdot 4 = 1320\mu\text{L}$  of Reaction Mix was needed. Again, to allow easier pipetting,  $1400\mu\text{L}$  was planned. Adapting **Table 9** to a  $1400\mu\text{L}$  reaction volume while maintaining the ratios, **Table 11**<sup>61</sup> results (using the factor  $\frac{1400}{8} = 175$ , values from the second column were multiplied by 175 for the third column).

**Table 11:** Reagent concentrations and volumes for final Master Mix

Reagents	Volume for 8 $\mu\text{L}$ [ $\pm 0.1\mu\text{L}$ ]	Volumes for 1400 $\mu\text{L}$ solution [ $\pm 0.1\mu\text{L}$ ]
SYBR PowerTrack Mix (2x)	5.0	875.0
PCR Forward/Reverse Primer Mix [3 $\mu\text{M}$ ]	0.5	87.5
ddH <sub>2</sub> O	2.5	437.5
total volume	8.0	1400.0

1. Follow **Steps 1 to 13** in **8.3.2**.
2. **Mind:** For **Step 2**, add  $157.5\mu\text{L}$  of water.
3. For **Step 4**, add  $437.5\mu\text{L}$  of ultrapure water,  $875\mu\text{L}$  of Master Mix, and  $87.5\mu\text{L}$  primer solution.
4. For **Step 5**, add  $110.0\mu\text{L}$  to each well.
5. Place the qPCR plates in both qPCR machines. Then, turn on the computers and start the qPCR program following the temperature targets in the protocol.

## 9. SAFETY AND ENVIRONMENTAL ISSUES

### 9.1. Environmental/Biological safety and ethical issues

All used solutions were discarded in the biosafety waste container to avoid chemical release in the environment. Extreme care was taken to avoid skin and eye contact by wearing a lab coat, gloves, and goggles. No organisms were harmed, as *D. villosus* and *D. polymorpha* would have been visible to the human eye in the samples. Therefore, only their eDNA (for example, detached cells) were manipulated in the laboratory. *E. coli* would not have been visible to the eye but was killed during the lysis. Only dead *E. coli* were hence amplified (as their DNA remains stable), meaning there was no risk of propagation or infection of this pathogen.

### 9.2. Material/Mechanical safety

Scalpels were discarded in SHARPSAFE containers. This author was supervised using the INOTECH Vortex Genie 2™ machine to ensure proper use and avoid injuries. In addition, this author was introduced to the centrifuge to prevent its incorrect use, then used it alone.

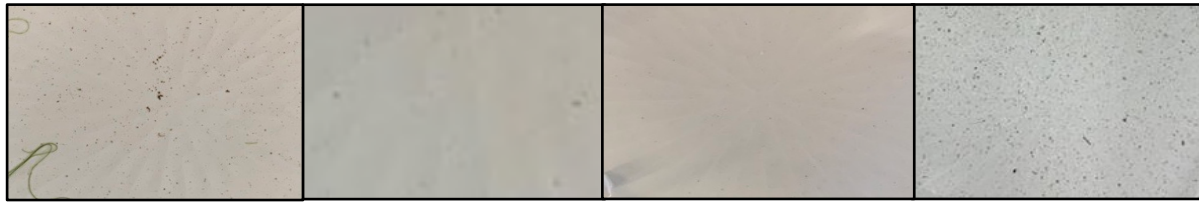
### 9.3. Chemical safety

Instructions concerning safety from the manufacturer of the different kits were read carefully before starting with the experimental procedures (for example for the inflammable ethanol).



## 10. DATA COLLECTION AND PROCESSING

### 10.1. Qualitative data from field sampling and water filtering



**Figure 13:** Qualitative data depicting four membrane filters after vacuum pumping (from left to right: Site 1 (Replicate 3), Site 2, (Replicate 2), Site 3 (Replicate 2), Site 4 (Replicate 1))

Overall, the fluvial water sampled from the river Limmat and the Lake of Zurich at all sites was of clear turbidity for the human eye, with only a few particles in suspension. As seen in **Figure 13**,<sup>62</sup> after filtrating the water with the vacuum pump, particles were collected on the Ø13.5cm membrane filter with 0.22µm pore size. Of note, elongated fibers were present on all filter membranes of Sites 1 and 2. The filter membrane of Site 3 appeared clear (no residues). On the filters of Site 4, a great particle density of black particles showed.

### 10.2. Quantitative data from DNA extraction

#### 10.2.1. Table Display

**Tables**<sup>63</sup> 12, 13, and 14 display the results from the DNA extraction using the following methods:

- 1) DNEASY® POWERWATER® KIT (so-called Method 1)
- 2) ZymoBIOMICS™ DNA/RNA Miniprep Kit (so-called Method 2)
- 3) Modified DNEASY® POWERWATER® KIT (so-called Method 3)

Again, for the Modified DNEASY® POWERWATER® KIT, the lysis step recommended by the manufacturer was modified to improve DNA recovery, notably by using a lysis tube containing beads and TissueLyser II machine from Extraction Method 2 (see 7.7 for further information).

After each DNA extraction step, the DNA concentrations in the samples were determined with a Qubit® 2.0 Fluorometer and a DNA-dependent fluorescent dye following the manufacturer's instructions.<sup>64</sup> This was done to ensure enough DNA was extracted before proceeding to and performing the qPCRs. Quantification of DNA using 260nm absorbance using Nanodrop could have been used, but this method is less sensitive (down to 10-20ng/mL), while Qubit® is more sensitive to lower DNA concentrations (down to 10pg/mL).<sup>65</sup> Total eDNA yield recovered from fluvial water reported from the previous publication "Freshwater monitoring by nanopore sequencing"<sup>66</sup> (2021) ranged from 2.5ng to > 3'000.0ng per 400ml of water. Filters in this Maturitätsarbeit were cut in four, and 1l of water was filtered per replicate. In comparison, there were no cuts, and 400ml of water was filtered per replicate in the publication. Hence the expected eDNA concentration per site replicate was expected to be in the range<sup>1</sup> of  $103.5\text{ng} \cdot 0.625$  to  $> 3'000\text{ng} \cdot 0.625$ , and hence 64.7ng to 1875.0ng. Given the low turbidity of the sampled water, it was also expected for the total eDNA yield to be in the lower range, justifying the use of Qubit fluorescent detection (also used in "Freshwater monitoring by nanopore sequencing").<sup>67</sup>

<sup>1</sup> $\frac{1}{4}$  (filters) ·  $\frac{1000}{400}$  (filtered water volume) = 0.625 (factor)

The site and replicate numbers are indicated in the first column of **Tables<sup>68</sup> 12, 13, and 14**. “S1, R1” stands for “Site 1, Replicate 1”; “NC” stands for “Negative Control”. Two negative controls, NC1 and NC2, were performed for each method to confirm that results did not occur from sample contamination during the experimental procedure.

In the second column, the DNA concentrations detected by the Qubit<sup>®</sup> 2.0 Fluorometer are recorded in [ng/ml]. A crossed cell in the table indicates that the DNA concentration was too low (< 0.50 ng/ml) to be detected by the Qubit<sup>®</sup> 2.0 Fluorometer, which was the case for all negative controls, indicating non-contamination and proper sample manipulation. Following manufacturer’s instructions of Qubit™ dsDNA Assay,<sup>69</sup> 5µl of DNA solution was mixed with 195µl of *working solution* for fluorescent analysis. There was a dilution factor of  $(195\mu\text{l} + 5\mu\text{l})/5\mu\text{l} = 40$ . In the third column, the values originating from the second column were multiplied by 40 to determine the DNA concentrations of the original DNA samples.

In the fourth column, the values of the third column were converted from [ng/ml] to [ng/µl] for easier data manipulation in further downstream processing. At the end of the different DNA extractions performed as part of this study, DNA was eluted from purification with 100µl of *elution buffer (EB)*. Hence, to obtain the absolute DNA yield of the samples in µg, the results from the fourth column were multiplied by 100 to obtain the absolute DNA yield of the sampled in ng, here-in reported in the fifth column. Standard deviations of DNA yield values were calculated for the three replicates of each site.

**Table 12:** Quantitative summary of DNA quantification concentrations extracted from 1000ml water sample using Qubit™ dsDNA HS Assay Kit (Extraction performed with DNEASY<sup>®</sup> POWERWATER<sup>®</sup> KIT)

Replicate	DNA concentration detected by Qubit [+/- 0.1ng/ml]	DNA concentration after dilution factor conversion [+/- 1ng/ml]	conversion to [+/- 0.001ng/µl]	DNA yield [+/- 0.1ng]	Standard deviation
S1, R1	14.3	572	0.572	57.2	27.1
S1, R2	26.1	1044	1.044	104.4	
S1, R3	14.4	576	0.576	57.6	
S2, R1	17.1	684	0.684	68.4	19.4
S2, R2	7.8	312	0.312	31.2	
S2, R3	10.1	404	0.404	40.4	
S3, R1	13.3	532	0.532	53.2	19.1
S3, R2	22.8	912	0.912	91.2	
S3, R3	17.3	692	0.692	69.2	
S4, R1	16.8	672	0.672	67.2	3.9
S4, R2	16.3	652	0.652	65.2	
S4, R3	18.2	728	0.728	72.8	
NC1/NC2					

**Table 13:** Quantitative summary of DNA quantification concentrations extracted from 1000ml water sample using Qubit™ dsDNA HS Assay Kit (Extraction performed with ZymoBIOMICS™ DNA/RNA Miniprep Kit)

Replicate	DNA concentration detected by Qubit [+/- 0.1 ng/ml]	DNA concentration after dilution factor conversion [+/- 1 ng/ml]	conversion to [+/- 0.001 ng/µl]	DNA yield [+/- 0.1 ng]	Standard deviation
S1, R1	16.1	644	0.644	64.4	9.1
S1, R2	19.5	780	0.780	78.0	
S1, R3	15.2	608	0.608	60.8	
S2, R1	18.1	724	0.724	72.4	13.0
S2, R2	23.9	956	0.956	95.6	
S2, R3	18.5	740	0.740	74.0	
S3, R1	20.8	832	0.832	83.2	23.0
S3, R2	32.3	1292	1.292	129.2	
S3, R3	27.2	1088	1.088	108.8	
S4, R1	26.0	1040	1.040	104.0	13.8
S4, R2	19.1	764	0.764	76.4	
S4, R3	22.9	916	0.916	91.6	
NC1/NC2					

**Table 14:** Quantitative data summary of DNA quantification concentrations extracted from 1000ml water sample using Qubit™ dsDNA HS Assay (Extraction performed with Modified DNEASY® POWERWATER® KIT)

Replicate	DNA concentration detected by Qubit [+/- 0.1 ng/ml]	DNA concentration after dilution factor conversion [+/- 1 ng/ml]	conversion to [+/- 0.001 ng/μl]	DNA yield [+/- 0.1 ng]	Standard deviation
S1, R1	18.2	728	0.728	72.8	5.2
S1, R2	19.5	780	0.780	78.0	
S1, R3	16.9	676	0.676	67.6	
S2, R1	15.5	620	0.620	62.0	8.3
S2, R2	16.6	664	0.664	66.4	
S2, R3	19.5	780	0.780	78.0	
S3, R1	32.3	1292	1.292	129.2	20.6
S3, R2	35.6	1424	1.424	142.4	
S3, R3	42.4	1696	1.696	169.6	
S4, R1	40.0	1600	1.600	160.0	23.5
S4, R2	32.5	1300	1.300	130.0	
S4, R3	28.4	1136	1.136	113.6	
NC1/NC2					

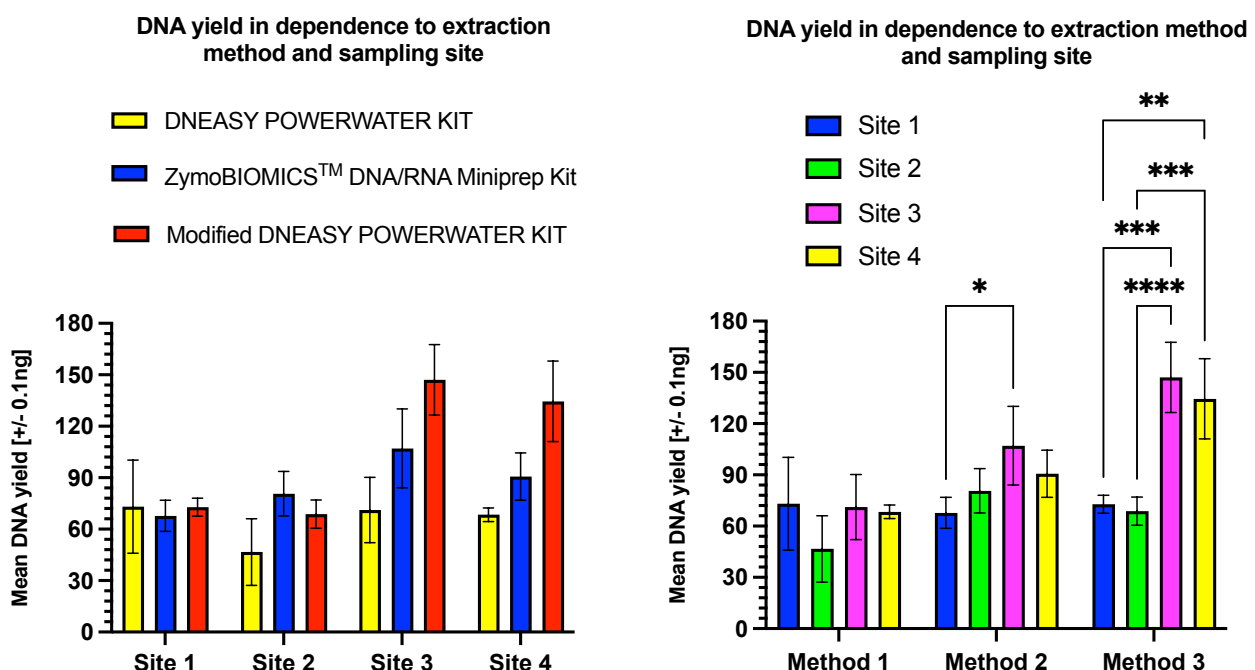
Table 15<sup>70</sup> includes the mean DNA concentration detected, mean DNA yield, and mean standard deviation for each method to enable comparison. Raw data was taken from Tables 12, 13, and 14.

**Table 15:** Mean data summary of DNA quantification concentrations using Qubit™ dsDNA HS Assay and three extraction methods

Extraction Method	mean DNA concentration detected [+/- 0.01 ng/ml]	mean DNA yield [+/- 0.01 ng]	mean standard deviation
DNEASY® POWERWATER® KIT	16.21	64.84	17.38
ZymoBIOMICS™ DNA/RNA Miniprep Kit	21.63	88.55	14.73
Modified DNEASY® POWERWATER® KIT	26.45	105.80	14.40

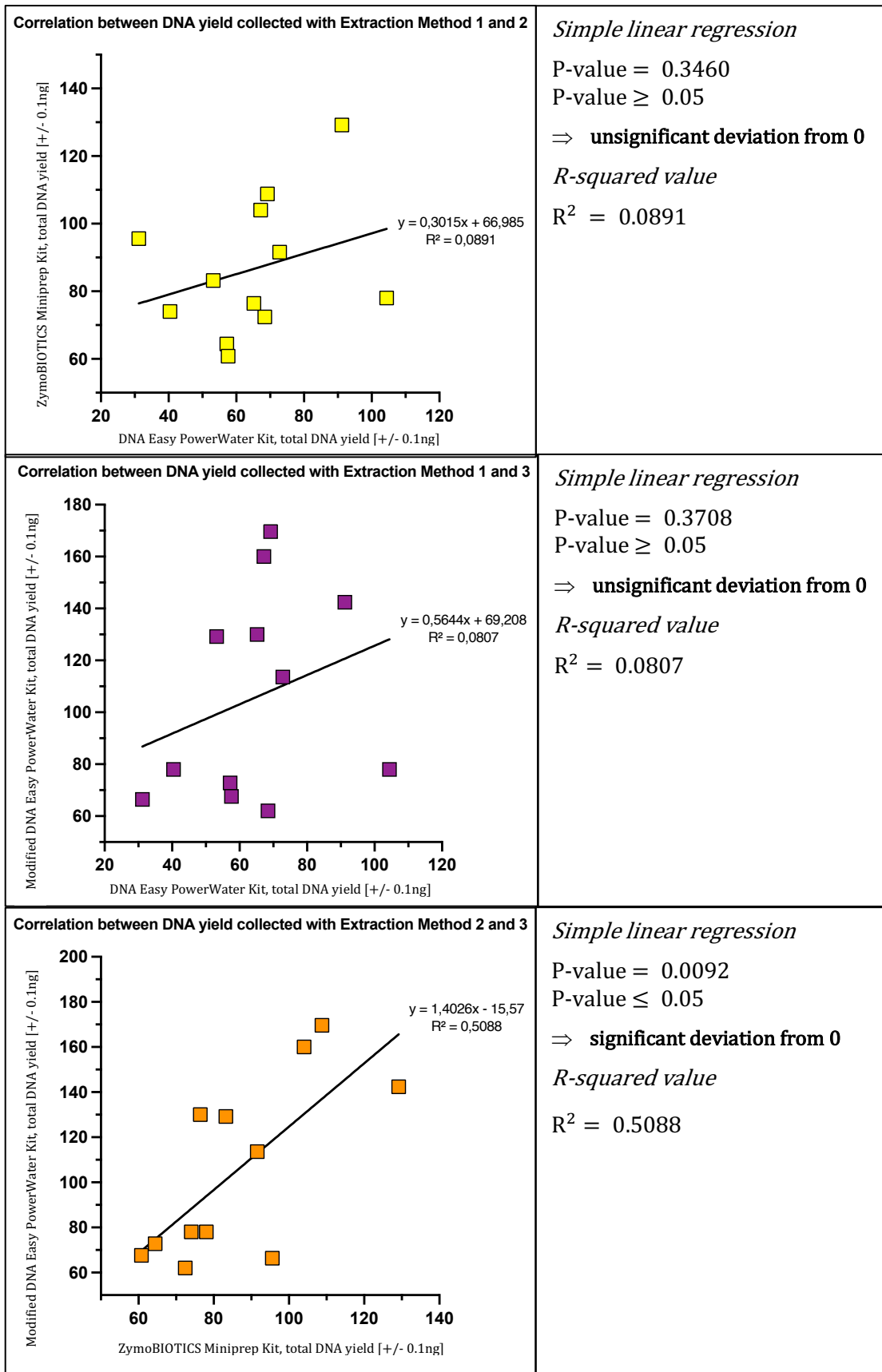
10.2.2. Graphical Display

Graph 1<sup>71</sup> (right) is a bar chart that visually displays the data from the “DNA yield” columns highlighted in purple in Tables 12, 13, and 14 for each extraction method and site, including the standard deviations for the site replicates. In Graph 1<sup>72</sup> (right), a two-way Analysis of Variance with a Šidák correction was performed on the data set using Prism software to conduct multiple comparisons to determine whether eDNA yield significantly differs between the sites. An asterisk indicates that the two sets of data differ significantly from each other.



**Graph 1:** Bar chart displaying the average DNA yield for each sampling site using all extraction methods including ANOVA for the right graph (\* = p ≤ 0.05, \*\* = p ≤ 0.01, \*\*\* = p ≤ 0.001) with error bars indicating standard deviation

**Graph 2<sup>73</sup>** shows the correlation between the DNA yield obtained from Method 1 and 2, 2 and 3, and 1 and 3, as X/Y plots, including a linear regression equation and its associated R-squared value.



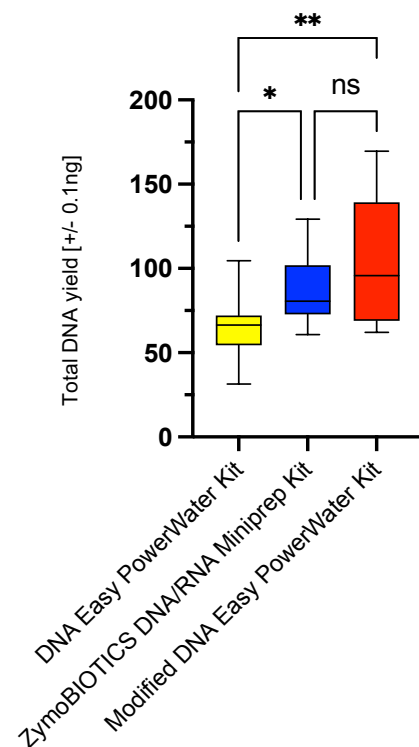
**Graph 2:** X/Y plots of inter-correlations between the eDNA yield of all extraction methods

The  $R^2$ -values of line equations in **Graph 2** define the relationship strength between the regression line and dependent variables on a 0 – 1 scale.<sup>74</sup> 0 indicates that the model explains none of the variability in  $y$ , and 1 indicates that it explains all variability in  $y$ .<sup>75</sup> A high  $R^2$ -value for graphs in **Graph 2** would indicate that data fit the linear regression line chosen well. Assuming that filters were homogeneous and the extraction methods efficient to 100%,  $R$ -squared would be 1 for all compared extraction methods. As four sampling sites were chosen, the variability of the filters was assumed to be averaged. Hence the  $R$ -squared value reflects the efficacy of the extraction methods.

Additionally, the  $p$ -values of the linear regression lines were calculated with PRISM GraphPad. If the  $p$ -value obtained by PRISM for each correlation  $\geq 0.05$ , the null hypothesis was accepted, stating that there was no significant deviation from 0 for the regression line. If the line differed significantly from 0, this could hint at a potential weak, moderate, or strong linear relationship between the variables.

### 10.2.3. Comparison of the extraction methods: Two-way Analysis of Variance (ANOVA)

**Total DNA yield using three extraction methods**



**Graph 3:** Box plot with ANOVA comparing the eDNA yield from the three extraction methods  
 (\* =  $p \leq 0.05$ , \*\* =  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$ )

**Graph 3**<sup>76</sup> is a box plot and displays descriptive statistics for the columns highlighted in purple in **Tables 12, 13, and 14** for each extraction method.

The plot includes first and second quartiles, minimum and maximum values, and the median.

A two-way Analysis of Variance with a Šidák correction was performed on the data set using Prism software to conduct multiple comparisons on the value of total eDNA yield between the extraction methods. “Ns” means that they do not differ significantly from each other and that  $H_0$ , stating that there is no correlation between the matched data, cannot be excluded.

### 10.2.4. Qualitative and quantitative comparison of the extraction methods

**Table 16**<sup>77</sup> summarizes qualitative and quantitative data for comparing of the extraction methods in terms of time, price, difficulty, and amount of DNA collected.



**Table 16:** Qualitative data summary including comparison of all three DNA quantification methods in terms of time, price, difficulty, and eDNA yield collected

Extraction Method	time needed [+/- 20min]	price for 14 samples [+/- 10 CHF]	difficulty	average DNA yield collected [+/- 0.1ng]
DNA Easy® PowerWater® Kit	220	175	+	64.8
ZymoBIOTICS DNA/RNA Miniprep Kit	300	76	++	88.6
Modified DNA Easy® PowerWater® Kit	230	175	+	105.8

### 10.3. Quantitative data from qPCR

Raw data originating from the preliminary experiment is shown in the appendix. This data was not processed as eDNA was mainly detected at very late amplification cycles (38-40), increasing the uncertainty of the measurements as these values are considered the threshold for DNA detection. Furthermore, no qPCR amplification was detected in 67% of the technical samples. The following sections display the raw data from the actual experimental trial. Melting curves are shown in the appendix.

The higher the amplification cycle value at which the organisms were detected, the smaller the initial *D. polymorpha*, *D. villosus*, or *E. coli* eDNA concentration. Indeed, if an initially small eDNA yield is amplified, it will need more amplification cycles to be detected by the qPCR machine.

#### 10.3.1. Tables: *D. polymorpha*

Tables<sup>78</sup> 17, 18, and 19 depict the  $C_q$  values (critical threshold value) at which *D. polymorpha* eDNA was detected in all samples with Extraction Method 2 (Table 17), Extraction Method 3 (Table 18) and for the negative controls with ultrapure water (Table 19). The mean and the standard deviation for each column are highlighted in blue. A crossed cell in the table means that no eDNA was detected. A red highlighted value means that eDNA was detected at a late amplification cycle ( $C_q \geq 38$ ), which increases the uncertainty of that particular measurement. T1/T2/T3 and T4 indicate the technical replicates. “S1, R1” means “Site 1, Replicate 1”.

**Table 17:**  $C_q$  (cycle threshold) values for *D. polymorpha* (Extraction Method 2 and negative controls)

Technical replicate	$C_q$ value [+/- 0.01]												NC2	NC6
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3		
T1	25.51	25.59	27.10	27.10	28.48	26.91	29.95	28.09	25.27	27.85	27.94	28.11		38.00
T2	25.28	25.52	27.03	26.99	28.32	26.84	30.54	28.43	25.53	27.76	27.88	27.93		
T3	25.45	25.28	27.10	26.94	28.23	27.17	30.24	28.65	25.27	27.92	27.81	27.94		
T4	25.54	25.60	27.04	27.15	28.12	27.03	30.48	28.57	25.54	27.85	27.83	27.86		38.00
Sd. of T1-T4 [+/- 0.01]	0.12	0.15	0.04	0.10	0.15	0.14	0.27	0.25	0.15	0.07	0.06	0.11		
Mean of T1-T4 [+/- 0.001]	25.445	25.498	27.068	27.045	28.288	26.9875	30.303	28.435	25.403	27.845	27.865	27.960		
Mean [+/- 0.001]	26.004			27.440			28.047			27.890				
Sd. [+/- 0.01]	0.92			0.73			2.47			0.06				

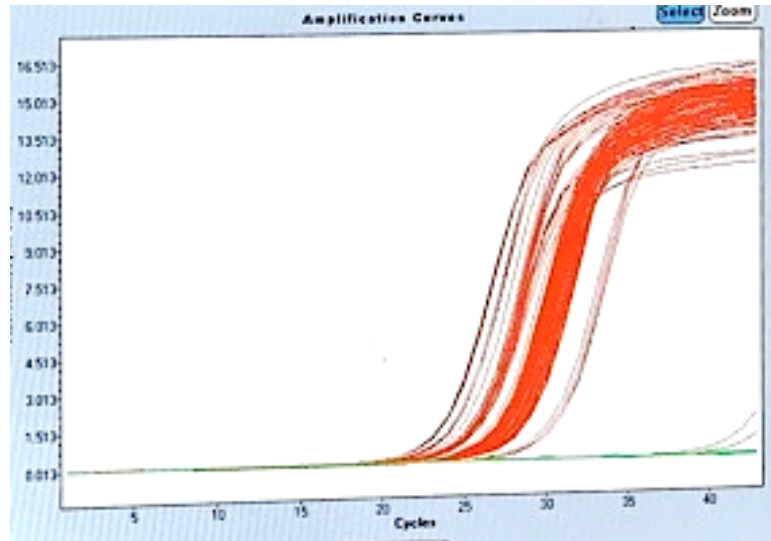
**Table 18:**  $C_q$  (cycle threshold) values for *D. polymorpha* (Extraction Method 3 and negative controls)

Technical replicate	$C_q$ value [+/- 0.01]												NC3	NC7
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3		
T1	26.54	26.80	27.29	23.34	26.86	25.48	27.10	27.76	24.28	27.51	28.45	27.22		
T2	27.53	26.44	27.21	23.54	27.29	25.47	27.22	27.92	24.07	27.55	27.91	27.27		
T3	27.28	26.76	27.30	23.45	27.27	25.45	27.55	27.70	24.78	27.63	27.84	27.83		
T4	26.64	26.95	27.46	23.54	27.06	25.59	27.44	28.45	24.20	27.71	28.44	27.77		
Sd. of T1-T4 [+/- 0.01]	0.48	0.21	0.10	0.09	0.20	0.06	0.20	0.34	0.31	0.09	0.33	0.32		
Mean of T1-T4 [+/- 0.001]	26.998	26.738	27.315	23.468	27.120	25.498	27.328	27.958	24.333	27.600	28.160	27.523		
Mean [+/- 0.001]	27.017			25.362			26.540			27.761				
Sd. [+/- 0.01]	0.29			1.83			1.94			0.35				

**Table 19:** C<sub>q</sub> (cycle threshold) values for *D. polymorpha* for negative controls with ultrapure water (UW)

Technical replicate	C <sub>q</sub> value [+/- 0.01]							
	UW	UW	UW	UW	UW	UW	UW	UW
T1								
T2								
T3								
T4								

The raw data from **Tables 17, 18, and 19** is depicted in **Figure 14**<sup>79</sup> as red amplification curves.



**Figure 14:** Photograph of Lightcycler application on computer with amplification curves of *D. polymorpha* (Extraction 2 and 3, negative controls)

**10.3.2. Tables: *D. villosus***

**Tables**<sup>80</sup> **20, 21, and 22** depict the C<sub>q</sub> values (critical threshold value) at which *D. villosus* eDNA was detected in all samples with Extraction Method 2 (**Table 20**), Extraction Method 3 (**Table 21**) and for the negative controls with ultrapure water (**Table 22**). Samples (not negative controls) where nothing was detected were given a C<sub>q</sub> value of 40.00 (detection threshold).

**Table 20:** C<sub>q</sub> (cycle threshold) values for *D. villosus* (Extraction Method 2 and negative controls)

Technical replicate	C <sub>q</sub> value [+/- 0.01]												NC2	NC6
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3		
T1	31.77	30.55	33.06	32.68	34.60	33.32	38.00	40.00	40.00	35.94	34.91	34.50		
T2	32.59	32.64	33.94	33.44	34.85	32.45	38.00	38.00	40.00	38.00	36.11	36.43		
T3	32.80	32.43	33.96	33.27	34.55	32.78	40.00	40.00	40.00	38.00	34.60	36.86		
T4	32.66	33.05	34.22	34.55	34.90	32.87	40.00	38.00	40.00	36.46	34.61	37.48		
Sd. of T1-T4 [+/- 0.01]	0.46	1.11	0.51	0.78	0.18	0.36	1.15	1.15	0.00	1.06	0.72	1.29		
Mean of T1-T4 [+/- 0.001]	32.455	32.168	33.795	33.485	34.725	32.855	39.000	39.000	40.00	37.100	35.058	36.318		
Mean [+/- 0.001]	32.806			33.688			39.333			36.159				
Sd. [+/- 0.01]	0.89			0.95			0.58			1.03				

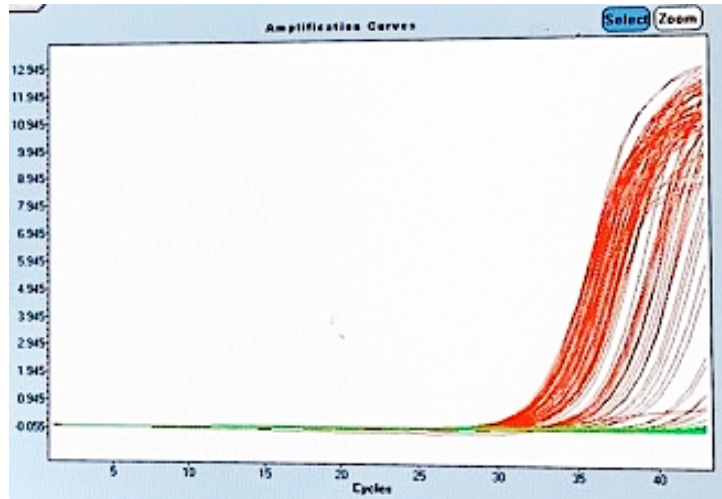
**Table 21:** C<sub>q</sub> (cycle threshold) values for *D. villosus* (Extraction Method 3 and negative controls)

Technical replicate	C <sub>q</sub> value [+/- 0.01]													NC3	NC7
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3			
T1	32.79	33.84	34.78	34.51	33.23	33.54	40.00	38.00	38.00	38.00	38.00	40.00			
T2	33.85	33.88	34.74	33.84	34.12	32.99	38.00	40.00	40.00	37.06	40.00	38.00			
T3	32.83	33.72	36.16	34.8	33.04	33.75	38.00	40.00	40.00	38.00	38.00	38.00			
T4	34.81	33.69	34.39	34.85	32.83	33.49	38.00	38.00	38.00	35.36	38.00	38.00			
Sd. of T1-T4 [+/- 0.01]	0.96	0.09	0.78	0.46	0.57	0.32	1.00	1.15	1.15	1.24	1.00	1.00			
Mean of T1-T4 [+/- 0.001]	33.570	33.783	35.018	34.500	33.305	33.443	38.500	39.000	39.000	37.105	38.500	38.500			
Mean [+/- 0.001]	34.124			33.749			38.833			38.035					
Sd. [+/- 0.01]	0.78			0.65			0.29			0.86					

**Table 22:**  $C_q$  (cycle threshold) values for *D. villosus* for negative controls with ultrapure water (UW)

Technical replicate	$C_q$ value [ $\pm$ 0.01]							
	UW	UW	UW	UW	UW	UW	UW	UW
T1								
T2								
T3								
T4								

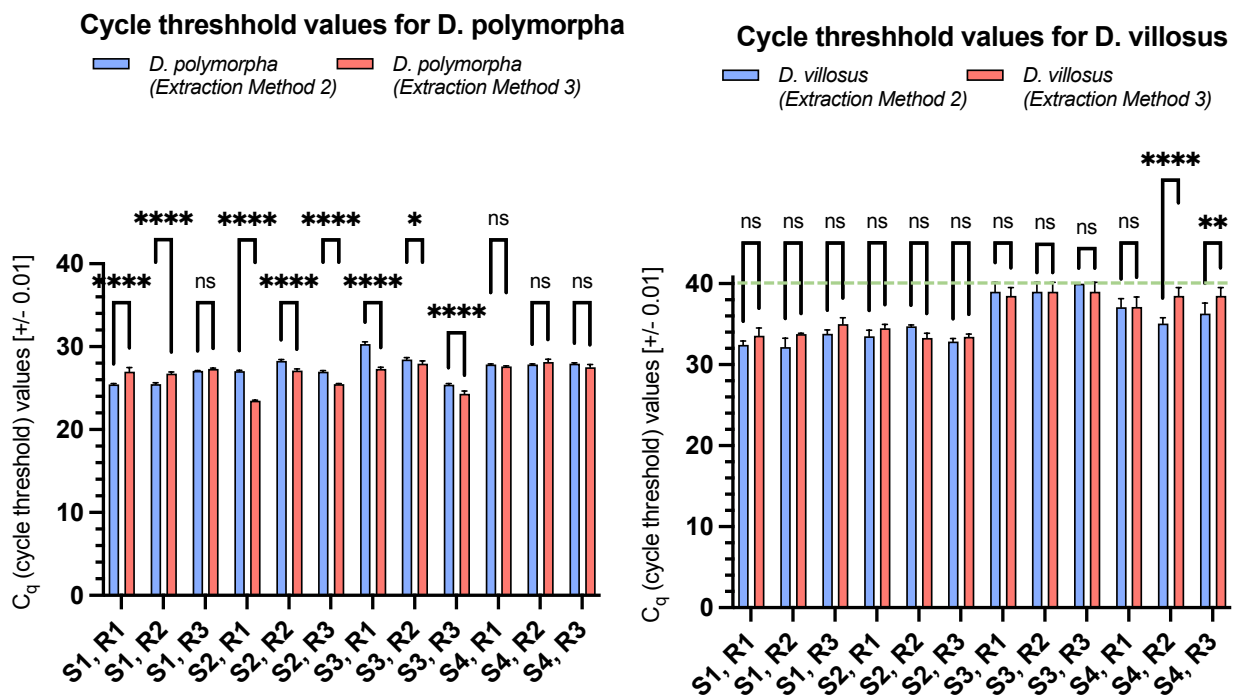
The raw data from **Tables 20, 21, and 22** is depicted in **Figure 15**<sup>81</sup> as red amplification curves.



**Figure 15:** Photograph of Lightcycler application on computer with amplification curves of *D. villosus* (Extraction 2 and 3, negative controls)

**10.3.3. Graphs: *D. villosus* and *D. polymorpha***

**Graph 4**<sup>82</sup> visualizes the data displayed in sections 10.3.1 and 10.3.2 for *D. polymorpha* and *D. villosus* (without the negative controls) for each extraction method. The value of 40.00 was attributed to each crossed-out cell in the graphs, defined at the threshold for detection (green dotted line).



**Graph 4:** Bar chart depicting the  $C_q$  value with error bars indicating standard deviation of the technical replicates for *D. polymorpha* (left) and *D. villosus* (right) for each site and extraction method, including ANOVA (\* =  $p \leq 0.05$ , \*\* =  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$ )

A two-way ANOVA with a Šidák correction was performed with PRISM Graphpad to determine whether the  $C_q$  values from both methods obtained differed significantly from another. In principle, the matched data sets should not differ from one another as the same original water samples were processed.

**10.3.4. Tables: *E. coli* (Positive control)**

Tables<sup>83</sup> 23, 24, and 25 depict the  $C_q$  values (critical threshold value) at which *E. coli* eDNA was detected in all samples with Extraction Method 2 (Table 23), Extraction Method 3 (Table 24), and for the negative controls with ultrapure water (Table 25).

**Table 23:**  $C_q$  (cycle threshold) values for *E. coli* (Extraction Method 2 and negative controls)

Technical replicate	$C_q$ value [+/- 0.01]													
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3	NC2	NC6
T1	33.54	34.17	32.05	33.2	38.00	33.51	38.00	35.18	33.10	33.47	38.00	36.17	34.54	
T2	33.29	34.69	35.65	34.11	36.77	35.26	34.52	35.22	35.39	36.03	34.15	38.00		
T3	32.86	35.16	33.81	35.58	35.69	31.97	38.00	34.71	38.00	38.00	33.98	38.00		
T4	33.43	33.85	35.12	35.41	34.15	38.00	38.00	35.62	35.47	38.00	38.00	36.65		
Sd. of T1-T4 [+/- 0.01]	0.30	0.58	1.60	1.13	1.63	2.59	1.74	0.37	2.00	2.15	2.27	0.94		
Mean of T1-T4 [+/- 0.001]	33.280	34.468	34.158	34.575	36.153	34.685	37.130	35.183	35.490	36.375	36.033	37.205		
Mean [+/- 0.001]	33.969			35.138			35.934			36.538				
Sd. [+/- 0.01]	0.62			0.88			1.05			0.60				

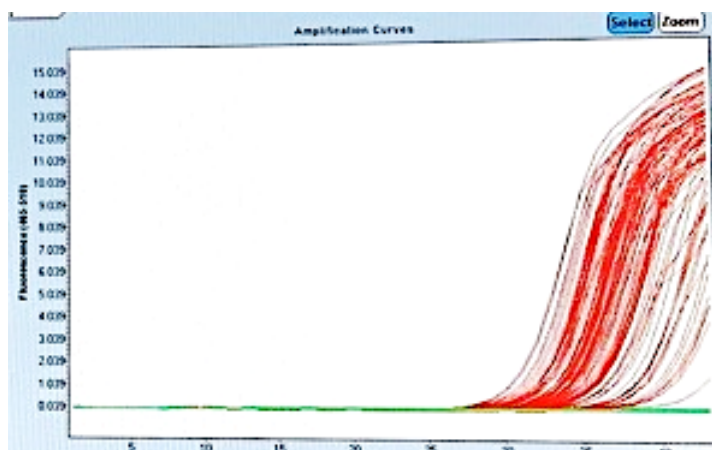
**Table 24:**  $C_q$  (cycle threshold) values for *E. coli* (Extraction Method 3 and negative controls)

Technical replicate	$C_q$ value [+/- 0.01]													
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3	NC3	NC7
T1	34.77	33.29	34.51	31.56	32.09	35.39	35.87	33.64	32.53	32.49	32.49	30.17		34.26
T2	31.60	33.79	38.00	32.49	31.31	33.84	35.67	38.00	33.72	32.54	33.95	30.97		
T3	32.98	32.71	33.64	32.30	30.99	34.52	32.09	33.63	35.11	32.44	34.15	30.60		
T4	31.96	32.56	33.87	35.64	31.92	33.61	34.72	33.87	34.54	32.13	33.44	30.85		
Sd. of T1-T4 [+/- 0.01]	1.42	0.56	2.03	1.81	0.52	0.80	1.74	2.15	1.12	0.18	0.74	0.35		33.25
Mean of T1-T4 [+/- 0.001]	32.828	33.088	35.005	32.998	31.578	34.340	34.588	34.785	33.975	32.400	33.508	30.648		
Mean [+/- 0.001]	33.640			32.972			34.449			32.185				
Sd. [+/- 0.01]	1.19			1.38			0.42			1.44				

**Table 25:**  $C_q$  (cycle threshold) values for *E. coli* for negative controls with ultrapure water (UW)

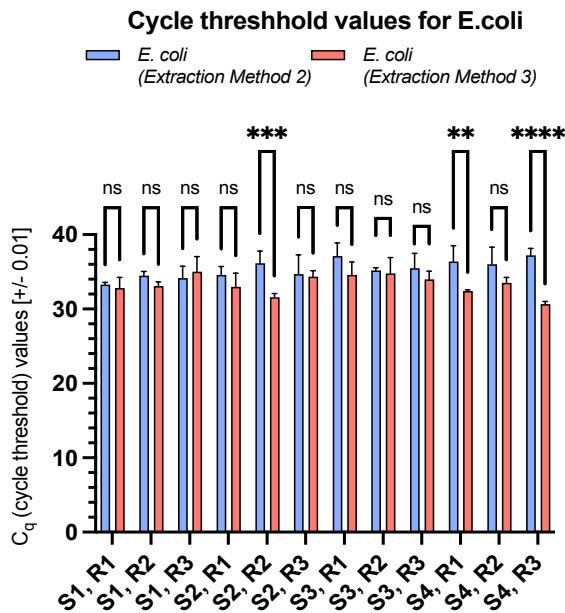
Technical replicate	$C_q$ value [+/- 0.01]							
	UW	UW	UW	UW	UW	UW	UW	UW
T1		38.00						
T2								
T3								
T4								

The raw data from Tables 23, 24, and 25 is depicted in Figure 16<sup>84</sup> as red amplification curves.



**Figure 16:** Photograph of Lightcycler application on computer with amplification curves of *E. coli* (Extraction 2 and 3, negative controls)





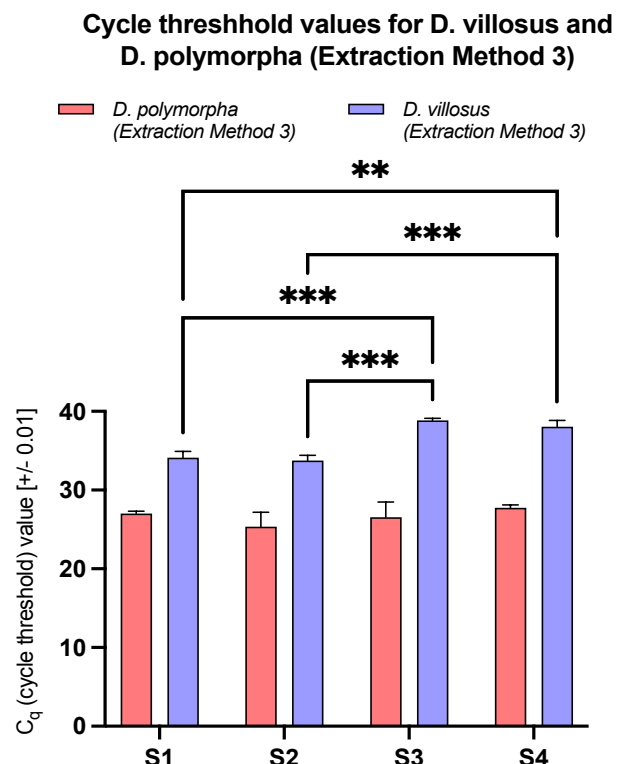
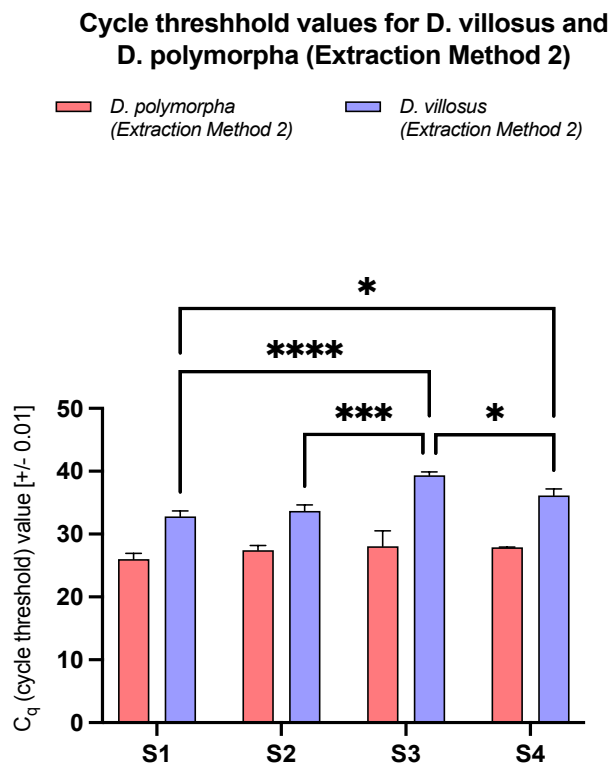
### 10.3.5. Graph: *E. coli*

Graph 5<sup>85</sup> visually displays the data displayed in section 10.3.4 for *E. coli* (without the negative controls) for each extraction method. The value of 40.00 was attributed to each crossed-out cell in the graphs, defined as the threshold for detection. Similar to 10.3.3, a two-tailed ANOVA with a Šidák correction was performed with PRISM to determine whether the C<sub>q</sub> values obtained differed significantly from another.

**Graph 5:** Bar chart depicting the C<sub>q</sub> value for *E. coli* for each site and extraction method with error bars indicating standard deviation of the technical replicates, including ANOVA (\* = p ≤ 0.05, \*\* = p ≤ 0.01, \*\*\* = p ≤ 0.001)

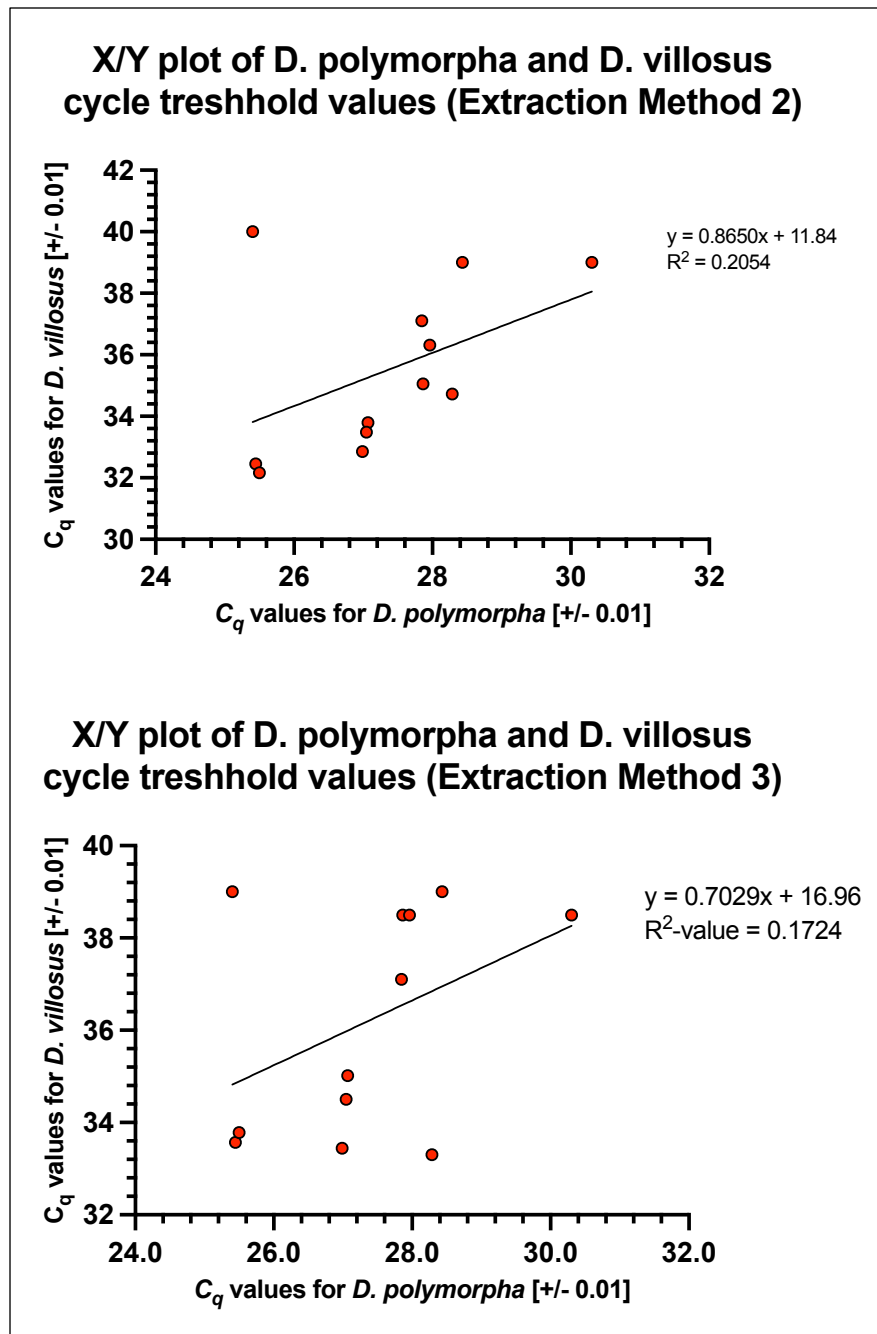
## 10.4. Testing for correlation using linear regression

Graph<sup>86</sup> 6 shows the cycle threshold value for both organisms for each site and method for a first visual impression. Similarly to 10.3.5, a two-tailed ANOVA with a Šidák correction was performed to determine whether *D. polymorpha* and *D. villosus* abundancy differed significantly at different sites. Of note, the C<sub>q</sub> values of *D. villosus* were significantly higher than the C<sub>q</sub> values of *D. polymorpha* at each site (all eight p-values ≤ 0.0001).



**Graph 6:** Bar chart showing *D. polymorpha* and *D. villosus* cycle threshold values for each replicate with Extraction Method 2 (left) and Extraction Method 3 (right), including ANOVA (\* = p ≤ 0.05, \*\* = p ≤ 0.01, \*\*\* = p ≤ 0.001)

**Graph 7<sup>87</sup>** displays the cycle threshold values of both organisms for both extraction methods as an X/Y plot, including a linear regression line and its associated R-squared value to test for a linear relationship between the two dependent variables.



**Graph 7:** X/Y plot of *D. polymorpha* and *D. villosus*  $C_q$  values including linear regressions and associated R-squared values (Extraction Method 2, above and Extraction Method 3, below)

The p-values of the linear regression lines were calculated with PRISM GraphPad. If the p-value obtained by PRISM for each correlation  $\geq 0.05$ , the null hypothesis was accepted, stating that there was no significant deviation from 0 for the regression line. If the line differed significantly from 0, this could hint at a potential weak, moderate, or strong linear relationship between the variables. The p-value for **Graph 7 (above)** was 0.1390, and for **Graph 7 (below)**, 0.1796.

## 10.5. Testing for correlation using Spearman's Rank coefficient

The statistical test **Spearman's rank correlation** coefficient was used to identify potential monotonic correlations between the  $C_q$  values of *D. polymorpha* and the  $C_q$  values of *D. villosus*. The strength and direction of potential correlations were calculated with PRISM GraphPad (see **Figure 17**).<sup>88</sup> If the p-value obtained by PRISM for each correlation  $\geq 0.05$ , as a 95% significance level was chosen, the null hypothesis was accepted, stating that no significant correlation was detected between the variables using the collected dataset.

Correlation	A	
	$C_q$ (cycle threshold) values for <i>D. polymorpha</i> (Extraction Method 2)	$C_q$ (cycle threshold) values for <i>D. polymorpha</i> (Extraction Method 3)
	Y	Y
<b>Spearman r</b>		
r	0.4659	0.2681
95% confidence interval	-0.1663 to 0.8266	-0.3781 to 0.7386
<b>P value</b>		
P (two-tailed)	0.1286	0.3961
P value summary	ns	ns
Exact or approximate P value?	Exact	Exact
Significant? (alpha = 0.05)	No	No
<b>Number of XY Pairs</b>	12	12

**Figure 17:** Calculations for Spearman's Rank correlation using PRISM Graphpad for  $C_q$  values of *D. polymorpha* and *D. villosus* for all site replicates

## 11. CONCLUSION AND EVALUATION

### 11.1. Results of extraction

#### 11.1.1. Price, time, and difficulty of the extraction methods (Table 16)

ZymoBIOTICS™ DNA/RNA Miniprep Kit was the most time-consuming extraction method (300 minutes, or 80, respectively 90 minutes more than the others) and the most difficult due to the frequent tube transfers and elutions. The DNA Easy® PowerWater® Kit and Modified DNA Easy® PowerWater® Kit were the most expensive (175CHF, or 99CHF more than the ZymoBIOTICS™ DNA/RNA Miniprep Kit). When calculating and integrating a price and time score ( $[\text{CHF}]^* [\text{min}]]/1000$ ) for the extraction methods to investigate their performance, whereby the calculated outcome should be the smallest possible, the Modified DNA Easy® PowerWater® Kit obtains **40.25**, the DNA Easy® PowerWater® Kit **38.50**, and the ZymoBIOTICS™ DNA/RNA Miniprep Kit **22.80**.

#### 11.1.2. eDNA yield per extraction method

The eDNA yield values obtained with the Qubit fluorometer reached from 31.2ng to 169.6ng using all extraction methods (Tables 12/13/14), which were outside the expected range of 64.7ng to 1875.0ng as expected from 10.2.1.

Table 15 and Graph 3 highlight that nearly twice as much eDNA was extracted using the Modified DNEASY® POWERWATER® KIT than the DNEASY® POWERWATER® KIT. However, the Modified DNEASY® POWERWATER® KIT did not significantly extract more eDNA than the ZymoBIOTICS™ DNA/RNA Miniprep Kit. As can be seen from Table 15 and Graph 1 (left), the standard deviation is highest for the DNEASY® POWERWATER® KIT and about equal for the ZymoBIOTICS™ DNA/RNA Miniprep Kit and the Modified DNEASY® POWERWATER® KIT.

#### 11.1.3. eDNA yield per site

Fluorometric quantification of purified DNA demonstrated that eDNA yield collected at Sites 3 (Frauenbadi) and 4 (Tiefenbrunnen) was higher compared with Sites 1 (Werdinsel) and 2 (Wipkingerwiese) for Method 3, as shown with the two-way ANOVA analysis with Šidák correction on Graph 1 (right). This is partially supported by the observation of the filter, as shown in Figure 13, where the highest particle density on the membrane filters was found for Site 4.

#### 11.1.4. Correlation of the extraction methods and the eDNA yield

Graph 2 shows no correlation between the eDNA yield obtained from the extraction methods DNA Easy® PowerWater® Kit and Modified DNA Easy® PowerWater® Kit and obtained from the DNA Easy® PowerWater® Kit and the ZymoBIOTICS™ Miniprep Kit, as tested with linear regressions ( $p$ -values  $0.346$  and  $0.371 \geq 0.05$ ). However, a positive correlation was found between the eDNA yield extracted by the Modified DNA Easy® PowerWater® Kit and the ZymoBIOTICS™ Miniprep Kit, as tested with linear regression ( $p$ -value  $0.0092 \leq 0.05$ ) and indicated by the increased R-squared value of 0.5088.

The two-way ANOVA analysis with Šidák correction with integrated pairwise comparisons in **10.2.3** supports this observation. The eDNA yield obtained differed significantly between the Modified DNA Easy® PowerWater® Kit and the DNA Easy® PowerWater® Kit and between the ZymoBIOTICS™ Miniprep Kit and DNA Easy® PowerWater® Kit, but not between the Modified DNA Easy® PowerWater® Kit and the ZymoBIOTICS™ Miniprep Kit. This suggests that both of these kits (Method 2 and 3) have the best performance for eDNA isolation.

## 11.2. Results of qPCR

Before proceeding to the display of the qPCR results, it must be noted that those must be interpreted with great prudence due to the limitations of the experimental design further discussed in the evaluation. For example, primer specificity and efficiency were not tested before, although they were taken from reliable previous studies, and human errors during the dilution and qPCR reagent preparation could have potentially biased the experimental outcome. Moreover, keep in mind that, in the following sections, when statements are made about the eDNA yield of the different organisms, this is an indirect and hence unreliable indicator for their abundance, especially when comparing organisms that have different sizes and belong to different paraphyletic groups (invertebrates and prokaryotes).

The higher the  $C_q$  value at which the organisms were detected, the smaller the initial *D. polymorpha*, *D. villosus*, or *E. coli* eDNA concentration in the analyzed sample. Indeed, suppose an initially small eDNA yield in the solution is amplified. In that case, it will need more amplification cycles to be detected by the qPCR machine and will have a higher  $C_q$  value.

Overall, and as seen in **Figure 14**, *D. polymorpha* eDNA is detected at small amplification cycles, meaning that it was an abundant species in the samples. Section **10.4** additionally suggests that at each site for both extraction methods, there was significantly more *D. polymorpha* eDNA than *D. villosus* eDNA, although this is, as mentioned, not an indicator of their respective abundance.

The standard deviations for the technical replicates during qPCR were the lowest for *D. polymorpha*, as reflected by the decreased standard deviation bars in **Graph 4 (left)** compared to **Graph 4 (right)** and **Graph 5**. Then follows the slightly higher standard deviation for *D. villosus*, and finally, the highest one for *E. coli*, which is reflected by the increased standard deviation bars on **Graph 5** compared to **Graph 4 (left and right)**.

There hence seems to be a positive trend between a more considerable standard deviation for the technical replicates and the tardive amplification of the samples.

### 11.2.1. Extraction methods

As seen in **Tables 17/18** and **Graph 6**, using two-way ANOVA analysis with Šidák correction, *D. polymorpha* eDNA yield at different sites did not differ significantly for both extraction methods. In contrast and as seen in **Tables 20/21** and **Graph 6** using two-way ANOVA analysis with Šidák correction, significantly more *D. villosus* eDNA was collected at Sites 1, 2, and 4 than at Site 3 with



Extraction Method 2. In addition, significantly more *D. villosus* eDNA was collected at Sites 1 and 2 than at Sites 4 and 3 with Extraction Method 3. *E. coli* eDNA, the positive control, was detected at all sites (Tables 23/24).

#### 11.2.2. Samples detected at higher uncertainties with cycle amplification values of $\geq 38.00$

*D. villosus* eDNA was detected at an amplification cycle of  $\geq 38.00$  (limit of detection) in 29.1% of Extraction Method 2 samples and 45.8% of Extraction Method 3 samples (Tables 20/21). *E. coli* eDNA was detected at an amplification cycle of  $\geq 38.00$  (limit of detection) in 25% of Extraction Method 2 samples and 4% of Extraction Method 3 samples at an amplification cycle of  $\geq 38.00$  (Tables 23/24).

#### 11.2.3. Negative controls

For *D. polymorpha*, eDNA (or primer dimers, further treated in the discussion) was detected in 2 negative controls from Extraction Method 2 (Tables 17/18). For *E. coli*, eDNA (or primer dimers) was detected in 3 negative controls from Extraction Methods 2 and 3. (Tables 23/24) Furthermore, *E. coli* eDNA was detected in a negative control sample with ultrapure water at an amplification cycle of 38.00 (Table 25).

#### 11.2.4. Correlation between the extraction methods and the site replicate $C_q$ values (Graphs 4/5)

Overall, it can be stated that the most significant positive correlation between the extraction method and the site replicate  $C_q$  values was found for *D. villosus* (10 of 12 paired  $C_q$  values did not differ significantly).

### 11.3. Testing for correlation with linear regression and Spearman's Rank Coefficient

The following statistical analyses answer the research question of this Maturitätsarbeit:

**Graph 7** shows no significant positive correlation between the  $C_q$  values of *D. villosus* and the  $C_q$  values of *D. polymorpha* for both extraction methods at the 95% confidence level as tested with linear regressions (p-values 0.1390 and 0.1796  $\geq 0.05$ ).

**Figure 17** shows no significant correlation between the  $C_q$  values of *D. villosus* and the  $C_q$  values of *D. polymorpha* for both extraction methods at the 95% confidence level using Spearman's Rank Coefficient (p-values 0.1286 and 0.3961  $\geq 0.05$ ).

### 11.4. Discussion of the eDNA extraction

#### 11.4.1. Total eDNA yield recovery at different sites

As mentioned in 11.1.3, significantly more eDNA was collected at the Frauenbadi and the Strandbad Tiefenbrunnen than at the Werdinsel and the Wipkingerwiese with Method 3.

In the research paper "Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds."<sup>89</sup> (2018) by L.R. Harper, the idea is put forward that eDNA accumulates in stagnant water bodies such as ponds due to the "lack of current and the small water volume."

This train of thought could be applied to the Lake of Zurich, as it does not have any significant current flow like a river, although it is not such a closed and small ecosystem as a pond. It must be noted that water from the Limmat was sampled in the actual streamflow and not in stagnant pools of water. Hence the sampling sites were exposed to the current, unlike Site 4 (Tiefenbrunnen).

With decreased current, eDNA has the opportunity to accumulate, leading to an increased eDNA concentration in the water and a higher eDNA recovery yield from sampling. Of note, the Bradshaw model<sup>90</sup> states that fluvial velocity and discharge, and hence river streamflow, increase with distance from a source for a river. The source of the Limmat being the Lake of Zurich, most eDNA was expected to be recovered in the Lake of Zurich (Site 4) and then at the sites nearest to the lake in the following order (with increased streamflow and distance from the lake): Site 3, Site 2, and finally Site 1. The data collected in this study (most eDNA recovered at Sites 3 and 4 for Method 3) would support this hypothesis.

Nevertheless, one should also consider that eDNA concentration in the water results from local aquatic organisms releasing mitochondrial and nuclear DNA in their surroundings,<sup>91</sup> as well as from bacteria and sediment resuspension (that is increased with current). Indeed, aquatic sediment may even contain more eDNA than water.<sup>92</sup>

In the Limmat, the constant flow would not allow for local eDNA accumulation but would, in contrast, favor sediment resuspension.

In the Lake of Zurich, the absence of flow would allow, as mentioned, the accumulation of eDNA but no natural sediment resuspension. Additionally, the large water volume would impair the diffusion of local eDNA from aquatic organisms.

However, while fluvial water was collected, for Sites 1 and 2, in the proximity of local bathing infrastructures (Flussbad Au-Höngg, Unterer Letten), it was collected in actual water basins (Badis) for Sites 3 and 4. Similarly to an increased current flow, it is likely that human activity in the water increases sediment resuspension by mechanical forces and hence the eDNA concentration present in the water.

All in all, when considering the factors that potentially impact eDNA concentration in the water above, most support the presence of a more significant eDNA concentration in Sites 3 and 4, increasing confidence in the data collected.

The range from the eDNA yield recovered from this Maturitätsarbeit does not fit the one in the paper "Freshwater monitoring by nanopore sequencing"<sup>93</sup> (2021). This could be explained by the different research locations (Cam (UK) *versus* Limmat (CH)).

The Cam is polluted by fecal bacteria from untreated sewage, and the presence of high phosphate levels from agriculture promotes algal blooms.<sup>94,95</sup> Both increase eDNA concentration and yield in the water.

In contrast, the water quality of the Limmat is excellent due to regular monitoring and adequate sewage treatment.<sup>96</sup> In this sense, it was expected for the Limmat to have a decreased eDNA concentration and yield than the Cam.

#### 11.4.2. Total eDNA yield recovery using different extraction methods

As stated in 11.1.2, the most efficient extraction methods with the best eDNA isolation performance were the Modified DNEASY® POWERWATER® KIT and the ZymoBIOMICS™ DNA/RNA Miniprep Kit (compared to the DNEASY® POWERWATER® KIT).

During the experimental procedure, it was observed that Extraction Method 2 recovered more eDNA yield than Method 1. This was surprising, as, on one side, due to the multiple tube transfers and elutions on different spin columns in Method 2, it was expected that more eDNA would be lost during the experimental procedure. However, on the other side, DNEASY® POWERWATER® KIT only uses mechanical lysis with bead beating. In contrast, ZymoBIOMICS™ DNA/RNA Miniprep Kit uses a **biomechanical** and **chemical lysis** (see protocols in 7.2 and 7.5), which, in turn, could have increased its eDNA yield extraction, fitting the obtained results.

All in all, this would confirm the initial statement in 7.7: the increased **biomechanical** and **biochemical** lysis (with a TissueLyser II machine here for five minutes) of the filter membrane was the decisive step for optimized extraction. Combining this with Method 1, **Method 3** was developed, which did not significantly extract more eDNA than Method 2 but extracted more in this investigation (**Table 15**). These observations follow the research paper “Methods to maximize recovery of environmental DNA from water samples”<sup>97</sup> (2017), which states that, *inter alia*, biochemical lysis is more efficient than mechanical lysis. Here, the advantage of having different methods is outlined: one can combine two methods to optimize them maximally.

An interesting follow-up investigation would be to perform lysis with the TissueLyser machine for a longer time (10min or 20min) to see if the recovered eDNA yield obtained is increased or decreased. Indeed, high-speed shaking of the samples could increase eDNA extraction, but the beads in the tube could also break the long eDNA helices. Furthermore, heat from the bead-based shaking or incubating the filter membrane too long in the lysis buffer could also denature the DNA. However, this would not be a problem for performing qPCRs as only small DNA fragments are needed for amplification. Nevertheless, further sequencing of fragmented samples using next-generation sequencing may not be possible as intact DNA may be needed for such analysis,<sup>98</sup> which could be problematic if one wanted to perform a complete genomic analysis.

#### 11.4.3. Comparison of the extraction methods

However, which one of these extraction methods would be the most appropriate to use if this experiment was performed again, not only in terms of eDNA yield recovered but also of money, time and difficulty? The time and price scores in 11.1.1, which must be minimized for experimental optimization, indicate that in those two terms, the ZymoBIOTICS™ DNA/RNA

Miniprep Kit (with a score of 22.80 *versus* 38.50 and 40.25) would deliver the best performance, as it did with Method 3 in the previous section with the increased eDNA yield. Nevertheless, if integrating the follow-up investigation mentioned above with the longer lysis time, Method 3 should be favored. Indeed, if neglecting costs and focusing on data reliability, the shorter experimental time and decreased step number of Method 3 would decrease the chance of human errors and allow more samples to be extracted in a given time. Hence the Modified DNEASY® POWERWATER® KIT (Method 3) should be favored for further similar investigations.

## 11.5. Discussion of qPCR

### 11.5.1. *D. polymorpha*

There was significantly more *D. polymorpha* eDNA than *D. villosus* eDNA at all sites and with both extraction methods in this Maturitätsarbeit, as stated in **10.4**. However, this does not mean that the absolute number and hence overall abundance of *D. polymorpha* organisms were higher than *D. villosus*, although this assumption would be supported by the results in the paper “Dikerogammarus-Monitoring im Zürichsee und in der Limmat/Bestandesmonitoring”<sup>99</sup> (2007) by AWEL, where the zebra mussel is more abundant than the killer shrimp in the Lake of Zurich and the Limmat.

*D. polymorpha* eDNA yield at different sites did not differ significantly from each other for both extraction methods. This statement follows the observations made in the paper “*Dikerogammarus villosus* im Zürichsee und in der Limmat.”<sup>100</sup> (2006). Indeed, in this paper, *D. polymorpha* was not even included in the evaluation, as the invasive mussels occurred at similar densities on all hard substrates at the sampling sites. This contrast to *D. villosus* (whose eDNA yield differed significantly between sites) could be explained by the fact that *D. polymorpha* invaded these freshwater bodies earlier than *D. villosus* (1969 *versus* 2006) and is fully established on all substrates in the river and the lake, leading to minor differences in eDNA yield collected at different sites. *D. villosus*, in contrast, is adapting to its environment and still has preferred habitats (difference in eDNA yield with sites).

The two negative controls found positive for *D. polymorpha* were suspected to be primer-dimers. “Primer-dimers occur when two PCR primers (either same-sense primers or sense and antisense primers) bind to each other instead of to the target. Melting curve analysis can identify the presence of primer-dimers because they exhibit a lower melting temperature than the amplicon.”<sup>101</sup>

Nevertheless, this assumption was not supported by the melting curve analysis in **Supplementary Figure 4**, where no product-unspecific peaks appear at a lower temperature than the prominent peak (in contrast to **Supplementary Figure 2**, where primer dimers occur). However, as the detection of the negative controls occurred at a late amplification cycle (38.00, **Table 17**), increasing the uncertainty of these particular measurements, and no other negative controls were

positive, the possibility of cross-contamination of the negative controls with a sample during qPCR preparation was favored. For example, a small eDNA volume from a nearby qPCR well could have landed in the negative controls during manipulation with the multi-channel pipette, contaminating it. This eDNA “trace” would also explain the late amplification cycle, as it would have needed an increased amplification cycle number to be detected.

#### 11.5.2. *D. villosus*

*D. villosus* eDNA yield acquisition was consistent for both extraction methods, with increased yield in Sites 1 and 2 compared to Sites 3 and 4. Assuming that here, eDNA yield could partially reflect the abundance of this organism, this result would contradict the results displayed in the research paper “Dikerogammarus-Monitoring im Zürichsee und in der Limmat/Bestandesmonitoring” (2007), where most *D. villosus* organisms were present in the lake (and not in the Limmat).

*D. villosus* may have continued to invade and colonize the Limmat since 2006, increasing its presence there. Indeed, according to the studies “Dikerogammarus villosus” (2022)<sup>102</sup> by S. Mastitsky and “Substrate preferences of coexisting invasive amphipods, *D. villosus* and *D. haemobaphes*, under field and laboratory conditions” (2018)<sup>103</sup> by K.E. Clinton et al., the preferred habitats of the killer shrimp were mats of algae near the surface and cobble substrates, which were abundantly present for Sites 1 and 2 along the Limmat.

Nevertheless, and most importantly, one should consider that eDNA is not a direct measure as it travels with the streamflow and can be found far away from its source. However, it would have been progressively diluted in the water.

When looking closer at the raw data obtained from *D. villosus*, and as stated in the result section, it strikes that for Sites 3 and 4, most of the *D. villosus* in the samples were detected at an amplification cycle of  $\geq 38.00$ , explaining, *inter alia*, the small standard deviation. Furthermore, when analyzing the melting curve of the qPCR (**Supplementary Figure 3**), the replicates of Sites 3, forming the red and turquoise blue curves,<sup>2</sup> demark themselves from the product-specific blue amplification curve, potentially indicating the presence of non-specific qPCR products. Hence, it is unclear whether it was *D. villosus* that was detected in the samples with amplification cycle values of  $\geq 38.00$ , decreasing the reliability of the results for Site 3.

#### 11.5.3. *E. coli*

Although *E. coli*, the positive control, was, as mentioned, detected at all sites, the high  $C_q$  values of 32.2 to 36.5 were unexpected, as more eDNA yield of this species was thought to be present in or near infrastructures where, for example, little children swim. Of note, with *E. coli*'s average length of 1.0 to 2.0  $\mu\text{m}$ ,<sup>104</sup> the bacteria would have been collected on the membrane with a pore size of

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<sup>2</sup>this was indicated in the LightCycler application



0.22 $\mu$ m during vacuum filtration. Unfortunately, no other studies discuss the eDNA abundance of *E. coli* in the bodies of water investigated; hence, no reference values are available for comparison. The high  $C_q$  values (25% of samples in Method 2 with cycle amplification value of  $\geq 38.00$ ) could nevertheless be explained by the inefficacy of the primers, which is further discussed in **11.7.4**. The primers may not have amplified and doubled *E. coli* eDNA optimally at each cycle; hence an increased number of cycles would have been needed for the samples to be detected by qPCR. Nevertheless, it must be noted that the primers were selected, as shown in the **Appendix**, from the research paper “A highly specific Escherichia coli qPCR and its comparison with existing methods for environmental waters” (2017),<sup>105</sup> where the specificity of the primers was explicitly validated. The three negative controls found positive for *E. coli* were suspected to be primer dimers. Indeed, three net peaks appear at a lower temperature than the prominent product-specific blue peak in **Supplementary Figure 2**. Hence the negative controls were indeed negative.

The slight differences between the extraction method and the  $C_q$  values of the site replicates in **Graph 5** could be explained by the fact that Method 2 is more suitable for extracting bacterial eDNA. Indeed, “the ZymoBIOMICS lysis system eliminates bias associated with unequal lysis efficiencies of different organisms (for example of gram-negative/positive bacteria, such as *E. coli*), making it ideal for microbial community profiling.”<sup>106</sup>

The trend mentioned between the more considerable standard deviation for the technical replicates and delayed amplification of the samples (for example, for *E. coli*) could be explained by the fact that, with more delayed amplification, PCR enzymes could become more and more altered due to temperature changes, resulting in the variability of their products.

#### **11.6. Correlation between the invasive species and implications**

No correlation was found between the eDNA concentration of *D. polymorpha* and *D. villosus* for both extraction methods at the 95% confidence level. This is in accordance with the results in the paper “Dikerogammarus-Monitoring im Zürichsee und in der Limmat/Bestandesmonitoring 2007”<sup>107</sup> (2007) performed by AWEL, as stated in the foreword.

Nevertheless, it can be stated that the eDNA of both invasive species was detected at most sites, meaning that *D. polymorpha* and *D. villosus* eDNA traces co-occur in the collected samples. Hence there is a correlation between the spatial distribution of the eDNA yield from *D. polymorpha* and *D. villosus* in the water samples from the four sites along the Lake of Zurich and the Limmat. Although this does not confirm the co-occurrence of the organisms themselves, as eDNA is only an indirect measure of their abundance, numerous other papers do support this co-occurrence using kick sampling.<sup>108</sup>

Despite this co-occurrence, it can be assumed that the impact of each invasive species investigated on its local biome will be mainly preserved. On one side, “predation of organisms by *D. villosus* could disrupt leaf-litter processes by curtailing the activity of macroinvertebrate taxa engaged in

leaf shredding. If not broken down, it would not be available to the rest of the community who might rely on it, resulting in even more biodiversity loss.”<sup>109</sup> On the other side, “*D. polymorpha* could cause the death of other local mussel communities, but be beneficial to macroinvertebrates as it provides adequate habitat structures.”<sup>110</sup>

However, as this Maturitätsarbeit investigates a correlation, one should also consider potential impacts of both co-occurring, interacting species on their local biome. Looking at one particular interaction in the next paragraph, it must be noted that many external environmental factors are left out. Nevertheless, this schematizes how the co-occurrence of two invasive species could affect an ecosystem.

The article “Important species interactions can destabilize aquatic ecosystems in response to nutrient inputs”<sup>111</sup> (2019) from the Eawag, the Swiss Federal Institute of Aquatic Science and Technology, could be considered as a case study for a better understanding of how “stabilizing effects [in an ecosystem] can be disrupted when the co-occurrence of species gives rise to complex interactions.”

The study investigated the impact of the mussel *Dreissena polymorpha* and watermilfoil aquatic plants on the local plankton community from the Lake of Greifen using a “pond facility” as an artificial mini-ecosystem. The results show that when either one of the organisms is present in the experimental ecosystem, “algal growth occurred after the addition of nutrients, but then also rapidly recovered.” However, when both species were present, “the algal biomass and turbidity was significantly higher after nutrient additions than in the ponds with neither [...]. In other words, algal biomass remained persistently high. In addition, in this case, a small cyanobacteria consistently took over and completely dominated these algal blooms, resulting in a loss of taxonomic and trait-level biodiversity.”

Indeed, as stated in the Eawag study, *D. polymorpha* increased the water clarity of the ponds by absorbing nutrients such as phosphorus or nitrogen, preventing excessive algal blooms. However, when the watermilfoil was additionally present, it inhibited the growth of green algae. Those green algae were the competitors of present cyanobacteria in the water that accordingly took over and led to a loss of biodiversity. Notably, cyanobacteria can be lethal to humans and aquatic organisms as they release toxins and carcinogens in the water.<sup>112</sup>

This reasoning could be applied to the co-occurrence of *D. polymorpha* and *D. villosus* in the Lake of Zurich and the Limmat and used as a follow-up investigation to determine the impact of the combined invasive species on fluvial biodiversity in a closed ecosystem, including the dynamic relationship between algae and cyanobacteria.

As stated in 5.2.1 and above, the killer shrimp and the zebra mussel both feed, *inter alia*, on microalgae. Hence the hypothesis would be that by reducing the abundance of this specific species in a particular location due to the co-occurrence of *D. villosus* and *D. polymorpha*, the presence of

cyanobacteria could be promoted in the closed ecosystem, and the overall biodiversity could be reduced not only by the cyanobacteria but also by both invasive species themselves, as stated in 5.2.1 and 5.2.2. Furthermore, this effect would be reinforced when considering that the Lake of Zurich and the Limmat will become more exposed to climate change and increased water temperatures in the future, promoting the growth of even more cyanobacteria,<sup>113</sup> resulting in a positive feedback loop. It must, however, be noted that this potential feedback loop will be affected by more dynamics than in the closed ecosystem, such as the invasion of new species as the quagga mussel *Dreissena bugensis* that was first recorded in 2014 in the Lake of Zurich and has already replaced *Dreissena polymorpha* in the Lake of Constance since its introduction in the 1960s.<sup>114</sup>

## 11.7. Evaluation/Reflection and improvements

### 11.7.1. Recovery of eDNA

It must be noted that the eDNA yield extracted from the original water samples did not contain eDNA fragments that were smaller than the filter membrane pore size of 0.22 $\mu$ m. Therefore, only cells, vesicles, debris, and bacteria such as *E. coli* (1-2 $\mu$ m),<sup>115</sup> bigger than 0.22 $\mu$ m, were collected on the filter. This could have led to an underestimation of the eDNA concentration of the two invasive species. Indeed, the eDNA yield extracted would not accurately reflect the total eDNA yield present in the original water samples (depending, for example, on how much more/less *D. villosus* eDNA fragments smaller than 0.22 $\mu$ m was present than *D. polymorpha* eDNA fragments smaller than 0.22 $\mu$ m).

To obtain as much eDNA pool from both investigated species as possible and limit this bias, the water collected in the lower bottle of the Stericup could undergo ultrafiltration using membranes with smaller pore sizes. The Vivaflow® Tangential Flow Filtration Cassettes<sup>116</sup> allow such ultrafiltration for sample volumes of up to five liters. The membrane filters and ultrafilters could then be extracted simultaneously in the same DNA LoBind Tube.

### 11.7.2. Human errors and dilution

The potential impact of human errors in the experimental procedure should not be neglected in this Maturitätsarbeit: The full results and statistical analyses assume and are based on the fact that the Qubit measurements for eDNA quantification were correctly assessed, the dilutions for the qPCR accurately calculated, and the pipetting step to obtain the right volume for each qPCR well correctly performed. If eDNA was not at an equivalent volume in all samples before the qPCRs, variations might have occurred in the  $C_q$  values. Indeed, reaction volume affects the time needed for each sample to reach the target temperature.<sup>117</sup> With a slightly greater qPCR well volume, a sample might need more time to reach the target temperature than others, resulting in different fluorescence and, as already mentioned, experimental variations in the  $C_q$  values.

To limit such errors, enough time should be planned to prepare for the qPCR to get acquainted with the procedure. An automated pipetting robot could be used to reach more consistent volumes in each qPCR well.

### 11.7.3. Primer efficiency

A theoretical assumption made in this study was the optimal efficiency of the specific primers and the fact that in each  $C_q$  amplification cycle, eDNA yield was doubled (primer efficiency = 2).<sup>118</sup> If this were not the case, the obtained  $C_q$  values would not accurately reflect eDNA yield at each cycle.

For example, if primer efficiency was optimal and hence 2, and the initial eDNA copy numbers were denoted as  $x$ , the  $C_q$  amplification value of 38 would represent  $2^{38} \cdot x$ . In contrast, if primer efficiency were, for example, 1.5, the  $C_q$  value 38 would represent  $1.5^{38} \cdot x$ , which is off by a factor of about 56'000 compared to the optimal primer efficiency. If comparing the eDNA yield of a singular organism at different sites, there would be no bias as the  $C_q$  values would be off by the **same** factor for each site. However, if comparing the eDNA yield of two distinct organisms with two specific primers with unknown efficiencies, the relative abundance of each eDNA in the samples could not be adequately assessed.

To prevent such bias, the efficiency of each primer should be assessed before the experimental procedure. Once the efficiency of the primers was assessed, the  $C_q$  values of both invasive species could be normalized accordingly. Qiagen proposes an experimental procedure for such testing:

<https://www.qiagen.com/ca/resources/faq?id=2d04d81f-ef1f-4d7e-aaa5-2f30767445b1&lang=en>.

### 11.7.4. Primer design and specificity for the target species

The following paragraph refers to pages 6 and 15 of the “Real-time PCR handbook”<sup>119</sup> by Life Technologies.

Primer specificity is crucial for qPCR to avoid, *inter alia*, the occurrence of primer dimers, which are non-specific products of amplification, as was the case for *E. coli* in this Maturitätsarbeit (**Supplementary Figure 2**). Indeed, “primer dimers in negative controls, as was the case here, hint that primer dimers are most probably also present in the actual samples, decreasing qPCR efficiency and biasing data.” Hence, once the primers are selected (in this paper, primer base sequences were taken from existing research papers (see **Appendix**)), they should be analyzed before starting the experimental procedure to avoid their complementarity. Indeed, “with complementary base sequences, both primers could hybridize, forming primer dimers.” The specificity of the primers could be evaluated using OligoAnalyzer™ Tool from IDT.<sup>120</sup>

Additionally, the tool Primer-BLAST from the National Library of Medicine (NIH) on the website <https://www.ncbi.nlm.nih.gov/tools/primer-blast/> was used (database = nr, leave blank in section “organism”) and could be used for further investigation to verify that primers are specific to the organisms investigated. The primers for *E. coli* were highly specific. However, they could also amplify some eDNA sequences from the bacteria *Shigella*, which is logical since *E. coli* and *Shigella* both belong to the *Enterobacteriaceae* and are genetically closely related.<sup>121</sup> The primers

for *D. polymorpha* were highly specific, although they could also amplify two sequences from the moth *Furcula furcula* on the genome 26. Finally, the primers for *D. villosus* were not specific to eDNA sequences from this organism, as eDNA sequences from dozens of other organisms could have also been amplified.

Suppose the primers, as in the case of *D. villosus*, are not considered specific. In that case, base sequences from other research papers could be tested, or one could develop his/her optimal primers to target a specific DNA sequence using the tool PrimerQuest™ from IDT.<sup>122</sup>

#### **11.7.5. Availability of similar research papers for data comparison and overall reliability**

To establish the significance of a study, it must be reproduced by teams differently and independently from the one at the origin of the publication and compared to existing studies.

Unfortunately, no published eDNA analyses known to this author characterize the correlation between both invertebrates in the Limmat and the Lake of Zurich. Until now, and as mentioned in this Maturitätsarbeit numerous times, this correlation has been assessed with the sampling and counting of each organism. Hence access to papers existent and singular to this research topic and area was limited. The data of this study was hence not optimally compared to other significant and recent studies. Nevertheless, this Maturitätsarbeit established the possibility of such an eDNA investigation. Of course, more sampling sites and replicates would be needed to establish a significant statement, but this paper lays the groundwork to incentivize and encourage further investigations using eDNA technologies.

#### **11.7.6. Nanopore sequencing**

When developing the experimental design of this experiment, Nanopore sequencing appeared, for a long time, next to qPCR, as an accurate method to assess the correlation in the research question. **Table 26**<sup>123</sup> summarizes the advantages and disadvantages of each method and refers to the comparison made by Oxford Nanopore Technologies for eDNA applications on the webpage <https://nanoporetech.com/applications/environmental-genomics>.



**Table 26:** Summary of main advantages and disadvantages of the MinION and qPCR

Oxford Nanopore Technologies (MinION)	qPCR
<b>Location</b>	
<p>(+) The MinION is a pocket-size, USB-powered, portable device allowing sequencing directly at the sampling source or in the lab. Hence eDNA in the sample has no time to degrade, and characterization of remote ecosystems is enabled.</p> <p>(-) DNA must first be extracted, and a 16S PCR must be performed on the samples before sequencing. Hence the same constraints as the qPCR remain, although the sequencing itself could be performed “in the field.”</p>	<p>(+) Conducting all procedures in a laboratory decreases the risk of aerosol contamination of the samples.</p> <p>(-) Real-time PCR can only be performed in well-equipped microbiology laboratories. In this Maturitätsarbeit, water samples (14 liters) from the field had to be transported to the laboratory, which was impracticable.</p>
<b>Costs</b>	
<p>(+) The MinION is a long-term investment as the machine and some equipment (such as flow cells) can be reused multiple times.</p> <p>(-) The starting costs are around 1000 dollars (including the kits) for the most basic equipment.</p>	<p>(+) The price per sample of the real-time PCR is lower than for the MinION in the short term.</p> <p>(-) Nevertheless, none of the reagents can be reused, and for bigger sample sizes and multiple experiments, the MinION is more cost-efficient per sample.</p>
<b>Availability of data and complexity</b>	
<p>(+) The MinION allows real-time data streaming with high accuracy. Whole fluvial microbiome eDNA can be sequenced using metabarcoding with a sample preparation time of as little as 10 minutes. In addition, the correlation between species is easier to assess as ratios between all the organisms in a sample are calculated.</p> <p>(-) Nevertheless, once the results are available, advanced skills in bioinformatics are needed to match the base sequences to online data banks and analyze them.</p>	<p>(+) qPCR results can be analyzed easily and do not require advanced bioinformatics skills.</p> <p>(-) Absolute quantification has numerous limiting factors and a high uncertainty when working with low eDNA concentration. The <math>C_q</math> values approximate only the eDNA concentration of a target species. Additionally, real-time PCR involves a laborious workflow, many procedural steps, increased experimental time, and hence also increased potential for human errors.</p>

In the end, the Nanopore Technology MinION device was not used for financial reasons. Nevertheless, an interesting follow-up investigation would be to use the MinION to assess the same correlation as stated in the research question, especially as some filter membranes from this Maturitätsarbeit remain. The results obtained by qPCR and the nanopore technology could be compared. Moreover, the entire microbiome community of the sites would be uncovered with the MinION, enabling the connections between the abundances of *D. villosus* and *D. polymorpha* and other organisms.

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## APPENDIX

All supplementary tables were created and calculated by Microsoft Excel. All supplementary figures were pictures or screenshots taken by the author of this research paper.

### 1. Oligonucleotide order from Microsynth

## Microsynth

### Technical Datasheet

Order Id 799739

Order Date 13.10.2022

DbuCOI3F				Oligo Id		4614695	
Type	DNA	Synthesis scale	Genomics	Molecular weight	6496.2 g/mol	Amount	9.18 OD
5' Modification	NONE	Purification	Desalted	Ext. coefficient	210.2 1/(mM·cm)		43.7 nmol
3' Modification	NONE	Length	21 nt	Tm (50 mM NaCl)	65.3 °C		283.9 µg
No internal modifications				Tm (NN-Method)	58.6 °C	Volume for 100 µM	437.0 µl
5'-GCT AAG GGC ACC TGG AAG CGT-3'							
DbuCOI3R				Oligo Id		4614696	
Type	DNA	Synthesis scale	Genomics	Molecular weight	6487.2 g/mol	Amount	8.76 OD
5' Modification	NONE	Purification	Desalted	Ext. coefficient	177.1 1/(mM·cm)		49.4 nmol
3' Modification	NONE	Length	22 nt	Tm (50 mM NaCl)	67.9 °C		320.7 µg
No internal modifications				Tm (NN-Method)	57.9 °C	Volume for 100 µM	494.4 µl
5'-CAC CCC CGAATC CTC CTT CCC T-3'							
DvillosusF1				Oligo Id		4614699	
Type	DNA	Synthesis scale	Genomics	Molecular weight	8241.3 g/mol	Amount	12.83 OD
5' Modification	NONE	Purification	Desalted	Ext. coefficient	249.8 1/(mM·cm)		51.4 nmol
3' Modification	NONE	Length	27 nt	Tm (50 mM NaCl)	68.2 °C		423.3 µg
No internal modifications				Tm (NN-Method)	62.6 °C	Volume for 100 µM	513.6 µl
5'-GGA GCT TCT TCA ATT CTC GGC GCA ATT-3'							
Dvillosus-R_OK				Oligo Id		4621769	
Type	DNA	Synthesis scale	Genomics	Molecular weight	8286.4 g/mol	Amount	9.87 OD
5' Modification	NONE	Purification	Desalted	Ext. coefficient	304.0 1/(mM·cm)		32.5 nmol
3' Modification	NONE	Length	27 nt	Tm (50 mM NaCl)	60.8 °C		268.9 µg
No internal modifications				Tm (NN-Method)	53.5 °C	Volume for 100 µM	324.6 µl
5'-AGC TGT AAT AAA CAT AGA CCA AAC AAA-3'							
coli_ybbW_F				Oligo Id		4621772	
Type	DNA	Synthesis scale	Genomics	Molecular weight	6157.0 g/mol	Amount	7.67 OD
5' Modification	NONE	Purification	Desalted	Ext. coefficient	198.4 1/(mM·cm)		38.7 nmol
3' Modification	NONE	Length	20 nt	Tm (50 mM NaCl)	58.4 °C		238.1 µg
No internal modifications				Tm (NN-Method)	53.7 °C	Volume for 100 µM	386.7 µl
5'-TGA TTG GCA AAA TCT GGC CG-3'							
coli_ybbW_R				Oligo Id		4621773	
Type	DNA	Synthesis scale	Genomics	Molecular weight	6055.0 g/mol	Amount	6.17 OD
5' Modification	NONE	Purification	Desalted	Ext. coefficient	198.4 1/(mM·cm)		31.1 nmol
3' Modification	NONE	Length	20 nt	Tm (50 mM NaCl)	58.4 °C		188.4 µg
No internal modifications				Tm (NN-Method)	53.6 °C	Volume for 100 µM	311.1 µl
5'-GAA ATC GCC CAA ATC GCC AT-3'							

**Supplementary Figure 1:** Oligonucleotide order from Microsynth AG for the specific primers

**DbuCOI3F & DbuCOI3R are the specific primers for *D. polymorpha* and were taken from the research paper:**

De Ventura, Lukas & Kopp, Kirstin & Seppälä, Katri & Jokela, Jukka. (2017). Tracing the quagga mussel invasion along the Rhine river system using eDNA markers: Early detection and surveillance of invasive zebra and quagga mussels. *Management of Biological Invasions*.

In: <https://www.researchgate.net/publication/314083474> Tracing the quagga mussel invasion along the Rhine river system using eDNA markers Early detection and surveillance of invasive zebra and quagga mussels; page 105.

**Table 2. Target species: Zebra mussel.** (18.12.22)

**DvillosusF1 & DvillosusR\_OK are the specific primers for *D. villosus* and were taken from the research paper:**

Mauvisseau, Quentin & Troth, Chris & Young, Emily & Burian, Alfred & Sweet, Michael. (2019). The development of an eDNA based detection method for the invasive shrimp *Dikerogammarus haemobaphes*. *Management of Biological Invasions*.

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Of note, to find the reverse primer, the sequence found in the paper was read in 3' to 5' direction and transformed into its complementary strand.

**coli\_ybbW\_F & coli\_ybbW\_R are the specific primers for *E. coli* and were taken from the research paper:**

Walker, David & Mcquillan, Jonathan & Taiwo, Michael & Parks, Rachel & Stenton, Craig & Morgan, Hywel & Mowlem, Matthew & Lees, David. (2017). A highly specific *Escherichia coli* qPCR and its comparison with existing methods for environmental waters. *Water Research*.

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## 2. Data from preliminary qPCR experiment

### 2.1. *D. polymorpha*

**Supplementary Table 1:** C<sub>q</sub> values for *D. polymorpha* (Extraction Method 2 and negative controls)

Technical replicate	C <sub>q</sub> value [+/- 0.01]												NC2	NC6
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3		
T1	33.06		36.15	33.75	40.00	40.00	35.21	37.71	36.03	36.09	34.13	40.00		
T2	35.29	40.00	40.00	40.00	40.00	40.00	36.11	34.64	35.00	33.68	35.52	40.00		
T3	40.00	36.55	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00		
T4	40.00	40.00		40.00	35.07	40.00	40.00	35.82	40.00		40.00	40.00		
Sd. [+/- 0.01]	3.48	1.99	2.22	3.13	2.47	0.00	2.53	2.34	2.62	3.19	3.04	0.00		
Mean [+/- 0.001]	37.088	38.850	38.717	38.438	38.768	40.000	37.830	37.043	37.758	36.590	37.413	40.000		

**Supplementary Table 2:** C<sub>q</sub> values for *D. polymorpha* (Extraction Method 3 and negative controls)

Technical replicate	C <sub>q</sub> value [+/- 0.01]												NC3	NC7
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3		
T1	35.95	36.12				35.08						35.14		
T2	40.00	40.00	40.00		40.00		40.00	40.00		40.00	40.00	40.00		
T3	40.00	40.00	40.00	40.00		40.00	40.00	40.00	40.00	40.00	40.00	40.00		
T4			40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00				
Sd. [+/- 0.01]	2.34	2.24	0.00	0.00	0.00	2.84	0.00	0.00	0.00	0.00	0.00	2.81		
Mean [+/- 0.001]	38.650	38.707	40.000	40.000	40.000	38.367	40.000	40.000	40.000	40.000	40.000	38.380		

**Supplementary Table 3:** C<sub>q</sub> values for *D. polymorpha* for negative controls with ultrapure water (UW)

Technical replicate	C <sub>q</sub> value [+/- 0.01]							
	UW	UW	UW	UW	UW	UW	UW	UW
T1								
T2						40.00		
T3								
T4				36.11	40.00			

### 2.2. *D. villosus*

There was no qPCR amplification for all samples of *D. villosus* using both extraction methods.

### 2.3. *E. coli*

**Supplementary Table 4:** C<sub>q</sub> values for *E. coli* (Extraction Method 2 and negative controls)

Technical replicate	C <sub>q</sub> value [+/- 0.01]												NC2	NC6
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3		
T1	40.00	36.83	40.00		40.00		40.00	40.00	40.00	40.00	40.00			
T2		40.00												
T3			38.02									40.00		
T4											40.00			



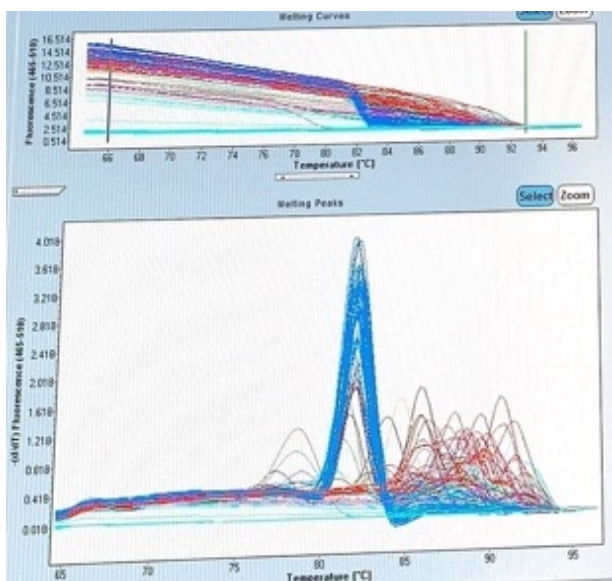
**Supplementary Table 5:** C<sub>q</sub> values for *E. coli* (Extraction Method 3 and negative controls)

Technical replicate	C <sub>q</sub> value [+/- 0.01]													
	S1, R1	S1, R2	S1, R3	S2, R1	S2, R2	S2, R3	S3, R1	S3, R2	S3, R3	S4, R1	S4, R2	S4, R3	NC3	NC7
T1														
T2														
T3										39.33		38.99		
T4							37.16			39.26				

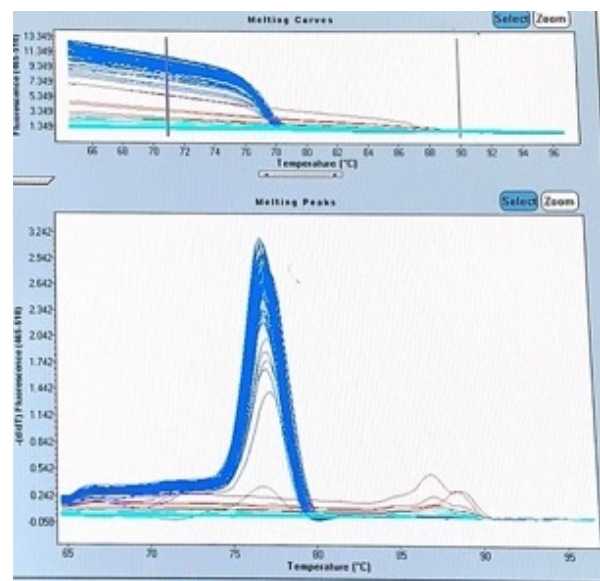
**Supplementary Table 6:** C<sub>q</sub> values for *E. coli* for negative controls with ultrapure water (UW)

Technical replicate	C <sub>q</sub> value [+/- 0.01]							
	UW	UW	UW	UW	UW	UW	UW	UW
T1								
T2								
T3								
T4							38.80	36.65

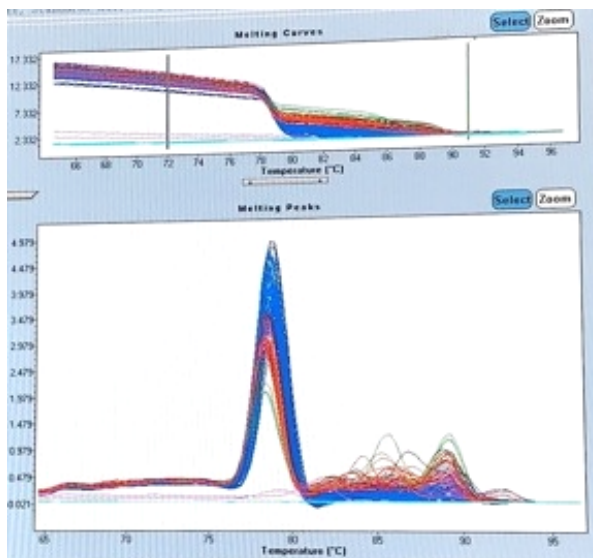
### 3. qPCR melting curves from qPCR experiment



**Supplementary Figure 2:** *E. coli* melting curve from qPCR



**Supplementary Figure 3:** *D. villosus* melting curve from qPCR



**Supplementary Figure 4:** *D. polymorpha* melting curve from qPCR