# Metagenomic Approaches to Develop Biological Control Strategies for Aquatic Invasive Species

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# Declaration of Authorship

I hereby affirm that this research work was written solely by me and that I have not previously submitted this work on another educational institution for the purpose of receiving an academic degree. In particular, contributions by other persons in this work have been appropriately cited and the data gathered through the methods described have been accurately reproduced.

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## Abstract:

This study focuses on the characterization of microbial communities associated with Eurasian watermilfoil (EWM) – an aquatic invasive species.

EWM samples were collected from three different sites at Cedar Lake and were analyzed by using a combination of culture-dependent and culture-independent approaches.

Bacterial identification using Next-Generation Illumina sequencing revealed a huge difference in the microbial community structure derived from the culture-based and metagenomic approaches. The dominant bacterial orders in cultivated samples were Aeromonadales, Pseudomonadales, Flavobacteriales, Enterobacteriales and Bacillales. In contrast, the dominant orders in metagenomic samples were Rickettsiales, Sphingomonadales, Burkholderiales and Rhodobacterales.

Furthermore, EWM were screened for waterborne human pathogens using selective cultivation approaches. No known pathogens were identified using Sanger sequencing of isolates.

The present study, however, provides insight into the structure of microbial communities associated with EWM. These results can help develope future biological control strategies against invasive species.

## Keywords:

Aquatic Invasive Species, Eurasian Watermilfoil, 16S rDNA, Next-Generation Sequencing

**Kurzzusammenfassung:**

Diese Arbeit beschäftigt sich hauptsächlich mit der Charakterisierung der mikrobiellen Gemeinschaft auf dem Ährigen Tausendblatt – einer aquatisch eingewanderten Art. Dazu wurden Proben des Ährigen Tausendblattes an drei verschiedenen Orten des Cedar Lakes gesammelt und durch die Kombination von kultivierungs-abhängigen und kultivierungsunabhängigen Verfahren untersucht.

Die Identifizierung von Bakterien mittels Illumina Sequenzierung zeigte einen großen Unterschied zwischen der kultivierungs-basierten und der metagenomischen Herangehensweise auf. Die dominierenden Bakterienordnungen in kultivierten Proben waren Aeromonadales, Pseudomonadales, Flavobacteriales, Enterobacteriales und Bacillales. Im Gegensatz dazu waren die dominierenden Ordnungen in den metagenomischen Proben Rickettsiales, Sphingomonadales, Burkholderiales und Rhodobacterales.

Außerdem wurde das Ährige Tausendblatt durch selektive Kultivierung auf wasserbürtige Humanpathogene untersucht. Es konnten keine bekannten Pathogene anhand von Sanger Sequenzierung identifiziert werden.

Diese Studie liefert dennoch einen Einblick in die Zusammensetzung von mikrobiellen Gemeinschaften, die mit dem Ährigen Tausendblatt in Verbindung gebracht werden können. Die Ergebnisse können zur Entwicklung von zukünftigen biologischen Steuerungsstrategien für invasive Arten beitragen.

**Stichworte:**

Aquatische Invasive Arten, Ähriges Tausendblatt, 16S rDNA, Next-Generation Sequencing

# List of Abbreviations

| | |
|---|---|
| *A*IA | *Aeromonas* Isolation Agar |
| AIS | Aquatic Invasive Species |
| *C*SA | *Campylobacter* Selective Agar |
| dd$H_2O$ | double-distilled water |
| DMSO | Dimethyl Sulfoxide |
| EMB Agar | Eosin Methylene Blue Agar |
| EWM | Eurasian Watermilfoil |
| ITS | Internal Transcribed Spacer |
| MEA | Malt Extract Agar |
| MHA | Mueller Hinton Agar |
| NA | Nutrient Agar |
| NB | Nutrient Broth |
| OTU | Operational Taxonomic Unit |
| PCA | Plate Count Agar |
| PDA | Potato Dextrose Agar |
| *P*IA | *Pseudomonas* Isolation Agar |
| R2A Agar | Reasoner's 2A Agar |
| RBA | Rose Bengal Agar |
| SD Agar | Sabouraud Dextrose Agar |
| *SS* Agar | *Salmonella Shigella* Agar |
| TSA | Tryptic Soy Agar |
| WA | Wort Agar |
| XLD Agar | Xylose Lysine Deoxycholate Agar |
| YPD Agar | Yeast Extract Peptone Dextrose Agar |

# Table of Contents

# 1 Background

## 1.1 Invasive Species

An invasive species is defined by Executive Order 13112 (1999) as 'an alien species whose introduction does or is likely to cause economic or environmental harm or harm to human health'. Invasive species are deemed a serious threat to native biodiversity, which is even greater than the overall threat posed by pollution, harvest and disease (McGinley, 2010). It is estimated that about half of the species in the United States that are in danger of extinction are endangered because of various impacts of invasive species (McGinley, 2010). For example, invading species acting as competitors or predators to local species can lead to drastic reductions in stock or even to extinction.

Invasive species may also have effects on biodiversity by altering habitats (McGinley, 2010). Such is the case with zebra mussels (*Dreissena polymorpha*) which were likely brought to the United States from Western Russia (McGinley, 2010). Due to their habitude to filter large quantities of water for nutritional use, they reduce the total available plankton serving as food for other animals and at the same time cause the growth of more aquatic vegetation resulting from the increased water clarity (MNDNR, 2015a).

In the case of a native species and an intruder being related, they may produce offsprings containing genes from both species (McGinley, 2010). This event is referred to as "hybridization". Especially, if the population of the invasive species has already exceeded that of the native species, following hybridizations can easily conduct in the replacement of the actual native genotype, causing the native species to become extinct.

Furthermore invasive species can vector diseases that can not only cause harm to local species, but also have serious impacts on human health (ISAC, 2008).

In addition, invading species negatively influence economy by restricting recreational and industrial use of land or waters as well as by decreasing property values (ISAC, 2008).

Minnesota which is known for its more than 11,000 lakes is especially threatened by invasion of non-native aquatic species. Up to date over 800 of Minnesota's water

bodies are known to be infested with at least one AIS accounting for about 7 % of the total count of water bodies, but this number is trending to increase as there are new lakes, rivers and wetlands being listed as infested every year (MNDNR, 2015c). In the year 2015 alone, 59 of Minnesota's water bodies have been added to the list of infested waters (MNDNR, 2015c).

## 1.2 Pathways for Spreading of Aquatic Invasive Species

AIS can spread quickly in new environments via animals, ships, commercial goods and equipment and through natural waterways (MISAC, 2009).

The primary pathway for the introduction and spread of aquatic invasive species is considered ships, which can transport a high density of species in their ballast water tanks, filled at the source port, and releasing them with the water at the destination port (Roman, 2010). Also, the intentional release of non-native fish species into new environments as food source or for the interest of sport fishing and the – deliberate or not – release from aquariums or commercial aquaculture are powerful pathways.

Natural waterways are pathways that allow the spreading of species without people's actions (MISAC, 2009), but might have been facilitated by humans constructing canals between previously isolated water systems (Roman, 2010).

The recreational use of water bodies makes an important contribution to the spread of invasive species as well: mussels like zebra mussels (*Dreissena polymorpha*) or aquatic plants like the Eurasian watermilfoil (*Myriophyllum spicatum*) can be transferred from infested lakes to other lakes by attaching to or getting entangled in boat parts or fishing equipment (MISAC, 2009).

The last-mentioned two species are ranked as severe threats by the Minnesota Invasive Species Advisory Council (MISAC) and have already strongly established in Minnesota. Together they are responsible for more than 550 infestations from the total of 820 infested waters (MNDNR, 2015c). It is consequently of crucial importance to gain control of AIS and to reduce their further spread.

## 1.3 Project Overview

Though different mechanical, chemical and biological management tools for AIS already exist, most of these approaches have been found to be controversial, i.e.

adversely affect the environmental health or simply are not effective enough. Nevertheless, biological control strategies, implying the use of parasitic or predatory organisms, bacteria, fungi or viruses seem to be the most promising approach in the battle against invasive species. One of the advantages of biocontrol agents is their high specificity towards a single species, which minimizes harmful impacts on nontarget species (Culliney, 2005). Furthermore, biocontrol agents distribute themselves and - once established - are self-sustaining and will adjust to changes in the density of the targeted species (Culliney, 2005). This makes the biological approach more cost effective compared to the other methods, as most costs only occur at the beginning of a program.

This work is part of a larger the project which aims to identify and develop microbiological agents for selective, cost-effective and rapid management strategies for the control of AIS in Minnesota waterways. Species targeted in the overall project include the Eurasian watermilfoil (*Myriophyllum spicatum*), zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*).

However, this study only focuses on EWM, since mussels were no longer available for analysis during the season.

## 1.4 Eurasian Watermilfoil

EWM is a submersed aquatic plant, native to Europe, Asia and northern Africa, that was first introduced to North America in the 1940s, most likely though the aquarium trade (Aiken et al., 1979). This plant, which is characterized by its feather-like looking leaves, can grow up to 7 meters from the lake bottom to the water surface where its stem branches, forming dense mats of vegetation (Aiken et al., 1979). These mats not only degrade the aesthetics of lakes and make recreational activities like swimming and boating difficult (Roley and Newman, 2008), but can also suppress the growth of native plants by reducing the available light intensity (Madsen et al., 1991). This negatively affects the floral biodiversity and can also change the chemical composition (Cross and McInerny, 2006). The competitive advantage of EWM over native aquatic plants is especially its ability to grow at low temperatures making their new seasons growth possible before that of native species (Aiken et al., 1979). Another advantage might be their ability to fix carbon through the utilization of bicarbonate (Grace and Wetzel, 1978).

EWM can reproduce by both sexual and asexual means, whereof the asexual reproduction through fragmentation is the more important one and is also responsible for the rapid spread of the plant in North America (Aiken et al., 1979). These fragments can be formed either by the plant itself or by human activities, like swimming or boating, which facilitates the spread even more.
The first line of defense against the establishment of EWM is preventing introductions, but it can be difficult to control the milfoil, once established (Roley and Newman, 2008).
In Minnesota, previous control strategies for the EWM included:
Mechanical control: Throughout the growing season of EWM, the plants get harvested regularly using a custom designed harvester (Melchior, 1997). However, this harvester can only remove about 2 meters of EWM making it necessary to harvest repeatedly. Another disadvantage of this method is that it is linked to a significant increase in the plants relative growth rate (Crowell et al., 1994).
Chemical control: EWM has been found to be susceptible to 2,4-dichlorophenoxyacetic acid, also referred to as 2,4-D, but the problem with the use of herbicides is the possible harm that can be caused to other native plant species and to the environment (Melchior, 1997). For this reason, the Environmental Protection Agency had set a limit to the use of 2,4-D which made it difficult to control watermilfoil due to not reaching desired treatment amounts.
Biological control: Usually, natural predators can be used for the management of invasive species, but bring the risk of introducing another exotic species (Melchior, 1997). For EWM a local weevil - *Euhrychiopsis lecontei* – worked out quite well. However, if this biological control strategy is combined with mechanical harvesting, most of the weevils get removed too.
In addition a fungi - *Mycoleptodiscus terrestris* - seemed promising in the use as a microbiological control agent, but it failed during field trials (Shearer et al., 2011).

As Roley and Newman (2008) predict that 18 -39 % of Minnesota's lakes will be infested by EWM in the future, there is clearly the need to develop new control strategies.

# 2 Microbial Community associated with Eurasian Watermilfoil

## 2.1 Introduction

### 2.1.1 Task Description and Objective

As previously mentioned, microorganisms have the potential to serve as effective biocontrol agents for aquatic invasive species. Their application, however, needs to reliably work in the field.

Chun et al.(2013) reported that some microbes have evolved to live in close association with macrophytes. In terms of toxin-producing bacteria or fungi with the ability to degrade plant components, these associations can also be pathogenic which might be useful for the development of microbiological control strategies.

The primary aim of this study was to characterize and understand the microbial community associated with EWM.

Previous research primarily focused on culture-dependent approaches, though it is believed that only about 1 % of microorganisms in environmental samples can be easily grown in the laboratory (Kaeberlein et al., 2002). Thus, a majority of microorganisms remains undetected.

In order to avoid this bias the community characterization in this study was achieved by amplicon-based Next Generation Illumina sequencing targeting the V4 region of the 16S rDNA. This variable region is commonly used for phylogenetic classifications.

However, also a cultivation-dependent approach was performed on the same samples for comparison purpose. To achieve this, different media were used to examine which media generated diversity profiles similar to that using culture independent approach.

In addition, fungi were cultivated on general media and identified by Sanger sequencing of the rDNA ITS2 region in order to characterize the fungal community associated with EWM.

### 2.1.2 Theoretical Background: Multiplexed Paired-End Illumina Sequencing

The Illumina sequencing platform works on the principle of sequencing-by-synthesis (Wang, 2016). In preparation for sequencing the gene of interest must be amplified using specific primers and appended with different index regions (for pooled samples) and with Illumina adapter regions.
The sequencing reaction itself proceeds in a flow cell which is a glass slide divided into microfluidic lanes covered with two types of oligonucleotide sequences (Wang, 2016). The Illumina adapter regions are complementary to these sequences allowing an immobilization of sequencing fragments on the flow cell. After immobilization the fragments are synthesized and the original strand is washed away through denaturation (Wang, 2016). The free end of the new strand can then fold over and bind to the second type of oligonucleotide sequence on the flow cell, forming a bridge, and is synthesized again (Wang, 2016). This process, called "bridge-amplification", is repeated several times resulting in clusters of identical copies of the templates in close proximity which are ready for parallel sequencing (Wang, 2016).
During sequencing, fluorescently labeled nucleotides with reversible terminators compete for incorporation and are detected by their fluorescence signals (Wang, 2016). After completion of the read the index regions are sequenced in order to assign sequencing results to the individual samples (Wang, 2016).
For paired-end Illumina sequencing both the forward and the reverse strand of a template are read in the same manner as described which allows for an even more precise alignment of reads. Data evaluation is performed using special bioinformatic platforms such as QIIME or mothur.

### 2.1.3 Theoretical Background Sanger Sequencing

Sanger sequencing, invented by Frederick Sanger, uses fluorescently labeled dideoxy terminators dyes to sequence DNA (Hartl and Ruvolo, 2011). The sequencing reaction is divided into four individual reactions, each containing all four types of nucleotides, whereof one is type is substituted by a dideoxynucleotide which can terminate the synthesis (Hartl and Ruvolo, 2011). In that way, each reaction yields in DNA fragments of different lengths, which are subsequently

separated by gel electrophoresis in a capillary tube. A detector reads the fluorescent label of each band that drops of the bottom of the capillary and generates a chromatogram of the fragments sequence (Hartl and Ruvolo, 2011).

## 2.2 Materials and Methods

### 2.2.1 Eurasian Watermilfoil Samples

All EWM samples analyzed in this study were collected from Cedar Lake which is located two miles east of Annandale in Wright County, Minnesota.
Cedar Lake was added to the list of infested waters in 2010, when EWM was first discovered (MNDNR, 2015b).
Milfoil samples were collected in November 2015 from three public accesses points on Cedar Lake.
EWM samples were processed previously by the supervisor as described below:
Microorganisms were elutriated from EWM by the use of a sterile ammonium pH 7 phosphate-gelatin solution and the supernatants were filtered through 5 µm filters and subsequently through 0.22 µm filters. The cells trapped on these filters were extracted again by agitation in pyrophosphate buffer. For the storage at -80 °C, 25 % glycerol was added.
In this report, samples from each Cedar Lake site have been referred to as MF1, MF2 and MF3.

### 2.2.2 Experimental Setup

Figure 1 shows the workflow for the assessment of bacteria and fungi associated with EWM.
All these steps have been addressed in more detail below.

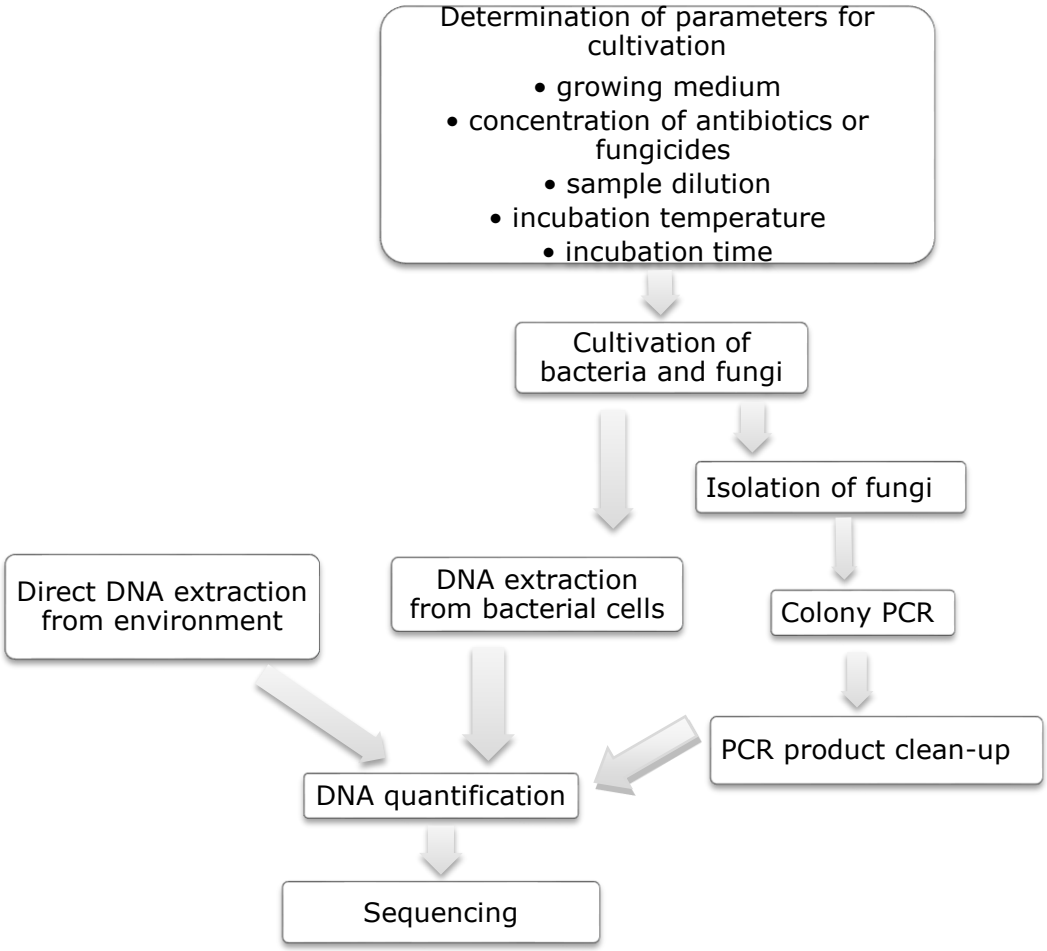


**Figure 1: Flowchart for the processing of EWM samples**

### 2.2.3 Selection of Media

In order to assess the greatest possible diversity of culturable bacteria, five different general media, namely Mueller Hinton Agar (MHA), Nutrient Agar (NA), Plate Count Agar (PCA), Reasoner's 2A (R2A) agar and Tryptic Soy Agar (TSA) were selected for the cultivation of the bacteria derived from EWM. The selection included nutrient low as well as nutrient rich media. NA and TSA which are both high in nutrients were additionally used in tenfold diluted versions, based on a publication of Azevedo et al. (2004). In this study, it was hypothesized that the non-cultivability of bacteria from environmental samples might be caused by a "state of shock" due to an oversupply of nutrients with respect to the conditions in the sampling site.

Cycloheximide was used to suppress fungal growth.

For the comprehensive cultivation of fungi the chosen media were Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouraud Dextrose (SD) agar, Wort Agar (WA) and Yeast Peptone Dextrose (YPD) agar which were all used in their original composition. Chloramphenicol was added to suppress bacterial growth. All mentioned media, their formulas and specifications can be found in Table 1.

**Table 1: Descriptions and compositions of general and universal growing media used for the cultivation of bacteria and fungi**

| **Medium** | **Composition per Liter** | **pH** | **Prepared from (Manufacturer; Reference number)** |
|---|---|---|---|
| MHA | 3.0 g infusion from beef<br>17.5 g casein acid hydrolysate<br>1.5 g starch<br>17.0 g agar | 7.3 ± 0.1 | Pre-mixed MHA (HiMedia Laboratories; M173) |
| NA | 3.0 g beef extract<br>5.0 g peptone<br>15.0 g agar | 6.8 ± 0.2 | Pre-mixed Difco™ NA (BD; 213000) |
| 1/10 Strength NA | 0.3 g beef extract<br>0.5 g peptone<br>15.0 g agar | 6.8 ± 0.2 | Difco™ NB (BD; 234000)<br>and<br>BBL™ Agar Granulated (BD; 11849) |
| PCA | 5.0 g pancreatic digest of casein<br>2.5 g yeast extract<br>1.0 g dextrose<br>15.0 g agar | 7.0 ± 0.2 | Pre-mixed Difco™ PCA (BD; 247940) |
| R2A Agar | 0.5 g yeast extract<br>0.5 g proteose peptone No. 3<br>0.5 g casamino acids<br>0.5 g dextrose<br>0.5 g soluble starch<br>0.3 g sodium pyruvate<br>0.3 g dipotassium phosphate<br>0.05 g magnesium sulfate<br>15.0 g agar | 7.2 ± 0.2 | Pre-mixed Difco™ R2A Agar (BD; 218263) |
| TSA | 15.0 g pancreatic digest of casein<br>5.0 g papaic digest of soybean<br>5.0 g sodium chloride<br>15.0 g agar | 7.3 ± 0.2 | Pre-mixed Difco™ TSA (BD; 263920) |
| 1/10 Strength TSA | 1.5 g pancreatic digest of casein<br>0.5 g papaic digest of soybean<br>0.5 g sodium chloride<br>15.0 g agar | 7.3 ± 0.2 | Pre-mixed Difco™ TSA (BD; 263920)<br>and<br>BBL™ Agar Granulated (BD; 11849) |
| MEA | 30.0 g malt extract<br>5.0 g mycological peptone<br>15.0 g agar | 5.4 ± 0.2 | Pre-mixed MEA (Sigma-Aldrich; 70145) |
| PDA | 4.0 g potato extract<br>20.0 g dextrose<br>15.0 g agar | 5.6 ± 0.2 | Pre-mixed PDA (Sigma-Aldrich; 70139) |

| Medium | Composition per Liter | pH | Prepared from (Manufacturer; Reference number) |
|---|---|---|---|
| SD Agar | 10.0 g mycological peptone<br>20.0 g dextrose<br>15.0 g agar | 5.6 ± 0.2 | Prepared from SD Broth (Sigma-Aldrich; S3306)<br>and<br>BBL™ Agar Granulated (BD; 11849) |
| WA | 15.0 g malt extract<br>1.0 g peptone (from casein)<br>12.5 g D-Maltose<br>2.5 g dextrin<br>1.0 g dipotassium hydrogenphosphate<br>1.0 g ammonium chloride<br>17.0 g agar<br>2.35 g glycerol | 4.8 ± 0.2 | Prepared from WA (Sigma-Aldrich; 70196)<br>and<br>Glycerine Lab Grade (Fisher Science; S25342B) |
| YPD Agar | 20.0 g bacteriological peptone<br>10.0 g yeast extract<br>20.0 g glucose<br>15.0 g agar | 6.5 ± 0.2 | Pre-mixed YPD Agar (Sigma-Aldrich; Y1500) |

### 2.2.4 Preparation of Media

Equipments and materials for the preparation of the diverse growing media are listed in Table 2.

**Table 2: Equipments and stock-solutions used for the preparation of nutrient media**

| Description | Manufacturer | Specifications |
|---|---|---|
| Mettler PM460 DeltaRange Balance | Mettler-Toledo, Colombus, OH 43240 | Capacity: 410 g/60 g<br>Readability: 0.01 g/0.001 g |
| Thermolyne Nuova II (S182525) Magnetic Stirrer | Barnstead International, Dubuque, Iowa 52001 | 120 V, 60 Hz, 0.3 A, 25 W,<br>Speed: 100-1000 rpm |
| Corning pH meter 430 | Corning Incorporated , Corning, NY 14831 | pH 0.00 to 14.00<br>Temp. 0 to 100 °C<br>Accuracy: pH ± 0.01 |
| Corning PC-320 Hot Plate Stirrer | Corning Incorporated , Corning, NY 14831 | 60-1100 rpm<br>25-500 °C |
| Amsco Lab 250 Steam Sterilizer | STERIS Corporation, Mentor, OH 44060 | |
| **Description** | **Specifications** | |
| Cycloheximide/DMSO Stock Solution (Fungicide) | c = 0.1 g/mL; prepared from Cycloheximide, 95 % (Acros Organics; 357420050) and Dimethyl sulfoxide, RegentPlus®, ≥ 99.5 % (Sigma-Aldrich, D5879) -> sterilized by filtering through 0.2 µm membrane filters (Pall Corporation; PN 4192) | |
| Chloramphenicol Stock Solution (Antibiotic) | c = 0.05 g/mL; prepared from Chloramphenicol, ≥98 % (Sigma-Aldrich; C0378) and Ethanol 190 proof, 95 % (Decon Laboratories, Inc.; 2805) | |

Pre-mixed media or individual ingredients as listed in Table 1 were weighed out for the desired amount and dissolved in the corresponding volume of $ddH_2O$ using a heated magnetic stirrer plate. The pH was measured and adjusted to the required value with a few drops of either saturated NaOH or 10 % HCl. All prepared media were autoclaved at 121 °C for 30 minutes (based on STERIS' recommended liquid cycle parameters) and subsequently put in the water bath to bring the temperature to about 55 °C. Once this temperature was reached the according volume of fungicide or antibiotic stock-solutions for the desired end-concentration was added with a pipette. The media were gently swiveled to avoid bubbling and poured into sterile petri dishes. For the solidification of the agar the petri dishes were allowed to cool for at least two hours.
Finished petri dishes were stored upside down at 4 °C until use.

### 2.2.5 Optimization of Cultivation Conditions

This step aimed at the selection of appropriate conditions for an optimal growth of bacterial/fungal species on the agar plates.
For this study's objective an optimal growth on plates was considered when there was a rather dense growth, but no merging of individual colonies. Depending on the size of the colonies the targeted number of bacterial colonies per plate was therefore about 3000 to 5000 which should ensure the traceability of bacteria available in low abundance.
Contrarily, for the larger fungi a number of about 50 colonies per plate was targeted.

Bacteria:
To cover a range from low to high nutritious media R2A agar, TSA and NA were used for the advance test. According to orderable media containing fungicides the mentioned media were supplemented with Cycloheximide/DMSO in a concentration of 50 mg/L.
Triplicates of MF1, MF2 and MF3 were spread on these plates in undiluted form as well as in 1:10 and 1:100 dilutions respectively. Per plate 100 µL of sample were applied and 15 to 20 autoclaved 3 mm glass beads (11-312A, Fisher Scientific, USA) were used for spreading to ensure an even and fast distribution. The plates

were incubated at high temperature (35 °C), medium temperature (28 °C) and at room temperature (~ 22 °C) for one day.
After incubation the growth was interpreted for the setup of a second test. This interpretation can be found in the results section 3.1.1.

The second experiment aimed to check the newly considered sample dilutions and the higher fungicide concentration in the media as well as to compare the bacterial growth on full and half strength media.
For this, NA and TSA media were selected - the composition of their half strength versions were 1.5 g beef extract, 2.5 g peptone and 15.0 g agar per liter for NA and 7.5 g pancreatic digest of casein, 2.5 g papaic digest of soybean, 2.5 g sodium chloride and 15.0 g agar per liter for TSA. Both versions of the media were supplemented with both 50 mg and 400 mg Cycloheximide/DMSO per liter.
Again 100 µL of the milfoil samples were spread with 3 mm glass beads, whereas MF1 was spread in 1:400 dilution and MF2 and MF3 were spread 1:50 diluted.
As this experiment was conducted for comparison purposes, all plates were only incubated at 30 °C for one day. However, the main cultivation was conducted at 20 °C, 25 °C, 30 °C and 35 °C.
After incubation the growth was interpreted again (see 3.1.2).

Fungi:
The cultivation parameters for fungi were tested on all selected media – MEA, PDA, SD agar, WA and YPD agar. A concentration of 50 mg Chloramphenicol per liter medium was used to suppress bacterial growth.
Respectively 100 µL of the milfoil samples MF1, MF2 and MF3 were spread on the media in different dilutions (undiluted, 1:10 and 1:100 diluted) with glass beads. The incubation was conducted at four different temperatures (20 °C; 25 °C, 30 °C and 35 °C) with humidity of 50 % and was planned for a maximum of seven days. The growth on the plates was checked every day. An interpretation of the growth results can be found in paragraph 3.1.3.

### 2.2.6 Cultivation

The final parameters for the cultivation of bacteria were as described below:

- Seven different media as described in 2.2.3

- A fungicide (Cycloheximide/DMSO) concentration of 50 mg per liter media
- Sample dilutions of 1:400 for MF1 and 1:50 for each MF2 and MF3

Spreading of the samples in triplicates was conducted in the same way as described in 2.2.5.

A total of four different incubation temperatures (20 °C, 25 °C 30 °C and 35 °C) was used in order to imitate the temperature range of the Cedar Lake throughout the growing period of the EWM.
Despite the fast bacterial growth the incubation time was prolonged to three days, so that slow growing bacteria could also be gathered in the sequencing results.

The variety of different parameters and the preparation of triplicates yielded in a total count of 252 plates.

The parameters for optimized fungal growth were:

- Five different media as described in 2.2.3
- A concentration of 50 mg antibiotic (Chloramphenicol) per liter media
- Undiluted samples for spreading
- Incubation temperatures: 15 °C, 20 °C, 25 °C and 30 °C at 50 % humidity
- An incubation time of five days

Because this part of the experiment was carried out at a later point of the study, time limitations did not allow the assessment of fungi depending on sample site. Therefore mixed samples consisting of equal parts undiluted MF1, MF2 and MF3 were used for spreading which was performed in triplicates as described in 2.2.5. All other parameters remained the same.

A description of the growth results of the 252 bacteria plates and the 60 fungi plates can be found in 3.1.4.
Due to arisen mold contaminations and time limitations it was not possible to submit fungal samples for Illumina sequencing. However, Sanger sequencing was performed.

## 2.3 Illumina Sequencing of Bacterial Samples

### 2.3.1 Preparation of Bacterial Colonies for DNA Extraction

All bacterial colonies had to be skimmed from the agar surface in order to convert them into a suitable condition for DNA extraction.
For this step, the agar surfaces of all plates were wetted with 4 mL of autoclaved dd$H_2O$ respectively and colonies were dissolved in the water using sterile plastic loops. The suspensions were aspirated with a pipette and transferred into sterile 15 mL centrifuge tubes, whereby the suspensions of triplicates were jointed into one single tube giving a total number of 84 samples. For a better overview the labeling and source of these samples can be found in Table 3.
A volume of 1.8 mL of the well mixed suspension of each tube was transferred to autoclaved 2 mL spin tubes and centrifuged at 10,000 x g for 16 minutes. The supernatant was decanted and the pellet was used for the extraction of the DNA.

**Table 3: Overview of parameters used for the cultivation of the plates for Illumina sequencing**

| Media | Sampling Site | Incubation Temperature [°C] |
|---|---|---|
| TSA | 3 | 20 |
| NA | | |
| PCA | | |
| R2A | | |
| MHA | | |
| 1:10 TSA | | |
| 1:10 NA | | |
| NA | | 25 |
| PCA | | |
| MHA | | |
| R2A | | |
| 1:10 NA | | |
| TSA | | |
| 1:10 TSA | | |
| MHA | | 30 |
| TSA | | |
| NA | | |
| R2A | | |
| 1:10 NA | | |
| 1:10 TSA | | |
| PCA | | |
| 1:10 TSA | | 35 |
| NA | | |
| 1:10 NA | | |
| R2A | | |
| PCA | | |
| TSA | | |
| MHA | | |
| MHA | 1 | 20 |
| R2A | | |
| 1.10 TSA | | |
| 1:10 NA | | |
| TSA | | |
| NA | | |
| PCA | | |
| 1:10 NA | | 25 |
| NA | | |
| TSA | | |
| R2A | | |
| PCA | | |
| MHA | | |
| 1:10 TSA | | |
| 1:10 TSA | | 30 |
| 1:10 NA | | |
| NA | | |
| TSA | | |
| MHA | | |
| PCA | | |
| R2A | | |

| Media | Sampling Site | Incubation Temperature [°C] |
|---|---|---|
| TSA | 1 | 35 |
| NA | | |
| R2A | | |
| MHA | | |
| PCA | | |
| 1:10 NA | | |
| 1:10 TSA | | |
| 1:10 NA | 2 | 20 |
| PCA | | |
| TSA | | |
| MHA | | |
| 1:10 TSA | | |
| R2A | | |
| NA | | |
| 1:10 NA | | 25 |
| NA | | |
| TSA | | |
| PCA | | |
| 1:10 TSA | | |
| R2A | | |
| MHA | | |
| MHA | | 30 |
| NA | | |
| 1:10 TSA | | |
| R2A | | |
| TSA | | |
| PCA | | |
| 1:10 NA | | |
| PCA | | 35 |
| 1:10 NA | | |
| MHA | | |
| R2A | | |
| 1:10 TSA | | |
| TSA | | |
| NA | | |

### 2.3.2 DNA Extraction and Quantification

For the extraction of the DNA from bacterial cells the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.; 12888-100) was used. DNA extraction of each pellet was performed according to the manufacturer's instructions.

The extracted bacterial DNA was prepared for quantification with the Quibit® dsDNA HS Assay Kit (Q32854, Thermo Fisher Scientific, USA). A mastermix of Quibit working solution was prepared using 198 µL of Quibit buffer and 2 µL of Quibit 200x reagent per sample and aliquoted to 198 µL into Quibit assay tubes. A volume of 2 µL of DNA sample was added per tube and mixed with the working

solution by gentle shaking. The DNA concentration was measured with the Qubit® 2.0 Fluorometer (Q32866, Thermo Fisher Scientific, USA) and documented.
The DNA extracts were stored at -20 °C till further analysis.

The same procedures were followed for samples directly obtained from milfoil.

### 2.3.3 Illumina Sequencing

The DNA samples were submitted for sequencing in a 96-well-plate. A total volume of 15 µL per DNA extract was loaded into each well. MF1, MF2 and MF3 were loaded in triplicates.
The DNA library was prepared by employees of the University of Minnesota Genomics Center: The V4 region of the 16S rRNA gene was amplified using region-specific primers and tagged with individual barcode sequences. The amplicons were paired-end sequenced at a read length of 300 bp on an Illumina/Solexa Sequencer (MiSeq).

## 2.4 Sanger Sequencing of Fungal Samples

### 2.4.1 Isolation of Fungi

For the isolation of fungi from the contaminated plates Rose Bengal Chloramphenicol Agar (RBA) was used because it 'suppresses the development of bacteria and restricts the size and the spreading of mould colonies' (Sigma-Aldrich).
Rose bengal medium was prepared as described in 2.2.4 following Sigma-Aldrichs' recipe using 5.0 g of select soytone (212488, BD, USA), 10.0 g of dextrose (DX0145-1, EM Science, USA), 1.0 g of potassium phosphate (P-0662, Sigma-Aldrich, USA), 0.5 g of magnesium sulfate (2504, J.T. Baker, USA), 0.05 g of rose bengal (R-3877, Sigma-Aldrich, USA), 15.5 g of BBL™ granulated agar (11849, BD, USA) and 0.05 g of Chloramphenicol per Liter of $ddH_2O$.
The prepared rose bengal agar plates were divided into four to six parts and fungal colonies from the contaminated plates were transferred to them using autoclaved toothpicks. In doing so only colonies that have not been totally overgrown by mold were picked and care has been taken to avoid touching mold hypheae.

All plates were incubated at 25 °C and 50 % humidity for three days. In order to make sure to get rid of all the contamination the fungal isolates were transferred to new rose bengal plates for three times in total.
Morphology of the isolates was documented over the whole period and only colonies that did not change their appearance after transferring and showed no evidence of mold formation were considered as pure.

### 2.4.2 Colony PCR

A total number of 68 fungal isolates was picked for colony PCR.
In order to avoid an overload of DNA in the PCR reaction all selected colonies were diluted in 30 µL of $ddH_2O$ beforehand using a pipette tip for transfer.
Each actual PCR was carried out on a total volume of 50 µL, using 5 µL of 10x standard Taq reaction buffer (B9014S, New England BioLabs, USA), 1 µL of 10 mM dNTPs (77102; 77104; 77106; 77108, affymetrx USB, USA), 1 µL of forward primer (IDT, USA), 1 µL of reverse primer (IDT, USA), .25 µL of Taq polymerase (M0273S, New England BioLabs, USA), 40,75 µL of nuclease-free water (AM9937, Ambion, USA) and 1 µL of the diluted colonies. Specifications of the primers can be found in Table 4.

**Table 4: Primer sets employed for PCR amplification**

| Target | Primer | Sequence | Concentration [pmol/µL] |
|---|---|---|---|
| ITS2 region of rDNA | Forward (5.8SR) | 5'-TCGATGAAGAACGCAGCG-3' | 31.3 |
| | Reverse (ITS4) | 5'-TCCTCCGCTTATTGATATGC-3' | 37.4 |

For the negative controls the 1 µL of diluted colonies was substituted with nuclease-free water.
The amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA) starting with 10 minutes at 95 °C to ensure opening of the cells, followed by 30 cycles of 30 seconds denaturation at 95 °C, 45 seconds annealing at 55 °C, 90 seconds extension at 72 °C and a final extension step at 72 °C for 10 minutes.

### 2.4.3 Gel Electrophoresis

All PCR products were verified through agarose gel electrophoresis.
For this, each 0.8 % agarose gel consisting of 0.8 g agarose per 100 mL of 1x TAE buffer was prepared by mixing of both the components and bringing them to boil in the microwave. After a short cooling period a final concentration of 5 µg/mL ethidium bromide was added and the solution was poured into a casting tray. After about 30 minutes the gel electrophoresis unit was filled with 1x TAE buffer and the gel was ready for loading.
For this, 2 µL of 6x gel loading dye (B7021S, New England BioLabs, USA) were added to 10 µL of each PCR product and to the negative sample respectively and transferred into the gel lanes. As reference 7 µL of a 100 bp DNA ladder (N0467S, New England BioLabs, USA) were added in the outermost lanes.
The gels were run for about 90 minutes at 130 V and DNA fragments were subsequently visualized using UV imaging.
Of all successfully amplified PCR products 48 fungal amplicons were selected for Sanger sequencing based on the morphology of the originating colony.
The chosen amplicons were purified by using the UltraClean® Clean-Up Kit (12500-250, MO BIO Laboratories Inc., USA) in accordance with the manufacturers' instructions.
The DNA concentration of the clean-up products was subsequently measured as described in 2.3.2.

### 2.4.4 Sanger Sequencing

The purified amplicons were submitted for High Throughput Sanger sequencing to the University of Minnesota Genomics Center in 96-well-plates.
Each well was filled with a total of 6 µL of sample consisting of 3 µL of forward primer as template and purified amplicon to reach the requested amount of approximately 10-20 ng.

# 3 Results and Discussion

## 3.1 Interpretation of Growth

### 3.1.1 Preliminary Analysis of Bacterial Growth

After the incubation of the plates growth of yellow and white colonies was present on all plates. However, there seemed to be less growth on the plates which were incubated at medium and low temperature.

The merging of colonies occurred on all plates of MF1, but only on the undiluted and 1:10 dilution plates of MF2 and MF3. The 1:100 MF2 and MF3 plates were not countable, but in order to reach the 3000 – 5000 colonies target growth could have been denser. Hence, a 1:400 dilution for MF1 and a 1:50 dilution for MF2 and MF3 were considered for the second advance test.

Regarding the concentration of fungicide in the media, there was no obvious fungal growth on neither of the plates. However, on three of them colonies had a fungi-like appearance with unequally hypheae-like margins, but it was not clear if these colonies were fungi as some bacterial species can also grow in hypheae-like formations.

A Cycloheximide/DMSO concentration of as high as 400 mg/L was considered for testing in a second test.

### 3.1.2 Second Analysis of Bacterial Growth

After the incubation growth could be observed on all plates in the desired extent of 3000 – 5000 colonies.

The fungi-like colonies also appeared on some of the plates that were supplemented with 400 mg of fungicide, suggesting that the colonies were either Cycloheximide-resistant fungi or just bacteria with fungi-like growth characteristics. For this reason, the fungicide concentration for the main cultivation approach was kept at 50 mg/L.

There was almost no difference between the growth on full and half strength media, but the colonies seemed to be bigger in size on full strength media.

Nevertheless, for the main cultivation approach a further dilution of NA and TSA to 1/10 was considered.

### 3.1.3 Preliminary Analysis of Fungal Growth

Fungal growth started on all plates on day three and incubation was stopped and on day five. Only the undiluted samples yielded in the targeted growth of about 50 fungi per plate, whereas the underpopulation of just 1-10 fungi on the 1:10 and 1:100 plates, despite the Chloramphenicol, left enough space for severe bacterial growth. Nevertheless, some bacteria also grew on the plates of the undiluted samples, but this number remained within an acceptable degree.
Overall, a difference in the size and color spectrum of the fungal colonies could be observed between the individual media, with the largest colonies being found on WA and on MEA. The incubation temperatures also had a noticeable impact on the abundance of some fungi. However there was effectively no difference between the 30 °C and 35 °C plates and it was therefore considered to replace the 35 °C incubation temperature with a lower temperature for the main cultivation.

### 3.1.4 Cultivation with Defined Parameters

The parameters worked very well for bacteria - all 252 plates had growth in the targeted colony number. As in the preliminary analysis the low nutrient media like R2A and the 1/10 diluted NA and TSA grew much smaller colonies than the other media throughout all sites. On the contrary to the preliminary analysis the number of colonies on low temperature plates was as high as on plates incubated at higher temperatures which might have been caused by the longer incubation time of three days. Furthermore, the low temperatures resembled the conditions in the sampling site more than the higher temperatures.
Nevertheless, a morphologic comparison of different media and sites was not possible as colonies appeared in the same mix of yellow and white on all media.

Unfortunately fungal growth did not work out as expected, since most colonies were overgrown by cottony mold within the fourth day of incubation. The dimension of this contamination can be seen in figure 3. Still it was possible to get an idea of the growth when looking at the bottom of the plates. As can be seen

from figure 4 the black colonies decreased with raising incubation temperatures. Reddish fungi only appeared at 25 and 30 °C. Especially MEA and WA were contaminated with mold, whereas YPD agar harbored the largest fungi. No correlation between media components and size of fungi could be observed. However, as MEA and WA were the only media that contained malt extract, it seems that molds were able to metabolize this substrate faster than the other fungi which caused the contamination.
Nevertheless, it was not possible to prepare the fungi plates for Illumina sequencing.

### 3.1.5 Growth of Fungal Isolates on Rose Bengal Medium

The rose bengal dye contained in the medium was able to suppress the growth of contaminating mold. After three steps of transferring the isolates to new media, the molds were almost completely gone and distinct fungal colonies which could be picked for colony PCR were recognizable again. A comparison between the fungal growth on the general media and the growth of the isolates can be seen in Figure 2.

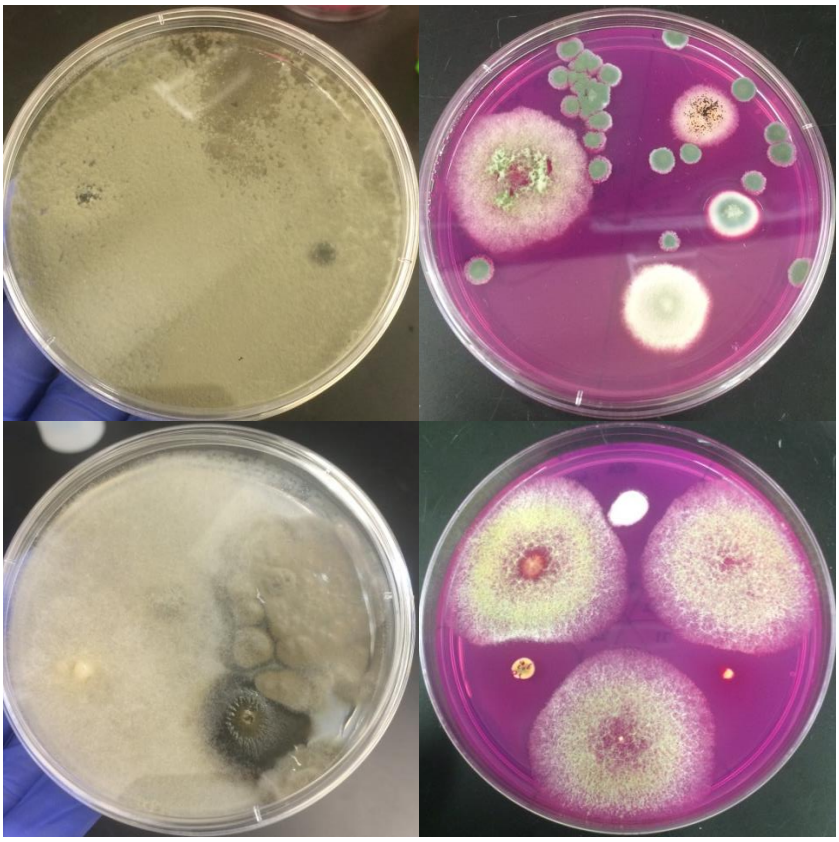

**Figure 2: Completely overgrown MEA plates due to mold contamination (left) and isolated fungal colonies on Rose Bengal Chloramphenicol Media (right)**

Overall, the diversity in the morphology of the individual fungal colonies seemed high, but as most colonies appeared in shades of green it was hard to distinguish whether colonies originated from the same fungus or not. Furthermore, some sporadic yellowish, red and orange colonies could be observed on the medium.

## 3.2 Verification of Fungal PCR Products

The amplification of the 300 - 400 bp long ITS2 region worked for about 75 % of the samples. An example of an agarose gel UV image can be seen in Figure 3.


**Figure 3: UV image of an agarose gel with bands of the amplified fungal ITS2 rDNA region: due to lower DNA concentrations the bands appear weak; some primer dimers can be seen underneath**

## 3.3 Illumina Sequencing

### 3.3.1 Evaluation of Sequencing Data

A total of 17,831,012 amplicon sequences were processed by the supervisor using the QIIME pipeline. 80.7 % of these sequences were high quality reads with a quality score of more than 30.

Sequences with at least 97 % similarity were combined into OTUs and OTU diversity from each sample was calculated. As can be seen in the resulting Figure 4 and Figure 5 the metagenomic samples were significantly more rich and more diverse than the corresponding cultivated samples.

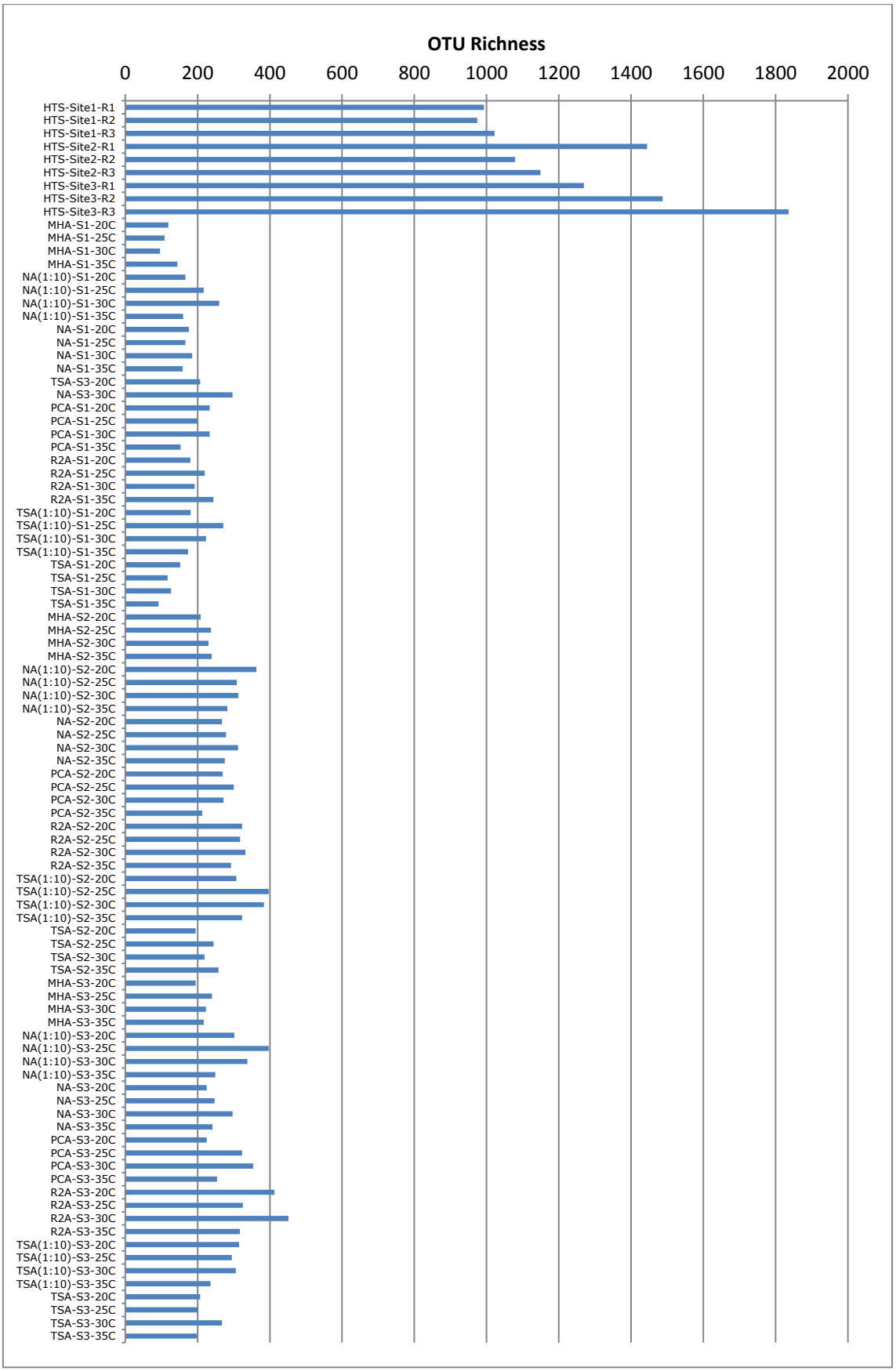


**Figure 4: OTU richness of microbial samples derived from EWM;**
HTS: High Throughput Sequencing, R1: Replicate 1, R2: Replicate 2, R3: Replicate 3, S1: Site 1, S2: Site 2, S3: Site 3, 20C: 20 °C, 25C: 25 °C, 30C: 30 °C, 35C: 35 °C, MHA: Mueller Hinton Agar, NA: Nutrient Agar, TSA: Tryptic Soy Agar, PCA: Plate Count Agar, R2A: Reasoner's 2A Agar

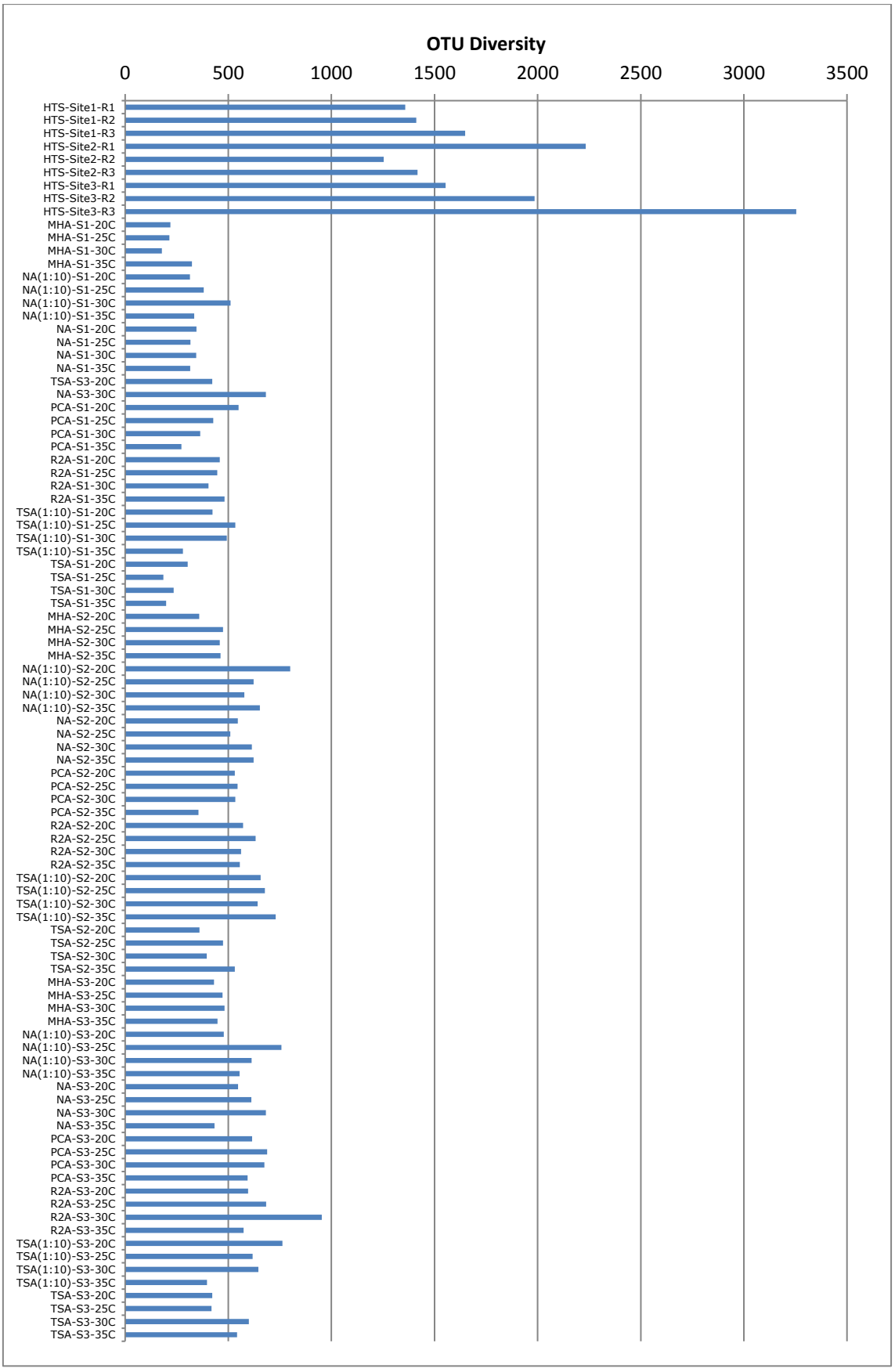


**Figure 5: OTU diversity of microbial samples derived from EWM**
HTS: High Throughput Sequencing, R1: Replicate 1, R2: Replicate 2, R3: Replicate 3, S1: Site 1, S2: Site 2, S3: Site 3, 20C: 20 °C, 25C: 25 °C, 30C: 30 °C, 35C: 35 °C, MHA: Mueller Hinton Agar, NA: Nutrient Agar, TSA: Tryptic Soy Agar, PCA: Plate Count Agar, R2A: Reasoner's 2A Agar

### 3.3.2 Microbial Community Analysis

A chart of the microbial community structure from the high throughput sequencing analysis of the metagenomic approach as well as of the cultivation approach can be found in Figure 6 and Figure 7.

The metagenomic microbial community structure on order level from site 1 was dominated by Rickettsiales ranging from 12 to 39 %, followed by Burkholderiales with 8-10% and Sphingomonadales with 4-13 %. The major orders of the metagenomic approach from site 2 and site 3 were Burkholderiales with 6-13 % and 6-9 %, Rhodobacterales with 9-22 % and 7-9 % and Sphingomonadales with 5-11 % and 6-11 % respectively. About 10 % of the bacterial sequences derived from each site were either unclassified or represented with less than 0.1 %.

The microbial community of the cultivation approach from site 1 consisted mainly of Aeromonadales throughout all media. Although the relative abundance of Aeromonadales ranged from a minimum of 31 % to a maximum of 91 % no obvious correlation between incubation temperatures and no media preference could be found. Furthermore, 3-35 % were Pseudomonadales, 0.1-38 % were Flavobacteriales, 0.5-7 % were Enterobacteriales and 0.2-6% were Bacillales.

The composition of cultivable bacteria of site 2 was similar to site 1 with 21-52 % Aeromonadales, 11-38 % Pseudomonadales, 1-57 % Flavobacteriales and 0.5-5 % Enterobacteriales. The relative abundance of Bacillales was a bit higher ranging from 1 to 19 %. In contrast to site 1, some Burkholderiales, Caulobacterales and Neisseriales were present ranging from 1-12 %, 0.01-9 % and 0.1-10 % respectively. Again, the fluctuations within the relative abundances of these orders seemed rather random.

The cultivable microbial community of site 3 differs even more from those of site 1 and site 2: Aeromonadales were present in a much lower abundance, ranging from a minimum of 5 % to a maximum of only 32 %. However, Enterobacteriales were present in a relatively high abundance ranging from 2-62 %. Furthermore, 4-42 % were Pseudomonadales and 3-40 % were Flavobacteriales, which is higher

compared to site 1 and site 2. Bacillales, Burkholderiales, Caulobacterales and Neisseriales ranged from 1-30 %, 0.1-18 %, 0.02-20 % and 0.5-7 % respectively.

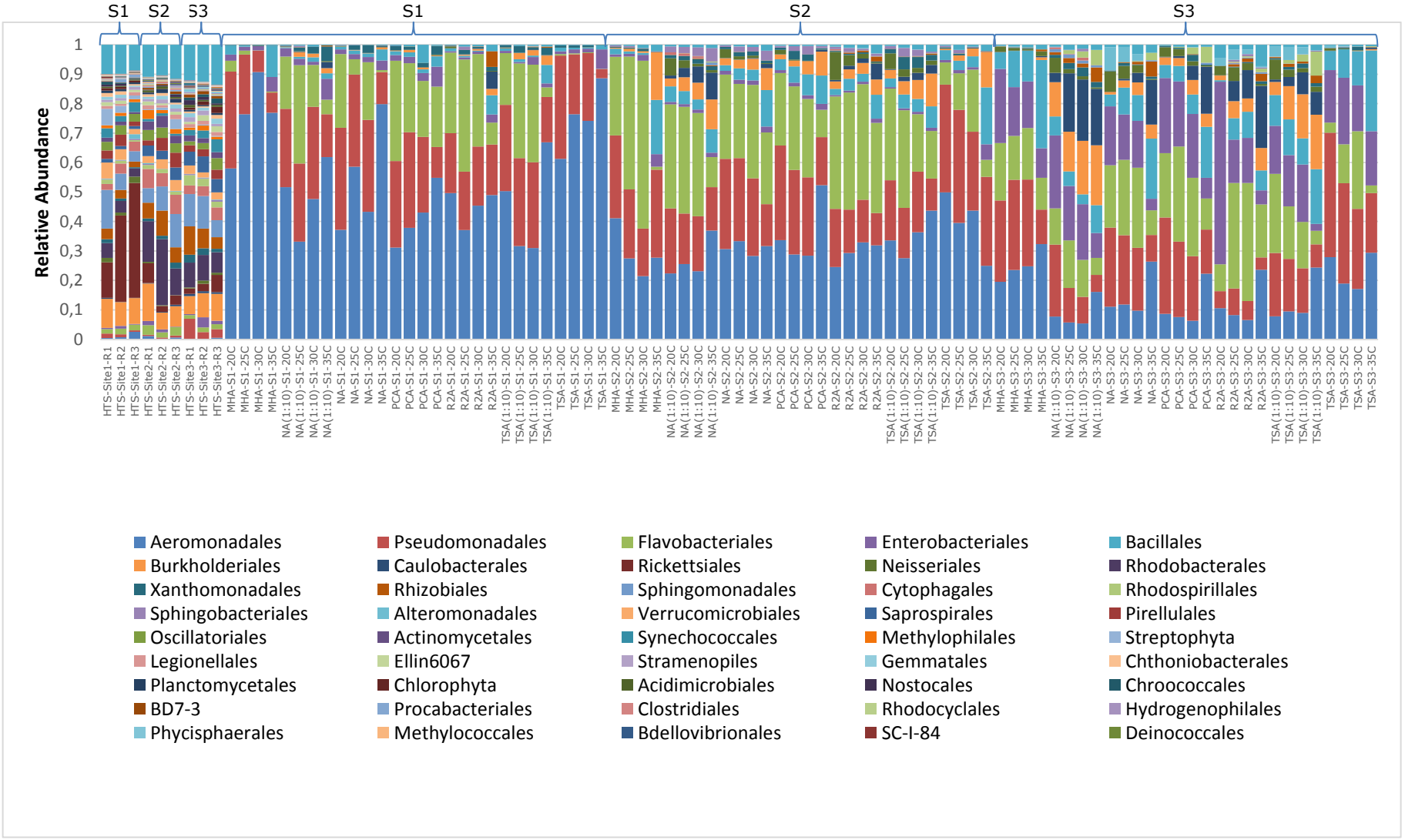


**Figure 6: Relative abundance of bacterial communities based on order level in EWM from metagenomic and from cultivated approach arranged by sampling site;**
HTS: High Throughput Sequencing, R1: Replicate 1, R2: Replicate 2, R3: Replicate 3, S1: Site 1, S2: Site 2, S3: Site 3, 20C: 20 °C, 25C: 25 °C, 30C: 30 °C, 35C: 35 °C, MHA: Mueller Hinton Agar, NA: Nutrient Agar, TSA: Tryptic Soy Agar, PCA: Plate Count Agar, R2A: Reasoner's 2A Agar

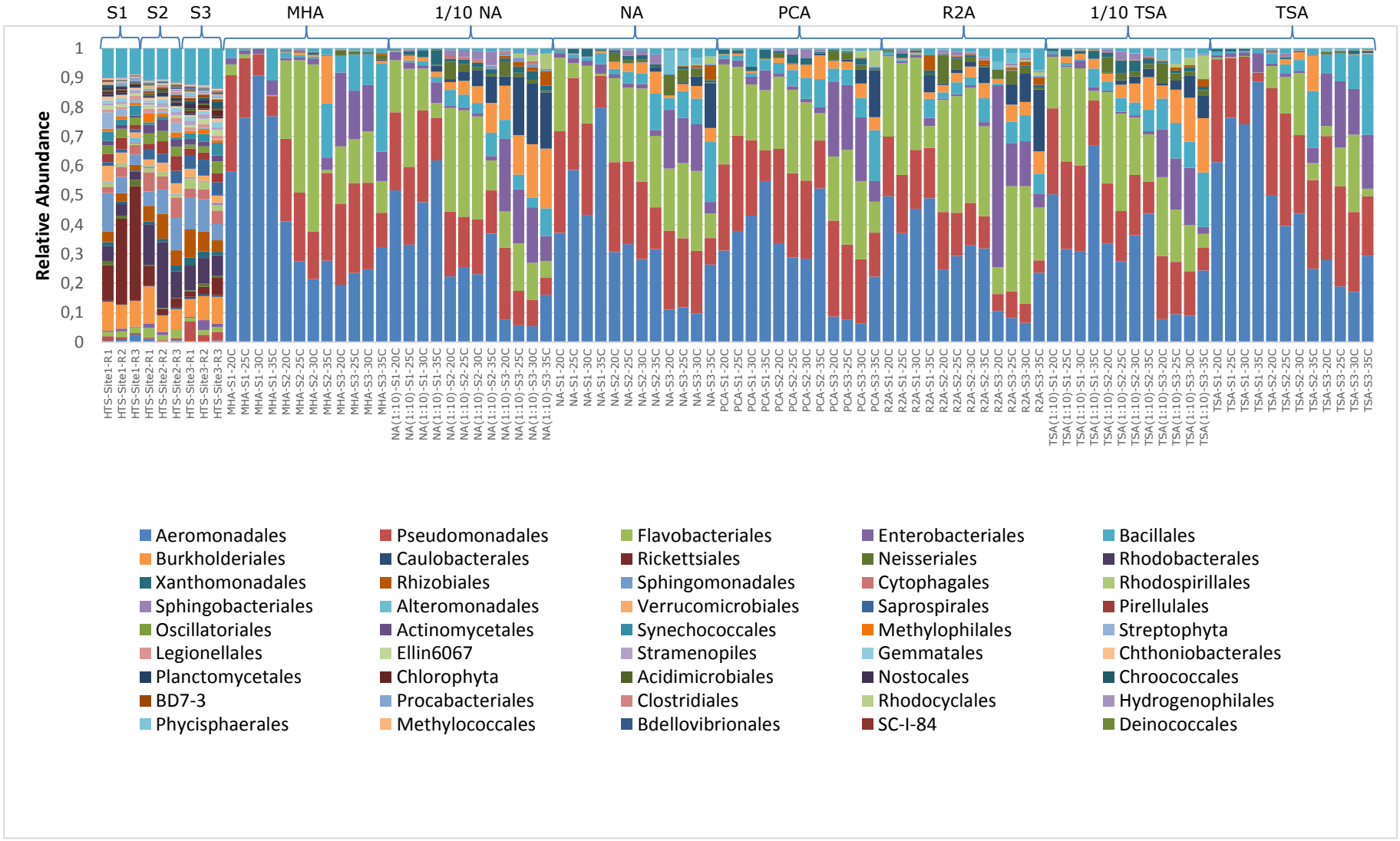


**Figure 7: Relative abundance of bacterial communities based on order level in EWM from metagenomic and from cultivated approach arranged by media;**
HTS: High Throughput Sequencing, R1: Replicate 1, R2: Replicate 2, R3: Replicate 3, S1: Site 1, S2: Site 2, S3: Site 3, 20C: 20 °C, 25C: 25 °C, 30C: 30 °C, 35C: 35 °C, MHA: Mueller Hinton Agar, NA: Nutrient Agar, TSA: Tryptic Soy Agar, PCA: Plate Count Agar, R2A: Reasoner's 2A Agar

### 3.3.3 Discussion

An in depth analysis of the bacterial community structure from the cultivation approach in Figure 7 it seems that neither the incubation temperature nor the media used for cultivation has had any influence on the relative abundances of the identified bacteria. In fact, solely the sampling site seems to have influenced the microbial community structure (Figure 6).
Nevertheless, the microbial structure of the cultivation approach was completely different from the metagenomic microbial structure including a much lower richness and diversity which is demonstrated by the OTU richness and diversity charts in Figure 4 and Figure 5.
There were also remarkable differences in the relative abundance of the major orders found in the samples using both approaches. Aeromonadales, for example was the most abundant order in the cultivated site 1 and site 2 samples, whereas this order was only represented with 1 % in the corresponding metagenomic samples. In contrary, Rickettsiales, Burkholderiales, Sphingomonadales and Rhodobacterales which were the major orders found in the uncultivated samples hardly grew on the media plates. A possible reason for this might be that the bacteria dominant in the cultivated samples can utilize nutritional recourses more efficient which allowed them to grow faster and to outcompete the other bacteria. It is also possible that the incubation conditions were not suitable for some bacteria, as they might usually grow at much lower or much higher temperatures than the selected range of 20-35 °C. Additionally, a longer incubation period could have resulted in a higher diversity of cultivated samples by allowing the assessment of very slow growing bacteria.
However, the presence of bacteria in a viable but non-culturable state might have also caused the low diversity compared to the metagenomic diversity.

Nevertheless, these results confirm that it is not feasible to decipher the entire microbial community of environmental samples using cultivation-based approaches.
Although, in this study very broad media were used for cultivation, no medium came close to representing the true metagenomic diversity from each sample, but due to fluctuations of the diversity within the different media was not apparent which medium would have come closest.

On the other hand, the diluted strength media which should imitate environmental conditions did as well not yield a noticeably higher diversity. For this reason the "nutrient-shock-theory" can not be confirmed in this study.
There might be, however, a difference between the diversity of the samples which were cultivated on TSA and the diversity of samples which were cultivated on 1/10 strength TSA (Figure 5), but more experiments would be necessary in order to confirm this.

## 3.4 Sanger Sequencing

### 3.4.1 Processing of Reads and Identification of Fungi

By checking the chromatograms of all reads using Finch TV 1.4.0, 9 samples were considered as contaminated due to overlapping signals. These samples are not included in the results.
Moreover, signals at the beginning of each read were not clear. For this reason, 25 bases were uniformly cut off the beginning of the reads. The lengths of the cut sequences ranged from 260 to 301 bases and were subsequently used for fungal classification with the Warcup Fungal ITS trainset on the RDP Classifier (www.rdp.cme.msu.edu).An overview of the results can be found in Table 5.

**Table 5: Description of fungal colonies selected for Sanger sequencing and heir identification on genus and species level; mentioned media were used before the isolation on rose bengal agar**

| Labeling | Medium | Temp. [°C] | Morphology | Warcup Classification | |
|---|---|---|---|---|---|
| | | | | **Genus [Similarity, %]** | **Species [Similarity, %]** |
| F1 | MEA | 30 | fair yellow, cottony | *Hypocrea/Trichoderma [99%]* | *Hypocrea lixii/Trichoderma aureoviride [98%]* |
| F2 | MEA | 20 | cream, white edge, large | *Talaromyces [95%]* | *Talaromyces purpurogenus [74%]* |
| F4 | MEA | 20 | olive green, rutted, large | *Aspergillus [100%]* | *Aspergillus awamori [88%]* |
| F5 | MEA | 15 | light olive green, rutted, velutinous | *Cladosporium [82%]* | *Cladosporium rectoides/Cladosporium tenuissimum [70%]* |
| F7 | MEA | 15 | cream edge, gray center, convex | *Phoma [45%]* | *Phoma fungicola [27%]* |
| F9 | WA | 30 | yellowish, cottony, green spores, small | *Aspergillus [100%]* | *Aspergillus awamori [97%]* |
| F10 | WA | 25 | yellow, crumbly, flat, large | *Trichoderma [56%]* | *Trichoderma harzianum [47%]* |
| F11 | WA | 25 | ochre colored, flat, large | *Penicillium [96%]* | *Penicillium oxalicum [90%]* |
| F12 | WA | 25 | light salmon colored, large | *Alternaria [100%]* | *Alternaria tenuis [100%]* |
| F13 | WA | 25 | white, dense, rutted, large | *Hypocrea/Trichoderma [98%]* | *Hypocrea lixii/Trichoderma piluliferum [55%]* |
| F14 | WA | 20 | orange edge, green center, small | *Talaromyces [92%]* | *Talaromyces purpurogenus [71%]* |
| F15 | WA | 20 | bright salmon colored, small | *Talaromyces [95%]* | *Talaromyces purpurogenus [78%]* |
| F16 | WA | 15 | light green, rutted, convex | *Penicillium [100%]* | *Penicillium sp PX_2011a [82%]* |
| F17 | WA | 15 | dark green, rutted, large | *Cladosporium [84%]* | *Cladosporium rectoides/Cladosporium tenuissimum [84%]* |
| F18 | WA | 15 | light green, convex | *Penicillium [100%]* | *Penicillium glandicola [13%]* |
| F20 | PDA | 15 | light green, convex, deeper in center | *Penicillium [100%]* | *Penicillium brevicompactum [97%]* |
| F21 | PDA | 30 | yellow edge, green center, small | *Penicillium [100%]* | *Penicillium oxalicum [100%]* |
| F22 | PDA | 30 | light green, small | *Penicillium [98%]* | *Penicillium oxalicum [95%]* |
| F23 | PDA | 25 | white, crumbly, large | *Trichoderma [100%]* | *Trichoderma asperellum [78%]* |
| F24 | PDA | 25 | yellowish, cottony, green spores, small | *Aspergillus [100%]* | *Aspergillus awamori [99%]* |

| Labeling | Medium | Temp. [°C] | Morphology | Warcup Classification | |
|---|---|---|---|---|---|
| | | | | **Genus [Similarity, %]** | **Species [Similarity, %]** |
| F25 | PDA | 25 | white, cottony, light green spores, small | *Aspergillus [99%]* | *Aspergillus clavatus [99%]* |
| F26 | PDA | 20 | yellowish, cottony, large | *Trichoderma [67%]* | *Trichoderma harzianum [64%]* |
| F27 | PDA | 15 | yellowish green, flat, small | *Cladosporium [77%]* | *Cladosporium rectoides/Cladosporium tenuissimum [73%]* |
| F28 | PDA | 15 | red edge, then white, green center, flat, small | *Penicillium [100%]* | *Penicillium sp PX_2011a [84%]* |
| F30 | YPD | 30 | white, gray center, rutted, huge | *Aspergillus [95%]* | *Aspergillus fumigates [89%]* |
| F31 | YPD | 30 | cream, crimpy surface | *Plectosporium [47%]* | *Plectosporium alismatis [47%]* |
| F33 | YPD | 15 | white edge, then gray, white center, rutted, large | *Penicillium [100%]* | *Penicillium brevicompactum [97%]* |
| F34 | YPD | 15 | cream edge, then gray, pink center, rutted, large | *Penicillium [100%]* | *Penicillium glabrum/Penicillium thomii [100%]* |
| F35 | YPD | 15 | gray edge, then white, gray center | *Davidiella [88%]* | *Davidiella macrospora/Davidiella tassiana [88%]* |
| F37 | SD | 30 | green, white edge | *Penicillium [99%]* | *Penicillium oxalicum [98%]* |
| F38 | SD | 25 | brownish, cottony, large | *Epicoccum [96%]* | *Epicoccum sorghi [94%]* |
| F39 | SD | 25 | ochre colored, cottony edge, large | *Fusarium/Gibberella [60%]* | *Fusarium asiaticum/Gibberella zeae [60%]* |
| F40 | SD | 25 | green, white edge | *Penicillium [100%]* | *Penicillium brevicompactum [99%]* |
| F41 | SD | 25 | green, crumbly | *Hypocrea/Trichoderma [96%]* | *Hypocrea lixii/Trichoderma aureoviride [90%]* |
| F42 | SD | 20 | red edge, then white, light green center | *Penicillium [100%]* | *Penicillium hirsutum/Penicillium verrucosum [97%]* |
| F44 | SD | 20 | white, cottony | *Fusarium/Gibberella [99%]* | *Fusarium cf equiseti MY_2011/Gibberella intricans [49%]* |
| F45 | SD | 15 | mixed colored (ochre + brown + pink), large | *Epicoccum [65%]* | *Epicoccum nigrum [62%]* |
| F46 | SD | 15 | turquoise, cottony, convex | *Penicillium [100%]* | *Penicillium biourgeianum [100%]* |
| F47 | SD | 15 | cream, dense, small | *Metschnikowia [100%]* | *Metschnikowia pulcherrima [100%]* |

### 3.4.2 Interpretation of Fungal Community Structure

All of the 39 classified fungal isolates ranked amongst the phylum Ascomycota whereof the genus *Penicillium* dominated with over 33 % followed by *Aspergillus* with an abundance of 13 %. The remaining 54 % consist of a variety of different genera (*Metschnikowia, Epicoccum, Alternaria, Davidiella, Cladiosporium, Talaromyces, Fusarium/Gibberella, Trichoderma and Hypocrea lixii/Trichoderma aureoviride*). However, the results are not very reliable as only 14 sequences of all isolates could be identified to species level with the desired certainty of 97 %. Furthermore, the results do not allow conclusions on the composition of fungi in Cedar Lake as the high diversity in genera, which were represented in some case by only one single isolate, suggest an even higher diversity of actual culturable fungi.

# 4 Screening Eurasian Watermilfoil for Potential Waterborne Pathogens

## 4.1 Introduction

### 4.1.1 Task Description and Objective

Besides microorganisms that can be harmful to the plant itself, the EWM could also harbor potential pathogens that may detach in water bodies and pose serious hazards to human health.
For this reason, the second objective of this study was the screening for both indicator organisms as well as for cultivable waterborne human pathogens associated with EWM. The aim was pursued by selective cultivation and subsequent Sanger sequencing of 16S rDNA amplicons from individual isolates.
Genera that have been previously found to be potentially pathogenic include *Aeromonas, Campylobacter*, *Salmonella*, *Pseudomonas* and *Shigella.*

### 4.1.2 Indicator Organism *E. coli*

Historically, the enumeration of various bacterial populations has been used for the suggestion of the presence of pathogens (WHO, 2011). Especially *E. coli* is considered the first organism of choice for the monitoring of water quality, as it occurs in high numbers in human and animal feces but its growth is usually not supported in water (WHO, 2011). Hence it provides only evidence of recent fecal contamination, which also infers that pathogens may be present.
For this reason *E. coli*, which is not necessarily a human pathogen, was screened for in this study in order to predict the presence of potential waterborne pathogens.

## 4.2 Materials and Methods

### 4.2.1 Experimental Setup

The identification of individual colonies was conducted in accordance with the flowchart shown in Figure 8.
The single steps are described in the following paragraphs.

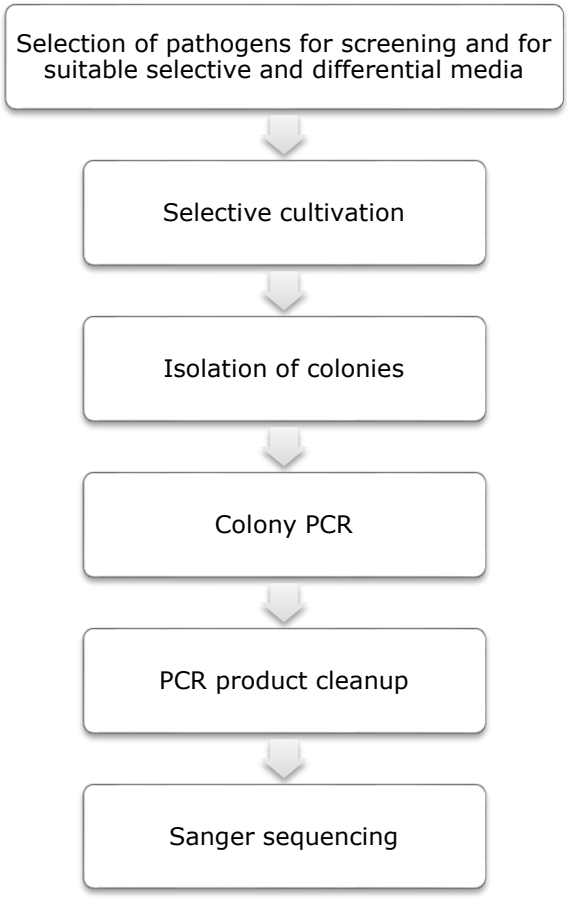


**Figure 8: Flowchart for the screening of cultivable potential pathogens**

### 4.2.2 Selection of Screening Media for Pathogens

The decision on which of the numerous water pathogens should be screened for was based on their family level abundance derived from the projects previously collected Illumina sequencing data.
Appropriate cultivation media were selected using the 'Media by Microorganism' search on the website of Sigma-Aldrich (www.sigmaaldrich.com), whereas the

search was limited to only selective and differential media. All selected pathogens and appendant media as well as incubation conditions recommended by the datasheet can be found in Table 6.

**Table 6: Selective media for different potential waterborne pathogens, including recommended incubation conditions**

| **Media** | **Microorganisms** | **Incubation Temperature [°C]** | **Incubation Time [h]** |
|---|---|---|---|
| *Aeromonas* Isolation Agar | *Aeromonas hydrophila* | 35-37 | 18-24 |
| *Campylobacter* Selective Agar | *Campylobacter coli, Campylobacter jejuni, Campylobacter laridis* | 37 | 24-48 |
| Chromogenic ECC Selective Agar | *Escherichia coli* and total Coliforms | 35-37 | 24 |
| Chromogenic Urinary Tract Infection Agar | Enteric bacteria (*Escherichia coli, Enterococcus ssp. Enterobacer ssp., Klebsiella ssp., Citrobacter ssp., Proteus ssp.*) | 35-37 | 24 |
| Eosine Methylene Blue (EMB) Agar | *Escherichia coli,* other *Enterobacteriaceae* | 35 | 24-48 |
| MacConkey No. 1 | Coliforms and intestinal pathogens (*Escherichia coli, Enterobacter aerogenes, Salmonella ssp. Shigella flexneri, Proteus vulgaris, Staphylococcus aureus, Enterococcus faecalis*) | 35-37 | 18-24 |
| *Pseudomonas* Isolation Agar | *Pseudomonas aeruginosa* | 35 | 24-48 |
| *Salmonella Shigella* (*SS*A) Agar | *Salmonella enteriditis, Salmonella typhi, Salmonella typhimurium, Shigella flexneri* | 35-37 | 18-24 |
| Xylose Lysin Desoxycholat (XLD) Agar | Gram-negative enteric pathogens (*Salmonella ssp., Shigella ssp., Escherichia coli, Klebsiella pneumonia, Streptococcus faecalis*) | 35 | 24 |

### 4.2.3 Preparation of Media

Most media were available in the lab inventory and those which did not were prepared using the individual ingredients according to Sigma-Aldrichs' media recipes. All media were prepared as described in 2.2.4, but the autoclaving step was skipped for *A*IA, Chromogenic ECC Selective Agar, *SS* agar and XLD agar. These media were dissolved and sterilized by one-minute boiling on the magnetic stirrer hot plate in order to spare the contained heat-sensitive components. All used ingredients for the preparation along with specification are listed in Table 7.

**Table 7: Descriptions and compositions of selective growing media used for the pathogen-screening**

| Media | Composition per Liter | pH | Prepared from(Manufacturer; Reference number) |
|---|---|---|---|
| *Aeromonas* Isolation Agar | 5.0 g special peptone<br>3.0 g yeast extract<br>3.5 g L-Lysine hydrochloride<br>2.0 g L-Arginine hydrochloride<br>2.5 g inositol<br>1.5 g lactose<br>3.0 g sorbose<br>3.75 g xylose<br>3.0 g bile salts<br>10.67 g sodium thiosulfate<br>5.0 g sodium chloride<br>0.8 g ferric ammonium citrate<br>0.04 g bromothymol blue<br>0.04 g thymol blue<br>12.5 g agar<br>0.005 g Ampicillin | 8.0 ± 0.2 | Prepared from AIA (Sigma-Aldrich; 17118)<br>and<br>0.005 g/mL Ampicillin stock solution [Ampicillin (Sigma-Aldrich; A9518) dissolved in $ddH_2O$ -> sterilized by filtering through 0.2 µm membrane filters (Pall Corporation; PN 4192)] |
| *Campylobacter* Selective Agar | 25.0 g nutrient broth no. 2<br>4.0 g bacteriological charcoal<br>3.0 g casein hydrolysate<br>1.0 g sodium deoxycholate<br>0.25 g ferrous sulphate<br>0.25 g sodium pyruvate<br>12.0 g agar<br>0.032 g Cefoperazone | 7. 4 ± 0.2 | Prepared from Campylobacter Blood Free Selective Agar Base (Oxoid; CM0739)<br>and<br>0.032 g/mL Cefoperazone stock solution [Cefoperazone (Sigma-Aldrich; C-4292) dissolved in $ddH_2O$ -> sterilized by filtering through 0.2 µm membrane filters (Pall Corporation; PN 4192)] |
| Chromagar ECC | 8.0 g peptone & yeast extract<br>5.0 g sodium chloride<br>4.8 g chromogenic mix<br>15.0 g agar | 7.2 ± 0.2 | Re-mixed Microbiology Chromagar ECC (CHROMagar; EF322) |
| Chromagar Orientation | 17.0 g peptone & yeast extract<br>1.0 g chromogenic mix<br>15.0 g agar | 7.0 ± 0.2 | Pre-mixed Microbiology Chromagar Orientation (CHROMagar; RT412) |
| EMB Agar | 10.0 g peptone<br>10.0 g lactose<br>2.0 g dipotassiumhydrogenphosphate<br>0.065 g methylene blue<br>0.4 g eosine Y<br>15.0 g agar | 7.1 ± 0.2 | Pre-mixed EMB (Sigma-Aldrich; 70186-500G) |

| **Media** | **Composition per Liter** | **pH** | **Prepared from(Manufacturer; Reference number)** |
|---|---|---|---|
| Mac Conkey No. 1 | 20.0 g peptone<br>10.0 g lactose<br>5.0 g bile salts<br>5.0 g sodium chloride<br>0.075 g neutral red<br>12.0 g agar | 7.4 ± 0.2 | Pre-mixed Mac Conkey No. 1 (Sigma-Aldrich; 70143) |
| *Pseudomonas* Isolation Agar | 20.0 g peptic digest of animal tissue<br>1.5 g magnesium chloride<br>10.0 g potassium sulfate<br>0.025 g Triclosan<br>13.6 g agar<br>20 mL glycerol | ~ 7,0 | Prepared from:<br>Proteose Peptone (BD; 211684),<br>Magnesium Chloride (Fisher Scientific; M33-500)<br>Potassium Sulfate (EMD; PX1595-1),<br>Triclosan (Sigma-Aldrich; PHR1338-1G),<br>BBL™ Agar Granulated (BD; 11849)<br>and<br>Glycerol (Fisher Scientific, REF: S25342B) |
| *SS* Agar | 5.0 g beef extract<br>2.5 g pancreatic digest of casein<br>2.5 g peptic digest of animal tissue<br>10.0 g lactose<br>8.5 g bile salts mixture<br>8.5 g sodium citrate<br>8.5 g sodium thiosulfate<br>1.0 g ferric citrate<br>0.025 g neutral red<br>0.00033 g brilliant green<br>13.5 g agar | 7.0 ± 0.2 | Pre-mixed BBL™ Salmonella Shigella Agar (BD; 211597) |
| XLD Agar | 3.0 g yeast extract<br>5.0 g L-Lysine<br>3.5 g xylose<br>7.5 g lactose<br>7.5 g saccharose<br>2.5 g sodium deoxycholate<br>0.8 g ferric ammonium citrate<br>6.8 g sodium thiosulfate<br>5.0 g sodium chloride<br>13.5 g agar | 7.4 ± 0.2 | Pre-mixed XLD Agar (BD; 278850) |

### 4.2.4 Cultivation

All culture-based experiments were conducted in a BSL 2 laboratory.
For each media, parameters regarding incubation were already set by the targeted microorganism. The only parameter that had to be determined before the main cultivation was the sample concentration for spreading of each individual site on each individual medium. This was done in several steps starting with the spreading of MF1, MF2 and MF3 undiluted and in 1:10 and 1:100 dilutions respectively on each selective media. Incubation was performed as per manufacturers' recommendations.
In order to avoid excluding the growth of bacteria present in the sample in low abundance, a total number of about 500 colonies per plate was targeted. In this way the colonies had enough space to grow and to enable differentiation.
The growth on the plates was checked after incubation: when the targeted colony number was achieved the used dilution was set for the main cultivation. If not, a new dilution of sample was used for spreading – either higher or lower- and plates were incubated again.

The main cultivation was conducted with the following parameters and sample dilutions:

- *A*IA: 37 °C, 1 day, MF1: 1:10, MF2: 1:200, MF3: 1:40
- *C*SA: 37 °C, 2 days, microaerophilic, MF1: 1x, MF2: 1x, MF3: 1x
- CHROMagar ECC: 37 °C, 1 day, MF1: 1:10, MF2: 1:50, MF3: 1:100
- CHROMagar Orientation: 37 °C, 1 day, MF1: 1:100, MF2: 1:10, MF3: 1:10
- EMB: 35 °C, 2 days, MF1: 1:10,000, MF2: 1:10,000, MF3: 1:10,000
- MacConkey No.1: 37 °C, 1 day, MF1: 1:50,000, MF2: 1:10,000, MF3: 1:20,000
- *P*IA: 35 °C, 1 day, MF1: 1x, MF2: 1x, MF3: 1x
- *SS* Agar: 37 °C, 1 day, MF1: 1:10, MF2: 1:100, MF3: 1:100
- XLD Agar: 35 °C, 1 day, MF1: 1:200, MF2: 1:400, MF3: 1:25

Spreading of the milfoil samples in triplicates was realized in the same manner as described earlier in 2.2.5. After incubation the color reactions on the plates were compared to media references in order to estimate possible pathogens.

### 4.2.5 Isolation of Individual Colonies

As Sanger sequencing requires pure colonies for a reliable identification, individual colonies were picked from the incubated plates for isolation based on their morphology – in total 42 colonies of each media and sampling site were picked. The colonies were transferred to matching media using autoclaved toothpicks and were incubated at the same conditions as the original plates.
The morphology of each colony was documented and for a better overview plates were divided into grids (Figure 9).

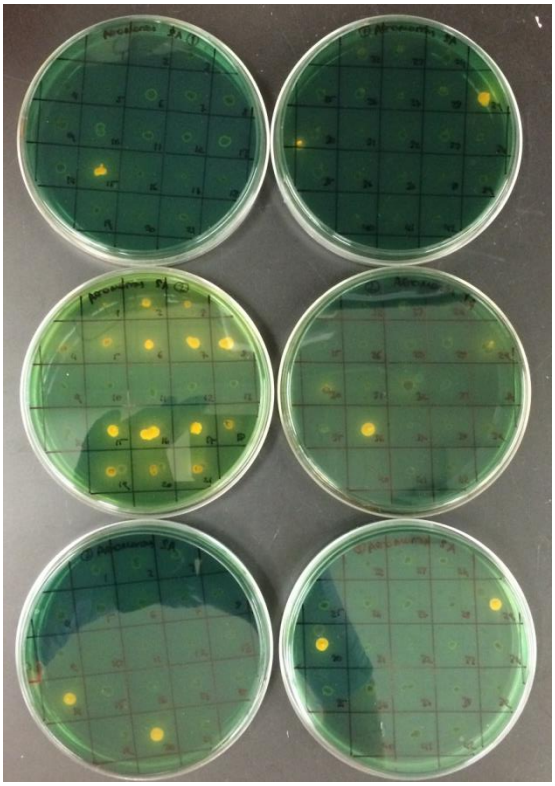

**Figure 9: Isolated colonies on *Aeromonas* isolation agar plates divided into grids**

Colonies were inoculated onto new grids two times and the morphology of the isolates was subsequently compared to that of the initial colonies. Only colonies which appeared on the last plates in the same color and morphology as the originally picked colonies were considered pure and were suitable for colony PCR.

### 4.2.6 Preparation of Samples for Sanger Sequencing

Altogether, 282 bacterial isolates were picked for the colony PCR -42 from Chromagar orientation and 30 from all other selective media respectively. To ensure the collection of all different possible species from each media, colonies appearing in the same morphology were picked at least in duplicates. As the growth on Chromagar orientation showed a higher variation in morphology more colonies were picked from this medium.

A total of three colony PCR runs of the selected colonies were performed according to 2.4.2, but instead of 55 °C a temperature of 57 °C was set for annealing. All the other parameters remained the same.
Specifications of the used primer set can be found in Table 8.

**Table 8: Primer set employed for PCR amplification**

| Target | Primer | Sequence | Concentration [pmol/µL] |
|---|---|---|---|
| 16S rDNA | Forward (27F) | 5'-AGAGTTTGATCMTGGCTCAG-3' | 20.5 |
| | Reverse (U1492R) | 5'-GGTTACCTTGTTACGACTT-3' | 28.7 |

The verification of the PCR products via gel electrophoresis was performed as described in 2.4.3.
142 successfully amplified DNA fragments were selected for Sanger sequencing based on the morphology of the originating colony.
Before submission for Sanger sequencing, these fragments were purified by using the UltraClean® Clean-Up Kit (12500-250, MO BIO Laboratories Inc., USA) according to the manufacturers' instructions and subsequently the DNA concentration of the clean-up products was measured as described in 2.3.2.

### 4.2.7 Sanger Sequencing

The purified amplicons were submitted for High-Throughput Sanger sequencing to the University of Minnesota Genomics Center in 96-well-plates.
Each well was filled with a total of 6 µL of sample consisting of 3 µL of forward primer as template and purified amplicon to reach the requested amount of approximately 10-20 ng of the 16S rDNA.

## 4.3 Results and Discussion

### 4.3.1 Comparison of Colony Characteristics from Different Sites

Overall the growth on the selective media seemed promising, as a comparison between the actual morphology of colonies and the medias' cultural characteristics references indicated the presence of some prospected pathogens.

Growth on the AIA plates from all three sites composed mainly of dark green colonies which should indicate *Aeromonas hydrophila*. However there were also some light green and black colonies which could not be assigned as these colors were not described in the cultural characteristics reference. The light green colonies occurred especially on the plates from site 1 and site 2.

The growth on the Chromagar ECC plates from site 1 and site 2 was also very similar. The majority of colonies was violet - indicating the presence of coliforms - and one to five blue colonies per plate inferred the possible presence of *E. coli*. In contrary, no blue colonies at all grew on the site 3 plates
However, no colony on neither of the Chromagar orientation plates indicated the possible presence of *E. coli.* Most colonies that grew on this medium where blue (*Entercoccus, Klebsiella, Enterobacter* or *Citrobacter*) Some violet and yellow colonies could be observed in a very low abundance, but are not mentioned in the cultural characteristics reference.

The colony morphologies observed on both EMB Agar and MacConkey No. 1 plates were not really informative. All colonies that grew on EMB agar appeared either brownish or in different shades of violet which are not covered in the reference. Colonies on the MacConkey No. 1 plates appeared only in different shades of pink which indicates several species (*E. coli, Enterobacter aerogenes, Staphylococcus aureus* or *Enterococcus faecalis*).

The possible presence of *Campylobacter* species is indicated on CSA by grey colonies, which could be found on the plates of all sites. However growth was very meager with the least growth of only three colonies in total on site 3 plates.

*Pseudomonas aeruginosa* would be characterized on *P*IA by green or blue colonies but the colonies on *P*IA plates from all sites only appeared white or cream. Solely two colonies that grew on the site 1 plates were bright greenish yellow.

Growth on *SS* agar indicated the possible presence *Shigella* species by colorless colonies. Furthermore, a few black colonies could be observed on site 1 and site 2 plates implying the possible presence of *Salmonella* species.

On XLD agar plates, contrarily, no colonies indicated a possible presence of *Salmonella*, but growth of yellowish and reddish colonies inferred a possible growth of *Klebsiella* and *Shigella.* Additionally, there was a noticeable fall of the pH of the media from site 1 plates into the acidic range (Figure 10) which could have been caused by carbohydrate-fermenting coliforms.

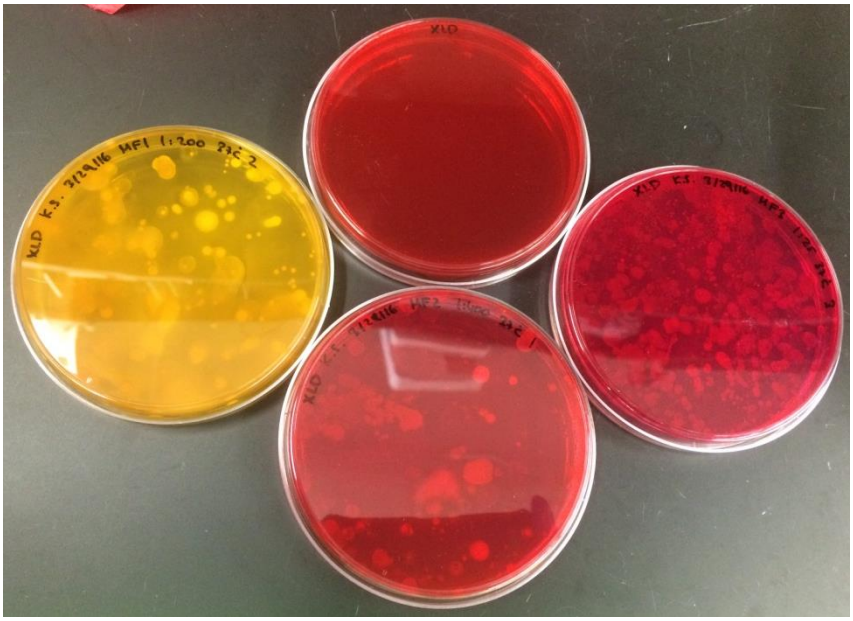

**Figure 10: Comparison of inoculated XLD agar plates from site 1 (left), site 2 (lower center) and site 3 (right) with fresh XLD medium (upper center): bacterial growth on the plate from site 1 shifted the pH into the acidic range which is indicated by color change**

### 4.3.2 Colony PCR Verification

About 75 % of all colony PCR runs yielded in amplicons of the predicted size which was about 1500 pb (Figure 11). The formation of some primer dimers occurred in most of the samples. However, they were removed in the clean-up step, so that they could not interfere with the sequencing step.

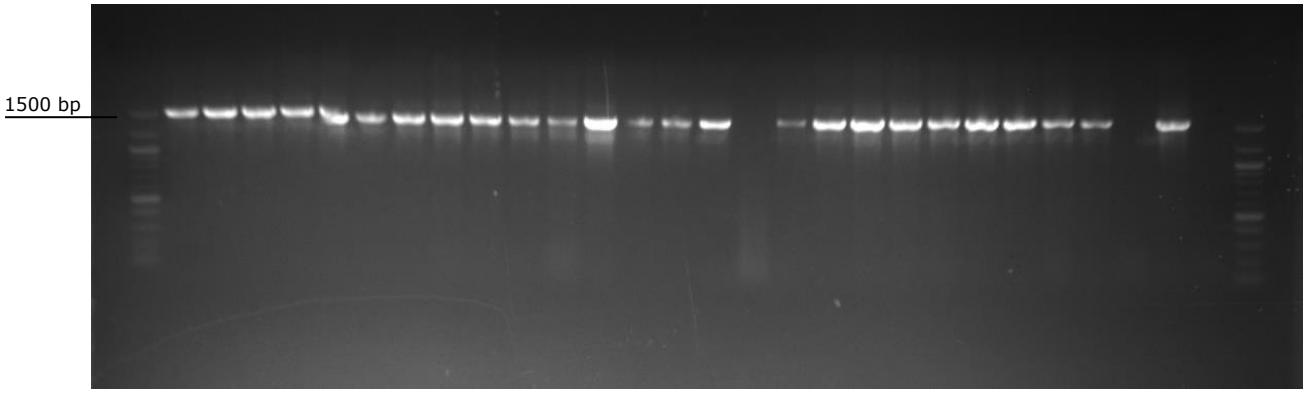


**Figure 11: UV image of an agarose gel with 16S rDNA amplicon bands: bands are bright due to high DNA concentrations; the blurred bands on the bottom of the lanes indicate the formation of some primer dimers**

### 4.3.3 General Overview of Sequencing Results

Before the evaluation of the sequencing data, chromatograms of all reads were checked for any overlaps or anomalies suggesting mixed samples or unreliable sequences by the use of Finch TV 1.4.0.

Of all 142 16S rDNA reads 10 chromatograms indicated a mixed sample - these samples are not included in the results.

The sequences of all other reads were subsequently cut on both ends. 25 bases from the start of each read were removed as they did not give clear signals in the chromatogram. Furthermore, the chromatograms of all bacterial samples started to level off after about 670-700 bases. For this reason sequences after base 675 were uniformly not considered reliable and were removed too. This process yielded in 86 bacterial sequences of 650 base lengths.

Bacteria were identified to species level using the RDP Seqmatch (www.rdp.cme.msu.edu).

An overview of the results can be found in Table 9: which also includes a comparison between the actual identified species and the species suggested by its morphology on the selective media.

**Table 9: Description of the individual bacterial colonies selected for Sanger sequencing, including estimations on possible species based on the cultural characteristics described on the medias' data sheets and the actual results**

| Labeling | Medium | Site | Morphology | Possible Species | RDP SEQMATCH Identification | Similarity of Sequences |
|---|---|---|---|---|---|---|
| B1 | *SS* Agar | 1 | cream, small | *Shigella* | *Aaeromonas veronii or Aeromonas hydrophila* | 1.000 |
| B2 | *SS* Agar | 1 | cream, large | *Shigella* | *Aaeromonas veronii or Aeromonas hydrophila* | 1.000 |
| B3 | *SS* Agar | 1 | cream, large | *Shigella* | *Aeromonas popoffii* | 0.995 |
| B4 | *SS* Agar | 1 | Black | *Salmonella/Proteus* | *Citrobacter braakii* | 0.997 |
| B5 | *SS* Agar | 1 | cream, large | *Shigella* | *Aaeromonas veronii or Aeromonas hydrophila* | 1.000 |
| B6 | *SS* Agar | 2 | Black | *Salmonella/Proteus* | *Citrobacter freundii* | 1.000 |
| B7 | *SS* Agar | 2 | Yellowish | *?* | *Aaeromonas veronii or Aeromonas hydrophila* | 1.000 |
| B8 | *SS* Agar | 2 | cream, large | *Shigella* | *Aaeromonas veronii or Aeromonas hydrophila* | 1.000 |
| B9 | *SS* Agar | 2 | Black | *Salmonella/Proteus* | *Citrobacter freundii* | 0.970 |
| B10 | *SS* Agar | 2 | Black | *Salmonella/Proteus* | *Citrobacter freundii* | 0.990 |
| B11 | *SS* Agar | 2 | Reddish | *E. coli/Enterobacter/Klebsiella* | *Pseudomonas fulva or Pseudomonas parafulva* | 1.000 |
| B12 | *SS* Agar | 3 | cream, large | *Shigella* | *Aeromonas veronii or Aeromonas hydrophila* | 1.000 |
| B13 | *SS* Agar | 3 | cream, small | *Shigella* | *Aeromonas veronii* | 0.990 |
| B14 | *SS* Agar | 3 | cream, large | *Shigella* | *Aeromonas hydrophila or Aeromonas veronii* | 0.990 |
| B15 | *SS* Agar | 3 | cream, small | *Shigella* | *Aeromonas allosaccharophila or Aeromonas veronii* | 0.964 |
| B16 | EMB Agar | 1 | Violet | *Enterococcus faecalis* | *Enterobacter hormaechei* | 0.965 |
| B17 | EMB Agar | 1 | Brownish | *?* | *Pantoea agglomerans* | 0.936 |
| B18 | EMB Agar | 1 | Brownish | *?* | *Pantoea ananatis* | 0.936 |
| B19 | EMB Agar | 1 | violet, small | *Enterococcus faecalis* | *Herbaspirillum huttiense* | 1.000 |
| B20 | EMB Agar | 1 | dark violet | *Enterococcus faecalis* | *Herbaspirillum huttiense* | 0.989 |
| B21 | EMB Agar | 2 | Brownish | *?* | *Enterobacter hormaechei* | 0.955 |
| B23 | EMB Agar | 2 | dark violet | *Enterococcus faecalis* | *Pantoea ananatis* | 0.990 |
| B24 | EMB Agar | 2 | light pink, small | *?* | *Herbaspirillum huttiense* | 0.989 |
| B25 | EMB Agar | 2 | Brownish | *?* | *Pantoea agglomerans* | 0.928 |
| B26 | EMB Agar | 3 | Brownish | *?* | *Enterobacter hormaechei* | 0.960 |
| B27 | EMB Agar | 3 | Brownish | *?* | *Herbaspirillum huttiense* | 0.998 |
| B28 | EMB Agar | 3 | Brownish | *?* | *Pantoea ananatis* | 1.000 |
| B29 | EMB Agar | 3 | dark violet | *Enterococcus faecalis* | *Pantoea ananatis* | 1.000 |
| B30 | EMB Agar | 3 | dark violet | *Staphylcoccus aureus/Enterococcus faecalis* | *Klebsiella pneumoniae* | 1.000 |

| Labeling | Medium | Site | Morphology | Possible Species | RDP SEQMATCH Identification | Similarity of Sequences |
|---|---|---|---|---|---|---|
| B32 | MacConkey No. 1 | 1 | pinkish cream | *Staphylcoccus aureus/Enterococcus faecalis* | *Pantoea ananatis* | 1.000 |
| B33 | MacConkey No. 1 | 1 | pinkish cream, large | *Staphylcoccus aureus/Enterococcus faecalis* | *Enterobacter hormaechei* | 0.959 |
| B35 | MacConkey No. 1 | 1 | light pink, small | *Staphylcoccus aureus/Enterococcus faecalis* | *Herbaspirillum huttiense* | 1.000 |
| B36 | MacConkey No. 1 | 2 | pinkish cream | *Staphylcoccus aureus/Enterococcus faecalis* | *Serratia ureilytica* | 0.990 |
| B37 | MacConkey No. 1 | 2 | pinkish cream | *Staphylcoccus aureus/Enterococcus faecalis* | *Pantoea ananatis* | 1.000 |
| B38 | MacConkey No. 1 | 2 | Cream | *Salmonella typhi/Salmonella enteritidis/Salmonella paratyphi A/Salmonella paratyphi B/Shigella flexneri/Proteus vulgaris* | *Pantoea ananatis* | 0.877 |
| B39 | MacConkey No. 1 | 2 | light pink, small | *Staphylcoccus aureus/Enterococcus faecalis* | *Herbaspirillum huttiense* | 0.997 |
| B40 | MacConkey No. 1 | 2 | cream, reddish center | *E. coli/Enterobacter aerogenes* | *Pantoea ananatis* | 0.917 |
| B41 | MacConkey No. 1 | 3 | pinkish cream | *Staphylcoccus aureus/Enterococcus faecalis* | *Enterobacter hormaechei* | 0.957 |
| B42 | MacConkey No. 1 | 3 | cream, reddish center | *E. coli/Enterobacter aerogenes* | *Pantoea ananatis* | 1.000 |
| B44 | MacConkey No. 1 | 3 | violet cream | *?* | *Ralstonia mannitolilytica or Ralstonia pickettii* | 0.990 |
| B45 | MacConkey No. 1 | 3 | cream, reddish center | *E. coli/Enterobacter aerogenes* | *Pantoea ananatis* | 0.904 |
| B46 | XLD Agar | 1 | Yellow | *Klebsiella pneumonia* | *Pantoea ananatis* | 1.000 |
| B47 | XLD Agar | 1 | yellow, large | *Klebsiella pneumonia* | *Pantoea ananatis* | 1.000 |
| B49 | XLD Agar | 1 | yellow, irregular edge | *Klebsiella pneumonia* | *Leclercia adecarboxylata or Enterobacter cloacae or Enterobacter ludwigii* | 0.997 |
| B50 | XLD Agar | 1 | yellow, irregular edge | *Klebsiella pneumonia* | *Pantoea ananatis* | 0.946 |
| B51 | XLD Agar | 2 | dark yellow | *Klebsiella pneumonia* | *Salmonella bongori or Escherichia hermannii or Kosakonia cowanii* | 0.990 |
| B52 | XLD Agar | 2 | light yellow | *Klebsiella pneumonia* | *Pantoea ananatis* | 0.979 |
| B53 | XLD Agar | 2 | Reddish | *Shigella flexneri* | *Pseudomonas moraviensis* | 1.000 |

| Labeling | Medium | Site | Morphology | Possible Species | RDP SEQMATCH Identification | Similarity of Sequences |
|---|---|---|---|---|---|---|
| B55 | XLD Agar | 2 | Orange | *?* | *Cronobacter sakazakii* | 1.000 |
| B56 | XLD Agar | 3 | pinkish yellow, slimy | *?* | *Pantoea agglomerans or Enterobacter cloacae or Enterobacter ludwigii* | 1.000 |
| B57 | XLD Agar | 3 | Reddish | *Shigella flexneri* | *Enterobacteriaceae bacterium or Serratia fonticola* | 0.989 |
| B58 | XLD Agar | 3 | Reddish | *Shigella flexneri* | *Plesiomonas shigelloides* | 0.994 |
| B59 | XLD Agar | 3 | yellow, translucent | *Klebsiella pneumonia* | *Citrobacter murliniae* | 0.997 |
| B60 | XLD Agar | 3 | Yellow | *?* | *Citrobacter freundii* | 1.000 |
| B61 | *A*IA | 1 | dark green, large | *Aeromonas hydrophila* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B62 | *A*IA | 1 | dark green, small | *Aeromonas hydrophila* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B63 | *A*IA | 1 | Yellowish | *?* | *Klebsiella oxytoca* | 0.990 |
| B64 | *A*IA | 1 | dark green, large | *Aeromonas hydrophila* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B65 | *A*IA | 1 | dark green, small | *Aeromonas hydrophila* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B66 | *A*IA | 2 | Black | *?* | *Citrobacter braakii or Citrobacter freundii* | 1.000 |
| B67 | *A*IA | 2 | yellowish brown | *?* | *Raoultella ornithinolytica* | 1.000 |
| B68 | *A*IA | 2 | Black | *?* | *Citrobacter freundii* | 0.989 |
| B69 | *A*IA | 2 | dark green | *Aeromonas hydrophila* | *Aeromonas hydrophila or Aaeromonas veronii* | 1.000 |
| B70 | *A*IA | 2 | dark green, light center | *Aeromonas hydrophila* | *Klebsiella pneumonia* | 1.000 |
| B71 | *A*IA | 3 | dark green | *Aeromonas hydrophila* | *Aeromonas hydrophila or Aaeromonas veronii* | 1.000 |
| B72 | *A*IA | 3 | dark green, light center | *Aeromonas hydrophila* | *Klebsiella pneumonia* | 0.994 |
| B74 | *A*IA | 3 | light green | *?* | *Klebsiella variicola* | 1.000 |
| B75 | *A*IA | 3 | dark green | *Aeromonas hydrophila* | *Aeromonas hydrophila or Aaeromonas veronii* | 1.000 |
| B76 | Chromagar ECC | 1 | Blue | *E. coli* | *Escherichia coli* | 0.987 |
| B77 | Chromagar ECC | 1 | Blue | *E. coli* | *Escherichia coli* | 0.990 |
| B78 | Chromagar ECC | 1 | Violet | *Other Coliforms* | *Enterobacter amnigenus* | 0.990 |
| B79 | Chromagar ECC | 1 | White | *Other Bacteria* | *Citrobacter freundii* | 1.000 |
| B80 | Chromagar ECC | 1 | Violet | *Other Coliforms* | *Enterobacter amnigenus* | 0.984 |
| B81 | Chromagar ECC | 2 | Blue | *E. coli* | *Escherichia coli* | 0.982 |
| B82 | Chromagar ECC | 2 | White | *Other Bacteria* | *Pseudomonas mendocina* | 0.975 |

| Labeling | Medium | Site | Morphology | Possible Species | RDP SEQMATCH Identification | Similarity of Sequences |
|---|---|---|---|---|---|---|
| B83 | Chromagar ECC | 2 | Blue | *E. coli* | *Escherichia coli or Shigella flexneri* | 1.000 |
| B84 | Chromagar ECC | 2 | White | *Other Bacteria* | *Escherichia coli or Shigella dysenteriae or Shigella boydii* | 1.000 |
| B85 | Chromagar ECC | 2 | Violet | *Other Coliforms* | *Citrobacter braakii* | 1.000 |
| B86 | Chromagar ECC | 3 | dark violet | *Other Coliforms* | *Pantoea agglomerans* | 0.921 |
| B87 | Chromagar ECC | 3 | Violet | *Other Coliforms* | *Pantoea agglomerans* | 0.869 |
| B88 | Chromagar ECC | 3 | White | *Other Bacteria* | *Pseudomonas plecoglossicida or Pseudomonas putida* | 1.000 |
| B90 | Chromagar ECC | 3 | White | *Other Bacteria* | *Pseudomonas mendocina or Pseudomonas pseudoalcaligenes* | 0.960 |
| B92 | Chromagar Orientation | 1 | mid-blue | *Klebsiella/Enterobacter/Citrobacter* | *Pantoea agglomerans or Enterobacter cloacae or Enterobacter ludwigii* | 0.995 |
| B93 | Chromagar Orientation | 2 | white, large | *Pseudomonas* | *Acinetobacter baylyi* | 0.928 |
| B95 | Chromagar Orientation | 3 | Turquoise | *Enterococcus* | *Bacillus thuringiensis or Bacillus cereus* | 1.000 |
| B96 | Chromagar Orientation | 3 | Blue | *Klebsiella/Enterobacter/Citrobacter* | *Erwinia persicina* | 0.984 |
| B97 | Chromagar Orientation | 3 | Yellow | *S. aureus* | *Chryseobacterium indologenes* | 0.990 |
| B98 | Chromagar Orientation | 2 | Turquoise | *Enterococcus* | *Bacillus thuringiensis or Bacillus cereus or Bacillus anthracis* | 1.000 |
| B99 | Chromagar Orientation | 2 | pinkish violet | *E. coli* | *Escherichia coli* | 0.949 |
| B100 | Chromagar Orientation | 2 | light violet | *S. saprophyticus* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B101 | Chromagar Orientation | 1 | dark blue | *Klebsiella/Enterobacter/Citrobacter* | *Citrobacter braakii or Citrobacter freundii* | 1.000 |
| B102 | Chromagar Orientation | 3 | violet, white edge | *S. saprophyticus* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B103 | Chromagar Orientation | 3 | pinkish violet | *E. coli* | *Citrobacter murliniae* | 1.000 |
| B104 | Chromagar Orientation | 2 | white, large | *Pseudomonas* | *Acinetobacter baylyi* | 1.000 |
| B105 | Chromagar Orientation | 3 | mid blue, translucent | *Klebsiella/Enterobacter/Citrobacter* | *Exiguobacterium indicum* | 0.990 |

| Labeling | Medium | Site | Morphology | Possible Species | RDP SEQMATCH Identification | Similarity of Sequences |
|---|---|---|---|---|---|---|
| B106 | Chromagar Orientation | 3 | mid-blue, translucent | *Klebsiella/Enterobacter/Citrobacter* | *Exiguobacterium acetylicum* | 1.000 |
| B107 | Chromagar Orientation | 3 | dark blue, large | *Klebsiella/Enterobacter/Citrobacter* | *Erwinia persicina* | 0.994 |
| B108 | *P*IA | 1 | White | *?* | *Citrobacter braakii or Citrobacter freundii* | 0.990 |
| B109 | *P*IA | 1 | White | *?* | *Aeromonas hydrophila* | 1.000 |
| B110 | *P*IA | 1 | yellowish, small | *Pseudomonas aeruginosa* | *Aeromonas hydrophila or Aeromonas veronii* | 0.986 |
| B111 | *P*IA | 1 | yellowish, large | *Pseudomonas aeruginosa* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B112 | *P*IA | 1 | Yellowish | *Pseudomonas aeruginosa* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B113 | *P*IA | 2 | White | *?* | *Escherichia vulneris* | 1.000 |
| B114 | *P*IA | 2 | white, small | *?* | *Citrobacter freundii* | 0.971 |
| B115 | *P*IA | 2 | white, large | *?* | *Aeromonas media or Aeromonas veronii* | 0.998 |
| B116 | *P*IA | 2 | White | *?* | *Citrobacter braakii or Citrobacter freundii* | 1.000 |
| B117 | *P*IA | 2 | White | *?* | *Citrobacter freundii* | 1.000 |
| B118 | *P*IA | 3 | Yellowish | *Pseudomonas aeruginosa* | *Aeromonas hydrophila or Aeromonas veronii* | 1.000 |
| B119 | *P*IA | 3 | Yellowish | *?* | *Aeromonas hydrophila or Aeromonas veronii* | 0.979 |
| B120 | *P*IA | 3 | greenish yellow, bright | *Pseudomonas aeruginosa* | *Pseudomonas otitidis* | 1.000 |
| B121 | *P*IA | 3 | greenish yellow, bright | *Pseudomonas aeruginosa* | *Pseudomonas otitidis* | 1.000 |
| B122 | *P*IA | 3 | white, large | *?* | *Aeromonas hydrophila or Aeromonas caviae* | 1.000 |
| B123 | *C*SA | 1 | grayish, very small | *Campylobacter jejuni* | *Acinetobacter lwoffii* | 1.000 |
| B124 | *C*SA | 1 | grayish, very small | *Campylobacter jejuni* | *Acinetobacter lwoffii* | 0.925 |
| B125 | *C*SA | 1 | Grayish | *Campylobacter jejuni* | *Acinetobacter lwoffii* | 0.913 |
| B126 | *C*SA | 1 | Grayish | *Campylobacter jejuni* | *Acinetobacter lwoffii* | 0.923 |
| B127 | *C*SA | 1 | grayish, very small | *Campylobacter jejuni* | *Acinetobacter lwoffii* | 0.923 |
| B128 | *C*SA | 2 | grayish, very small | *Campylobacter jejuni* | *Acinetobacter lwoffii* | 1.000 |
| B129 | *C*SA | 2 | white, large | *Campylobacter laridis* | *Acinetobacter calcoaceticus or Acinetobacter rhizosphaerae* | 0.923 |
| B130 | *C*SA | 2 | brownish grey, large | *Campylobacter coli* | *Comamonas aquatic* | 1.000 |

| Labeling | Medium | Site | Morphology | Possible Species | RDP SEQMATCH Identification | Similarity of Sequences |
|---|---|---|---|---|---|---|
| B131 | *C*SA | 2 | grayish, small | *Campylobacter jejuni* | *Acinetobacter beijerinckii* | 1.000 |
| B132 | *C*SA | 2 | white, large | *Campylobacter laridis* | *Acinetobacter calcoaceticus or Acinetobacter rhizosphaerae* | 1.000 |
| B133 | *C*SA | 2 | brownish grey, large | *Campylobacter coli* | *Comamonas aquatic* | 1.000 |
| B134 | *C*SA | 2 | grayish, small | *Campylobacter jejuni* | *Acinetobacter beijerinckii* | 1.000 |
| B135 | *C*SA | 2 | white, large | *Campylobacter laridis* | *Acinetobacter calcoaceticus or Acinetobacter rhizosphaerae* | 1.000 |
| B136 | *C*SA | 2 | brownish grey, large | *Campylobacter coli* | *Comamonas aquatic* | 1.000 |
| B137 | *C*SA | 3 | cream-grey | *Campylobacter coli* | *Roseomonas cervicalis* | 1.000 |
| B138 | Chromagar Orientation | 2 | white, large | *Pseudomonas* | *Acinetobacter baylyi* | 0.990 |
| B139 | Chromagar Orientation | 3 | dark blue | *Klebsiella/Enterobacter/Citrobacter* | *Aeromonas hydrophila or Aeromonas caviae* | 1.000 |
| B140 | Chromagar Orientation | 3 | blue, white edge | *Klebsiella/Enterobacter/Citrobacter* | *Enterobacteriaceae bacterium or Serratia fonticola* | 0.989 |
| B141 | Chromagar Orientation | 2 | violet, white edge | *S. saprophyticus* | *Acinetobacter baylyi* | 0.990 |
| B142 | Chromagar Orientation | 1 | dark blue | *Klebsiella/Enterobacter/Citrobacter* | *Citrobacter braakii or Citrobacter freundii* | 0.997 |

### 4.3.4 Classified Bacteria Within and Between Sites

The 132 classified sequences comprised 3 different phyla with a total of 21 different genera. The most dominant phylum was by far Proteobacteria which accounted for over 96 % of all sequences. Members of the phyla Firmicutes and Bacteroides were represented in a low abundance, whereas these phyla could only be found on the Chromagar orientation media.
On genus level *Aeromonas* dominated with over 21 % of all sequences, followed by *Pantoea* (13.6 %), *Citrobacter* (12.9 %), *Acinetobacter* and *Enterobacter* (11.4 % each). The remaining 30 % of all sequences consisted of *Pseudomonas, Escherichia/Shigella, Herbaspirillum, Comamonas, Exiguobacterium, Bacillus, Serratia, Klebsiella, Erwinia, Chryseobacterium, Roseomonas, Ralstonia, Salmonella/Kosakonia, Cronobacter, Plesiomonas* and *Raoultella*.

Identifications on species level were only considered reliable when there was at least 97 % similarity between the sequence of the DNA amplicon and the database sequence.

All isolates from site 1 belonged to *Proteobacteria.* More precisely, 12 isolates were represented by the genus *Aeromonas*, seven by *Enterobacter,* six by both *Pantoea* and *Acinetobacter,* five by *Citrobacter,* three by *Herbaspirillum,* two by *Escherichia/Shigella* and one by *Klebsiella.* Although some of these genera would fit to the searched waterborne pathogens, it was not possible to isolate neither of them. Solely two isolates of the indicator organism *Escherichia coli* derived from Chromagar ECC could be identified. Even though a high number of the *Aeromonas* sequences were 100 % identically with *Aeromonas hydrophila* database sequences, it was not possible to confirm the presence of this pathogen because the same sequence is also 100 % identically with A*eromonas veronii.*
One isolate of the pathogen *Acinetobacter lwoffii* which has been reported to be able to cause serious illness (Roy et al., 2015) could be identified. However, this possible pathogen was not subject of the screening.

Sequences from site 2 isolates composed of nine *Citrobacter* and nine *Acinetobacteria,* six *Pantoea,* five *Aeromonas, f*our *Escherichia/Shigella,* three *Comamonas* and three *Pseudomonas,* two *Herbaspirillum* and two *Enterobacter.*

Furthermore, one sequence of each *Klebsiella, Salmonella/Kosakonia, Cronobacter, Raoultella* and *Bacillus* were identified.
As in site 1 none of the targeted pathogens could be identified. The problem of a multiple match of sequences occurred again within *Aeromonas* species and within possible S*higella* and *Salmonella s*pecies. One *Escherichia coli* isolate could be verified with a certainty of 98.2 % and another *Acinetobacter lwoffii* could be identified.

The isolates from site 3 were most diverse and consisted of 11 *Aeromonas,* six *Pantoea,* four *Pseudomonas,* three *Citrobacter* and three *Enterobacter s*equences, two sequences of each *Erwinia, Klebsiella, Serratia* and *Exiguobacterium* as well as of one sequence of *Chryseobacterium, Roseomonas, Ralstonia, Herbaspirillum, Plesiomonas* and *Bacillus.*
In despite of the diversity none of the identified species were pathogenic and in contrast to the other two sites no *Escherichia coli* could be isolated.

## 4.4 Discussion

The results show, on the one hand, that 16S rDNA sequencing is principally suitable for the identification of different bacteria on species level. On the other hand, an accurate identification is highly dependent on the sequenced section of the 16S rDNA. This is especially demonstrated by matches of individual sequences with multiple different species as occurred several times in this study. In order to clarify this issue, another Sanger sequencing step of the same amplicons, yielding in a longer 16S rDNA read, would be necessary.

Pathogens were not detected in this study with the used selective media. Most of them did not support the growth of the microorganisms they actually should target and solely *Aeromonas* isolation agar and Chromagar ECC performed as expected. However, it might be possible that the targeted pathogens were displaced on their media by species dominating in abundance as long as media formulation fit to their nutritional requirements. This is especially indicated by *Aeromonas* species which were found not only on *A*IA but also on Pseudomonas isolation agar, *Salmonella Shigella* agar and on Chromagar orientation. Furthermore, the inoculum could have been not concentrated enough for the cultivation of pathogens.

Although in this study it was not possible to cultivate the targeted pathogens it is still possible that they exist in EWM in a viable but non-culturable state.

# 5 Conclusion

The results of this study demonstrate that there is a huge gap between the ascertainability of bacterial communities using culture-dependent and culture-independent methods. Only about 25 % of the metagenomic bacterial diversity associated with the EWM could be captured by cultivation techniques.

What is particularly interesting is the fact that both the general and the selective cultivation approach resulted in similar bacterial community structures within the sampling sites. This suggests that these identified bacteria were the most competitive and therefore suppressed the growth of other bacteria. Nevertheless, it seems that most of the bacteria associated with the EWM are simply not cultivable in the laboratory.

This study provides a good insight into the microbial communities associated with the EWM and presents a base for further investigation towards the identification and development of biological control agents.

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