Kingdom of Morocco

Ministry of National Education, Vocational Training,
Higher Education and Research

MOHAMMED V UNIVERSITY OF RABAT

FACULTY OF MEDICINE AND PHARMACY OF RABAT

MASTER'S THESIS

MASTER OF MEDICAL BIOTECHNOLOGY
OPTION: BIOMEDICAL

Theme

Exploring Microbial Diversity of Sebou's river using a Metagenomic approach

Presented by:
Ms Ikram GARBOUNI

Supervised by :
Pr. Hassan GHAZAL

Promotion: November 2020.

Jury members:

President: IBRAHIMI Azeddine, Professor, Faculty of Medicine and Pharmacy of Rabat
Supervisor: GHAZAL Hassan, Professor, National Center for Scientific and Technical Research
Examiner: ALLALI Imane, Professor, Faculty of Sciences of Rabat
Examiner: HAMMANI Khalil, Professor, Sidi Mohamed Ben Abdellah University, Fès
Examiner: CHAHBOUNE Rajae, Professor, Faculty of Medicine and Pharmacy of Tangier

Abstract

Sebou river is the second largest river in Morocco, starting from a source in the middle Atlas at an altitude of 2800m, traversing 600Km, and draining 40,000Km^2, and flows into the Atlantic Ocean near the city of Kenitra. This study aims to explore the microbial communities in the Sebou river and its variation between two seasons, Summer and Winter, and during three consecutive years. This will help to determine the impact of pollution on Sebou's microbial communities, and defining any sources of pathogenic infection might come from this river. In order to identify Operational Taxonomy Units (OTUs) from the 6 samples, the Metagenomics Micca package was used. Phyloseq bioinformatic tool has been used to determine the alpha and beta diversities of the river samples, as well as the taxonomy profile of each sample. Results showed that alpha diversity decreased from 2014 to 2016 in both summer and winter. Indeed, Shanon Index for summer went from 9 (2014) to 8 (2015), to 6.8 (2016)) while for winter, it went from 8 (2014) to 6.7 (2015), to 3 (2016). The alpha diversity of summer's communities was always higher than winter's one. BOD5, COD, and NO^{3-} increased from 2014 to 2016, indicating that the diversity most probably decreased due to the high pollution levels in the river sampling areas. The taxonomic profiles of Sebou's river include 36 families and 58 Genera. Taxonomy profiles showed the dominance of the Moraxellaceae family in 2016, especially the genus Acinetobacter, these are known as pathogenic agents that might cause pneumonia, bloodstream infection, and urinary tract infections. Such findings may contribute to characterizing Moroccan the microbial river inventories.

Keywords: Sebou river, Metagenomics, Pollution, Taxonomy profiling, Biodiversity, Microbiome.

Résumé

Le fleuve Sebou est le deuxième plus grand fleuve du Maroc, partant d'une source du Moyen Atlas à une altitude de 2800m, traversant 600Km, drainant 40000Km2, et se jette dans l'océan Atlantique près de la ville de Kénitra. Cette étude vise à explorer les communautés microbiennes de la rivière Sebou et sa variation entre deux saisons, été et hiver, et pendant trois années consécutives, ce qui permettra de déterminer l'impact de la pollution sur les communautés microbiennes de Sebou et de définir les sources d'infection pathogène éventuelles. Nous avons utilisé le package de Métagénomique Micca afin d'identifier les unités taxonomiques opérationnelles (OTU) à partir des 6 échantillons. L'outil bioinformatique Phyloseq a été utilisé pour déterminer les diversités alpha et bêta des échantillons fluviaux, ainsi que le profil taxonomique de chaque échantillon. Les résultats ont montré que la diversité alpha a diminué de 2014 à 2016 aussi bien en été qu'en hiver. En effet, l'indice de Shannon pour l'été est passé de 9 (2014), 8 (2015), à 6,8 (2016) tandis que pour l'hiver, il est passé de 8 (2014), 6,7 (2015), à 3 (2016). La diversité alpha des communautés d'été était toujours plus élevée que celle de l'hiver. La DBO5, la DCO et la concentration en NO^{3-} ont augmenté de 2014 à 2016, ce qui indique que la diversité a diminué fort probablement en raison des niveaux de pollution élevés dans les zones d'échantillonnage des rivières. Les profils taxonomiques de la rivière Sebou comprennent 36 familles et 58 genres. Ces profils de taxonomie présentent une domination de la famille des Moraxellaceae en 2016, en particulier le genre Acinetobacter, ces derniers sont connus comme des agents pathogènes susceptibles de provoquer une pneumonie, une infection de la circulation sanguine et des infections des voies urinaires. Ces résultats contribuent à la détermination des inventaires microbiens fluviaux au Maroc.

Mots clés : La rivière de Sebou, Métagénomique , Profil taxonomique, Biodiversité, Microbiome

Acknowledgement

I would like to express my special thanks of gratitude to my advisor **Professor Hassan GHAZAL** who gave me the golden opportunity to do this wonderful project on the topic, which also helped me in doing a lot of Research and I came to know about so many new things. I am really thankful to them.

I would like to extend my gratitude to **Professor Azeddine IBRAHIMI** for giving me a chance to continue my studies in this Master. I acknowledge from my depth all those who have helped me to put these ideas, well above the level of simplicity and into something concrete.

I would like to express my special thanks of gratitude to **Professor HAMMANI Khalil** as well as **Professor Imane ALLALI**, and **Professor Rajae CHAHBOUNE** who gave me numerous remarks and generous information to help me with my thesis.

I would also like to thank my parents and family who were my emotional and financial support throughout my work.

List of figures

List of tables

List of abbreviations

BOD5: Biochemical Demande of Oxygen in 5 Days

COD: Chemical Demande of Oxygen

OTUs: Operational Taxonomic Units

PCA: Principal Components Analysis

PE: Polyethylene

RSD: River Sampling Day

Table of content

Introduction

Sebou river is the second largest river in Morocco [1], starting from the middle Atlas at an altitude of 2,800m and flowing in the Atlantic ocean [2]. It is related to many water dams, and it's used as a water source for all the population around it, and also for irrigation [2], hence, it has an important social economical value [2].

Sebou river is known for its interesting biodiversity, in terms of microfauna it has more than 52 families [3], in addition to fungal communities [4], and interesting fishes such as *Anguilla anguilla* [5]. Unfortunately, this biodiversity is influenced by pollution caused by industrial, and agronomic activities near to the river [4,6].

The river pollution presents a real threat to public health, the industrial activities increase the number of heavy metals in those aquatic sources [1], those are highly toxic elements that threaten all populations around rivers [1]. The agronomic activities increase the amount of nitrogen and phosphor elements in water, which has an impact on the biodiversity of those ecosystems and public health [7]. Changes in biodiversity due to pollution might integrate pathogenic species like parasites, those can be transmitted to humans either via water directly, or via fishery resources [8].

Sebou is indeed infected by agronomic and industrial activities, which increased the number of nitrogen elements, mainly NH3, phosphor and heavy metals in it [9]. The pollution of this site influenced its faunistic and fungal diversity [4,6].

Metagenomics term stands for the study of microbial communities, their structures and functions [10], this field aims not only to study culturable strains but to unhide the incurable strains [11]. The starting key point of this field was in the 1970s when Woese revealed the ability to study rRNAs genes to find unculturable bacterias [11]. With the development of the bioinformatic field, those works led to an approach in metagenomics called amplicons studies [11]. It consists of studying the similarities and differences between variable regions in rRNAs genes in order to determine the composition of a microbial community [11].

This study took part in the River Sampling Day (RSD) initiative, which aims to explore the composition of river microbial communities around the world [12], as for our study, we aim to explore the changes in Sebou's microbial community caused by pollution, in addition to revealing any public health threat might come from these communities.

1. The river of Sebou

The river of Sebou is the second largest river in Morocco [1], it drains 40,000 km2 and travels some 600 km, starting from a source at an altitude of 2,800m in the middle Atlas mountains, and flows in the Atlantic ocean near to Kenitra city (Figure 1) [1].

Sebou river has an important social and economical value, this is due to its use as a source for irrigation, and the presence of water dams related to it [2].

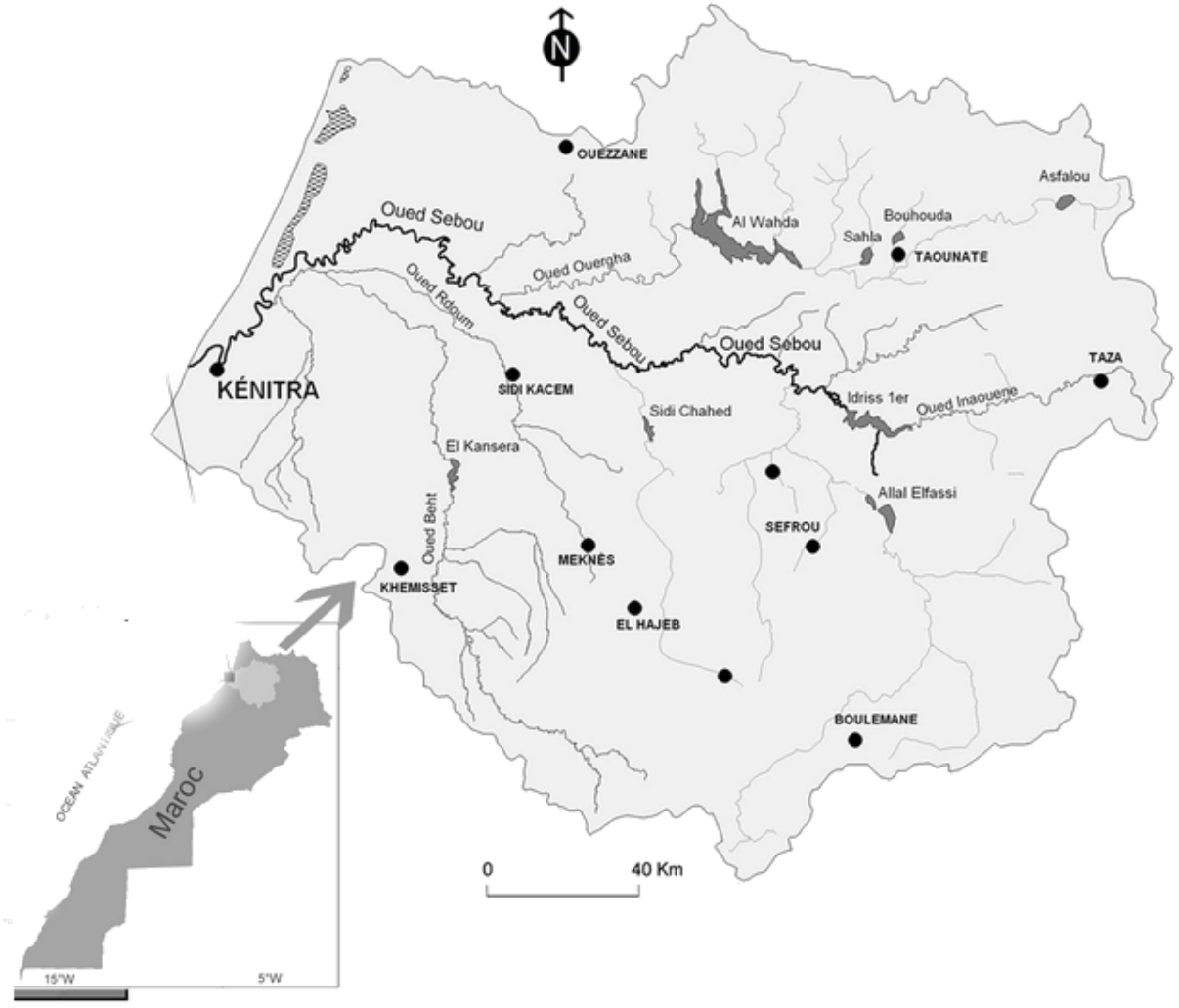

Figure 1: The Localization of the river of Sebou, **the bold black** line represents the river of Sebou [3].

2. The biodiversity of Sebou's river

The river of Sebou is known for its high biodiversity, its fauna contains more than 52 families and 80 taxons, they belong mainly to three groups, Annelids, Molluscs, and arthropods [7]. This river also has high fungal biodiversity, mainly characterized by Ascomycetes, Dematic, and Mucedinace [4], in addition to its fish community that is composed mainly of *Anguilla anguilla* [6].

This biodiversity gives also an important ecological, and social economical value to this basin [7], unfortunately, this biodiversity is influenced by pollution, which led to distrust habitat of some species and caused their disappear [6], the biodiversity of this river indeed has changed, many fungal characteristic species to pollution appeared in Sebou ecosystem [4].

3. River pollution and public health

Many studies revealed the effect of pollution of rivers in increasing the amount of highly toxic components. A study by Laissaoui et al. [1] revealed the increasing of heavy metals, such as Pb, Fe, Co and Cu from the source to the estuary, this is due to the industries deployed near to the river in the region of Gharb [1].

The river pollution also influences the composition of their ecosystem, such things lead to integrate some pathogenic fungal [4], or parasites to those ecosystems, such species can be transmitted to humans not just via water, but also through its fishery resources [5].

The Sebou River indeed is known by severe pollution downstream from the city of Fez, notably Nitrogenous components, mainly NH4, and phosphoric components. The most polluted sites are the ones that are directly under the influence of domestic and industrial wastewater inputs, in particular tannery effluents. Obviously, the concentrations measured at these locations defy all environmental quality standards. Pollutant loads are very heavy in the Sebou River and can contaminate the river course for kilometres. Hence, as the water of the Sebou River is used for the irrigation of vegetables, serious problems of public health could arise [13].

4. RSD initiative

River Sampling Day (RSD) is an initiative led by the global marine sampling campaign which is part of the EU-funded Micro B3.

This program was launched in order to discover the microbial communities in rivers, since they play important roles in biogeochemical cycles of matter, while there is a huge gap in knowledge of those communities.

RSD consist of a simultaneous sampling campaign of the world's rivers, the pilot study started 2013, the sampling day takes place on the solstices (June 21st and December 21st), collected samples of each year are contextualized by the same environmental parameters, and they created one dataset, which provides a good view of the biodiversity of rivers microbial communities, and their functions [12].

5. Overview about metagenomics

Metagenomics is the analysis of data produced through randomly sequencing fragments from a DNA pool extracted from environmental samples [10], it is mostly applied to study the composition and functions of microbial communities in their native environment [10].

Metagenomics helps to study not just culture dependent microorganisms, but also uncultured microorganisms [10]. The era of this field started with Carl Woese in the 1970s when he worked on small subunit ribosomal in order to unhide the non cultured microorganisms [11], his works on those units led with the development of bioinformatics to create an important approach in metagenomics studies called amplicons study [11].

Small ribosomal subunits genes are characterized by the presence of constants and variable regions, the first generally remain stable between different taxa, while the second is different, this structure was used to identify taxa by only using those genes sequences [11].

The amplicons approach consist on finding similarities between variable regions in small ribosomal subunits genes by mapping them to each other, this lead to creating clusters of sequences called Operational Taxonomic Units (OTUs), the alignment of OTUs clusters with predefined databases can lead to discovering the composition on a microbial community [11].

The use of OTUs can not just help in discovering microbial communities' composition, but also the abundance each OTU in the pool refers to the abundance of each species in the community [11] (Figure 2).

The second approach of metagenomic is the whole microbiome analysis, unlike the amplicons approach [14], whole microbiome approach does not study specific genomic regions, it is based on sequencing the while microbiome [14], assemble it, then performing the phylogenetic analysis and the annotation, this approach leads not just to discover the composition of the microbial community, but understanding all its functions and pathways (Figure 2) [14].

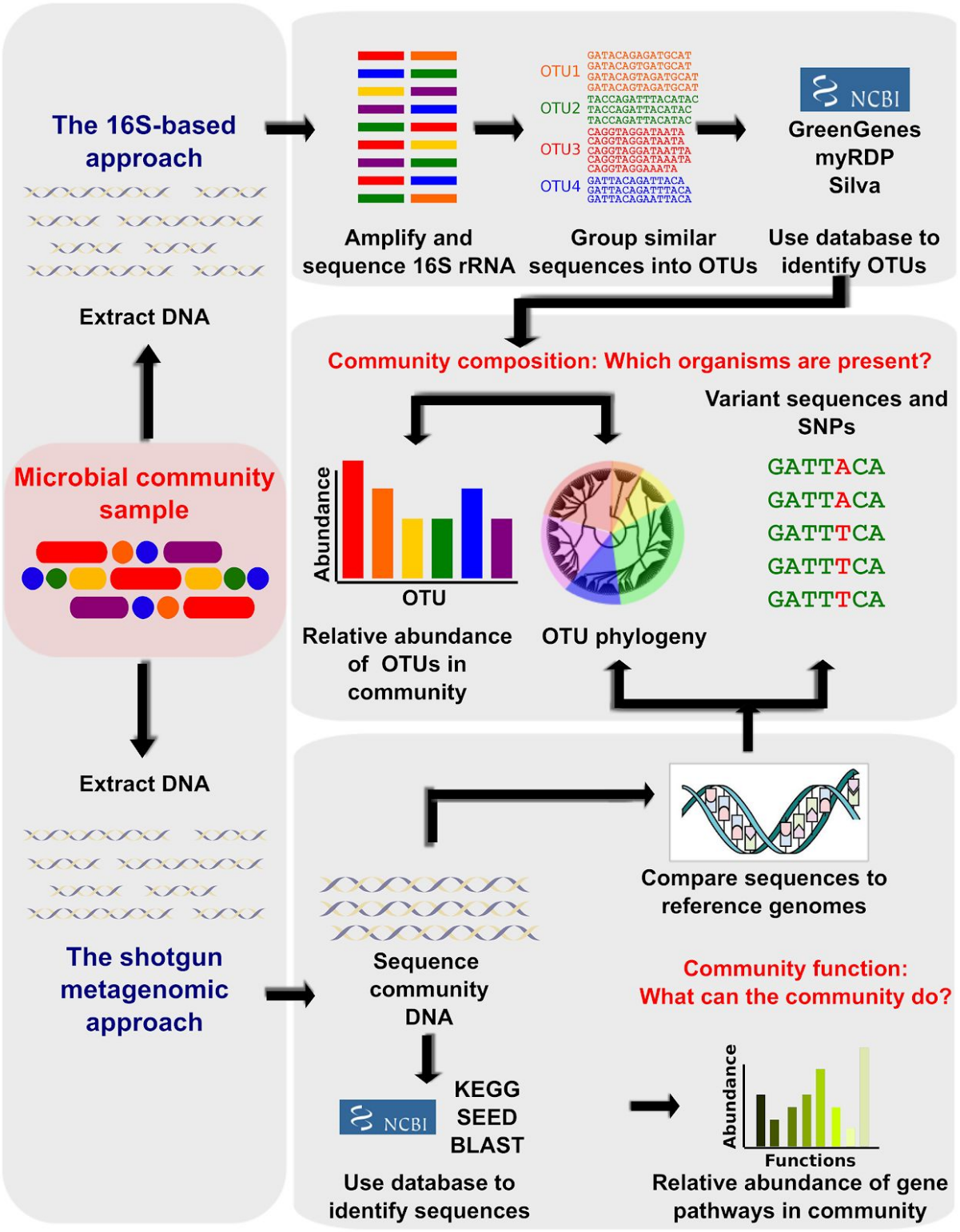

Figure 2: *An overview of the metagenomic workflow to analyse the microbial diversity in a given sample [15].*

6. Aim of the study

This study is a part of the RSD initiative, it aims to discover the bacterial population of Sebou river, and the influence of pollution on this population, which might help to define the sources of infections coming from this river.

7. Material and Methods

7.1. Sample Collection

The sampling of freshwater from Sebou (Coordinates:34.3002267 N 5.04079777 W) was performed during both winter and summer seasons (June 21st and December 21st) in the course of three years: 2014, 2015 and 2016. The samples were collected in polyethylene (PE) bottles, which were washed with 10% acid before use. About 5L of freshwater was collected from the surface (1-2 m) of each site between 10 pm to 2 pm for each time zone. These sample bottles were stored at 4°C then transported to the laboratory for further processing.

7.2. Physical and chemical measurements

Physical and chemical parameters of the site were taken in situ close to the date/time when the samples are collected using a multivariate calibrated probe. Those parameters were mainly, concentration of the Nitrate (NO3-), and the pH. The Biochemical Oxygen Demand (BOD) for each sample was recorded during 5 days at 20°C using an incubation flask (OxiTop ® Control measuring system). The Chemical Oxygen Demand (COD) was determined using a strong oxidizing reagent (potassium dichromate $Cr_2O_7^{2-}$) under acidic condition.

7.3. DNA extraction

Freshwater samples that were collected, were filtered through a 0.22 µm Sterivex™ filter (Merck Millipore, US). About 5L of freshwater samples were pumped using a vacuum system in order to collect microbial communities. The filters were stored immediately at -80°C till DNA extraction.

Microbial DNA extraction was performed using DNeasy PowerWater Sterivex Kit (QIAGEN, Germany) as described in the protocol. For each sample, one Sterivex filter was

used for DNA extraction. The concentration and quality of eluted DNA were determined using a spectrophotometer (NanoDrop3300, Thermo Fisher Scientific, USA).

7.4. DNA sequencing

16S rRNA genes were amplified using the primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 909R (5'- CCCCGYCAATTCMTTTRAGT-3') targeting variable regions V4-V5 [16]. Amplicons were sequenced using Illumina MiSeq (Illumina, USA) using a MiSeq Reagent kit v3 following the standard guidelines for preparing and loading samples on the MiSeq (Illumina, USA).

7.5. Data preprocessing

7.5.1. Quality profiling

Before starting the processing of the raw data, we merged each sample's forward and reverse sequences into one merged file using the command "mergepaires" of Micca package [17], we then profiled the quality of each merged sample using the command "stats" from the same package, this command generates three plots, one shows the distribution of read length, one for the distribution of quality score per position in read, and one for the average quality score per position in read.

7.5.2. Data preprocessing

In order to filter the quality of the merged data, we first used the command "filterstats" from Micca package [17], this command calculates the error rates and the distribution of read size, which helps in choosing filtering parameters. The filtration was done using the command "filter" of the same package.

7.6. OTU clustering and taxonomy assignment

In order to pick OTUs from the filtered data, we used the command "otu" from Micca package [17], we clustered the OTUs with a similarity of 97% using *de novo* clustering, then we removed chimeric sequences from the OTUs table using the same command with the parametre "rmchim". we assigned then the taxonomy to the resulting OTUs using rdp classifier [18], which uses the rdp database as a reference database in order to assign taxonomy. At the end, we generated plots of the most abundant taxa per year and per season,

this was performed by retrieving the ten most abundant OTUs for each sample using Phyloseq [19], then plot the corresponding taxa of each OTU using ggplot2 in R.

7.7. Alpha and Beta Diversity

In order to study the alpha and beta diversity, I fed the resulted Biom file intoPhyloseq [19] package in R, I then generated alpha diversity plots using "Chao-1" and "Shannon" indices, the first index refers to the abundance of each specie, hence, it increases when the number of species' individuals is high. While the second one is related to the number of species, it increases when the number of species is high. The beta diversity was reported using Principal Component Analysis (PCA), which presents the diversity between samples depending on the abundance of OTU, water temperature, BOD5, COD, and the concentration of NO3-. Plots were generated using the ggplot2 package in R.

8. Results

8.1. Quality profile and data preprocessing

The distribution of read length shows that the amount of reads decreases with read size, where it becomes less than 40% when the size becomes higher than 200 (Figure 3).

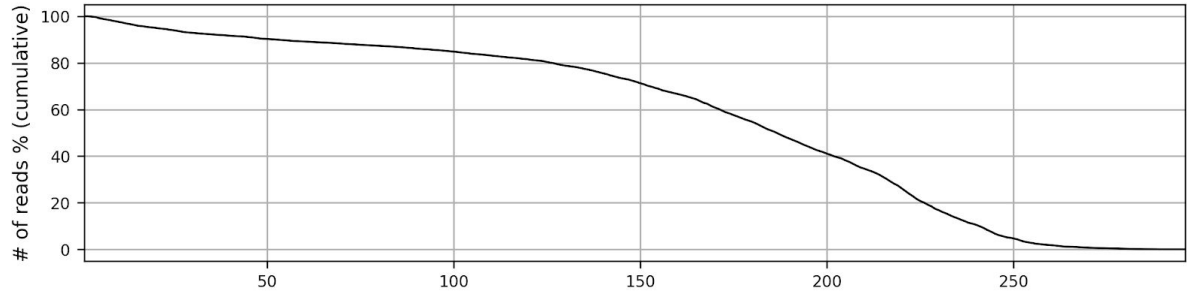

Figure 3: *Distribution of read length of the raw data.* ***Horizontal axis*** *presents the read length.* ***Vertical axis*** *presents the percentage of reads.*

The average quality distribution per read position seems to be higher than 30 for all bases in positions below 200, then it starts to decrease for the bases after this position (Figure 4)

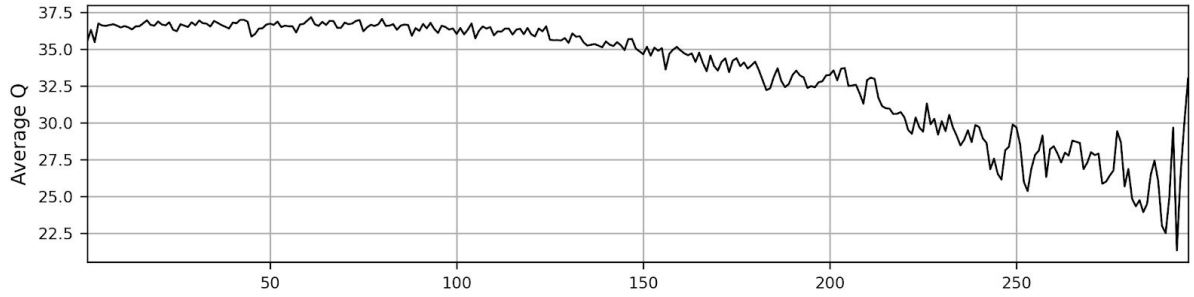

Figure 4: *Distribution of the quality average per position of raw data.* ***Horizontal axis*** *presents the read position.* ***Vertical axis*** *presents the average of quality.*

The average of expected error rate shows that the rate of errors generally is lower than 0.1% among the data, while it increases in the extremities to reach 0.25% in the starts of reads and 0.15% at the end of reads (Figure 5)

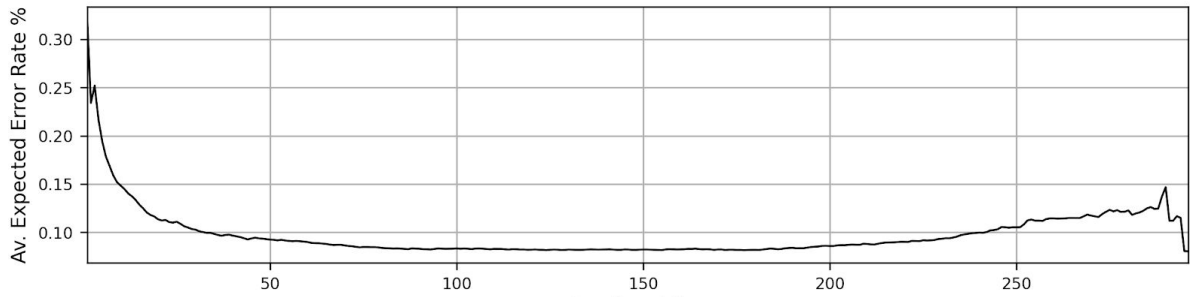

Figure 5: *Distribution of expected rate error per position of raw data.* ***Horizontal axis*** *presents the read position.* ***Vertical axis*** *presents the expected error rate.*

After the filtration, read lengths were adjusted to remove small sequences, only sequences with a size higher or equal to 150b were kept (Figure 6)

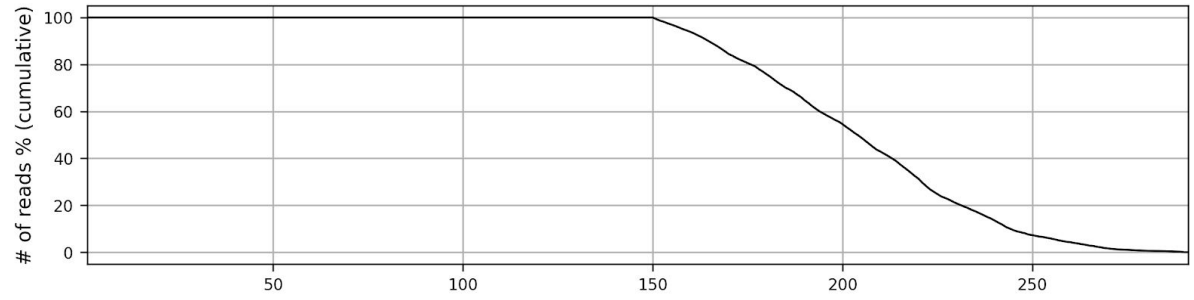

Figure 6: *Distribution of read length of the filtered data.* ***Horizontal axis*** *presents the read length.* ***Vertical axis*** *presents the percentage of reads.*

The average quality per position was adjusted to be higher than 30 for almost all sequences, the average quality decreased below 30 only for bases after position 250 (Figure 7).

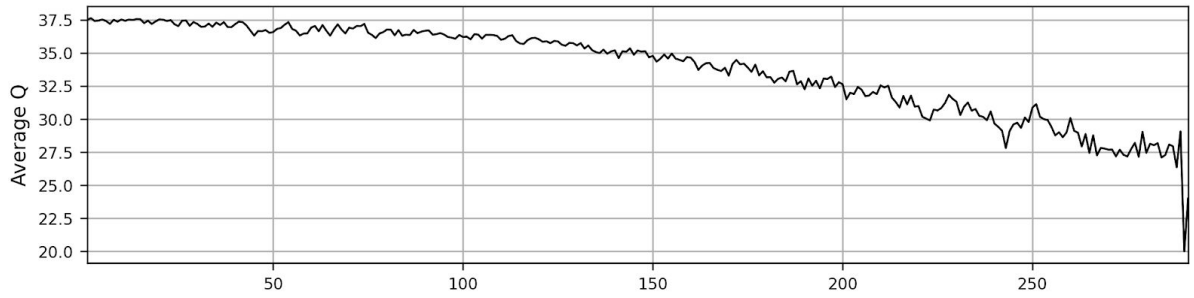

Figure 7: *Distribution of the quality average per position of filtered data.* ***Horizontal axis*** *presents the read position.* ***Vertical axis*** *presents the average of quality.*

After the filtration, the expected error rate was reduced to be below 0.14 for all sequences (Figure 8).

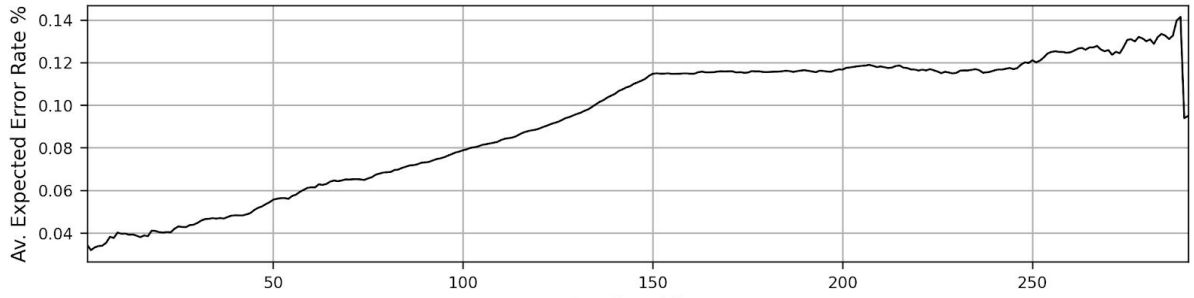

Figure 8: *Distribution of expected rate error per position of filtered data.* ***Horizontal axis*** *presents the read position.* ***Vertical axis*** *presents the expected error rate.*

8.2. OTU clustering

OTU clustering shows that the number of OTU's in summer's samples is always higher than winter's one. The number of OTU's decreased from 2014 to 2016 The frequency of chimeric sequences seems to be lower than 10% (Table 1).

Table 1: *Number of OTUs per sample.*

Saison	**Year**	**Number of OTUs**	**Frequency of Non-Chimeric sequences**
Summer	2014	14,033	95%
Winter	2014	5,763	93%
Summer	2015	11,373	94%
Winter	2015	1,172	91%
Summer	2016	4,311	92%
Winter	2016	388	95%

8.3. Alpha diversity

Chao-1 index is a diversity index that refers to the abundance of species in each community. The Chao-1 index decreased from 2014 to 2016, and the summer's index is always higher than winter's one (Figure 9).

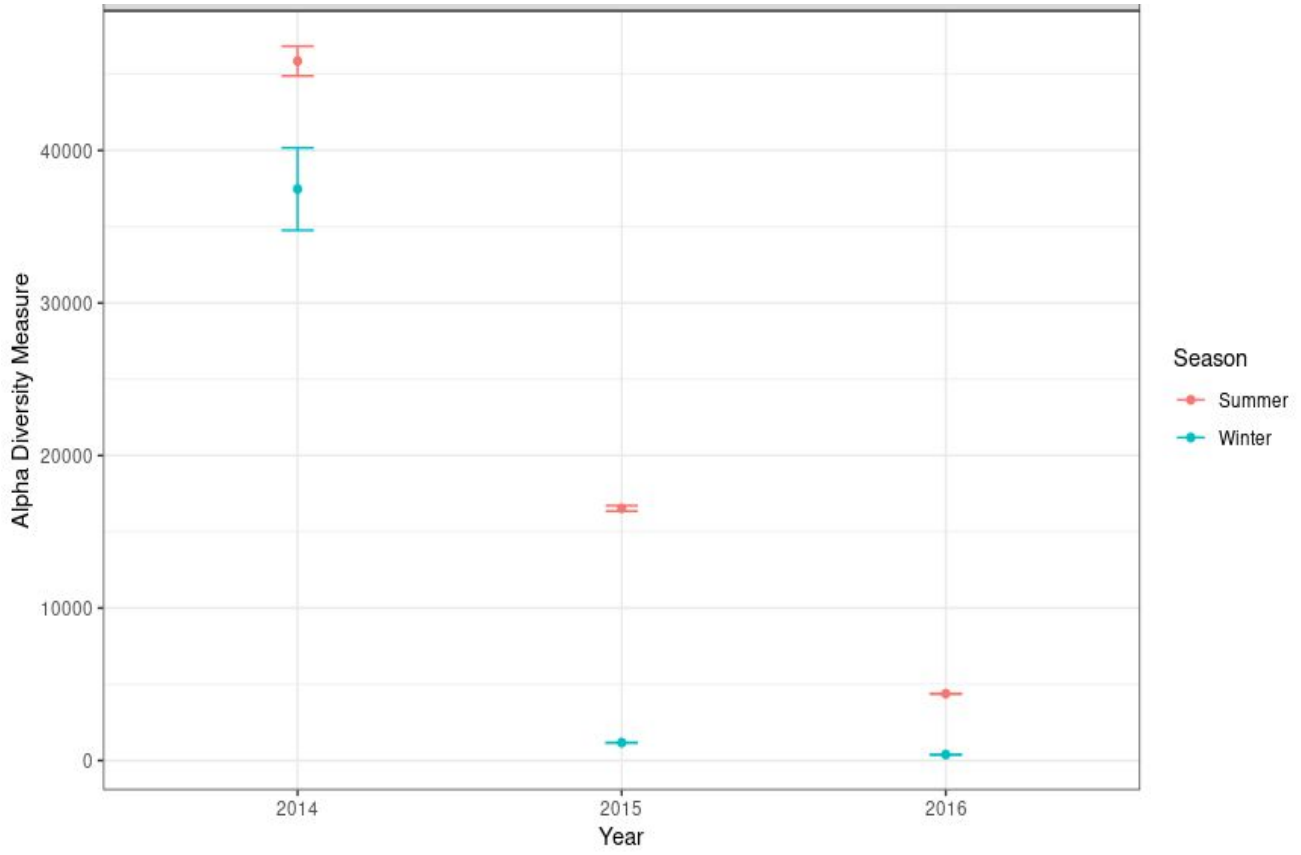

Figure 9: *Alpha diversity of samples using Caho-1 index.* ***Red*** *points represent summer's samples.* ***Blue*** *points represent winter's samples.* ***Points*** *présent the median abundance of species.* ***Upper line*** *presents the third quartile.* ***Lower*** *line presents the first quartile.*

Shannon index estimates the diversity in terms of the number of different species. The Shannon index decreased from 9 in 2014 to 3 in 2016, where summer's samples had a higher Shannon index than winter's samples (Figure 10).

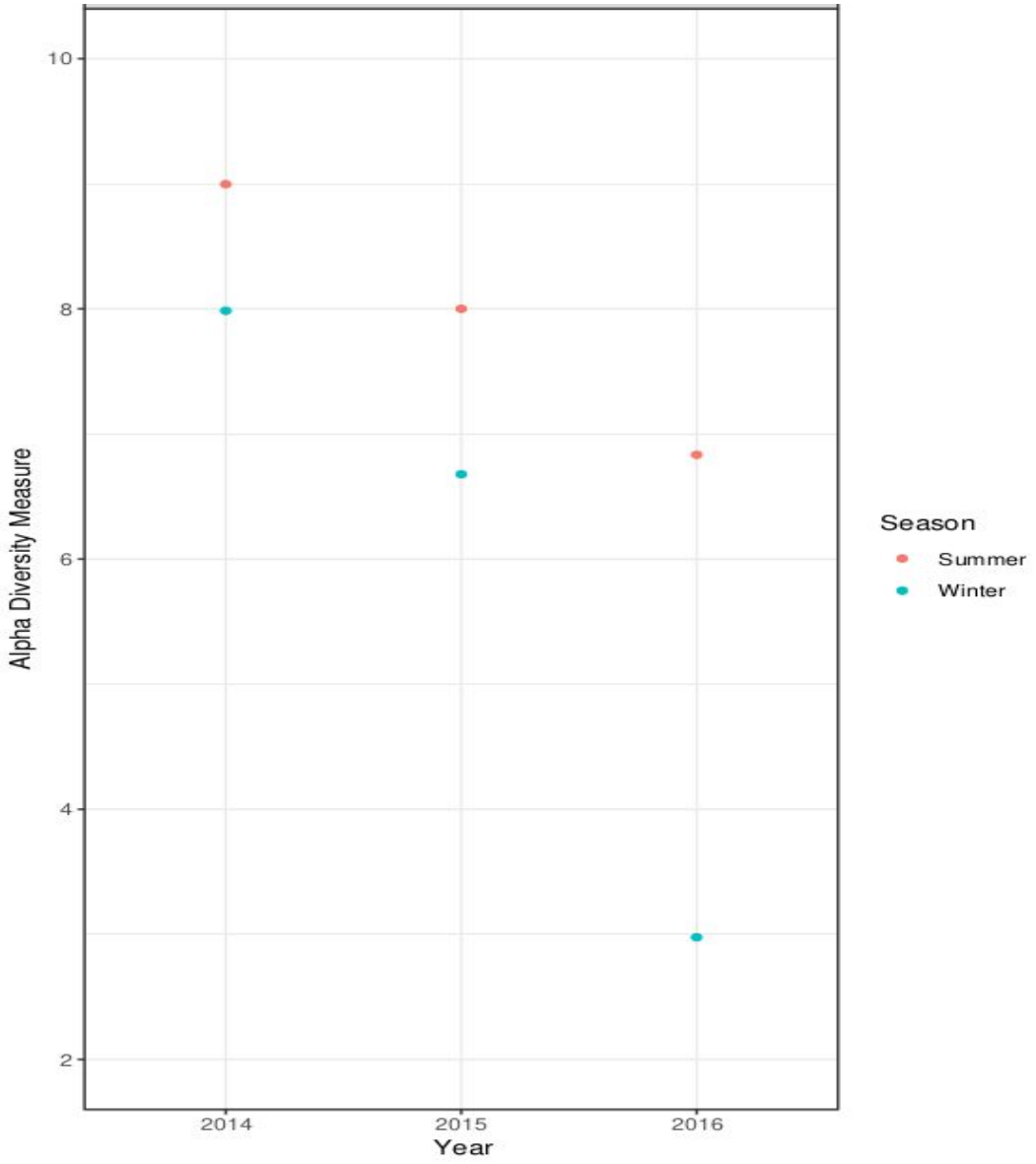

Figure 10: *Alpha diversity of samples using Shannon index.* ***Red*** *points represent summer's samples.* ***Blue*** *points represent winter's samples.*

8.4. Beta diversity

The PCA plot shows that the heterogeneity between summer's communities is higher than heterogeneity between winter's communities, the difference between summer and winter's samples is influenced mainly by pH and the concentration of nitrates, while the difference between 2014's, 2015's and 2016's samples is influenced by water temperature, COD, and BOD5. we observe that the number of OTUs decreased with the increase of nitrate concentration, COD, pH, and BOD5 (Figure 11).

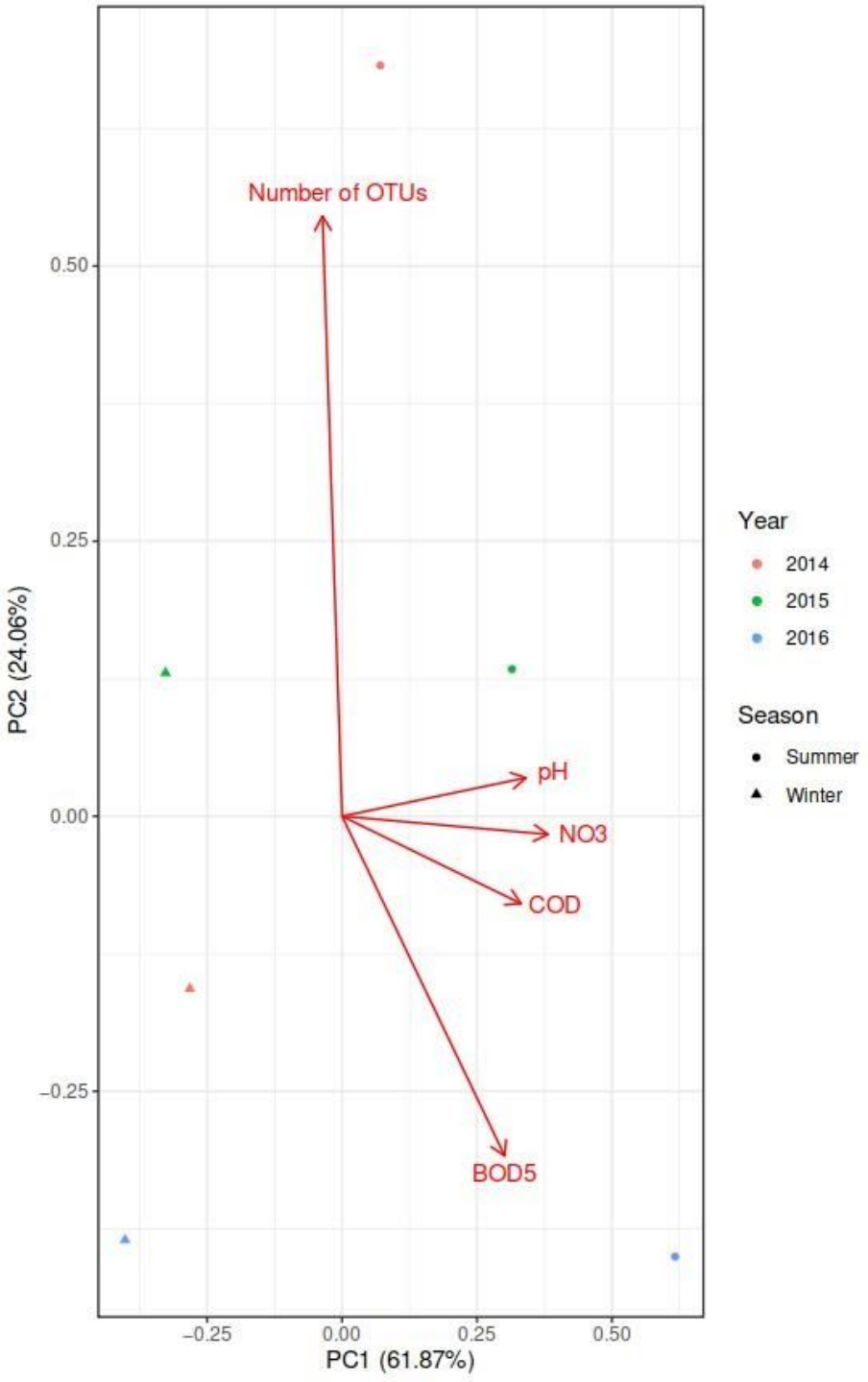

Figure 11: *PCA plot showing the beta diversity.* ***Red*** *points represent 2014's samples.* ***Green*** *points represent 2015's samples.* ***Blue*** *points represent 2016's samples.* ***Circular dots*** *represent summer's samples.* ***Triangle dots*** *represent winter's samples.* ***Red arrows*** *represent physical and chemical parameters.*

8.5. Taxonomy profiling

Taxonomy study reveals the presence of seven Phylum among all samples, where five of them are present in 2016 summer's sample and two in winter, six of them in 2015 summer's sample and three in winter, all of them are present in 2014 summer's sample and three in winter. The Proteobacteria exist in all samples, but its abundance decreases from 2014 to 2016, while the Acidobacteria phylum exists only in the 2016 summer's sample. There are ten classes, seven of them exist in 2016 summer's sample and two in winter, seven exist in 2015 summer's sample and four in winter, and eight classes exist in 2014 summer's sample and five in winter. The total number of orders is 27, seven of them are present in 2016 summer's sample and two in winter, ten orders exist in 2015 summer's sample and four in winter, while thirteen orders exist in 2014 summer's sample and five in winter. The total number of families is about 36, ten of them exist in 2016 summer's sample, eleven in 2015 summer's sample, and thirteen in 2014 summer's sample, as for winter's samples, six families exist in 2016's sample, six in 2015's sample, and eight in 2014's sample. As for genera, there is a total number of 58 in all samples, where fifteen exist in 2016's summer sample and eight in winter's one, thirteen exist in 2015 summer's sample and eight in winter's one, and eighteen exist in 2014 summer's sample and nine in winter's one (Table 2).

Table 2: *Number of taxa per sample.*

Year	Season	Number of Phyla	Number of classes	Number of orders	Number of families	Number of genera
2014	Summer	7	8	16	13	18
2014	Winter	3	5	8	8	9
2015	Summer	6	7	11	11	13
2015	Winter	3	4	6	6	8
2016	Summer	5	7	8	10	15
2016	Winter	1	2	6	6	8

The profile of the most abundant families per year shows that the Sporichthyaceae is the most abundant families in 2014, in 2015 Comamonadaceae, and Caulobacteraceae are the most abundant ones, while in 2016 Moraxellaceae, are the most abundant ones. (Figure 12).

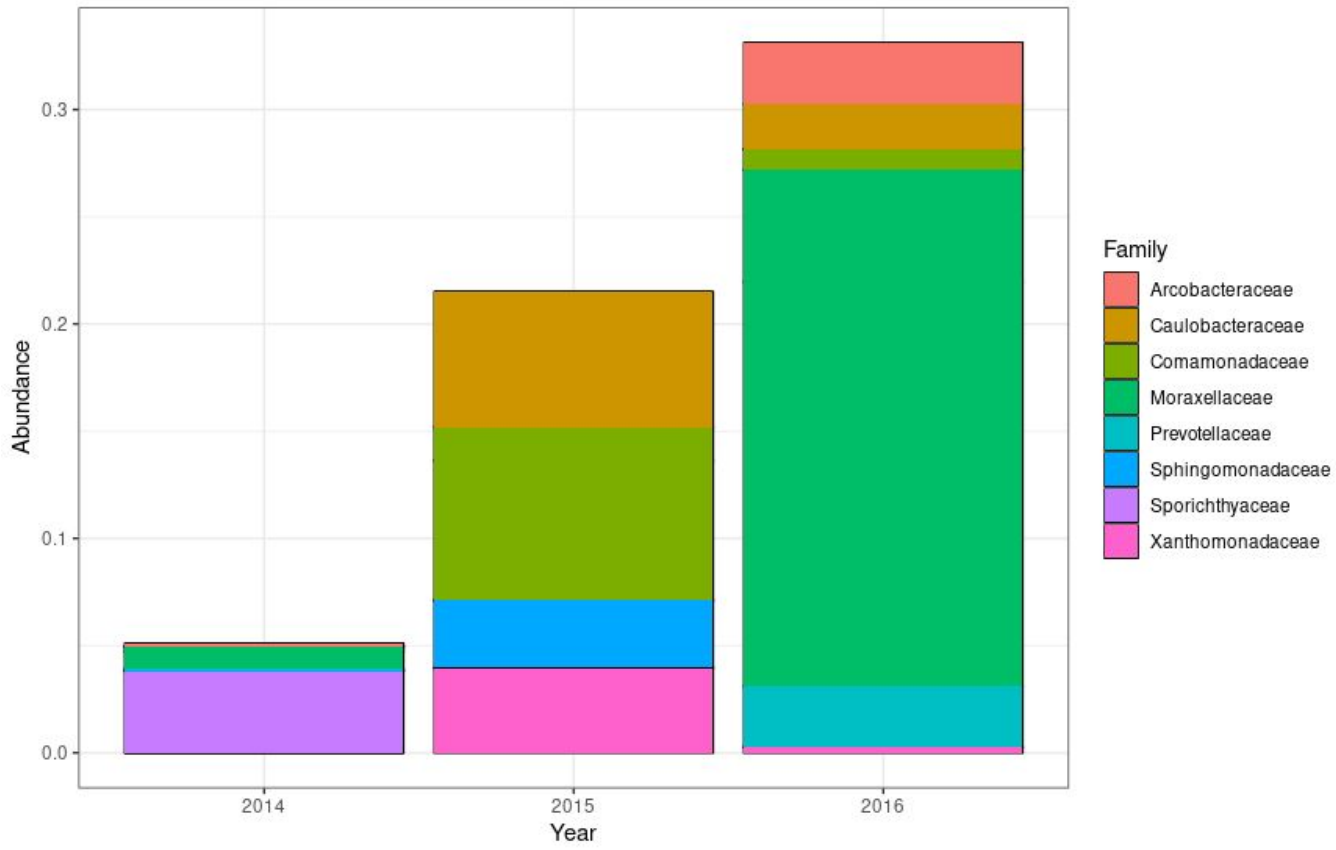

Figure 12: *Plot of the most abundant families each year.*

In the genus level, we observe a high abundance of hgcI clade in 2014, the abundance of Phenylobacterium and Idonella in 2015, and the abundance of Acinetobacter in 2016 (Figure 13).

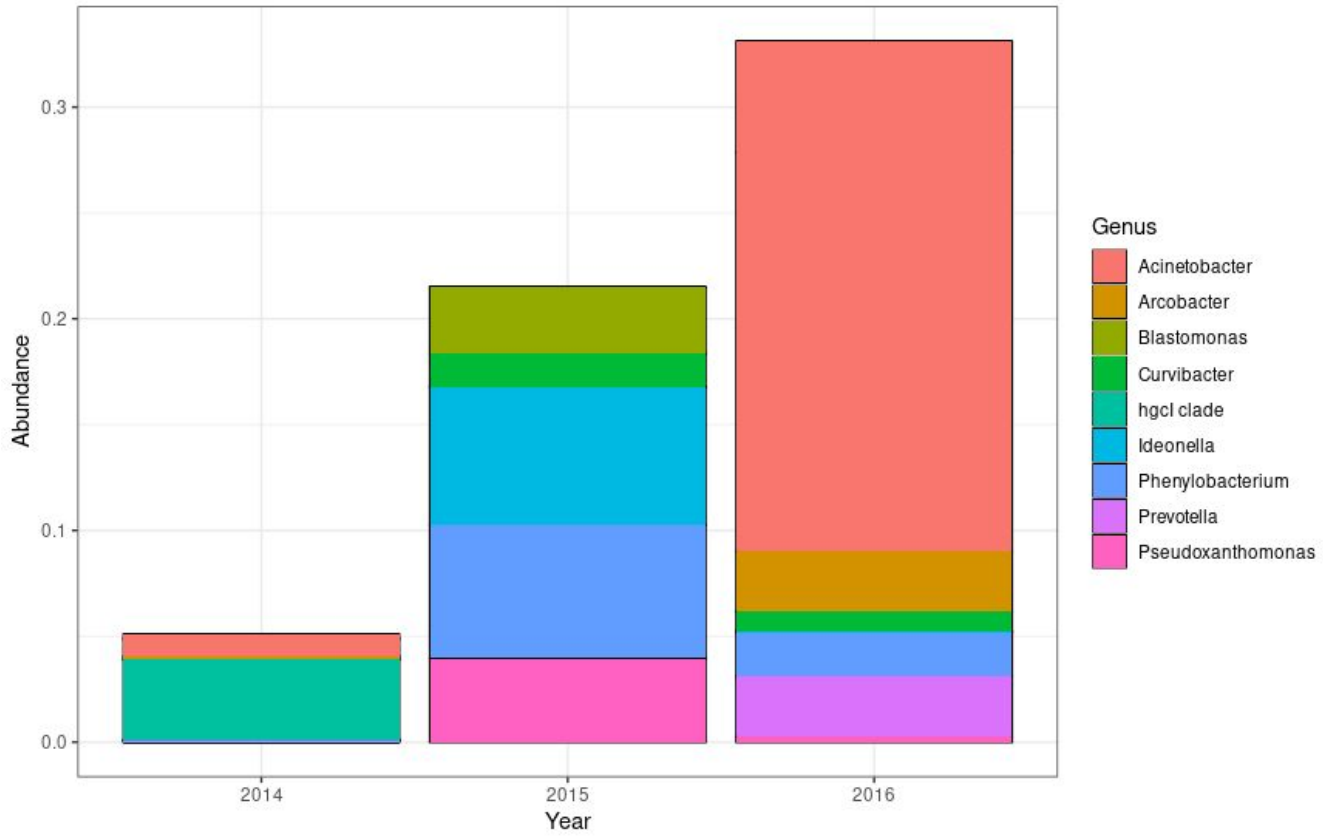

Figure 13: *Plot of the most abundant genera for each year.*

As for seasons, we observe that winter has none of the most abundant families (Figure 14) neither genera (Figure 15), while summer's samples contain eight high abundant families, mainly Moraxellaceae (Figure 14), which is present by the genus Acenitobacter (Figure 15).

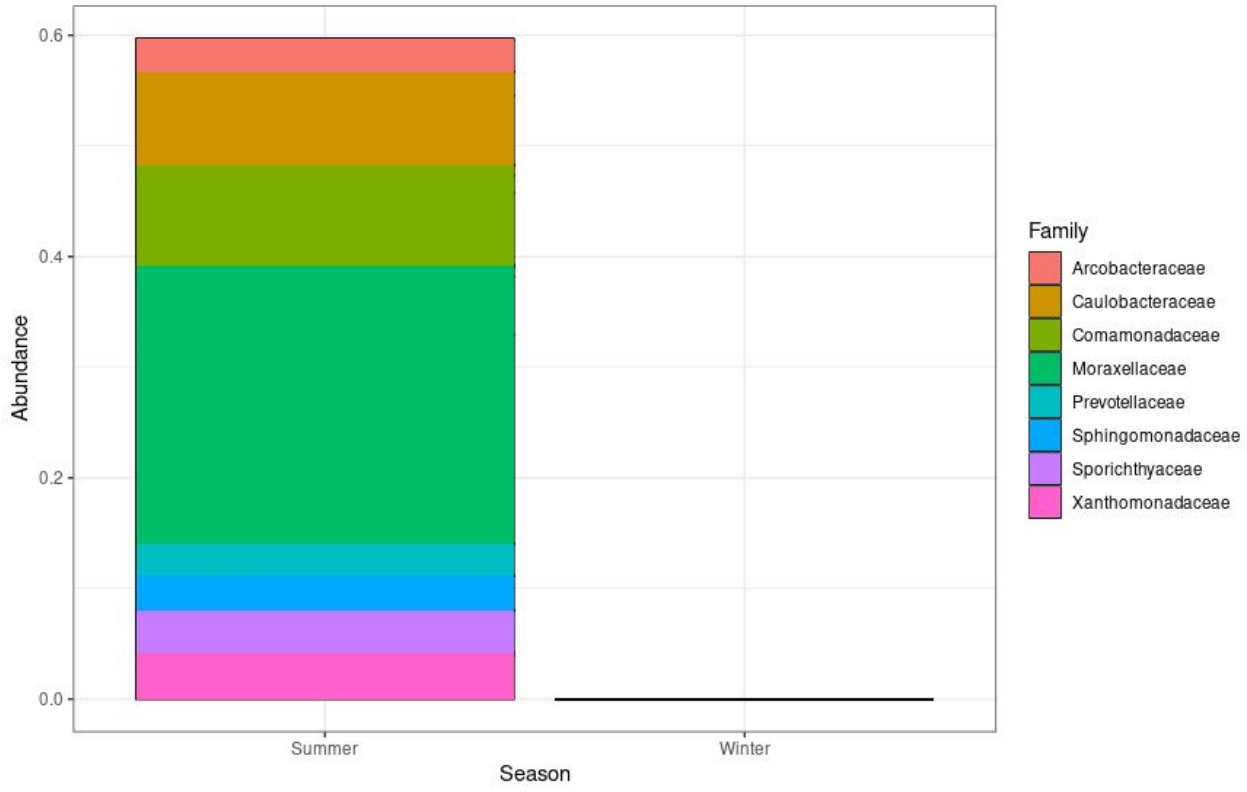

Figure 14: *Plot of the most abundant families per season.*

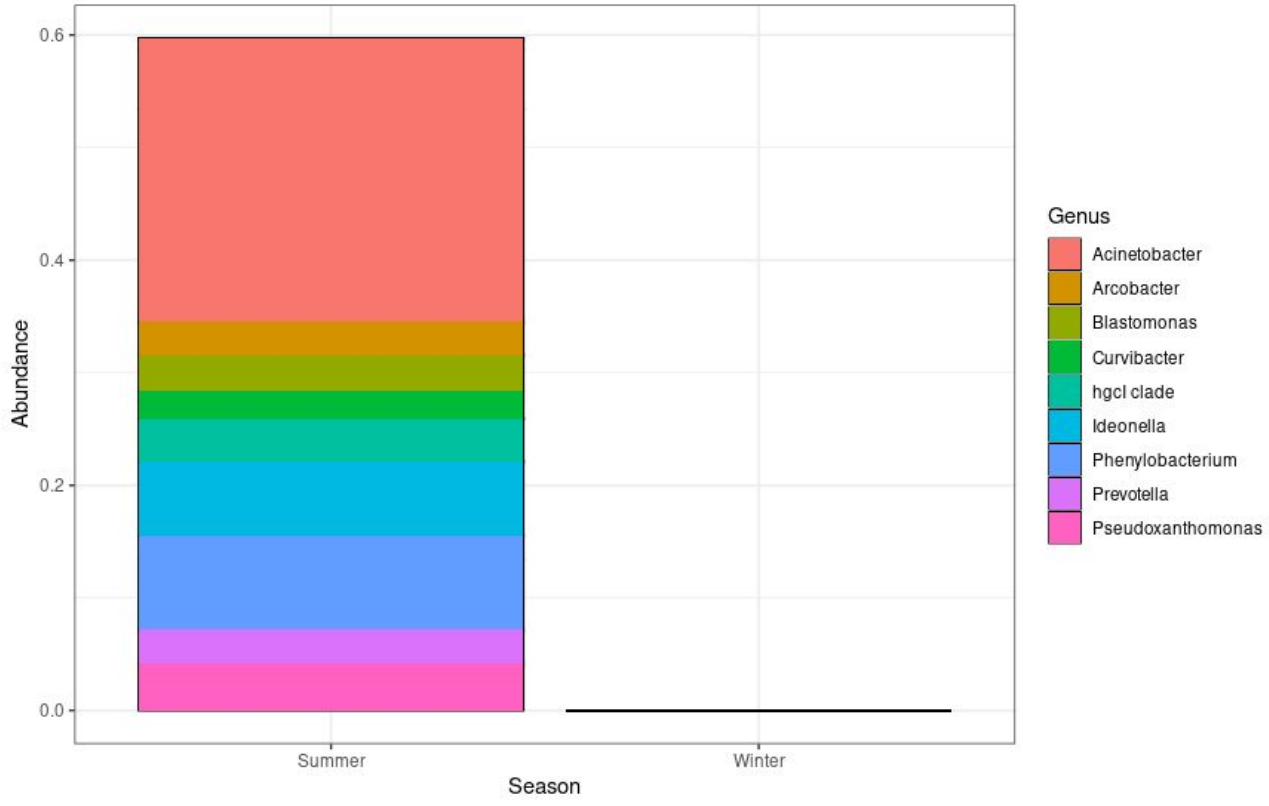

Figure 15: *Plot of the most abundant genera per season.*

9. Discussion

The river of Sebou is an important river in Morocco, it is the second largest river, and it plays an important role as a water source, either for irrigation, or for common uses, as there are many water reserves related to it. Hence, the study of its microbial diversity is important.

This study aimed to discover the diversity of microbial communities of Sebou river in three years (2014 to 2016), and two seasons (winter, and summer), such exploration will help determine all sources of infections might come from this basin.

The Chao-1 index decreases from 2014 to 2016, this reveals that the abundance of species decreases from 2014 to 2016 and tends to not be adjusted between species while decreasing of the Shannon index reveals that the diversity decreases from 2014 to 2016.

The beta diversity showed that the heterogeneity between summer's samples is higher than the difference between winter's samples.

The biodiversity each sample tends to decrease with the increase of BOD5, COD, pH, and Nitrate concentration, this means that the pollution reduces the biodiversity of the river.

The biodiversity of winter's samples was always lower than summer's samples, this means that the biodiversity of this river is influenced by season, probably the microbial community of the river prefers high temperature.

The taxonomy profile shows the abundance of Moraxellaceae family in 2016 samples, mainly the genus Acinetobacter, this genus is known as an infectious genus that can cause many diseases, mainly pneumonia, bloodstream infections and urinary tract infections [20].

The aforementioned results suggest that the increase of pollution in the basin of Sebou does not just reduce its biodiversity, which influences the whole ecosystem of this river, but it might also cause the increase of infectious bacteria in the reserves related to it, which require the application of a treatment strategy for this basin.

Conclusion

The river of Sebou has an important ecological, and social economical value, unfortunately, due to pollution its biodiversity is negatively influenced.

Through this study, we discovered the composition of the microbial community of the Sebou river and the impact of pollution on this composition.

River pollution reduces the diversity of its microbial community, and it changes its composition; it might also cause the integration of pathogenic bacteria which Threatens the public health of all populations around the river.

The aforementioned results suggest the importance of applying treatment and protection strategies for the river of Sebou.

References

[1] Laissaoui A, Mas JL, Hurtado S, Ziad N, Villa M, Benmansour M. Radionuclide activities and metal concentrations in sediments of the Sebou Estuary, NW Morocco, following a flooding event. Environ Monit Assess 2013;185:5019–29. https://doi.org/10.1007/s10661-012-2922-4.

[2] Snoussi M, Haïda S, Imassi S. Effects of the construction of dams on the water and sediment fluxes of the Moulouya and the Sebou Rivers, Morocco. Reg Environ Change 2002;3:5–12. https://doi.org/10.1007/s10113-001-0035-7.

[3] Ahizoun M, Maire E, Haida S, Darrozes J, Probst J-L. Estimation des changements de la ligne de rivage de la zone côtière sablonneuse de Kénitra au Maroc. Afr Sci 2010;5. https://doi.org/10.4314/afsci.v5i2.61727.

[4] Sage L, Bennasser L, Steiman R, Seigle-Murandi F. Fungal microflora biodiversity as a function of pollution in Oued Sebou (Morocco). Chemosphere 1997;35:751–9. https://doi.org/10.1016/S0045-6535(97)00196-3.

[5] Aprahamian MW, Bagliniere J, Sabatie MR, Alexandrino P, Thiel R, Aprahamian CD. Biology, status, and conservation of the anadromous Atlantic twaite shad Alosa fallax fallax. vol. 35, 2003, p. 103–24.

[6] Wariaghli F, Tilghman-Sibille A, El Abidi A, El Hamri H, Fekhaoui M, Yahyaoui A. Anguilla anguilla L.: Evaluation of the degree of heavy metal contamination in the Sebou estuary and in Moulay Bousselham lagoon reserve (Morocco). Int J Aquat Sci 2013;4:69–82.

[7] Abbou F, Fahde A. Structure et diversité taxonomique des peuplements de macroinvertébrés benthiques du réseau hydrographique du bassin du Sebou (Maroc). Int J Biol Chem Sci 2017;11:1785–806. https://doi.org/10.4314/ijbcs.v11i4.29.

[8] Williams M, Hernandez-Jover M, Shamsi S. Fish substitutions which may increase human health risks from zoonotic seafood borne parasites: A review. Food Control 2020;118:107429. https://doi.org/10.1016/j.foodcont.2020.107429.

[9] El Morabet R, Aneflouss M, El Mouak S. Waterborne Diseases in Sebou Watershed. In:

Naddeo V, Balakrishnan M, Choo K-H, editors. Front. Water-Energy-Nexus—Nature-Based Solut. Adv. Technol. Best Pract. Environ. Sustain., Cham: Springer International Publishing; 2020, p. 293–6. https://doi.org/10.1007/978-3-030-13068-8_73.

[10] Denef VJ. Peering into the Genetic Makeup of Natural Microbial Populations Using Metagenomics. In: Polz MF, Rajora OP, editors. Popul. Genomics Microorg., Cham: Springer International Publishing; 2019, p. 49–75. https://doi.org/10.1007/13836_2018_14.

[11] Salam LB. Chapter 5 - Metagenomic insights into the diversity and functions of microbial assemblages in lakes. In: Bandh SA, Shafi S, Shameem N, editors. Freshw. Microbiol., Academic Press; 2019, p. 175–223. https://doi.org/10.1016/B978-0-12-817495-1.00005-0.

[12] Bom. Ocean Sampling Day: River Sampling Day Call. Ocean Sampl Day 2013. http://oceansamplingday.blogspot.com/2013/05/river-sampling-day-call.html (accessed November 11, 2020).

[13] Perrin JL, Raïs N, Chahinian N, Moulin P, Ijjaali M. Water quality assessment of highly polluted rivers in a semi-arid Mediterranean zone Oued Fez and Sebou River (Morocco). J Hydrol 2014;510:26–34. https://doi.org/10.1016/j.jhydrol.2013.12.002.

[14] Hugenholtz P, Tyson GW. Metagenomics. Nature 2008;455:481–3. https://doi.org/10.1038/455481a.

[15] Morgan XC, Huttenhower C. Chapter 12: Human Microbiome Analysis. PLOS Comput Biol 2012;8:e1002808. https://doi.org/10.1371/journal.pcbi.1002808.

[16] Tuan NN, Chang Y-C, Yu C-P, Huang S-L. Multiple approaches to characterize the microbial community in a thermophilic anaerobic digester running on swine manure: A case study. Microbiol Res 2014;169:717–24. https://doi.org/10.1016/j.micres.2014.02.003.

[17] Albanese D, Fontana P, De Filippo C, Cavalieri D, Donati C. MICCA: a complete and accurate software for taxonomic profiling of metagenomic data. Sci Rep 2015;5:9743. https://doi.org/10.1038/srep09743.

[18] Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7. https://doi.org/10.1128/AEM.00062-07.

[19] McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 2013;8:e61217. https://doi.org/10.1371/journal.pone.0061217.

[20] Visca P, Seifert H, Towner KJ. Acinetobacter infection – an emerging threat to human health. IUBMB Life 2011;63:1048–54. https://doi.org/10.1002/iub.534.