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ASSESSING MICROBIAL COMMUNITY RESPONSES IN A CHEMICALLY STRESSED FRESHWATER ECOSYSTEM USING ENVIRONMENTAL DNA

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Degree project for Master of Science (120 hec) with a major in Ecotoxicology
ES2520 Master Thesis in Ecotoxicology (60 hec)
Second cycle
Semester/year: Autumn 2022/ Spring 2023
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Abstract

Microbial communities represent the fundamental cornerstones of riverine ecosystems because they are involved in numerous processes which mediate ecosystem services. However, riverine ecosystems are currently massively altered by a variety of anthropogenic activities which includes changes in land-use and chemical pollution. Particularly, chemicals of emerging concern (CECs) such as pesticides and pharmaceuticals are ubiquitously found in rivers as a consequence of continuous agricultural runoff as well as domestic and industrial sewage discharge. Nonetheless, there is a tremendous lack of knowledge about bacterial communities in lotic environments, especially in developing countries like Kenya. The primary objective of this study was to analyse the structural and functional responses of bacterial communities to environmental parameters, most notably CECs, derived from land-use in five Kenya riverine ecosystems. For this purpose, environmental DNA metabarcoding was applied to take a snapshot of the bacterial community structure in surface water and sediment in two different seasons. Furthermore, to assess the impact of CECs, the antimicrobial toxic stress was calculated for the detected organic chemicals. The main results of this study revealed that the variation in bacterial community structure and functional diversity can primarily be attributed to differences in land-use and their seasonal variability. The measured environmental and chemical parameters, on the other hand, displayed low nutrient load and antimicrobial toxicity in all rivers in both seasons indicating overall uniformity from a chemical perspective. Consequently, antimicrobial stress was unable to adequately explain the significant differences between riverine ecosystems, however, this was mostly attributed to the lack of biological replication, thus statistical power. Ultimately, this study suggests the adaptation of bacterial community composition and ecological functioning to land-use as well as the associated chemical pollution.

Acknowledgements

I would like to thank Prof. Dr. Thomas Backhaus for allowing me to participate in this project within the FRAM centre. My gratitude goes to Isaac Cheruiyot Tanui and Dr. Faith Kandie for the conduction of the sampling campaigns and the chemical analysis. I would also like to give my thanks to Sonja Schaufelberger for always being willing to help with bioinformatic issues. Ultimately, special thanks to my supervisor Dr. Pedro Inostroza for his continuous support and guidance throughout the entire study.

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List of abbreviations

ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
ARB	Antibody-resistant bacteria
ARG	Antimicrobial-resistance gene
ASV	Amplicon Sequence Variant
BR	Broad range
CA	Concentration addition
CECs	Contaminants of emerging concern
db-RDA	Distance-based redundancy analysis
DS	Downstream
ECHA	European Chemicals Agency
EF	Extraction factor
FEF	Fundamental ecological function
HS	High sensitivity
HTS	High-throughput sequencing
LOD	Limit of detection
LVS	Lake Victoria South
MIC	Minimum inhibitory concentration
MSC	Minimal selective concentration
NMDS	Non-metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
PAH	Polycyclic aromatic hydrocarbons
PCoA	Principal Coordination Analysis
PERMONAVA	Pairwise Permutional Multivariate Analysis of Variance
PPCPs	Pharmaceuticals and personal care products
PPS	Pathogens, parasites and symbionts
SFU	Sterivex filter unit
SOB	Sulphur-oxidizing bacteria
SPE	Solid Phase Extraction
SRB	Sulphate-reducing bacteria
TDS	Total dissolved solids
TU	Toxic unit
US	Upstream
VIF	Variance Inflation Factor
WPGMA	Weighted Par Group Method with Arithmetic Mean
WWTP	Wastewater treatment plant

1. Introduction

1.1 Ecological relevance of microbial communities

Microbial communities play an essential role in global biogeochemical cycles by largely driving the biological fluxes of hydrogen, nitrogen, oxygen, carbon, sulphur and partly phosphorus - the six major building blocks for all biological macromolecules (Falkowski et al. 2008). Consequently, microbes are the fundamental cornerstones of terrestrial and aquatic ecosystems (DeLorenzo et al. 2001; Morris and Blackwood 2014; Sagova-Mareckova et al. 2021; Šantl-Temkiv et al. 2022). Microbial communities facilitate various processes which mediate ecosystem services such as decomposition and mineralization, inorganic nutrient cycling, causation and suppression of diseases, removal of pollutants as well as the establishment of mutualistic symbiosis with various eukaryotic organisms (Bissett et al. 2013; Russell et al. 2014). More precise examples of microbial processes are primary and secondary productivity (photosynthesis and heterotrophic growth, respectively), carbon sequestration, biological transformation of greenhouse gases, pest regulation, natural attenuation of contaminants as well as the mitigation of pathogens and the dissemination of antimicrobial resistance (Brandt et al. 2015).

1.2 Ecological relevance of microbial communities in freshwater ecosystems

1.2.1 Carbon cycling

The carbon cycle in freshwater ecosystems is mainly regulated by microbes which are driving the decomposition of organic matter, whole-stream respiration as well as the carbon flow to higher trophic levels (Zeglin 2015). For instance, phototrophic and chemo-lithotrophic bacteria and archaea process and fixate globally significant amounts of carbon dioxide, while heterotrophic pelagic and benthic bacteria and archaea consume the organic carbon which is available for respiration. In addition, within the anoxic layers of water and sediment methanogen populations contribute to the decomposition of organic matter. Methanotrophic bacteria and archaea, on the other hand, metabolize the subsequently produced methane or even capture atmospheric methane as their carbon and energy source. Consequently, freshwater ecosystems represent a hotspot for carbon cycling as well as a single large sink of organic carbon that is comparable to marine habitats with respect to its size (Sagova-Mareckova et al. 2021; Pernthaler 2013).

1.2.2 Nitrogen cycling

Microbial communities also play a significant role in various processes of the nitrogen cycle which includes nitrification and denitrification, anaerobic ammonium oxidation and dissimilatory reduction of nitrate to ammonium. The fixation of nitrogen (N_2) can be carried out by many cyanobacteria but also heterotrophic, chemo-lithotrophic and chemo-organotrophic bacteria and archaea showing that there is a widespread potential for N_2 fixation in sediment. Furthermore, microorganisms participate in the nitrification process where available ammonia is oxidised to nitrate. In the reverse process, the

denitrification, microbial communities denitrify the nitrate received from the catchment and emit the generated nitrous oxide or N_2 into the atmosphere. Consequently, the nitrogen budget in a freshwater ecosystem is significantly determined by microbial processes which affect the balance between nitrogen sinks (e.g. denitrification, riverine output and sediment burial) and nitrogen sources (e.g. N_2 fixation, sediment release, riverine inputs and atmospheric deposition) (Sagova-Mareckova et al. 2021; Battin et al. 2016).

1.2.3 Sulphur cycling

Microorganisms are an essential part of the freshwater sulphur cycle through a variety of metabolic processes including sulphate reduction, sulphide oxidation as well as disproportionation. The latter describes the turnover of intermediate-valence species such as elemental sulphur, sulphite or thiosulphate via simultaneous reduction and oxidation into two different products which are usually sulphate and hydrogen sulphide (Fike et al. 2015; Strauss et al. 2016). Sulphate-reducing bacteria (SRB) play a vital role in the fermentation and anaerobic oxidation of organic matter (Muyzer and Stams 2008). In the subsurface of freshwater sediments sulphate is reduced by SRBs under anoxic conditions. The generated dissolved sulphide is subsequently oxidized by sulphide-oxidizing bacteria to more oxidized sulphur forms at the anoxic-oxic transition zone (Strauss et al. 2016). Furthermore, sulphur-oxidizing bacteria (SOB) are common in freshwater sediments (Tang et al. 2009; Wu et al. 2021). Some SOBs internally store elemental sulphur in granules which can then be further oxidized to sulphate (Tang et al. 2009). In summary, microbes play a pivotal role in the biogeochemical cycling of sulphur in freshwater ecosystems.

1.2.4 Phosphorous cycling

Phosphorous is the primary nutrient in limiting long-term algal and plant growth in freshwater ecosystems because, unlike carbon and nitrogen, phosphorous has no stable gaseous form which can be captured and utilized from the atmosphere by primary producers (McMahon and Read 2013). Therefore, phytoplankton and heterotrophic prokaryotes that can utilize phosphorous-containing compounds play a vital role in phosphorous cycling in freshwater environments (Withers and Jarvie 2008; Sagova-Mareckova et al. 2021). These microorganisms assimilate the pool of dissolved inorganic and organic phosphorous (DIP and DOP, respectively), which enters the catchment or becomes available through biomass degradation, and converts it into biomass through proliferation. Subsequently, the phosphorous is transferred to higher trophic levels via grazing and microbivory. Moreover, microbes regulate the flux of phosphorous across the water-sediment interface through its uptake, liberation from organic matter, the alteration of redox conditions at the sediment surface affecting its solubility and the conversion to refractory organic phosphorous compounds that become terminally deposited in the sediment. Within the sediment phytoplankton and heterotrophic

prokaryotes mediate the transformation processes from particulate inorganic phosphorous (PIP) to DIP and particulate organic phosphorous (POP) to DOP and then DIP. Under anoxic conditions DIP is highly soluble and therefore, released into the freshwater ecosystem. Consequently, the availability of phosphorous in the freshwater environment is significantly driven by microbial communities which can act both as sink and source (Withers and Jarvie 2008; McMahon and Read 2013; Sagova-Mareckova et al. 2021).

1.2.6 Removal of pollutants

Natural microbial communities in sediment and freshwater are key players in various processes which control ecosystem quality and regulate the fate of natural and anthropogenic pollutants released into the environment (Barra Caracciolo et al. 2015). New metabolic pathways are constantly developed by microorganisms to utilize such compounds for energy and carbon acquisition or as detoxification mechanisms. However, microbial transformation or degradation of an environmental pollutant can only occur under favourable conditions. For instance, the rate and degree of degradation is affected by the microbial community composition, environmental conditions such as temperature and pH, favourable redox conditions as well as the bioavailability, enzymatic accessibility, concentration and toxicity of the pollutant and its metabolites (Al-Khazrajy et al. 2018; Kolvenbach et al. 2014). Consequently, many pollutants that enter freshwater ecosystems cannot be eliminated through microbial degradation (or abiotic processes such as photodegradation), thereby exhibiting high environmental persistence. Especially freshwater sediments seem to act as a sink for heavy metals, pharmaceuticals and other organic pollutants like high molecular weight polycyclic aromatic hydrocarbons (PAHs) (Monteiro and Boxall 2010; Li 2014; Zhang et al. 2014; Ghosal et al. 2016; Patel et al. 2019).

Heavy metals

Microbial communities can drive the removal of some heavy metals from freshwater ecosystems through several biological processes including biosorption, intracellular sequestration, volatilization, transformation, and degradation. Biosorption describes the surface complexion of metals onto the microbial cell wall via extracellular polymeric substances (EPS) such as polysaccharides, glycoprotein, liposaccharides, or soluble peptides. These EPS possess functional groups that interact with metal molecules through van-der-Waals forces, electrostatic interactions, complexation or surface precipitation to physically adsorb the metal to the cells surface layers (Kikuchi and Tanaka 2012; Shah 2020). Some microorganisms can capture, sequester and store metals using metallothioneins or precipitate metals as oxides, sulphides, or protein aggregates in close association with the cytoplasmatic cell membrane (Gupta et al. 2017). Volatilization, on the other hand, represents a detoxification mechanism where, for instance, selenium oxyanions or inorganic mercury are

enzymatically reduced, transformed into volatile alkylselenides or elemental mercury, respectively, and subsequently emitted into the atmosphere or nearby environment for further transformation. Microbial biotransformation and -degradation are usually enzymatically driven via oxidative or reductive processes (Kikuchi and Tanaka 2012). Examples include the conversion of inorganic mercury to the more bioavailable methylmercury by sulphate- and iron-reducing bacteria (Marvin-Dipasquale et al. 2009; Dranguet et al. 2017), the removal of Fe (II) and Mn (II) by iron- and manganese-oxidizing/reducing bacteria (Nealson 1997; Kikuchi and Tanaka 2012) or the reduction of arsenic, selenic or chromic oxyanions (Kikuchi and Tanaka 2012).

Organic pollutants

Communities of microorganisms also play an important role in the elimination organic pollutants such as hydrocarbons originating from petroleum as well as some pharmaceuticals and endocrine disrupting compounds (EDCs). For example, microbial communities have shown their ability to degrade EDCs such as bisphenol A and nonylphenol in river water and sediment (Peng et al. 2015; Zhang et al. 2016). Various bacteria and archaea utilize low molecular weight PAHs like naphthalene, phenanthrene and pyrene as well as other petroleum compounds such as alkanes, benzene, toluene, ethylbenzene and several xylenes as carbon source and biodegrade those molecules under aerobic and anaerobic conditions (Bamforth and Singleton 2005; Ghosal et al. 2016; El-Naas et al. 2014; Haritash and Kaushik 2009; Das and Chandran 2011; Ghattas et al. 2017). Furthermore, some freshwater green algae and algal-bacterial communities have also shown their ability to degrade PAHs (Haritash and Kaushik 2009). With respect to pharmaceuticals, various compounds appear to be pseudo-persistent because their constant release into the environment outpaces their removal through microbial communities and abiotic processes (Patel et al. 2019; Barra Caracciolo et al. 2015). In addition, some pharmaceuticals including acetaminophen, atenolol, carbamazepine, ibuprofen, mefenamic acid and propranolol display actual resistance against microbial degradation in river water (Yamamoto et al. 2009). Nonetheless, some pharmaceuticals are potentially biotransformed through metabolic or particularly co-metabolic pathways (Li 2014; Barra Caracciolo et al. 2015). For instance, diltiazem, cimetidine and ranitidine in river sediment (Al-Khazrajy et al. 2018) as well as naproxen and diclofenac in water (Barra Caracciolo et al. 2015; Pal et al. 2014).

1.2.5 Microbial resistance

Chemicals with antimicrobial properties occur naturally in the environment which includes heavy metals and antibiotics (e.g., β-lactamase) produced by bacteria and fungi to compete for resources and habitat through the mitigation of pathogenic and non-pathogenic competitors. These compounds provide a natural source of selective pressure on microbial communities to develop and maintain antimicrobial resistance phenotypes even in the most pristine environments. That is because many

microorganisms can exhibit intrinsic resistance to the exposure to naturally present antimicrobial chemicals through changes in gene expression, e.g. by changing the cellular permeability to the compound (Davies and Davies 2010; Vaz-Moreira et al. 2014; Scott et al. 2016). However, environmental concentrations of antimicrobial substances – natural or anthropogenic origin - which exert high selective pressure on microbes can result in the evolution and dissemination of antimicrobial resistance (AMR) genes (Grenni 2022). Moreover, microbial resistance against metals and antibiotics is often genetically linked with multiple resistance genes being commonly found on the same plasmid, conferring co-resistance (Stepanauskas et al. 2006). The primary mechanism for the distribution of AMR genes between bacteria is horizontal gene transfer which can be conducted through transduction, transformation or conjugation (Davies and Davies 2010). The exchange can not only occur within and between environmental microbial communities but also with pathogenic and non-pathogenic microbes that contaminate the ecosystem. Therefore, freshwater microbial communities may not only represent the origin of AMR gene evolution but also act as reservoir for already acquired antimicrobial resistance and as a bioreactor which facilitates the transmission of resistance genes between pathogenic and non-pathogenic bacteria (Scott et al. 2016; Grenni 2022). The persistence and dissemination of AMR genes in freshwater ecosystems also increases the risk of disease outbreaks in other organisms caused by common and emerging resistant pathogens (Nnadozie and Odume 2019).

1.3 Stressors of freshwater ecosystems

Freshwater ecosystems are currently, as a consequence of ever-increasing anthropogenic activities and climate change, among the most altered ecosystems on Earth which threatens both the harboured biodiversity and compromises the provision of essential ecosystem services (Carpenter et al. 2011; Vörösmarty et al. 2010).

1.3.1 Stressors of riverine ecosystems

Particularly riverine ecosystems are dramatically affected by human activities through modifications of hydrologic flow (e.g., damming, river channelization and diversion), changes in land-use and introduction of invasive species. In addition, chemical and biological pollutants originating from non-point sources like agricultural runoff and point sources such as untreated and treated sewage put the microbial diversity in rivers under massive pressure (Carpenter et al. 2011; Mansfeldt et al. 2020). These pollution sources shape the chemical and biological composition of the receiving water body through the provision of excess nutrients, organic matter, and microbes but concurrently through the discharge of heavy metal and chemicals of emerging concern (CECs) such as pesticides and pharmaceuticals. Especially small streams and rivers are affected because the effluent is less diluted and constitutes for a significant proportion of the annual flow in comparison to bigger lotic ecosystems (Tiirik et al. 2021; Barbosa et al. 2016; Hladilek et al. 2016). Consequently, this affects important

ecosystem services provided by microbial communities, for instance, by altering biogeochemistry of nutrients and toxicants, through the modification of carbon storage and ecosystem metabolism, loss of native species as well as emergence of novel and common disease through the dissemination of AMR genes (Carpenter et al. 2011; Mansfeldt et al. 2020).

1.3.2 Contaminants of emerging concern

Contaminants of emerging concern are substances of either natural or anthropogenic origin such as pharmaceuticals, personal care products, pesticides, disinfectants, and various industrial compounds including plasticizers, flame retardants and surfactants. These chemicals and their transformation products are released into the aquatic environment during their life cycle from various sources (e.g., agricultural runoff, domestic and industrial wastewater, among others) and at concentrations between few ng/L and several µg/L (Barbosa et al. 2016; Oberg and Leopold 2019; Pal et al. 2014). In the past five decades CECs have been found ubiquitously in aquatic ecosystems worldwide. Especially highly persistent compounds acting as global pollutants and the continuous emission of chemicals, whose degradation products exhibit a higher toxicity, are concerning for the exposed environment (Schwarzenbach et al. 2006). The assumption that trace-pollutants are harmless is heavily questioned because the receiving environment is not exposed to single substances but rather chronically exposed to complex mixtures of hundreds of compounds simultaneously. Particularly, the transformation of contaminants, which is rarely a full mineralization, leads to an uncountable number of substances that might be present at low concentrations (Oberg and Leopold 2019). Furthermore, the environmental concentrations of CECs are subjected to large spatial and temporal variations depending on their usage and environmental factors such as precipitation, temperature or UV radiation which can affect their dilution or degradation, respectively (Petrie et al. 2015; Oberg and Leopold 2019). Although CECs in aquatic ecosystem have been researched for several decades, they still pose a serious threat to the aquatic environment. This is because removal technologies from e.g., wastewater are still insufficient, whereas the detection and effect assessment of these chemicals and their transformation products is reaching its analytical and methodological limits; especially because the development, production, usage and discharge of existing and novel compounds is always outpacing the capacities for assessing their risk (Oberg and Leopold 2019; Fischer et al. 2019).

1.3.3 Contaminants of emerging concern in Africa

While the risk assessment and monitoring of CECs is increasingly conducted in high-income countries, this research field is - to a great extent - unexplored in developing countries worldwide. Africa and particularly Kenya is a low-income country in which chemical pollution and its detrimental consequence for sustainable development are often ignored. In addition, only few studies went beyond the usual monitoring of urban areas with a restricted, sometimes even obsolete, list of organic

chemicals (Kandie et al. 2020b). The main classes of CECs which are released into the environment are pesticides, pharmaceuticals, and personal care products (PPCPs). This is due to the (re)emergence of diseases and epidemics as well as the importance of agriculture as one of the main economic branches in Kenya whose growth entails an increasing demand for plant protection products. Consequently, agricultural runoff represents one of the major emission of CECs into the environment. PPCPs, on the other hand, are reaching aquatic ecosystem through the discharge of usually untreated domestic and industrial sewage (Kandie et al. 2020a; Odhiambo et al. 2023; Shehu et al. 2022). In low-income countries only 8% of the generated municipal and industrial wastewater is treated in any kind before being discharged into the environment. In Kenya, one of the main challenges is the lack of infrastructure for wastewater collection and treatment which entails the pollution of, in most cases, limited surface- and groundwater resources (UN World Water Development Report, 2017).

Although monitoring of CECs in Kenya is scarce, research has shown the ubiquitous presence of these substances in surface water, sediment, and biota of Kenyan freshwater ecosystems. The detected CECs included dozens of pesticides, PPCPs, microplastics and industrial chemicals in concentrations ranging from several ng/L to g/L. The pollution was linked to untreated or inadequately treated municipal wastewater, lack of sanitation and the excessive or uncontrolled use of agrochemicals (Kandie et al. 2020a; Kandie et al. 2020b; Shehu et al. 2022).

1.4 Responses of bacterial communities to different land-use types

The structural and functional responses of bacterial communities to CECs derived from land-use, as shaping drivers of microbial biodiversity, remain unknown. Although several studies on the effect of different land-uses on riverine bacterial communities were conducted, the focus was majorly on the impact of released nutrients and heavy metals, or physical water quality parameters. The studied land-uses include agricultural, rural, urban, industrial, and natural sites as well as various mixes between them. Sometimes a differentiation between the degree of anthropogenic impact at each site was additionally made. In general, the responses of bacterial communities to different types of land-use can be summarized in the following way:

1.4.1 Structural responses

No significant relationships between land-use and the species richness of bacterial communities have been observed in surface waters, sediment, or biofilm of riverine ecosystems. However, sites exposed to higher degrees of chemical pollution tended to exhibit lower species richness in comparison to sites which are less affected (Lear and Lewis 2009; Ancion et al. 2010; Lear et al. 2011; Ibekwe et al. 2016; Laperriere et al. 2020; Pan et al. 2022; Liu et al. 2023; Li et al. 2023). On the other hand, significantly different bacterial community compositions were displayed by different types of land-use in surface water, sediment, and biofilm. Most of the studies observed significant shifts in beta-biodiversity

between agricultural and urban, agricultural and natural, urban and natural as well as urban and rural sites, among others (Lear and Lewis 2009; Ancion et al. 2010; Lear et al. 2011; Staley et al. 2013; Gibbons et al. 2014; Dang et al. 2021; Zhao et al. 2021; Li et al. 2023). The same outcome was observed when sites with different degrees of chemical pollution were compared with each other (Lear et al. 2011; Dang et al. 2021; Pan et al. 2022). In addition, rural and urban areas were the major source for faecal-indicator bacteria and ARGs as well as a bioreactor for the transmission and dissemination of ARGs and mobile genetic elements along the river. Consequently, the respective natural bacterial communities experienced massive shifts due to e.g., the proliferation of agroecosystem-adapted bacteria originating from animal faeces being used as fertilizer or ARB favoured by urban conditions (Kang et al. 2010; Muurinen et al. 2022).

In conclusion, shifts in bacterial community composition can be considered as a more sensitive indicator to reflect different types of land-use (Li et al. 2023). This is because the respective environmental conditions are putting the microbial community under pressure, forcing strong resource competition and selection which entails the development of tolerant community (possibly with limited species diversity) even in chronically polluted environments (Ancion et al. 2010; Muurinen et al. 2022).

1.4.2 Functional responses

Very few studies examined functional responses of bacterial communities to land-use. For instance, agricultural and urban areas resulted in a stimulation of metabolic pathways associated with nitrogen removal such as nitrification and denitrification. The abundance of microbial functional groups linked to nitrogen cycling and removal were found to be positively correlated to the nutrient load in the respective ecosystem (Qu et al. 2017; Liu et al. 2023). Dang et al. (2021), on the other hand, showed that agricultural and urban land-use with a high degree of anthropogenic activity may destabilize stream ecosystems through a decline in metabolic efficiency of microbial communities.

1.5 Assessment of biodiversity and ecological status

The assessment of ecological status and the monitoring of water quality of river ecosystems is usually following a traditional morphology-based species identification approach (Li et al. 2018). However, assessing biodiversity by surveying species richness and relative abundance based on physical, visual, or even acoustic methods is not without limitations. For instance, the invasive nature of conventional physical methods can lead to destructive impacts on the studied environment and its natural community which complicates e.g. the application in a conservative context (Deiner et al. 2017). In addition, the identification of species requires high taxonomic expertise, while being exceedingly time-consuming and labour-intensive (Li et al. 2018; Pawlowski et al. 2020). Consequently, this renders the upscaling of morphology-based methods in space and time, to satisfy the growing demand for

environmental monitoring and the subsequent informed ecosystem management, impossible. Moreover, conventional methods focus solely on biodiversity, which is morphologically identifiable, thereby overlooking inconspicuous microbial domains including small-bodied and elusive specimen. Finally, the disregard of cryptic biodiversity and misidentification especially at lower taxonomic ranks can result in inconsistent and noisy inventories impeding ecological assessments (Cordier et al. 2021). In conclusion, this highlights the necessity for more objective, robust, cost-effective and faster approaches to assess biodiversity and ecosystem functioning (Deiner et al. 2017; Cordier et al. 2021).

A promising alternative to the traditional approach is environmental DNA (eDNA) metabarcoding which couples PCR amplification and high-throughput sequencing (HTS) with the taxonomic identification of multiple species extracted from an environmental sample (Deiner et al. 2017). Within this approach, DNA is directly isolated from various environmental matrices such as water, sediment, air, soil, or faeces meaning that no prior isolation of the target organism is necessary. As a result, eDNA constitutes a complex mixture of DNA shed from a variety of microbial, meiofaunal and macrobial taxa in the form of skin cells, mucus, faeces, organelles, gametes or extracellular DNA (free DNA released through lyses of dying cells) (Altermatt et al. 2020; Creer et al. 2016; Deiner et al. 2017; Pawlowski et al. 2020). As such, sampling of eDNA is non-invasive to the natural community and its utilization in metabarcoding enables the upscaling of biodiversity surveys and biomonitoring in space and time (Deiner et al. 2017). It is particularly suitable to assess biodiversity in riverine ecosystems because of the relatively short persistence of DNA in the water column (maximally 1-2 weeks) which makes it a highly contemporary method, the simplicity of sampling which is easily automatable, and the possibility to spatially integrate the biodiversity information due to the downstream transport of eDNA. Moreover, eDNA metabarcoding displays a high sensitivity to detect species as well as a general applicability showing an increase in taxonomic precision while being less labour-intensive at the same time (Altermatt et al. 2020). Therefore, it can overcome the limitations of conventional approaches through the targeting of different species, sampling of greater diversity and enhancing the resolution of taxonomic identification, thereby facilitating the detection of e.g. elusive or invasive species (Creer et al. 2016; Deiner et al. 2017). However, eDNA metabarcoding is not without limitations. The presence of eDNA at a certain site does not necessarily imply the presence of the respective alive species because the eDNA can also originate from extracellular DNA as well as dead or inactive organisms. At the same time, the eDNA molecule might have just been transported to the site from further upstream -estimated travel distance for eDNA in rivers ranges between 0.25 and 12 km – which might create an inference challenge in time and space (Altermatt et al. 2020; Deiner et al. 2017). Furthermore, the taxonomic identification of species is mainly restricted by the lack of adequate and complete reference sequence databases (Altermatt et al. 2020). These databases also remain distorted towards certain taxonomic groups and geographical locations (Cordier et al. 2021). However, the availability of well-

defined bacterial barcoding regions and comprehensive bacterial reference sequence databases (such as SILVA) allowed eDNA metabarcoding to become a powerful tool to routinely uncover bacterial biodiversity information and is therefore, a suitable method for the biomonitoring and assessment of ecological status of bacterial communities in riverine ecosystems (Altermatt et al. 2020; Deiner et al. 2017; Glöckner et al. 2017).

1.6 Aim, objectives, and hypotheses

The essential role of bacterial communities within riverine ecosystems and the tremendous lack of knowledge about their structural and functional responses to CECs does not fit well together considering rivers represent one of the most chemically exposed ecosystems on Earth. Nonetheless, bacterial communities in lotic environments remain helplessly understudied, especially in developing regions such as Africa and particularly Kenya, which emphasizes the need for further research within this area.

The aim of this project is to analyse structural and functional changes of bacterial community biodiversity to environmental parameters, with a special focus on CECs, in river ecosystems of a developing country, Kenya. Environmental DNA metabarcoding is used to assess chemicals derived from land-use as shaping driver of bacterial biodiversity metrics – alpha-/beta-diversity and community composition – as well as to qualitatively assess changes in ecological functioning in bacterial communities affected by different types of land-use and consequently, varying degrees of anthropogenic activity.

1.6.1 Hypotheses

- Composition and functional profile of bacterial communities in river ecosystems is expected to change based on environmental parameters derived from land-us

2. Methods

2.1 Case study, chemical analyses and eDNA sampling

2.1.1 Lake Victoria South Basin

The Lake Victoria South (LVS) basin encompasses a catchment area of 26.906 km^2 in the western part of Kenya and borders Lake Victoria to the East as well as Tanzania to the South (see **Figure 1**). The LVS basin is characterised by an equatorial hot and humid climate with a bi-modal rainfall pattern – long rains between March and May, and short rains from October to November. Here, the northern and central parts of the basin obtain higher rainfalls, whereas the precipitation in the southern parts is considerably lower. Between April and June all rivers in the basin display a pronounced high runoff season. The rivers in the central and northern part in the LVS basin exhibit a constantly elevated flow from July to December, while between January and March the flow is low. In the southern part the rivers show low runoff from July to November and high runoff in December (LVS Integrated Water Resource Management and Development Plan, 2020).

The population in the basin was estimated to be 8.57 million in 2019, which corresponds to approximately 19% of Kenya´s total population, although it only covers about 5% of its total area. The majority of the population resides in rural settlements, while only a small proportion of the population living in urban areas (LVS Integrated Water Resource Management and Development Plan, 2020). In the LVS basin land use consists of scattered urban and residential areas, forest, grassland/rangeland, and agricultural (mostly rainfed crops such as maize, rice, and sugarcane) as well as industrial use, e.g. sugarcane factories (Kandie et al. 2020a). About half of the total population of the LVS basin is directly supplied with water from unimproved drinking water sources, while only 12% of the citizens obtain piped water from water service providers. The remaining 40% receive water from springs and boreholes. In rural areas, there are no sewerage systems installed, whereas in urban settlements only about 4 % of the population is connected to formal sewerage systems (LVS Integrated Water Resource Management and Development Plan, 2020).

Anthropogenic activities in the LVS basin are one of the reasons for the declining water quality in Lake Victoria. This includes the continuous conversion of rangelands into agricultural fields, unsustainable farming practices and poor management of croplands, poor waste disposal and sedimentation of water bodies as well as the inadequate monitoring and compliance control. The runoff from the LVS basin commonly contains industrial effluents from major towns as well as municipal/domestic sewage from urban settlements. Additionally, non-treated sewage is directly discharged into the running waters. Pesticide residues and nutrients from agriculture areas and agro-based industries end up in nearby waterbodies (LVS Integrated Water Resource Management and Development Plan, 2020).

2.1.2 Sampling strategy

The sampling was conducted within the SENTINEL-II project (DFG 299273352) which aims to understand the environmental factors which are relevant to the infection rate of freshwater snails with the parasite *Schistosoma sp*. (SENTINEL, 2020). The sampling area included the five Kenyan rivers Rangwena, Rangwe, Lambwe, Asao, and Sare (see specific description in **Box 1**).

Box 1. SAMPLING AREA

Rangwena (K1) is closely located to the East of Homabay town, while being surrounded by agricultural fields. The upstream site is characterized by horticultural farming of maize and cotton. Therefore, the land-use can be described as a mix of primarily agricultural with some urban influence, and the degree of anthropogenic activity is defined as intermediate.

Rangwe (K2) passes through a semi-urban area where the primary occupation of the inhabitants consists of subsistence farming of maize and livestock. Consequently, the land-use can be described as mostly agricultural with some urban influence, while the degree of anthropogenic activity is defined as intermediate.

Lambwe (K3) originates in Ruma National Park and is surrounded by natural vegetation and agricultural fields where subsistence farming of maize is carried out. The land-use can therefore be described as a mix of natural and agricultural with a low degree of anthropogenic activity.

Asao (K4) is characterized by subsistence farming of maize as the main crop during the long wet season as well as a tea growing area at the headwaters. The land-use can be described as agricultural with an intermediate degree of anthropogenic activity.

Sare (K5) passes through Sare town where several businesses are located. The river receives WWTP effluent from the nearby Sony sugar company as well as most of the domestic waste because the inhabitants are not connected to a sewerage system. Along the river large-scale sugarcane and small-scale maize farming are practiced. Consequently, the land-use can be described as a mix of urban and agricultural with a high degree of anthropogenic activity.

Three field campaigns - October 2021 (wet season), February 2022 (dry season and May 2022 (wet season) - were conducted in the sampling area. Water samples for chemical characterisation of the different rivers were collected at five equidistant sites (S1 – S5) along the course of 2 km (in total 25 sampling sites). The sampling was conducted at all rivers in October 2021 and February 2022 as well as in May 2022 only at Rangwena and Asao. On the other hand, microbial characterisation was conducted using environmental DNA from water and sediment for each river only at two sampling sites (ie., S1 and S5) - the most upstream and the most downstream site, respectively (in total ten sampling sites, see **Figure 1** and **Table 1**).

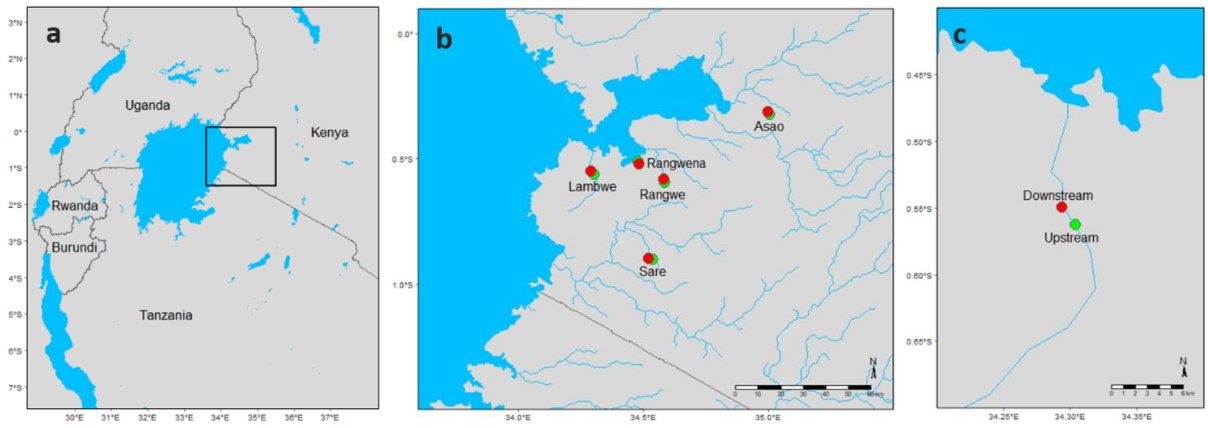

Figure 1. Sampling strategy*. The sampling was conducted in the LVS basin which is situated in the East African country Kenya (a). eDNA samples from water and sediment were taken from the five rivers Rangwena, Rangwe, Lambwe, Asao, and Sare (b). Each river was sampled in an upstream and downstream setting (c).*

Table 1 Sampling sites, geo-location (WGS84), land use, expected discharges and predicted degree of anthropogenic impact.

River	Site	Latitude N	Longitude E	Land-use	Expected discharges	Predicted degree of impact
Rangwena	US	-0.511802	34.481537	Agriculture Urban	Agricultural runoff Domestic sewage	Intermediate
	DS	-0.519796	34.482900	Agriculture	Agricultural runoff	
Rangwe	US	-0.593827	34.587787	Agriculture Urban	Agricultural runoff Domestic sewage	Intermediate
	DS	-0.582528	34.582272	Agriculture	Agricultural runoff	
Lambwe	US	-0.562709	34.303661	Natural Agriculture	Agricultural runoff	Low
	DS	-0.549385	34.293562	Agriculture Natural	Agricultural runoff	
Asao	US	-0.323101	35.007005	Agriculture	Agricultural runoff	Intermediate
	DS	-0.313919	35.000071	Agriculture	Agricultural runoff	
Sare	US	-0.903089	34.537552	Agriculture (Urban)	Agricultural runoff (Domestic sewage)	High
	DS	-0.898537	34.523174	Urban Agriculture	Domestic and industrial sewage Agricultural runoff	

2.1.3 Chemical analyses

Surface water was grab sampled in 500 mL Nalgene bottle for Solid Phase Extraction (SPE) as well as in 1 mL duplicates in 2 mL amber autosampler glass vials for direct injection into a Liquid Chromatographer coupled to a High-Resolution Mass Spectrometer (LC-HRMS).

The 500 mL water samples were vacuum-filtered to remove suspended particles before the sample extraction was conducted using SPE manifold with cartridges preconditioned with 5 mL of Ethyl acetate, 5 mL of LC- grade methanol and 10 mL of LC-MS grade water. Each cartridge contained 200 mg of sorbent hydrophobic polystyrene divinylbenzene copolymer (Chromabond HR-X, Macherey-Nagel). Following this, the cartridges were air-dried on the extraction manifold using vacuum for 30 min. The subsequent elution of the cartridges was performed using 5 mL of Ethyl acetate, 5 mL of LC- grade methanol, 4 mL MeOH with 1% formic acid (98–100%, p.a., Merck) and 4 mL MeOH with 2% 7N Ammonia in MeOH (Sigma- Aldrich) into one vial. In the same step blanks were prepared by extracting 10 mL of LC-MS grade water. Afterwards, the collected eluent was evaporated to approximately 200 µL using a stream of pure nitrogen gas. The collection vials were rinsed with methanol before being blow-dried with nitrogen. Each extract was reconstituted with methanol to a final extraction factor (EF) of 1000 and stored at -20 °C until further analysis.

For LC-HRMS measurement, an aliquot from each extract (with EF = 1000) was prepared by mixing 50 µL sample with 15 µL MeOH, 30 µL water and 5 µL internal standard mix (c = 1 µg/mL). The measurement was conducted both in positive and negative ionisation modes. All chemical analyses

were conducted by the Department of Effect-Directed Analysis of the Helmholtz Centre For Environmental Research (UFZ).

2.1.4 Physico-chemical parameters

Additional surface water was grab sampled in 125 mL bottle from each sampling site, stored at 4 °C and transported to the laboratory for the subsequent measurement of carbonate hardness, phosphate-, nitrate, nitrite, and ammonium concentration. *In situ* measurements of turbidity using WTW TURB 355 IR Turbidimeter as well as conductivity, pH, temperature, and total dissolved solids (TDS) using HANNA HI9811-5 were also performed. The concentration of the different nutrients and carbonate hardness was determined by using semi-quantitative test strips (Quantofix). The value of each parameter was determined visually through its comparison to the colour scale on the package.

2.1.6 Sampling of environmental DNA (eDNA)

eDNA was collected in different seasons (i.e., wet and dry) and in distinct environmental compartments (i.e., water and sediment). Water samples were collected in October 2021 (wet) and February 2022 (dry), while sediments were collected in February 2022 (dry) and May 2022 (wet). Water was sampled using Sterivex™ filter (Merck) and volumes ranged between 200 to 500 mL per replicate accordingly to the water conditions (i.e., turbidity). Roughly 5 g of superficial sediment samples (0-5 cm) were collected per biological replicate using a sterile 50 mL syringe (with the tip being cut off before sampling) and placed in sterile plastic bags. All eDNA samples were immediately stored at -4 °C in the field, before being transported to the lab and stored at -20 °C until subsequent DNA extraction in premises of the Department of Biological and Environmental Sciences at the University of Gothenburg in Sweden. The number of Sterivex™ filter units (SFUs) and sediment bags are shown in **Table 2**.

Table 2. Number of received Sterivex filter units and sediment bags*. For river water, each biological replicate was splitted up into up to three SFUs.*

	Sterivex filter units	Sediment bags
Wet season (October 2021)	55	0
Dry season (February 2022)	42	30
Wet season (May 2022)	0	12
Total	97	42

2.2. Analysis of chemical data

The effect of CECs on bacterial communities was assessed in two ways by differentiating between the role of compounds explicitly used as antibiotics as well as other substances which also exhibit antimicrobial properties but are used for other purposes. Ecotoxicological data specifically affecting microorganisms was mined for the chemicals with antimicrobial properties in the European Chemicals Agency (ECHA) database. The respective effect data was converted into chronic EC10 equivalents following the recommendations of (Warne et al. 2018).

In both scenarios a component-based approach was applied using the concentration addition (CA) mixture toxicity concept (Loewe and Muischnek 1926). For antibiotics the following formula was used:

$$\mathrm{TU}_{MIC} = \sum_{i=1}^{n} \frac{MEC_i}{MIC_i} = \sum_{i=1}^{n} TU_i$$

where MEC_i denotes the measured environmental concentration of the CEC *i*, while MIC_i represents the corresponding minimum inhibitory concentration (MIC) of antibiotic *i*. The ratio MEC_i/MIC_i provides a dimensionless measure of the individual toxicity contribution of each antibiotic present in the sample. Their sum is termed a "toxic unit" (TU) and describes the comprehensive antibiotic stress within the sample, henceforth TU_{MIC} is equal to the toxic stress exhibited by antibiotics. The MIC values for each antibiotic were obtained from (Bengtsson-Palme and Larsson 2016). Bengtsson-Palme and co-authors predicted the lowest MIC for each antibiotic that had an entry in the public database of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The lowest MIC was selected because antibiotic concentrations below the MICs have shown to select for resistant bacteria (Andersson and Hughes 2012; Gullberg et al. 2014). For instance, (Gullberg et al. 2011) reported minimal selective concentrations (MSC) – the minimum concentration of an antibiotic at which resistant bacterial strains possess a competitive advantage – to range between 1/230 and 1/4 of the corresponding MIC depending on the antibiotic.

Analogously, the antimicrobial stress exhibited by the group of chemicals derived from ECHA was termed TU_{ECHA}, although in this case the individual chronic EC10 equivalent (instead of the MIC_i) of each CEC *i* was used for the calculation:

$$\mathrm{TU}_{ECHA} = \sum_{i=1}^{n} \frac{MEC_i}{EC10_i^{chronic}} = \sum_{i=1}^{n} TU_i$$

The concept of CA is based on the notion that the components of a mixture share the same mode of action, while disregarding possible synergistic or antagonistic effects (Cedergreen 2014). The TU approach is prevalent in the risk assessment of chemical mixtures (e.g., pesticides, pharmaceuticals, industrial compounds among others) on aquatic ecosystems (Gustavsson et al. 2017; Spilsbury et al. 2020).

2.3 Environmental parameters

Regarding the measured physico-chemical parameters, ammonium and TDS were removed from the dataset. The ammonium concentrations were below the LOD for all sampling sites, while TDS was only measured at approximately half of the sampling sites. The quantification of the nitrite and nitrate concentration resulted in several values being below the LOD and consequently, reported as "zero".

However, due to the usage of semi-quantitative test strips and the subsequent visual comparison to a colour scale the measurement lacked accuracy. Consequently, instead of using “zero” the respective LOD was divided by two and the result was used as value for the respective nitrite and nitrate concentrations.

2.4 DNA extraction from environmental samples

2.4.1 DNA extraction from Sterivex filters

The DNeasy® PowerWater® Sterivex™ Kit was used to isolate the DNA from the river water samples. The extraction of the eDNA followed an in-house protocol (Chiang and Inostroza 2021), which is a slightly modified version of the protocol provided by the manufacturer in order to improve DNA yield from Sterivex™ filters.

Procedure

Prior to the extraction, the remaining liquid in the SFUs was removed using sterile syringes and the outlet was capped with fresh Parafilm to avoid any leakage throughout the process.

The first step of the extraction process was the release of the microbes from the membrane into the solution so that they can be lysed later. Lysis was chemically facilitated by the addition of a cell-release solution and mechanically by the usage of a vortex. SFUs were rotated in 90° steps to ensure higher lysis performance. Afterwards, the lysate, which contained both intact and lysed microbes, was transferred from the SFUs to a tube containing PowerBeads which mechanically broke the cell walls in another vortexing step. The samples were then centrifuged, and the supernatant was separated and removed from sample debris and PowerBeads. Next, the samples were incubated at 37 °C with RNAse to digest the RNA in the isolated supernatant, before a reagent was added to the solution to remove non-DNA organic and inorganic materials such as humic acids, cell debris and proteins. The samples were centrifuged again, and the supernatant was separated from the pellet that contained additional non-DNA organic and inorganic materials.

A high-concentration salt solution was added and mixed with the supernatant and the samples were loaded onto a spin column filter membrane which was connected to a vacuum source. The high salt concentration facilitated the selective binding of DNA molecules to silica in the column, while the remaining contaminants including non-DNA organic and inorganic materials were passing through the filter membrane. The supernatant extracted from SFUs that together form one biological replicate were merged in this step by loading them onto the same column. Through several washing steps with alcohol-based solutions residual contaminants and salts were removed from the column to increase DNA yield and purity. Afterwards, the columns were centrifuged to completely dry the membrane and remove all traces of the washing solutions. The DNA was then eluted from the column through the

addition of an elution buffer and centrifugation. To increase the DNA yield, the column was loaded with the eluted DNA solution and centrifuged again. The DNA samples were stored at -20 °C.

2.4.2 DNA extraction from sediment samples

The DNeasy® PowerSoil® Pro Kit was used to isolate the DNA from the river sediment samples. The extraction of the eDNA from sediment was conducted following the protocol provided by the DNeasy® PowerSoil® Pro Kit handbook. An additional incubation step with RNAse was added to the extraction process.

Procedure

The sediment in the plastic bag was squeezed around for better homogenization before up to 250 mg of sediment were transferred with a sterile plastic spoon to a tube containing beads. A buffer was then added facilitating the dispersal of sediment particles, the dissolvement of humic acids as well as the protection of nucleic acids from degradation. In the next step, the suspension was homogenized, and the microbial cells were lysed using a TissueLyser II. Here, the random shaking of the beads in the presence of a disruptive agent results in collisions between beads and microbial cells leading to the cells breaking up.

Following this, the samples were centrifuged, and the supernatant was isolated before being incubated with RNAse at 37 °C in the added digestion step. Afterwards, a reagent was added that precipitated non-DNA organic and inorganic material such as humic acids, cell debris, and proteins. These contaminants were then removed through centrifugation with the supernatant being isolated and mixed with a high-concentration salt solution. The samples were then loaded onto spin column filter membrane and centrifuged. The high salt concentration facilitated the selective binding of DNA molecules to silica in the column, while the remaining contaminants including non-DNA organic and inorganic materials were passing through the filter membrane. Two washing steps ensured the removal of protein, residual salts, humic acids, and other non-aqueous contaminants, while the DNA remained retained to the silica membrane. Afterwards, the columns were centrifuged to completely dry the membrane and remove all traces of the washing solutions. The DNA was then eluted from the column through the addition of an elution buffer and centrifugation. To increase the DNA yield, the column was loaded with the eluted DNA solution and centrifuged again. The DNA samples were stored at -20 °C.

2.5 Measurement of DNA purity and yield

The quality and yield of the DNA samples were analysed using a Nanodrop 2000C spectrophotometer and a Qubit 3.0 fluorometer, respectively. DNA purity was measured using 1 µL of sample. The absorption ratios 260 nm/280 nm and 260 nm/230 nm were used to evaluate DNA quality (**Table 3**).

DNA yield was measured using 2 µL of sample and by following the protocol of the Qubit 3.0. The high sensitivity (HS) kit was used for most of the samples due to their low DNA concentration. If the DNA concentration was too high for the HS kit, the broad range (BR) kit was used.

The biological replicates of all samples exhibited a low DNA concentration and therefore, the biological replicates of each respective sample were merged prior to the clean-up step.

Table 3. Thresholds for the evaluation of absorption ratios.

Absorption ratio 260 nm/280 nm: 1.8 – 2.0 for pure DNA	
Ratio < 1.8	Contamination with substances that absorb at 280 nm: -> Presence of organic contaminants such as proteins and phenol or glycol
Ratio > 2.0	High share of RNA
Absorption ratio 260 nm/230 nm: 2.0 – 2.2 for pure DNA	
Ratio < 2.0	Contamination with substances that absorb at 230 nm: -> salts, humic acids, peptides, aromatic compounds, urea, guanidine, thiocyanates
Ratio > 2.2	High share of RNA or phenol, high turbidity, dirty instrument, wrong blank

2.6 Clean-up

The DNA samples were cleaned up and concentrated using AMPure XP clean-up kit following the manufacturers protocol. However, the clean-up was not conducted in 96-well plates but in 1.5 mL Eppendorf tubes. The kit utilizes an optimized buffer to selectively bind DNA fragments of 100bp or larger to paramagnetic beads. Contaminations such as nucleotides, salts, enzymes, and smaller DNA fragments are removed in the process, resulting in a more purified extraction product.

Procedure

The DNA samples were mixed with 1.8 times AMPure XP of their reaction volume and incubated for 5 min at RT, before the tubes were placed in the Dynal bead separations rack. After 2 min the beads separated from the solution which was then removed and discarded. The beads were washed with enough 70% ethanol and incubated for 30 s at RT, before the ethanol was removed. This washing step was repeated once. Following this, as much ethanol was removed from the sample through aspiration and a dry time of approx. 1 min. The samples were removed from the magnetic rack and the beads were mixed with 50 µL of elution buffer and incubated for 2 min at RT. Finally, the samples were placed on the magnetic rack and incubated for 1 min for the beads to separate from the solution, before the eluate was removed and placed in a new tube. Lastly, the DNA concentration was measured again using the Qubit fluorometer before aliquoting for sequencing.

2.7 Preparation for dispatch

For the amplicon sequencing, the company Novogene Co. (Cambridge, UK) required at least 20 µL of a 10 ng/µL DNA solution from each sample for each amplicon. DNA eluents were concentrated using a SpeedVac if necessary and re-eluted in a smaller volume of elution buffer to fit the requirements.

2.8 Amplicon sequencing

The targeted region 16S rRNA (V3-V4) was PCR amplified by using specific primers (**Table 4**) connected with barcodes. The PCR products with proper size were selected via 2% agarose gel-electrophoresis. From each sample the same amount of PCR product was pooled, end-paired, A-tailed, and additionally ligated with Illumina adapters. The library was quantified with Qubit and real-time PCR, while the size distribution was checked with bioanalyzer. The quantified libraries were pooled and sequenced using a paired-end Illumina platform generating 250bp paired-end raw-reads.

Table 4. Target region and primer sequence for 16S amplicon following the recommendation of (Yu et al. 2005).

Amplicon	Target region	Forward	Reverse
16S rRNA	V3-V4	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT

2.9 Amplicon data processing

The processing of the raw sequences was performed on resources provided by the Swedish National Infrastructure for Computing (SNIC) in UPPMAX (Uppsala Multidisciplinary Center for Advanced Computational Science) at Uppsala University. Raw sequences were analysed using QIIME™ 2 (Bolyen et al. 2019), a next-generation microbiome bioinformatics platform. For this purpose, the paired-end reads were uploaded to the cluster without barcodes and primers (provided by Novogene) and then imported into Qiime2 using a manifest file. The algorithm DADA2 was used for the denoising – the removal of sequencing errors – of the marker gene amplicon dataset (Knight et al. 2018). In addition, dada2 utilizes error profiles to resolve the sequence data into exact sequence features (ASVs) instead of clustering sequences (usually based on 97% sequence similarity) into single features (OTUs). This ensures the identification of subtle and real biological variation and discrimination between closely related but distinct taxa. Within the algorithm, the settings for the truncation of the length of forward and reverse strand and the threshold for the number

Box 2. RAREFACTION

Biological data can be rarefied to account for the differences in library size and heteroscedasticity (unequal variability between samples) within the dataset. However, rarefying biological count data is statistically inadmissible. It requires the omission of available valid data, which leads to a loss of power or decreased sensitivity (increase in Type-II error). This is evident in sample-wise comparisons when fractions of a sample or even whole samples are discarded to generate rarefied counts but also in differential abundance analysis which expects the inclusion of moderate to rare ASVs that are more likely to be part of the omitted data. Additionally, rarefying does not address overdispersion among biological replicates which results in an underestimation of uncertainty due to an unacceptably high rate of Type-I errors and therefore, decreased specificity. Furthermore, the selection of the minimum threshold for the library size is arbitrary which influences downstream inference. Finally, the random subsampling step in rarefying adds additional uncertainty to the biological data (McMurdie und Holmes 2014).

of maximum expected errors were varied to find the balance between the number of retained raw reads and encountered features/ASVs. Ultimately, no truncation at all and a maximum expected error of 2 lead to the best results. Afterwards, the SILVA database (Version: 138 SSURef NR99) was used as a reference to perform the taxonomic assignment and to construct a phylogenetic tree (Quast et al. 2013; Yilmaz et al. 2014). Pre-formatted, QIIME-compatible SILVA reference sequence and taxonomy files, provided by Robeson et al., were used for these tasks (Robeson et al. 2020; Yilmaz et al. 2014; Quast et al. 2013). Finally, the data was exported for further downstream analysis in R.

2.10 Downstream analysis

The downstream analysis of the ASV data was conducted in R using the {phyloseq} package (McMurdie and Holmes 2013). For this purpose, the exported data from Qiime2 and the metadata was imported into R and transferred into a phyloseq object.

Box 3. Alpha diversity metrics

The most basic alpha diversity metric, the Observed richness, simply represents the number of different species in a sample (Kemp und Aller 2004).

Chao1, on the other hand, is an abundance-based estimator of the species richness in a community. This nonparametric method is based on the following formula:

$$S_{Chao1} = S_{obs} + \frac{F_1 \times (F_1 - 1)}{2 \times (F_2 + 1)}$$

where F_1 and F_2 are the number of singletons and doubletons, respectively, and S_{obs} represents the number of observed species. Consequently, Chao1 is based on the concept that rare species conclude the most information about the number of missing species and therefore, only singletons and doubletons are used to calculate their number (Kim et al. 2017). The estimate of this index can be interpreted as the lower estimate of the species richness that is consistent with the dataset (Haegeman et al. 2013).

Shannon-Weaver index (H) is an estimator of species richness and species evenness, thereby putting more weight on species richness. The additional consideration of the species´ relative abundances provides more inference about the composition of the community than simply species richness or evenness alone. The metric is based on the following formula:

$$H = \sum_{i=1}^{s} (p_i \times \ln(p_i))$$

where s represents the number of ASVs, and p_i displays the proportion of the community represented by ASV i. The Shannon diversity index increases when the number of observed species increases or the distribution of individuals between the species becomes more even (Kim et al. 2017).

Pielou Evenness (J) measures the deviation from an even distribution of individuals within a community that consists of different species (Ricotta und Avena 2003). Consequently, Evenness considers the abundance at which each species exists within a specific area (Johnston und Roberts 2009). The index is based on the following formula:

$$J = \frac{H}{\log(N)}$$

where H represents Shannon-Weaver index and N is the total number of species. The value for Evenness ranges from 0 (totally unequal distribution of individuals among species) to 1 (totally equal distribution) (Ricotta und Avena 2003).

2.10.1 Data preparation

Initially, ASVs without taxonomic assignment at phylum level as well as chloroplast-derived and mitochondrial sequences were removed from the dataset (Knight et al. 2018), whereas singletons and doubletons were retained. The biological data was not rarefied as suggested by McMurdie and Holmes (2014) (See **Box 2**).

2.10.2 Alpha diversity

For the analysis of alpha diversity, four different metrics (Observed richness, Chao1, Shannon-Weaver, and Pielou Evenness) were calculated for all samples (see **Box 3** for specific information on each metric). Afterwards, the role of each individual land-use was investigated by comparing US with DS site of each river in both seasons, respectively. The alpha diversities were also compared between seasons and, if possible, between environmental compartments. The non-parametric Wilcoxon-test was used to

assess significant differences between groups of samples. In addition to the calculation of alpha diversity indices, Venn diagrams were generated for each river and available environmental compartment to determine the number of shared and unique ASVs between sites, seasons, and matrices.

Box 4. UniFrac as distance metric

The most widely used distance metric is the UniFrac distance (unweighted or weighted), which determines the phylogenetic dissimilarity of samples using an evolutionary tree constructed by the estimation of the sequence similarity in all samples. UniFrac measures the phylogenetic distance between two communities as the fraction of branches of the phylogenetic tree which does not lead to descendants of both communities but is unique to one of them (Washburne et al. 2018; Lozupone und Knight 2008). UniFrac follows the intuition that communities which are comprised of more phylogenetically distinct species are more different that communities constituted of more closely related species. As a result, UniFrac usually provides interpretable biological patterns and therefore, is a more biologically meaningful measurement to compare the dissimilarity between communities than standard Euclidean and Bray-Curtis distances (Washburne et al. 2018; Knight et al. 2018).

While unweighted UniFrac distance only considers the absence or presence of a species, weighted UniFrac additonally incorporates the their abundance (FUKUYAMA et al. 2012). The latter is typically used to analyse the community as a system and therefore, perfectly suited to unveil differences between communities which are a result of changes in relative taxon abundance, e.g. different availability of nutrients or exposition to pollutants (Lozupone und Knight 2008)

2.10.3 Community composition

For the analysis of the community composition, the relative abundance of ASVs was calculated. Species with the same taxonomy at phylum level were merged and the least abundant phyla (< 1%) were put into one group. The data was represented in bar plots to determine differences between sites, rivers, seasons, and environmental compartments.

2.10.4 Beta biodiversity

Weighted Pair Group Method with Arithmetic Mean (WPGMA) was used to classify the different samples and to study their similarity. Weighted UniFrac (see **Box 4**) was used as distance metric to generate the WPGMA cluster tree and beta-diversity analysis.

For beta-diversity, the ASV data was graphically represented in a principal coordination analysis (PCoA) and non-metric multidimensional scaling (NMDS) using weighted UniFrac as distance metric following the recommendations of Washburne et al. (2018; see Box 4). The distance matrix was produced using the {rbiom} package in R. The accuracy of the calculation was verified by the simultaneous generation of the weighted UniFrac distance matrix in Qiime2. The comparison of both results showed that they are identical. Dissimilarities were assessed between seasons as well as season-specific between rivers and sampling sites using Pairwise Permutational Multivariate Analysis of Variance (PERMANOVA), implemented in the {pairwiseAdonis} package, and Analysis of Similarity (ANOSIM), implemented in the {vegan package}, for PCoA and NMDS, respectively (Martinez-Arbizu 2020).

Distance-based redundancy analysis (db-RDA) was used to analyse the relationship between beta diversity and environmental parameters. The analysis was performed for environmental matrices and seasons, respectively. For this purpose, the ASV data was first transformed by applying robust centered log ratio transformation, or robust Aitchison, as suggested by (Martino et al. 2019). On the other hand, the metadata, consisting of physico-chemical parameters and nutrients, was transformed using

Hellinger transformation according to (Legendre and Gallagher 2001). Both datasets were linked in the db-RDA using weighted UniFrac as a distance metric. The Variance Inflation Factors (VIF) were calculated for each constraining variable in order to determine whether the constraints were redundant. Explanatory variables with a VIF score > 10 were excluded from the analysis to build the "best" db-RDA model. The significance of each non-redundant parameter was assessed using an Analysis of Variance (ANOVA, 500 permutations) and insignificant parameters were removed from the model. Finally, ANOVA (500 permutations) was conducted to assess the global significance of the "best" db-RDA model. The {vegan} R package was used to perform the db-RDA as well as all subsequent permutations tests (Oksanen et al. 2020).

2.10.5 Assessment of functional profile of microbial communities

The functional microbial profile within each sample was assessed by mapping the identified taxa to established metabolic and ecologically relevant functions using the FAPROTAX database (Louca et al. 2016) and the {microeco} R package. Each species within the database is not restricted to only one functional group but can exhibit several ecological functions. Therefore, some identified taxa were mapped to more than one functional group. The identified functional groups in each sample were weighted against their relative abundances. For clear visualization, functional groups with a relative abundance of less than 1% were removed from the dataset. Furthermore, the assessment focussed on types of energy sources and ecological functions within biogeochemical cycles, thereby excluding functional groups related to e.g., pathogens, parasites, and dark oxidation. The data was graphically represented in bar plots by comparing the sampling sites of each individual river per season and environmental compartment.

3. Results and discussion

3.1 DNA extraction, clean-up, and dispatch for sequencing

The DNA extraction resulted in relatively low concentrations for the majority of water and sediment samples. With respect to water, this is because of the low volume that was filtered through the SFU. On average 230 mL of surface water were filtered per biological replicate which was not sufficient to yield at least 200 ng of DNA needed for amplicon sequencing. Consequently, the biological replicates of each sampling site were merged prior to the purification step in order to reach the minimum concentration for sequencing, which entailed a lack of statistical power in the downstream analysis. Nonetheless, two samples – Lambwe US and DS in the dry season - did not reach the minimum concentration and thus were excluded from sequencing. With regards to sediment, 80% of the samples basically contained no DNA at all since the concentration measurement with the HS Qubit kit displayed values below the LOD of 0.1 ng. The exact reason for this poor outcome is unknown but it can be a consequence of sampling errors or insufficient cooling during the storage and dispatch of samples. The only samples which provided sufficient DNA for amplicon sequencing were from river Asao. The biological replicates were merged prior to the clean-up step nonetheless and were afterwards split up into technical replicates for 16S rRNA amplicon sequencing. A summary of the extracted and dispatched samples in shown in **Table 5**.

Table 5. Summary table of eDNA extraction and dispatch.

	Surface water	Sediment
Sampling sites	10	10
Wet season – October 2021	10*	0
Dry season – February 2022	10*	10*
Wet season – May 2022	0	4*
Total sampling sites	20*	14*
Enough DNA extracted	18	4
Not enough DNA extracted	2	10
Dispatched for 16S rRNA sequencing	18	4**

* In replicates ** In technical replicates

3.2 Amplicon data sequencing and processing

The 16S rRNA amplicon sequencing provided a high number of high-quality raw reads for all samples (see **Table 6**) with an average sample size of 120,000 and 110,000 raw sequences for water and sediment samples, respectively. The processing of the amplicon data- which included filtering, denoising, merging of forward with reverse read and the removal of chimeric sequences – resulted in a final dataset of 2,654,267 sequences including 17,810 ASVs, 1776 doubletons (10.0%) and 774 singletons (4.3%). The quality control was on average passed by approximately 80% and 70% of raw

reads for water and sediment samples, respectively. **Table 6** shows that the discrepancy between the samples of both matrices occurred during the denoising (removal of sequences with errors) and merging of forward with reverse strand. The more complex environmental conditions in sediment, which can facilitate DNA denaturation and interference processes, might be the reason for this outcome. Nonetheless, the sediment samples displayed a higher species richness (number of ASVs) and micro-diversity (number of singletons and doubletons), which is consistent with previous observations (Torsvik et al. 2002; Lozupone and Knight 2007). The rarefaction curves (see **Figure 2**) highlight that sufficient sequencing depth was reached in all samples which indicates that the complete biodiversity in each sampling sites is represented by the ASV dataset. This also corresponds well to the high similarity of Observed richness and the species richness predicted by Chao1 in the alpha diversity analysis (see **Figure 4**). Finally, the taxonomic assignment using the SILVA database as a reference performed well, identifying approximately 85% of the taxa at genus level and around 30% at species level.

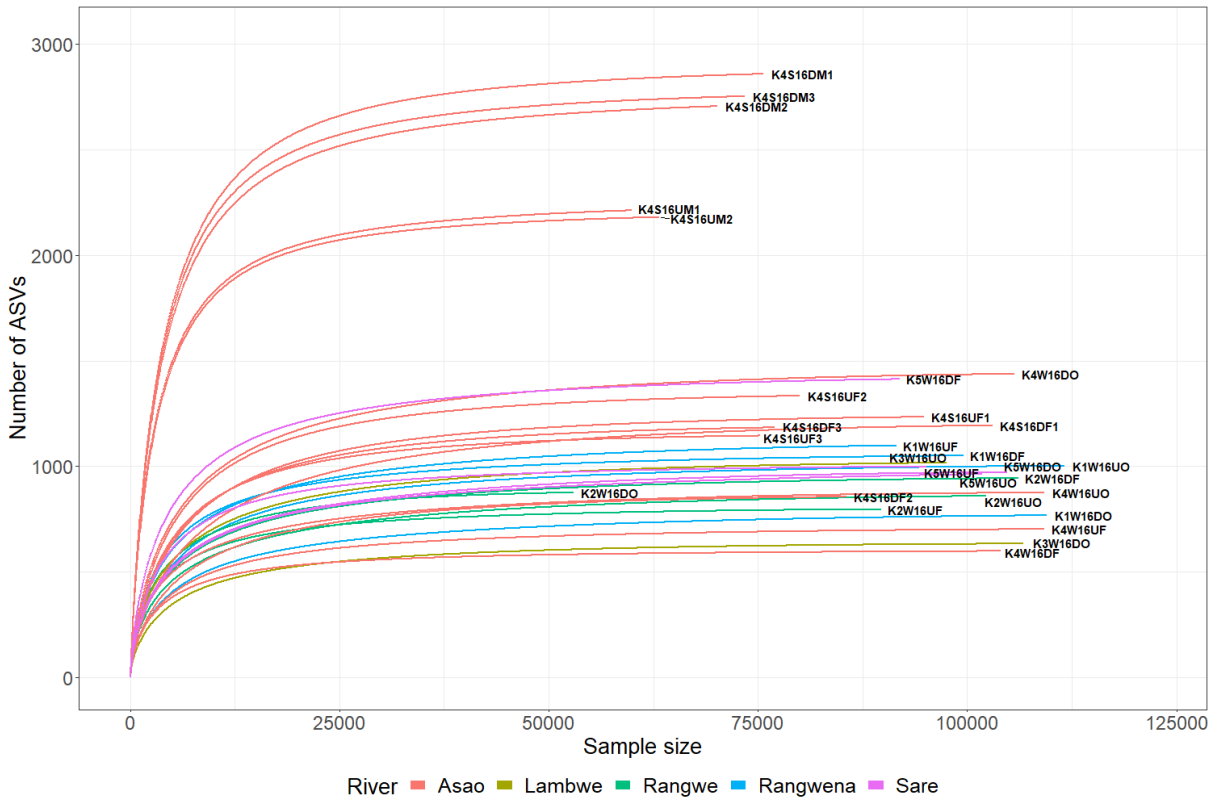

Figure 2. Rarefaction curves for 16S rRNA samples.

***Table 6. Results of 16S rRNA amplicon data processing**. The table shows the number of raw reads, the number of reads which passed the filtering, denoising, merging and chimera removal steps, respectively, as well as the number of ASVs, unique species, singletons, and doubletons in each sample.*

Sample	Raw Reads	Filtered	Filtered [%}	Denoised	Merged	Merged [%]	Non-chimeric	Non-chimeric [%}	ASVs	Unique species	Single-tons	Double-tons
K1W16DF	125269	115383	92.11	113105	106548	85.06	99773	79.65	1074	370	19	41
K1W16DO	134004	123863	92.43	121041	115293	86.04	110475	82.44	848	342	19	53
K1W16UF	115680	106767	92.3	104153	98723	85.34	91618	79.2	1113	444	31	70
K1W16UO	137138	127011	92.62	124714	117665	85.8	111601	81.38	1005	427	14	54
K2W16DF	138812	128919	92.87	125738	119852	86.34	106478	76.71	987	422	22	62
K2W16DO	69371	63928	92.15	61731	57323	82.63	54066	77.94	977	398	21	68
K2W16UF	111904	103933	92.88	102307	97984	87.56	89733	80.19	809	314	8	35
K2W16UO	129855	120490	92.79	117824	110683	85.24	102575	78.99	879	364	21	70
K3W16DO	126067	117146	92.92	115543	111738	88.63	106761	84.69	639	268	11	34
K3W16UO	118830	110566	93.05	108128	102823	86.53	97767	82.27	1028	422	26	59
K4S16DF1	132761	121043	91.17	117018	109137	82.21	103193	77.73	1213	627	32	102
K4S16DF2	109479	99404	90.8	96418	90354	82.53	85719	78.3	875	473	19	72
K4S16DF3	100974	92564	91.67	88913	81345	80.56	77079	76.34	1202	631	27	68
K4S16DM1	122490	110974	90.6	104970	85502	69.8	75808	61.89	2874	1027	55	82
K4S16DM2	113215	102680	90.69	96976	78926	69.71	70268	62.07	2726	961	65	69
K4S16DM3	118879	107744	90.63	101698	82794	69.65	73569	61.89	2775	993	50	76
K4S16UF1	121824	110648	90.83	106482	98621	80.95	95040	78.01	1266	646	26	64
K4S16UF2	106488	97395	91.46	93247	84078	78.96	80182	75.3	1352	708	36	80
K4S16UF3	101302	92082	90.9	87786	78729	77.72	75409	74.44	1161	604	24	66
K4S16UM1	103834	92079	88.68	85113	68289	65.77	60274	58.05	2296	776	51	75
K4S16UM2	100754	90534	89.86	85867	71493	70.96	63414	62.94	2213	762	28	53
K4W16DF	124260	117558	94.61	116600	110813	89.18	104015	83.71	600	237	9	7
K4W16DO	137618	127828	92.89	123441	112917	82.05	106180	77.16	1497	609	33	86
K4W16UF	130809	120959	92.47	119235	115405	88.22	109277	83.54	715	291	13	28
K4W16UO	132881	123744	93.12	121840	116552	87.71	109239	82.21	884	276	13	49
K5W16DF	122995	114588	93.16	110941	102139	83.04	92258	75.01	1438	577	29	71
K5W16DO	131571	122703	93.26	119698	113867	86.54	104938	79.76	992	404	21	81
K5W16UF	117732	109643	93.13	107829	102335	86.92	95259	80.91	1037	356	9	30
K5W16UO	131498	121411	92.33	116740	109805	83.5	102299	77.8	1038	387	42	59

Legend for sample labels:

K1= Rangwena, K2= Rangwe, K3= Lambwe, K4= Asao, K5= Sare W= water, S= sediment 16 = 16rRNA amplicon D= DS, U= US F= February, O= October, M= May

3.3 Environmental parameters and chemical data

Three sets of environmental parameters were analysed within this project: nutrients, physico-chemical parameters and the toxic stress exhibited by organic chemicals with antimicrobial properties (see **Figure 3**). Overall, there was not much variation between the US and DS sites for all parameters. The biggest difference between seasons was exhibited by nutrients and carbonate hardness which displayed higher values in the dry season as well as river flow which was higher in the wet season. More precisely, the measured values for nitrate and nitrite in the wet season were usually below the LOD, whereas in the dry season these nutrients were detected. The figure also shows that the variation across all rivers was marginal for most environmental parameters. The only noticeable abnormalities were relatively high levels of conductivity, nitrate, and carbonate hardness at all Rangwena sites as well as elevated conductivity and carbonate hardness at Asao in the dry season. The number of detected antibiotics and ECHA-derived antimicrobial substances were 9 and 61, respectively, in the wet season and 7 and 79, respectively, in the dry season. However, the respective environmental concentrations and therefore, the calculated TUs for both chemical groups were fairly low at all sampling sites. Nevertheless, the toxic stress exhibited by antibiotics (TU_MIC) was considerably higher compared to the group of chemicals derived from ECHA (**Figure 3**).

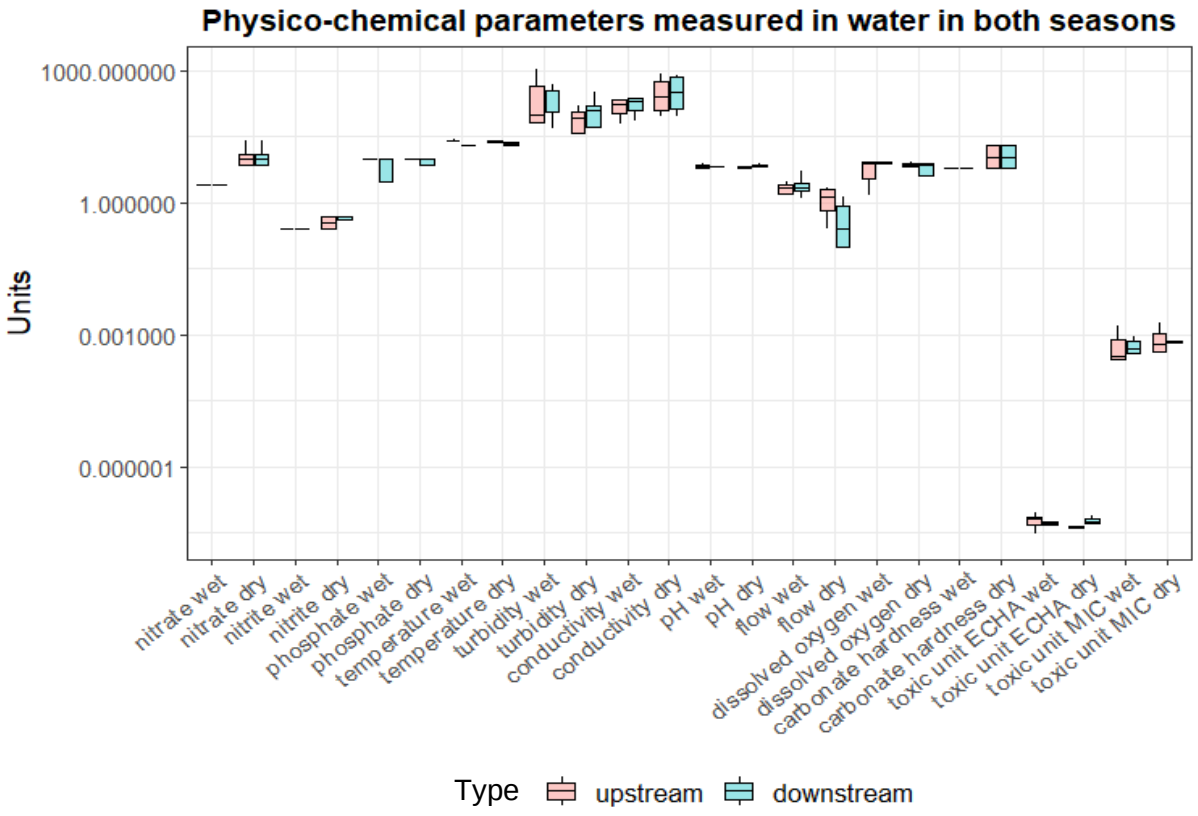

Figure 3. ***Physico-chemical parameters measured in water across all rivers in wet and dry season. The boxplots are based on five samples in the wet season and four samples in the dry season. The boxes delineate the 25% and 75% percentile, the horizontal line within the box the median value and the whiskers the minimum and maximum values. The environmental parameters were measued in the following units: nitrate [mg/L], nitrite [mg/L], phosphate, [mg/L], temperature [°C], turbidity [], conductivity [µS], pH [], flow [m/s], dissolved oxygen [mg/L], carbonate hardness [°d], TU_MIC [] and TU_ECHA [].***

3.4 Alpha diversity

3.4.1 Water

No significant differences between samplings sites, rivers or seasons were observed for all alpha diversity metrics in the water matrix due to a lack of biological replication, thus statistical power. Overall, within sample diversity (alpha-diversity) was homogeneous within and between the sampling sites except for selected sampling sites like Asao and Lambwe during the wet season and Sare in the dry season (see **Figure 4**). In the wet season, Asao displayed considerably lower alpha diversity in the upstream site, however a distinct pattern was observed for Lambwe. Similarly, in the dry season Sare showed lower alpha diversity in the upstream site. The respective Venn diagrams supported these observations (see **Figure 5**). With respect to Asao, the DS site in the wet season displayed two to three times as many unique ASVs than the US site, whereas Lambwe, on the other hand, observed more than twice as many unique ASVs US compared to DS in the same season. In the dry season, Sare´s DS site exhibited a considerably higher number of unique ASVs than the US site showing that the micro-diversity at these three rivers confirms the changes in alpha diversity.

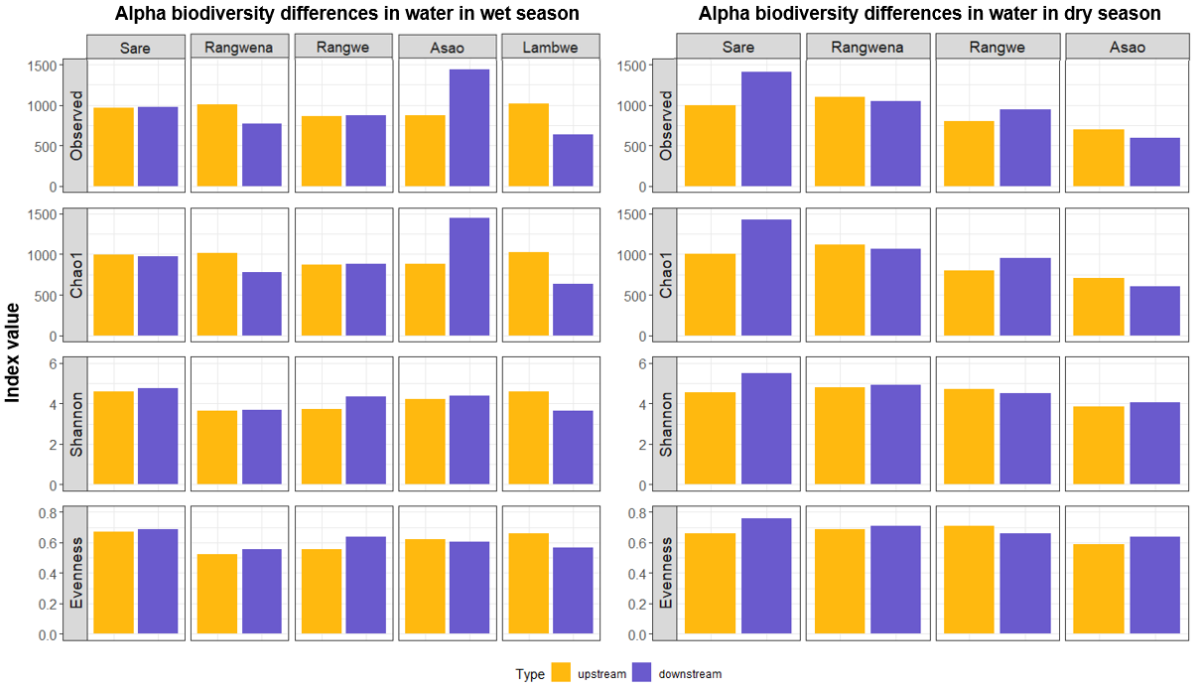

Figure 4. Alpha biodiversity differences in surface water of several rivers in wet and dry season collected in October 2021 and February 2022, respectively. Each bar represents one sample.

The changes in alpha diversity at Lambwe and Asao in the wet season as well as in Sare in the dry season can be linked to the respective land-use. With regards to Lambwe, the US site is in relatively close proximity to its wellspring in the Ruma National Park. Headwaters have shown to display high endemic bacterial species richness which observes a decline DS with increasing distance to the pristine headwaters (Staley et al. 2013; Altermatt et al. 2020; Blackman et al. 2022). With respect to Asao, the high agricultural runoff in the wet season may have resulted in an introduction of numerous taxa at

both sampling sites, and particularly in the downstream site, leading to a considerably higher species richness compared to the dry season. Moreover, the contribution of new species was substantially higher at the DS site due to geographical circumstances (e.g., downward gradient), which was confirmed by the two-fold river flow DS compared to US. Sare, on the other hand, showed an introduction of numerous taxa most likely from domestic sewage but only in the dry season. This is because the discharged wastewater is less diluted due to the lower river flow and therefore, relevant enough to considerably impact the bacterial species richness.

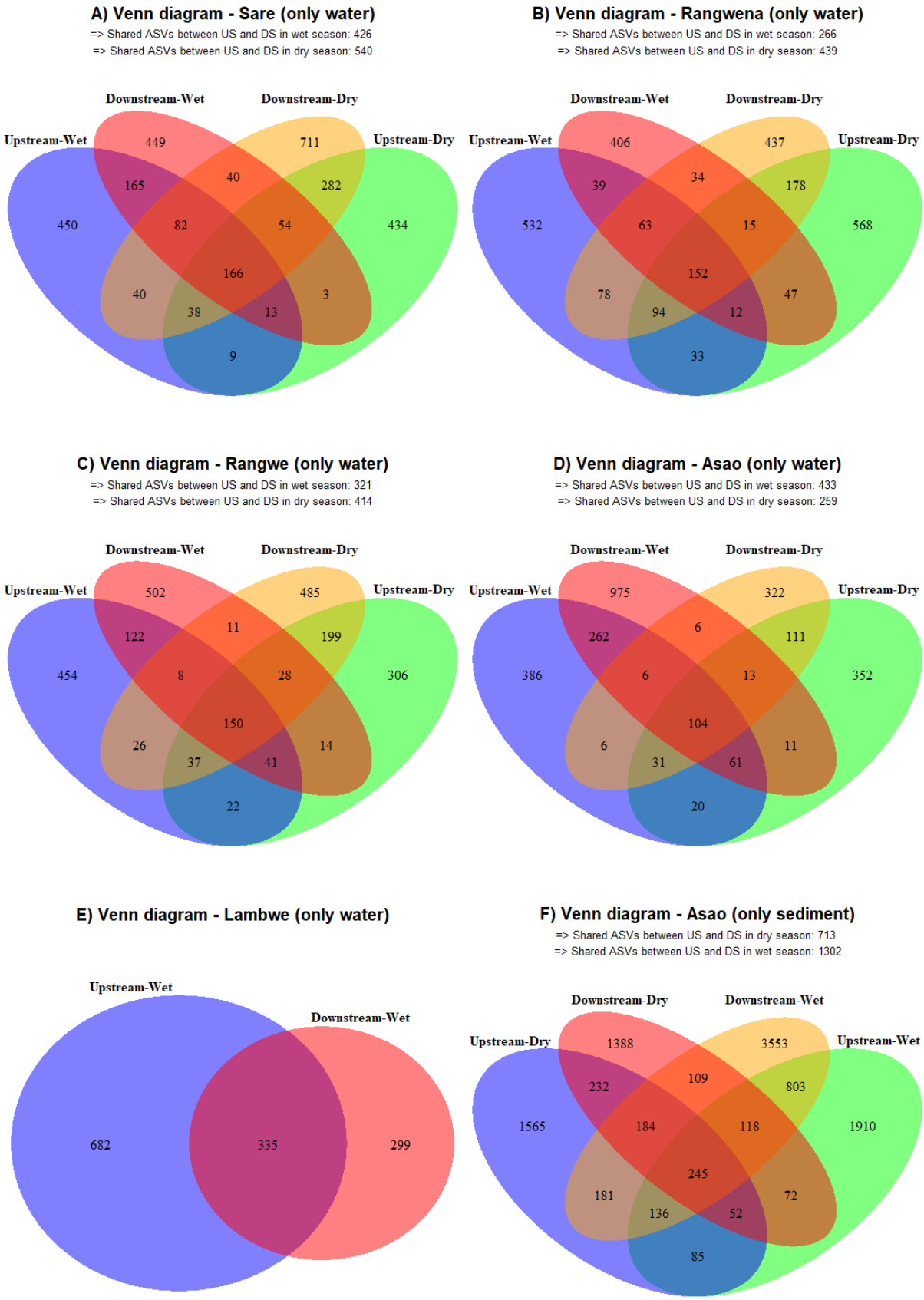

Figure 5. Venn diagrams showing the number of shared and unique ASVs for each river, season and matrix.

3.4.2 Sediment

The analysis of alpha diversity in sediment revealed considerable changes from dry to wet season as well as from US to DS in the wet season (see **Figure 6**). In the dry season, the alpha diversity varied only marginally between the sampling sites. However, higher alpha-diversity was determined in the wet season with an increase of up to 150% in both sites (**Figure 6A)**. Regarding the upstream and downstream setting, the dry season showed similar levels of alpha diversity (**Figure 6B**), nevertheless, clear differences were observed during the wet season. These observations were supported by the Venn diagram (see **Figure 5**) displaying nearly twice as many shared ASVs between US and DS in the wet season. In addition, while the number of unique ASVs between the US sites only showed little variation, the DS site in the wet season observed more than three times as many unique ASVs compared to DS in the dry season. A plausible explanation to these findings, both between sites and season, is linked to the role of the river flow as physical stress altering the alpha diversity of sediments. That is, lower river flow did not disturb superficial sediments promoting sediment stability in the dry season. The contrary can be observed during the wet season, where overall alpha-diversity is higher likely due to higher agricultural runoff contributions from headwaters and from runoff between the sampled sites. Agricultural runoff may have introduced numerous species into the river at both sampling sites which explains the tremendously higher species richness compared to the dry season.

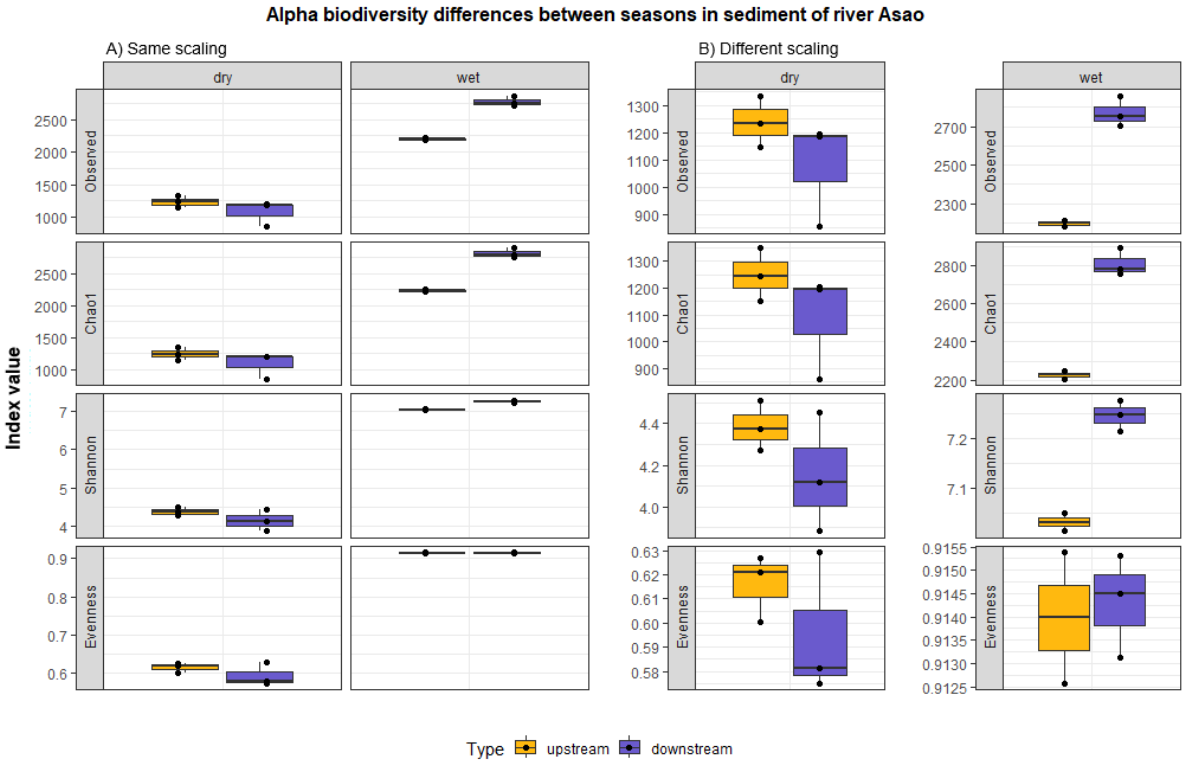

Figure 6. ***Alpha biodiversity differences in sediment. The boxplots are based on three samples in the dry season as well as two samples' DS and three samples' US in the wet season. The boxes delineate the 25% and 75% percentile, the horizontal line within the box the median value and the whiskers the minimum and maximum values.***

3.5 Community composition

3.5.1 Water

The bacterial community composition in the water matrix of the five rivers is visualized in **Figure 7**. Overall, no considerable differences were observed between upstream and downstream sites of each river, however, most bacterial communities underwent moderate changes from wet to dry season. Moreover, rivers exposed to the same land-use, in particular agricultural, showed high similarity in their community composition.

In the wet season only minor changes in the relative abundance of phyla were observed from US to DS. However, the community structure of Rangwena, Rangwe and Asao was similar and was primarily dominated by Proteobacteria (up to 90%) as well as Actinobacteriota and Bacteroidota, these latter, at considerably lower abundances. This trend is plausible because the rivers are majorly exposed to agricultural land-use with an intermediate degree of anthropogenic activities. On the other hand, Sare and Lambwe distinguished themselves with a comparably lower relative abundance of Proteobacteria (up to 70%) but higher proportions of Actinobacteriota and Patescibacteria. The additional influence of urban land-use with high anthropogenic impact at Sare and natural land-use with low anthropogenic impact at Lambwe, respectively, might be the reason for their differentiation from pure agricultural sites.

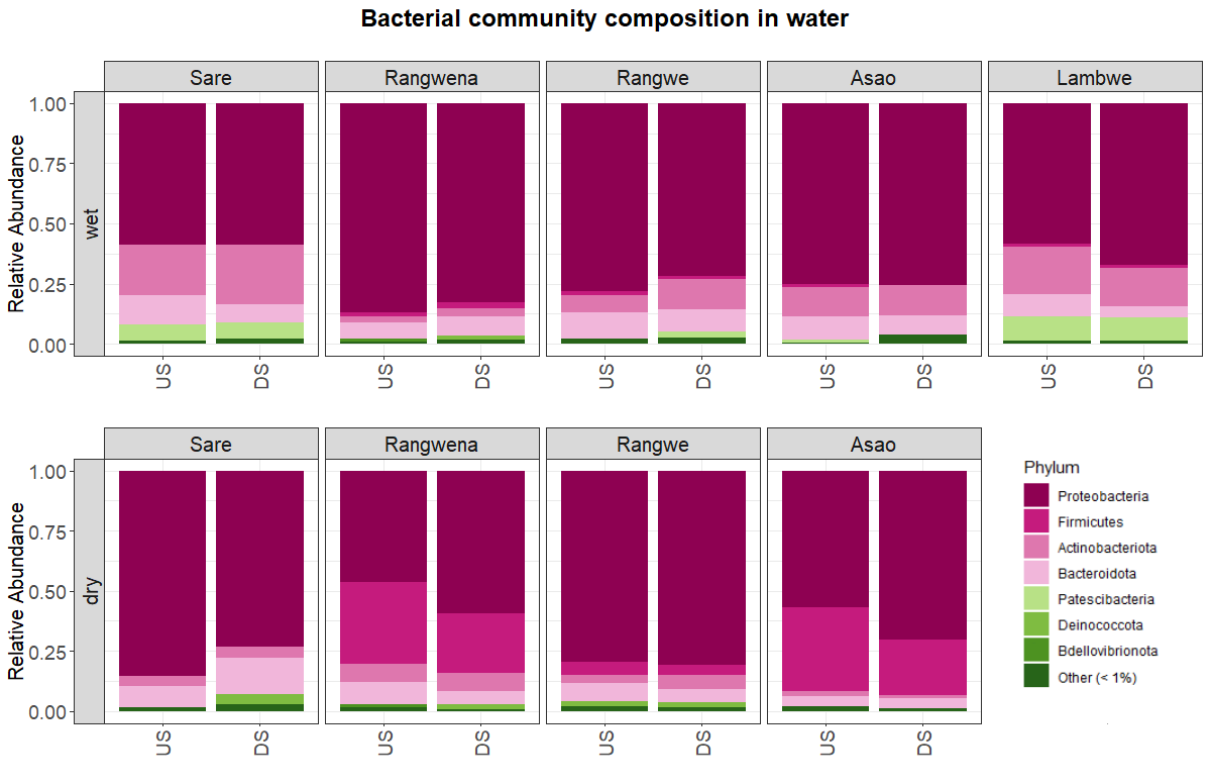

Figure 7. Community composition of bacteria in water. Each bar is based on one sample.

Conversely, the differences between US and DS were more pronounced in the dry season, especially for the rivers Sare, Rangwena and Asao. Nonetheless, the community composition was again shaped by the respective land-use. Rangwena and Asao, both primarily exposed to agricultural land-use, were dominated (up to 95%) by Proteobacteria and Firmicutes. Sare, on the other hand, observed a distinct community structure with comparably higher proportions of Protebacteria and an increase composition of Bacteroidota from upstream to downstream. Interestingly, Firmicutes were absence. In addition, Deinococcota increased from US to DS (see **Figure 7**). This corresponds well with the changes in alpha diversity which revealed that Sare is mainly exposed to urban land-use in the dry season where the discharged sewage is less diluted due to the lower river flow and consequently, has a considerable effect on both species' richness and community composition.

Finally, Rangwe´s community structure was the most similar to Sare (high proportion of Proteobacteria) but also showed, to some extent, similarity to sites exposed to agricultural land-use due to the presence of Firmicutes. This observation suggests that in the dry season the land-use around Rangwe is a mix of primarily urban with some agricultural influence. This assumption is plausible because both sampling sites are in close proximity to a semi-urban area where, similarly to Sare, domestic sewage is discharged into the river. Due to the lower river flow in the dry season, the wastewater effluent seems to be relevant enough to considerably shape the bacterial community composition.

3.5.2 Sediment

The analysis of the bacterial community composition in sediment revealed high similarity within technical replicates as well as between US and DS sampling sites for both seasons (see **Figure 8**). In contrast, considerable differences were observed between dry and wet season. In the dry season the community was primarily dominated by Firmicutes and Proteobacteria which accounted for about 90% of the relative abundance in all samples. The relative abundances of the present phyla also varied only marginally between sites. In comparison, the community in the wet season displayed much more phyla diversity, although Proteobacteria and Actinobacteriota made up approximately 70% of the total relative abundance. Gemmatimonadota, Desulfobacterota and Verrucomicrobiota appeared at the DS sampling sites, whereas Myxococcota and Chloroflexi increased in abundance from US to DS thereby evening up the decrease of Actinobacteriota. Firmicutes experienced a tremendous decline in relative abundance from dry to wet season which was compensated for by emergence of new and rise of less abundant taxa such as Gemmatimonadota, Verrucomicrobiota and Myxococcota which were only present in the wet season. The seasonal variation in sediment community structure of Asao is not only conclusive with the respective findings in the water matrix but also the observations of the alpha diversity analysis. In the dry season, the lower river flow results in less disturbance of bacterial

communities in the superficial sediment which seems to favour the phylum of Firmicutes. In contrast, the high agricultural runoff along the river did not only result in higher flow which diminished sediment stability and consequently, the abundance of Firmicutes, but also the introduction of new and promotion of less abundant phyla in the sediment community.

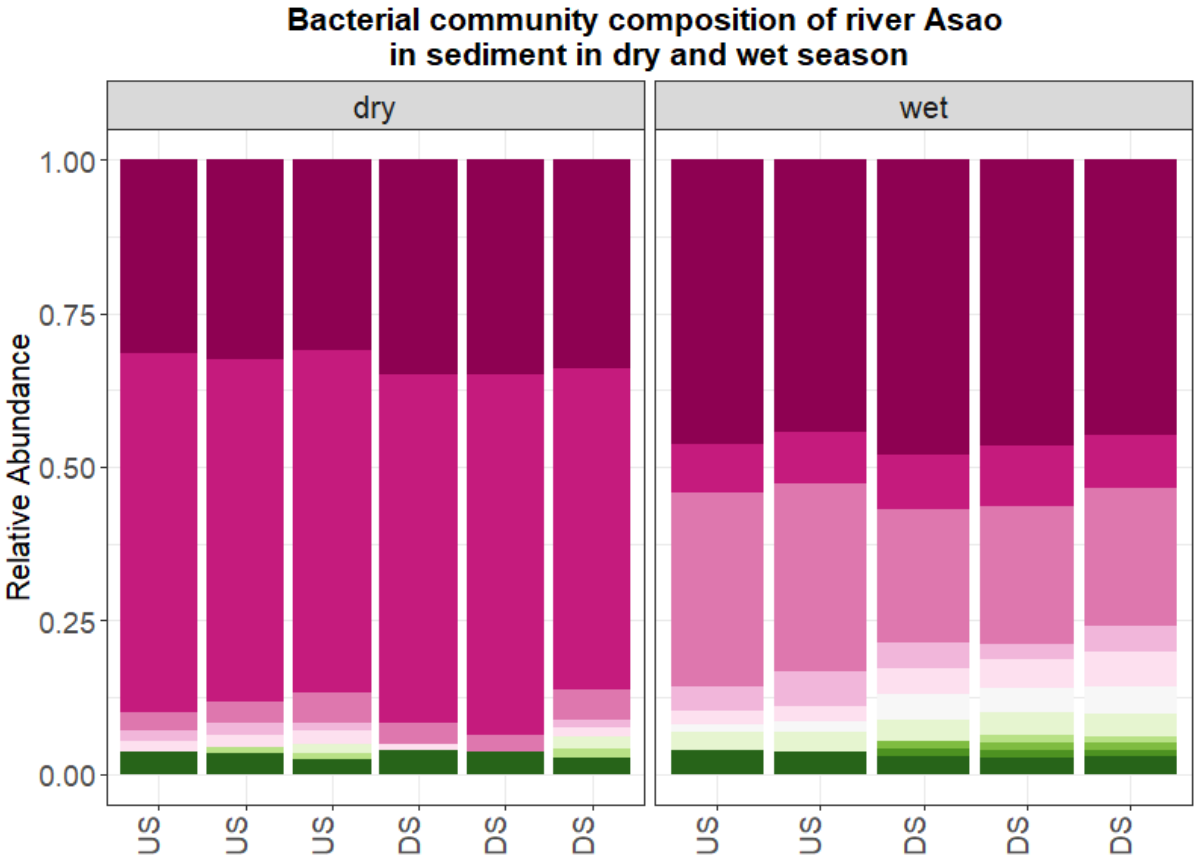

Figure 8. Composition of bacterial communities of river Asao in sediment in dry and wet season. The bars in the dry season are based on three samples, while in the wet season the bars are based on two samples US and three samples' DS.

3.6 Beta biodiversity

3.6.1 Water

Dissimilarity analysis

Significant differences between the rivers in both wet (ANOSIM, p = 0.03, R = 0.72) and dry (ANOSIM, p = 0.009, R = 0.8125) season are supported by the NMDS analysis (see **Figure 9**). Both seasons displayed low stress (< 0.03) which implied an excellent representation of the data in reduced dimensions, while the respective R-values indicated that the rivers in both seasons were well separated. However, due to a lack replicates it is unclear which rivers were significantly different from each other in each season. Nonetheless, the NMDS showed that in both seasons the rivers grouped together based on their primary land-use which was also supported by the WPGMA cluster tree (see **Figure 10**).

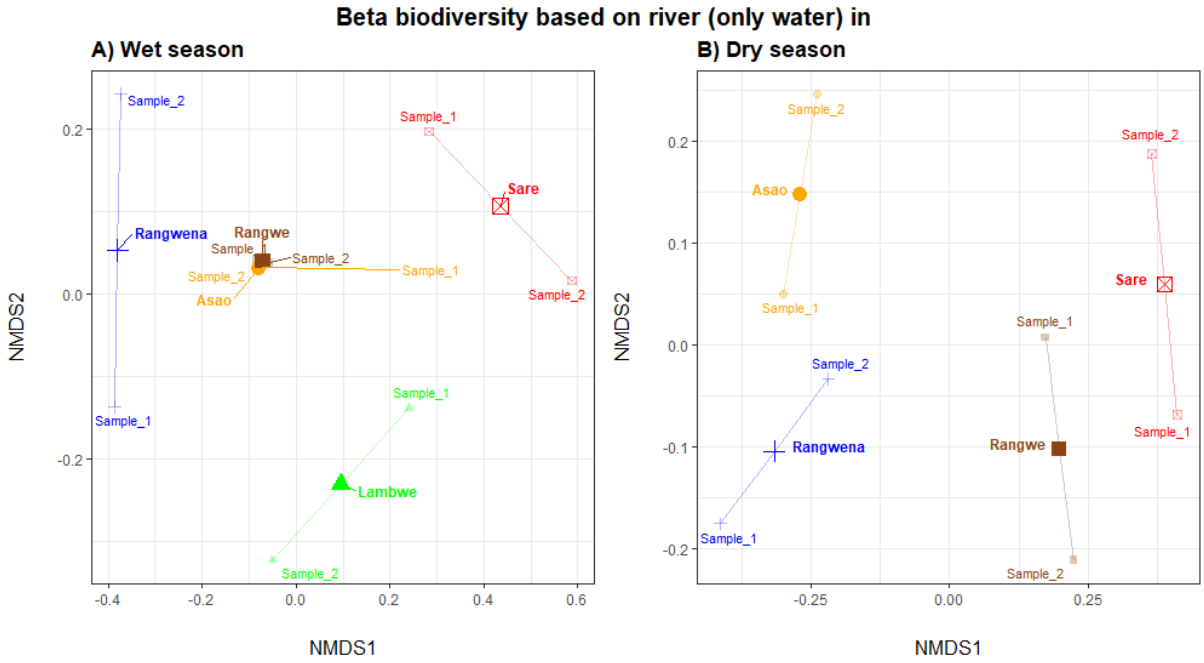

Figure 9. Beta diversity of river water compartments in A) wet season, B) dry season.

In the wet season (see **Figure 9A**), the centroids of Asao and Rangwe overlapped, and all four samples were plotted in close proximity showing high similarity between the two rivers. This is consistent with the WPGMA tree where the two rivers exhibited the closest phylogenetic distance between all rivers in the wet season. This outcome is conclusive because both rivers are primarily exposed to agricultural land-use and an intermediate degree of anthropogenic activities. Both analyses also showed that Rangwena was the most similar river to the pair of Asao and Rangwe. In the NMDS Rangwena´s centroid was in relatively close vicinity to the centroids of Asao and Rangwe, while in the WPGMA tree Rangwena was part of the same cluster as the other two rivers. This seems plausible because Rangwena is exposed to the same type of land-use and degree of anthropogenic impact. The only distinction between Rangwena and the other two rivers is the considerably higher dissimilarity between US and DS site which is also shown by the bigger phylogenetic distance between both sites in the WPGMA. The same observation was made for the rivers Lambwe and Sare. The large distance between the sites of each river is clearly visible in the NMDS and the WPGMA tree. Although the two rivers formed one cluster in the WPGMA, it was clearly discernible that they exhibited high dissimilarity from each other, and the other three rivers. This makes sense because the land-use at Lambwe is a mix of natural and agricultural with a low degree of anthropogenic activities, while Sare is primarily exposed to urban land-use with some agricultural influence and a high degree of anthropogenic impact.

In the dry season (see **Figure 9B**), there was no centroid overlap or clustering of sampling sites. Although Asao and Rangwe displayed high resemblance in the wet season, the rivers were very dissimilar in the dry season. Moreover, the US and DS site of each river showed dissimilarity which is

also depicted by the large phylogenetic distances between them in the WPGMA cluster tree. The only partial grouping in the NMDS was Asao and Rangwena on the left and Rangwe and Sare on the right side of the plot. This grouping was also visible in the WPGMA where Rangwe and Sare as well as Asao and Rangwena each formed a cluster. The reason for was most likely the predominant agricultural land-use at Rangwena and Asao, while Sare and Rangwe were primarily exposed to urban land-use.

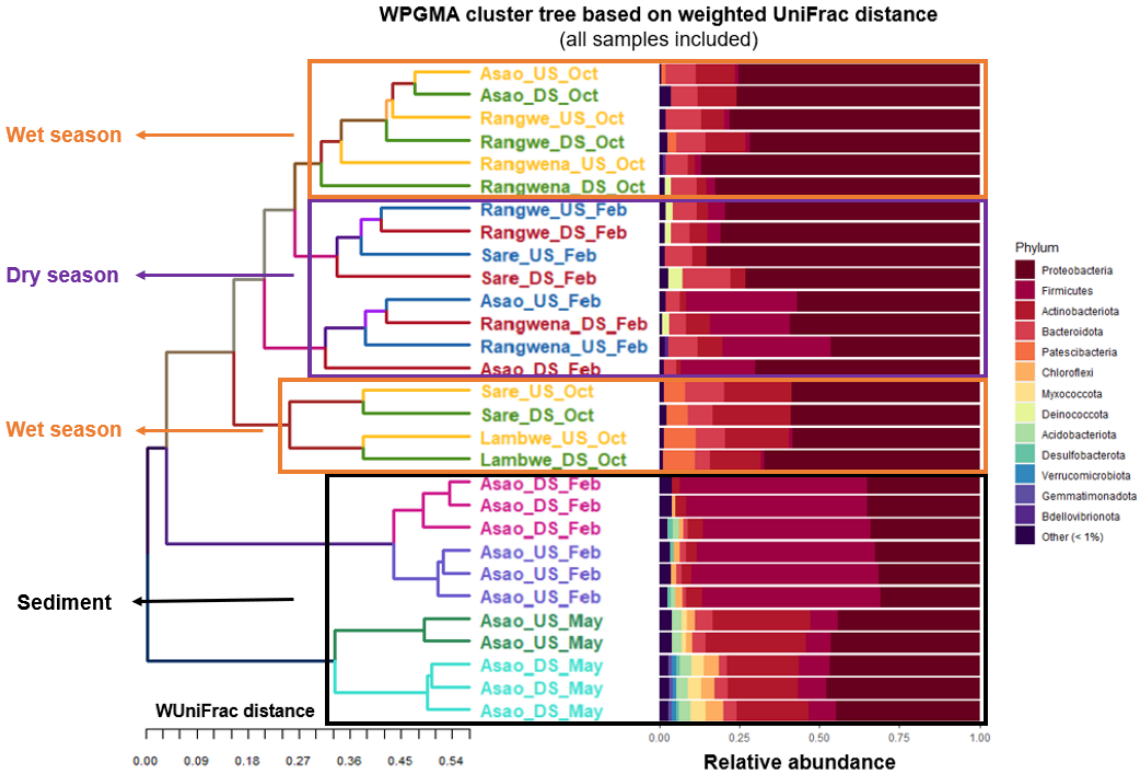

Figure 10. WPGMA cluster tree using weighted UniFrac distance.

The analysis of beta diversity using PCoA showed a similar allocation of rivers and samplings sites. However, in both scenarios the pairwise comparison of rivers observed no significant difference (PERMANOVA, $p > 0.05$) which was due to the lack of replicates and therefore, statistical power.

Impact of environmental parameters

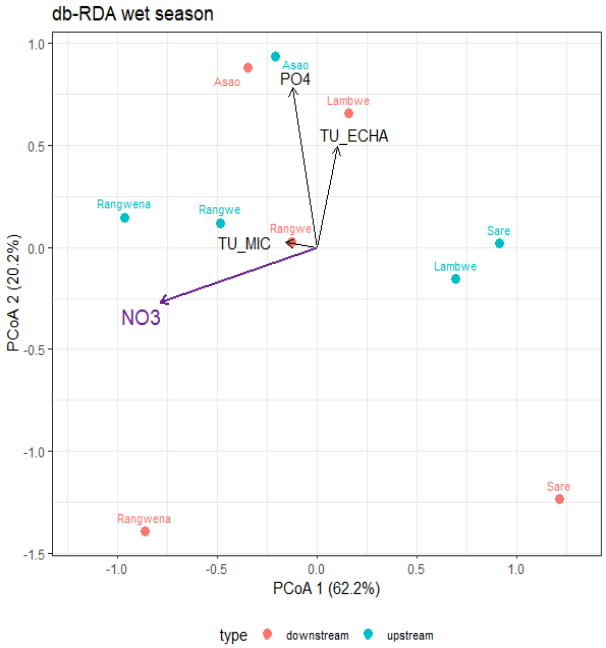

Figure 11. db-RDA of water samples in wet season.

The role of the measured environmental parameters was assessed through a distance-based redundant analysis (db-RDA), which revealed that in both seasons the variation between the rivers was best explained by nutrients (see **Figure 11**). In the wet season a significant model was built on four parameters of which only nitrate exhibited statistical significance itself. However, both nutrients – nitrate and phosphate – correlated positively with the sampling sites that were primarily affected to agricultural land-use - both sites at Rangwena, Rangwe and Asao as well as the DS site of Lambwe.

The antimicrobial stress exhibited by antibiotics and ECHA-derived organic chemicals also seemed to play a role. For instance, the toxic stress exhibited by organic compounds derived from ECHA correlated positively with Lambwe DS. However, the lack of statistical power prevented a confirmation of significant correlation between antimicrobial stress and the rivers. In the dry season the significant model was only build on one parameter and therefore, a graphical visualization was not possible. The only non-inflated parameter was phosphate which also exhibited statistical significance.

These findings were consistent with the overall lack of variation of environmental parameters between the rivers. Nutrients which displayed the most variability were the only group of parameters to exhibit significant correlation with certain rivers. The fact that nutrients only correlated with rivers majorly exposed to agricultural land-use is also conclusive. However, antimicrobial toxic stress, in particular the chemical group derived from ECHA, showed positive, albeit insignificant, correlation with some sites as well, although the environmental concentrations were relatively low. This suggests that CECs did, to some extent, play a role in shaping the beta diversity of bacterial communities.

3.6.2 Sediment

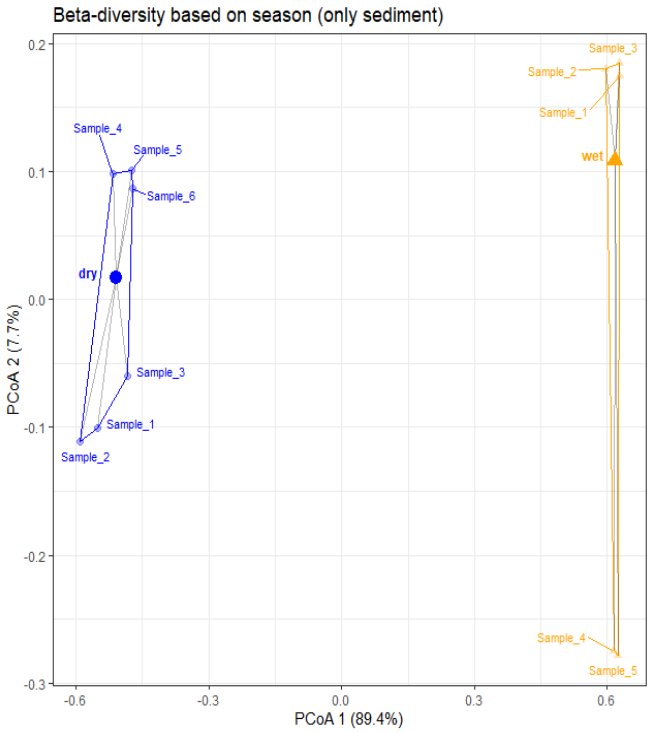

Figure 12. Beta diversity in sediment of river Asao.

The PCoA (see **Figure 12**) showed that the sediment in dry and wet season were significantly different from each other (PERMANOVA, p = 0.001). Furthermore, a clear distinction between US (sample 1-3) and DS (samples 4-6) was visible for both seasons. Both findings were supported by the WPGMA tree which showed separate clusters for the two seasons as well as a clear differentiation between US and DS sites in wet and dry season. The variation with the dataset was well represented since PCoA1 and PCoA2 comprised around 97% of the dataset´s variation.

The NMDS showed a similar sample distribution, however, the stress was almost zero indicating insufficient data for the analysis.

3.7 Functional diversity

3.7.1 Water

The assessment of the functional profile of bacterial communities revealed a similar structure for all rivers in both seasons. The most abundant ecological functions were energy sources, most notably aerobic chemoheterotrophy, followed by carbon cycling, particularly aromatic compound degradation. The latter also represented the functional group which showed the highest variation between rivers in both seasons. Nitrogen cycling, on the other hand, played only a marginal role in all surface waters. For instance, ureolysis was the most prominent functional group within the nitrogen cycle, although usually only being present at relative abundance between less than 1% and 2%. Moreover, functional groups linked to biogeochemical cycling of sulphur and metals showed nominal proportions of less than 1% at all sampling sites. See **Figure 13**, the functional profile of Lambwe in the wet season, as an example, whereas the other bar plots can be found in the appendix (**Figure 15** to **Figure 22**). Nonetheless, the analysis revealed some trends of functional changes shared by rivers which are exposed to the same land-use.

In the wet season, the two most abundant functional groups- aerobic chemoheterotrophy and aromatic compound degradation – displayed higher abundances at sampling sites that were primarily exposed to agricultural land-use. This included both sites at Rangwena, Rangwe, Asao as well as DS of

Lambwe. Particularly, the proportion of aromatic compound degraders was considerably lower at Sare, the only urban river in the wet season. However, all river except Lambwe observed a decrease in the relative abundance of the two most prominent ecological functions from US to DS. In contrast, Lambwe experienced an increase for both groups and in particular for aromatic compound degraders. In addition, Lambwe exhibited by far the highest abundance of aliphatic non-methane and aromatic hydrocarbon degradaers – two ecological functions associated with carbon cycling. The reason for this observation could be that the degree of anthropogenic impact at Lambwe is expected to be relatively low compared to the other four rivers where it is presumed to be either moderate or high. Lastly, ureolysis displayed a higher proportion in the wet season and most notably, at the US sites of each river.

In the dry season, aerobic chemoheterotrophy exhibited higher relative abundances at sampling sites primarily exposed to urban land-use – both sites of Sare and Rangwe. This constitutes a clear distinction to the wet season. Furthermore, there was as clear difference in the functional changes from US to DS between rivers majorly impacted by urban land-use and rivers predominantly exposed to agriculture which included Rangwena and Asao. The proportion of the most abundant ecological functions decreased or remained constant in urban rivers, while in agricultural rivers their relative abundances increased. Finally, photoheterotrophy and fermentation displayed an overall higher relative abundance in the dry season and most notably, in rivers exposed to urban land-use.

In summary, carbon cycling seemed to play a more important role in agricultural rivers in the dry and especially in the wet season. Ecological functions linked to energy sources, on the other hand, were of higher importance at rivers primarily exposed to agriculture in the wet season, but more important at urban rivers in the dry season.

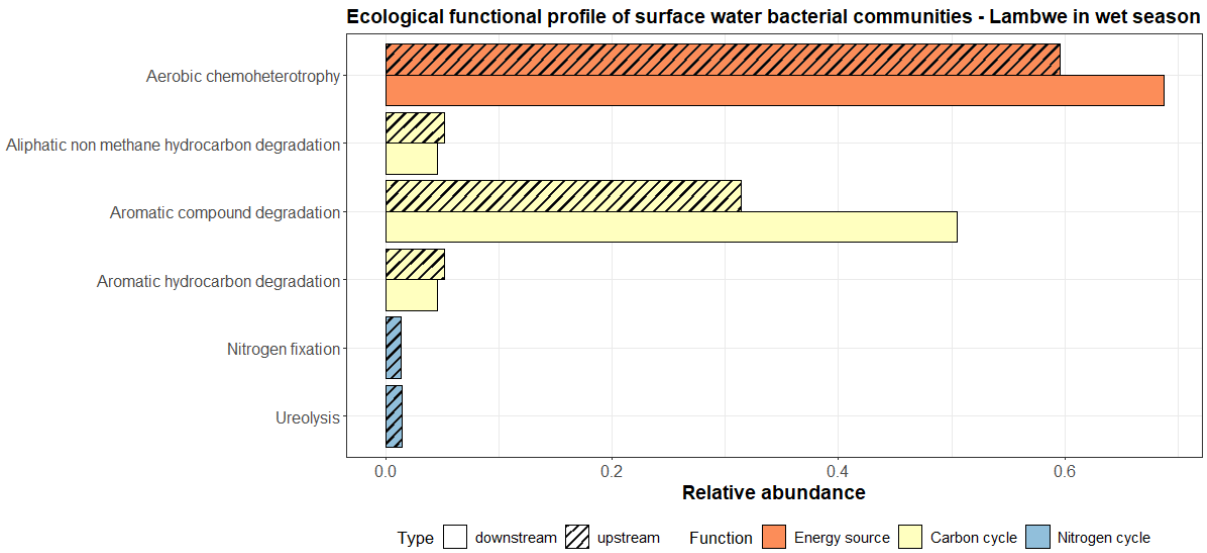

Figure 13. Functional profile of bacterial communities in surface water of Lambwe in wet season.

3.7.2 Sediment

The analysis of the functional profile of bacterial communities in the sediment revealed considerable differences between dry and wet season. Most notably, ecological functions associated with energy sources and nitrogen cycling were much more prevalent in the wet season (see **Figure 14**). In contrast, functional groups linked to carbon cycling, in particular aromatic compound degradation, observed considerably higher relative abundances in the dry season (see **Figure 23** in appendix). Furthermore, the variation between technical replicates and sampling sites was largely marginal with the exception of aromatic compound degradation and fermentation whose relative abundance increased by the factor of two to three from US to DS in the wet season.

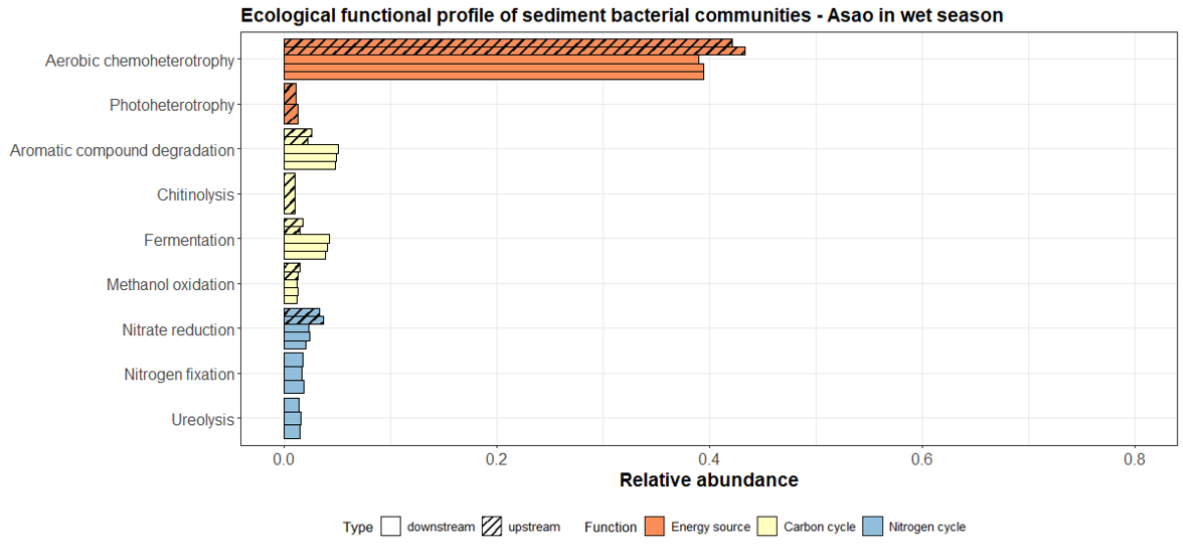

Figure 14. Functional profile of bacterial communities in sediment of Asao in the wet season.

4. Conclusion

In conclusion, eDNA metabarcoding allowed to take a snapshot of several freshwater bacterial communities and their structural and functional responses to CECs derived from land-use. The analysis revealed limited variation of biodiversity metrics within riverine ecosystems but significant structural differences between rivers exposed to different land-use. Furthermore, bacterial community structure also displayed considerable variation between seasons. The assessment of functional diversity observed the presence of core ecological functions at all sampling sites which varied, to some extent, in relative abundance based on land-use and season. Finally, a significant correlation between bacterial community responses and their exposure to CECs could not be established. This can either be due to the low chemical pressure or because of a suboptimal sampling design and a lack of statistical power. For this reason, further studies – with an optimized sampling design and sufficient sampling effort – must be conducted to, on the one hand, verify the general trends and differences observed in this study and, on the other hand, analyse in greater detail the impact of CECs on the structure and functioning of bacterial communities in freshwater ecosystems.

5. Publication bibliography

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6. Appendix

6.1 Material

6.1.1 Chemicals and reagents

Chemicals	**Manufacturer/Supplier**
Clear Line DES 70 (for disinfection)	Solveco (Rosersberg, Sweden)
RNAse A (100mg/mL)	QIAGEN GmbH (Hilden, Germany)
Ethanol 99.5%	Solveco (Rosersberg, Sweden)
Water, RNase & DNase free	Thermo Fisher Scientific (Ward Hill, MA, USA)
Kits	**Manufacturer/Supplier**
DNeasy® PowerWater® Sterivex™ Kit	QIAGEN GmbH (Hilden, Germany)
DNeasy® PowerSoil® Pro Kit	QIAGEN GmbH (Hilden, Germany)
Qubit™ dsDNA HS Assay Kit, 500 assays	Thermo Fisher Scientific (Eugene, Oregon, USA)
Qubit™ dsDNA BR Assay Kit, 500 assays	Thermo Fisher Scientific (Eugene, Oregon, USA)
Agentcourt AMPureXP	Beckman Coulter, Inc. (Brea, CA, USA)

6.1.2 Consumables and laboratory instruments

Consumable	**Manufacturer/Supplier**
Pipettes (10 µL, 100 µL, 200 µL, 1000 µL)	Eppendorf (Hamburg, Germany)
Pipette tips	Sarstedt (Nürnbrecht, Germany)
smartSpatula®	LevGo, Inc. (Berkeley, PA, USA)
10 mL syringe (luer lock tip)	Terumo (Tokyo, Japan)
Qubit™ assay tubes	Thermo Fisher Scientific (Eugene, Oregon, USA)
PCR tubes (PCR-05-C)	Corning (Reynosa, Mexico)
Sterivex filter units without filling bell	Merck (Darmstadt, Germany)
Instrument	**Manufacturer/Supplier**
Centrifuge 4-16KS	Sigma Aldrich (Darmstadt, Germany)
Centrifuge 5424	Eppendorf (Hamburg, Germany)
VortexGenie2	Scientific Indutries Inc. (Bohemia, NY, USA)
Heating Block Model 111002	Boekel Industries Inc. (Feasterville, PA, USA)
Water bath julabo 6A/U3	Julabo (Seelbach, Germany)
TissueLyserII	QIAGEN GmbH (Hilden, Germany)
TissueLyserII adapter	QIAGEN GmbH (Hilden, Germany)
Oven U90	Memmert (Schwabach, Germany)
Vortex Adapter for 6 (5 % 15 mL) Tubes	QIAGEN GmbH (Hilden, Germany)
Nanodrop 2000C spectrophotometer	Thermo Fisher Scientific (Eugene, Oregon, USA)
Qubit 3.0 fluorometer	Thermo Fisher Scientific (Eugene, Oregon, USA)
Vortex Lab dancer S40	VWR (Radnor, PA, USA)
Dynal bead separations	Thermo Fisher Scientific (Eugene, Oregon, USA)
VacValve Vacuum System	QIAGEN GmbH (Hilden, Germany)
Vacuum Pump VCP80	VWR (Leuven, Belgium)
SpeedVac SVC100	Thermo Fisher Scientific (Eugene, Oregon, USA)
Refrigerated VaporTrap RVT400	Thermo Fisher Scientific (Eugene, Oregon, USA)
SpeedVac Dryness Controller	Thermo Fisher Scientific (Eugene, Oregon, USA)
Vacuum Pump VP100	Thermo Fisher Scientific (Eugene, Oregon, USA)

Software	Supplier
Nanodrop 200	Thermo Fisher Scientific (Eugene, Oregon, USA)
RStudio 2023.03.0	RStudio (Boston, MA, USA)
QIIME2-2022.8	Qiime2 Development Team

6.2 Plots

6.2.1 Functional diversity

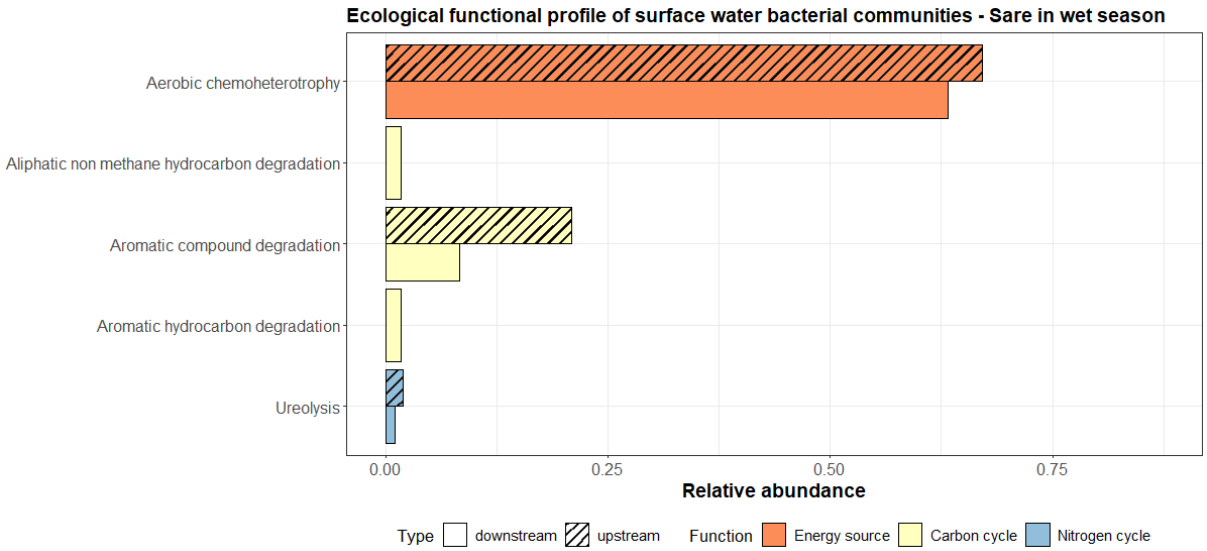

Figure 15 Functional profile of bacterial communities in surface water of Sare in wet season.

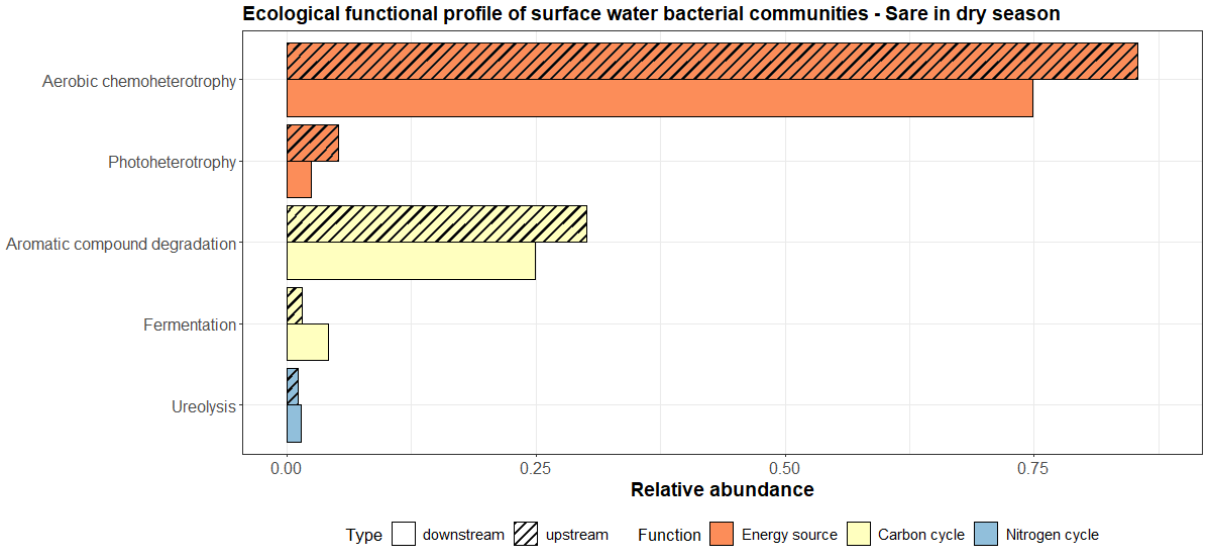

Figure 16. Functional profile of bacterial communities in surface water of Sare in dry season

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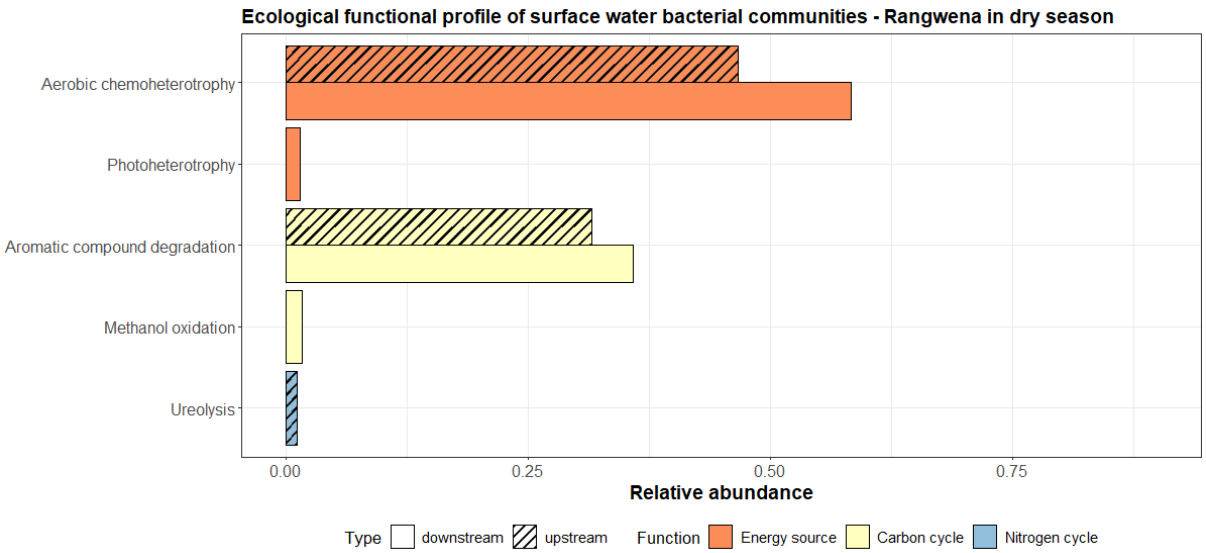

Figure 17. Functional profile of bacterial communities in surface water of Rangwena in wet season.

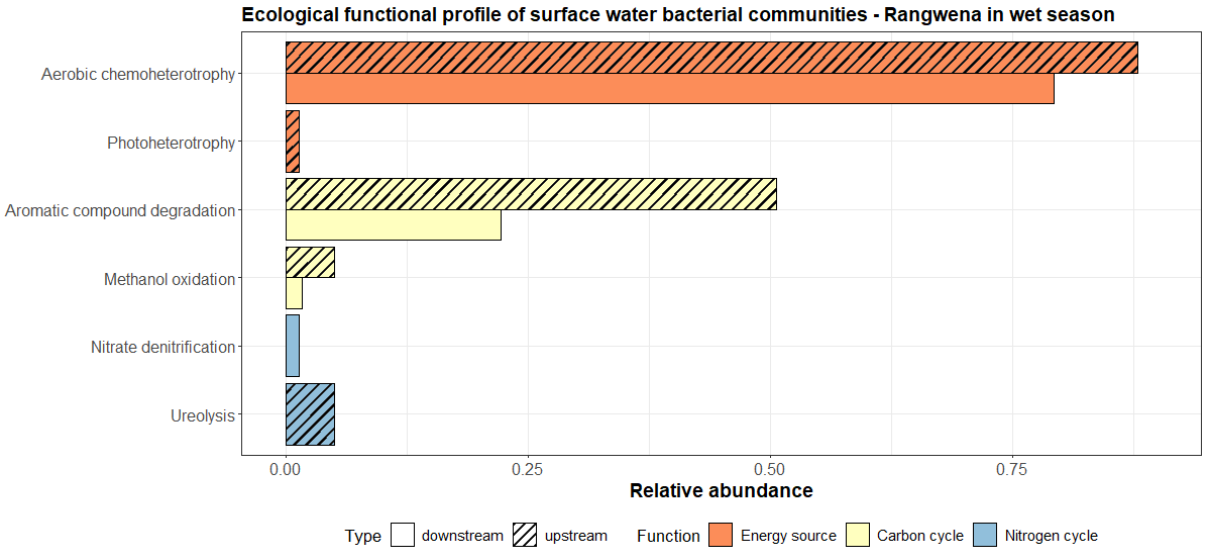

Figure 18. Functional profile of bacterial communities in surface water of Rangwena in dry season.

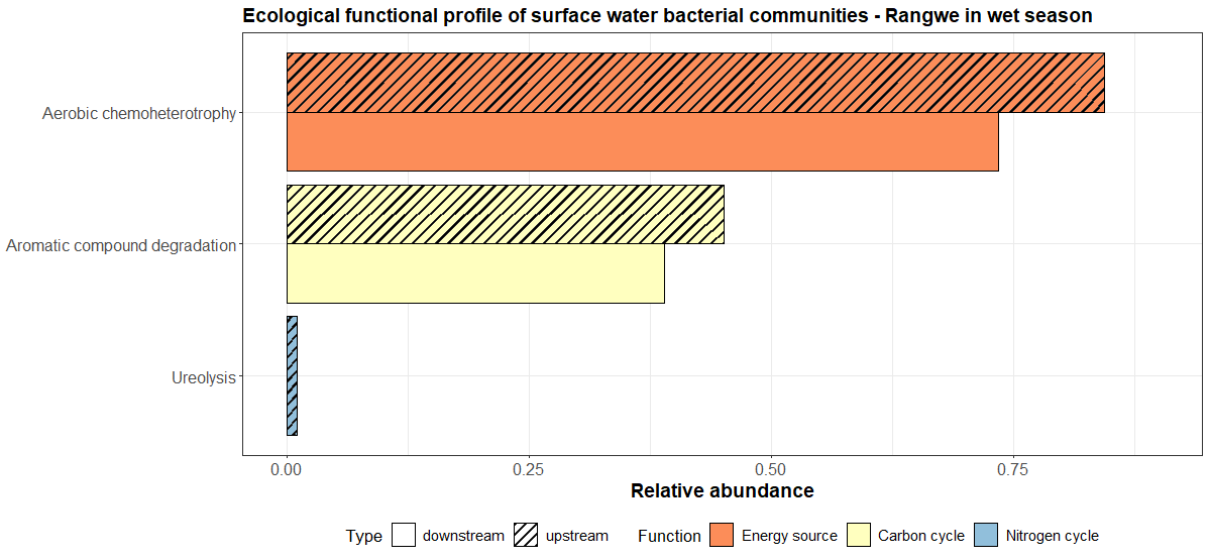

Figure 19. Functional profile of bacterial communities in surface water of Rangwe in wet season.

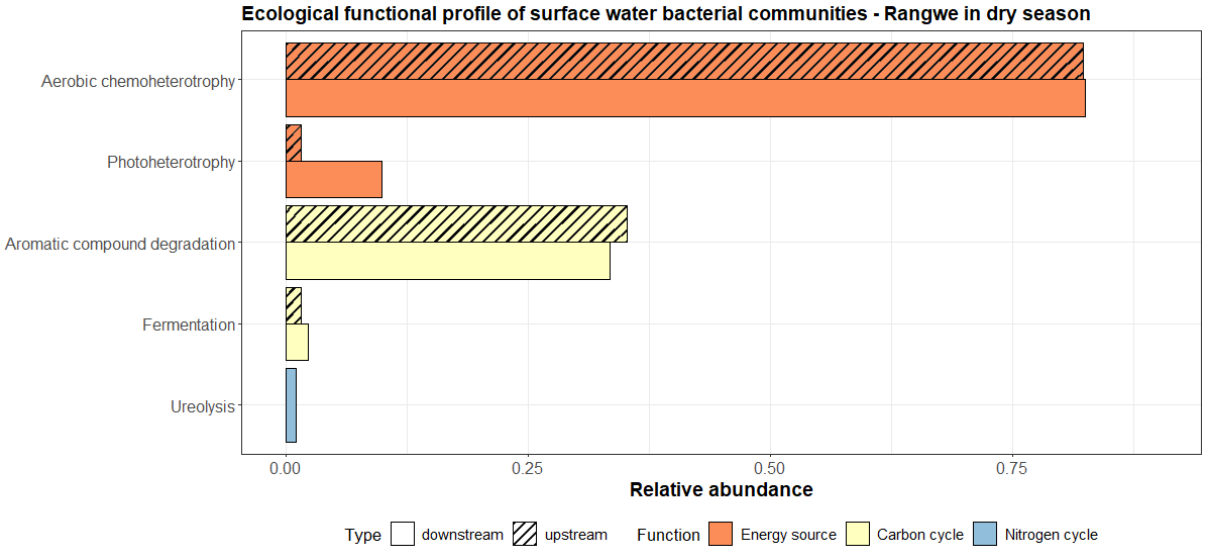

Figure 20. Functional profile of bacterial communities in surface water of Rangwe in dry season.

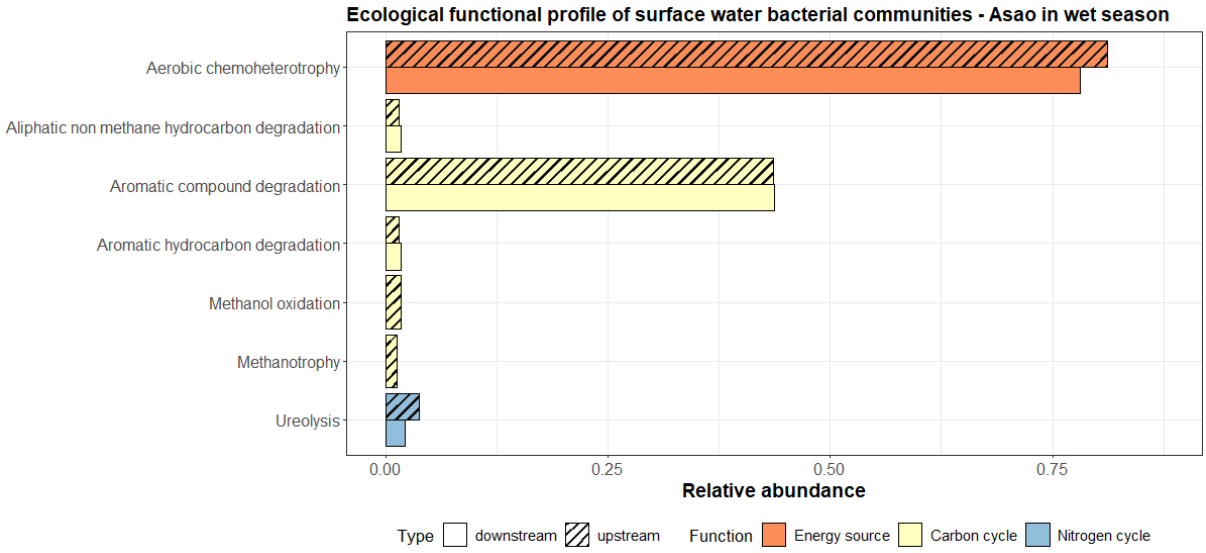

Figure 21. Functional profile of bacterial communities in surface water of Asao in the wet season.

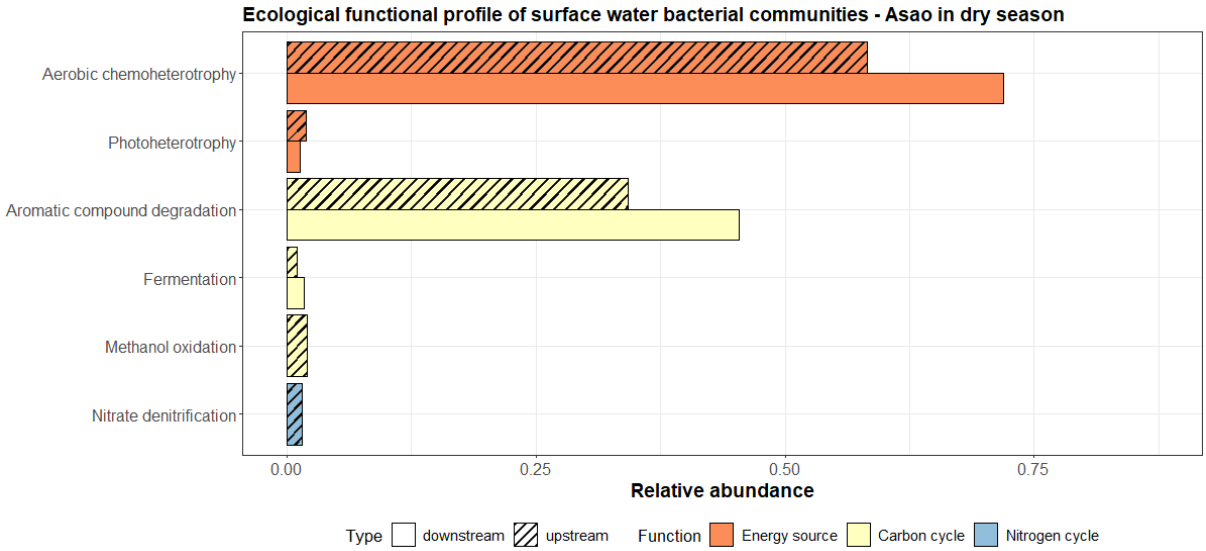

Figure 22. Functional profile of bacterial communities in surface water of Asao in the dry season.

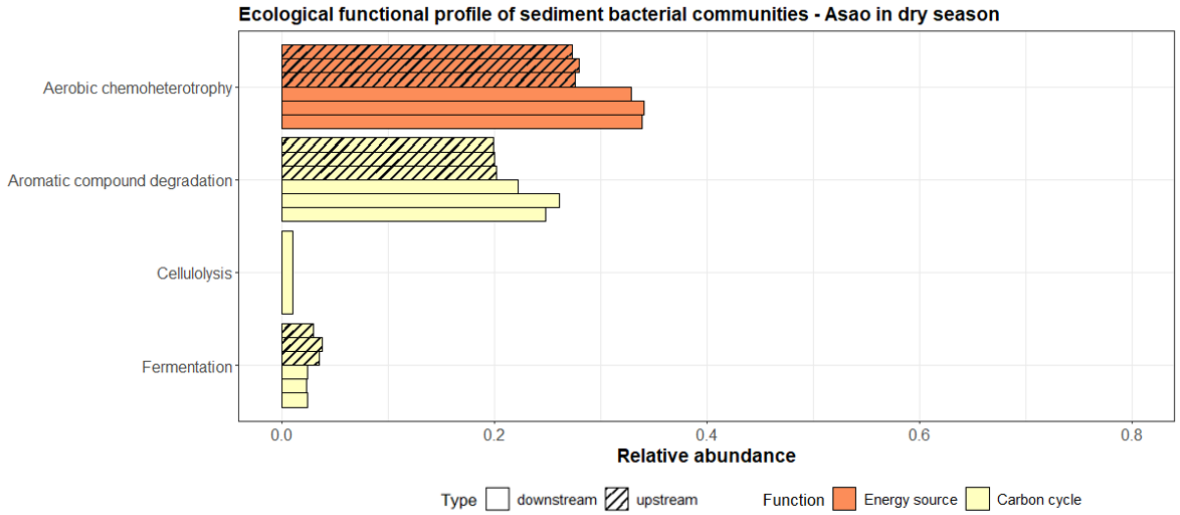

Figure 23. Functional profile of bacterial communities in sediment of Asao in the dry season.