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& INNOVATION

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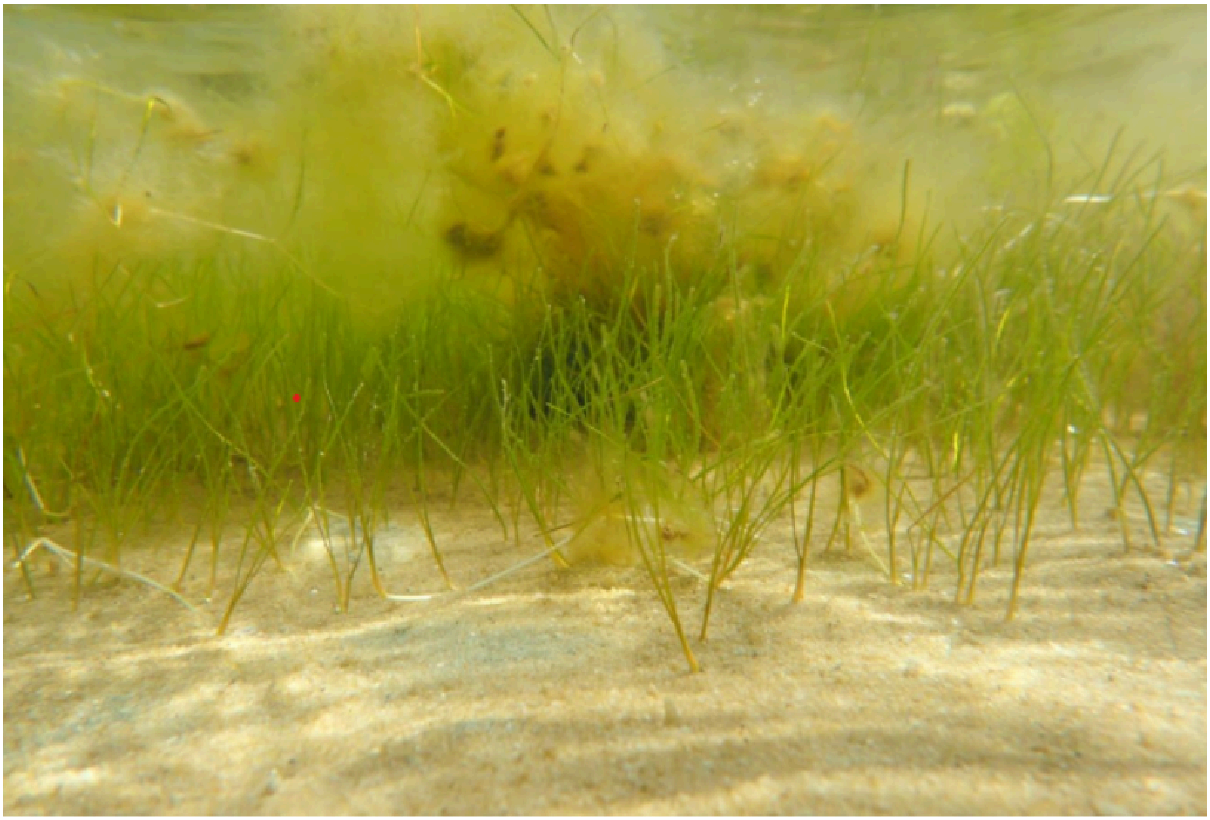
Investigating the role of communities of prokaryotes and eukaryotes contributing to the microbiome of Ruppia



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List of abbreviations

- **DNA :** Desoxyribonucleic Acid
- **RNA :** Ribonucleic Acid
- **ASV :** Amplicon Sequence Variants
- **PERMANOVA :** PERMutational ANalysis Of VAriance
- **AGRF :** Australian Genome Research Facility
- **SIMPER :** species contribution to sample SIMilarities
- **dbRDA :** distance based Redundancy analysis
- **PCA :** Principal Component Analysis
- **PCoA:** Principal Coordinates Analysis

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Introduction

The Coorong is a lagoon ecosystem in South Australia. It is known for its rich biodiversity, which is influenced by variations in hydrology and salinity. These factors can affect the composition and abundance of species present in the aquatic environment, and consequently have an impact on terrestrial species. One of the key species in this ecosystem is *Ruppia*, a seagrass that plays an essential role in stabilising sediments, water quality and is a pillar of local biodiversity.

Climate change and reduction of freshwater in the Coorong have disturbed the ecological balance and reduced the abundance and distribution of Ruppia seagrass meadows. Understanding these dynamics is essential for the conservation and management of this habitat.

The aim of this internship is therefore to study the eukaryotic communities associated with *Ruppia* to gain a better understanding of its interactions with its environment. It is also important to understand how those communities change with different environmental conditions. In this context, statistical tools such as PRIMER 7 have been used to analyse environmental data in order to assess ecological variation and the factors influencing the aquatic communities associated with *Ruppia*.

About Flinders University

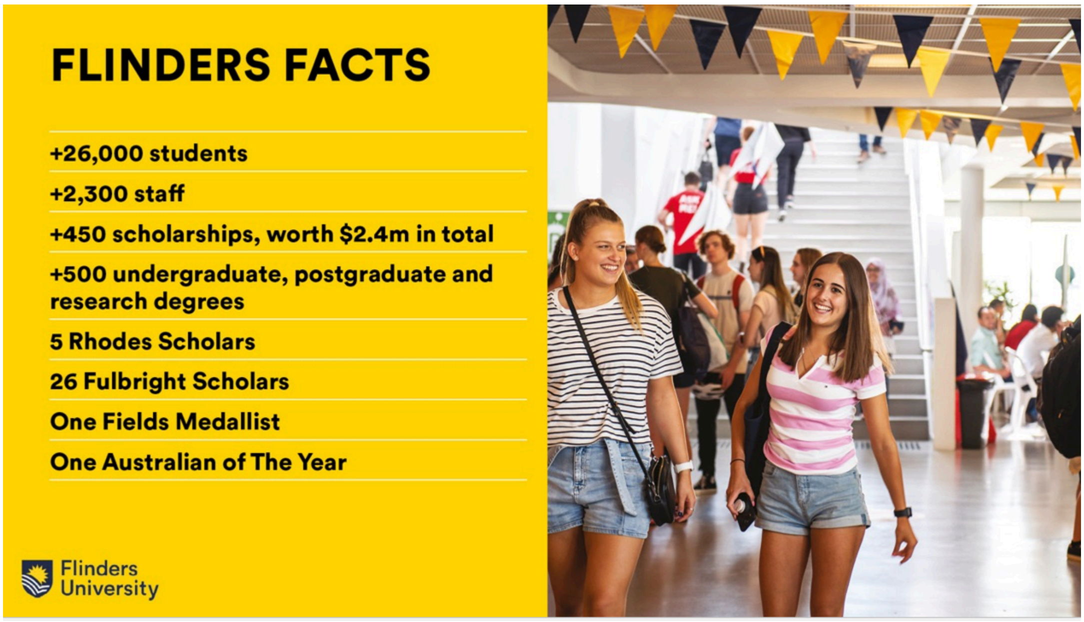
Flinders University, in Adelaide, is a university established in 1966 and is well-known for its commitment to cutting-edge research, quality education, and student support. The university provides a broad range of programs, which include biotechnology, medicine, and marine sciences, with an interdisciplinary and practical approach.

Adelaide is located in the State of South Australia, one of the 6 states and 2 territories in Australia (Figure 1).

Figure n°1: Map of Australia from (https://www.mappr.co/counties/australia)

Figure n°2: Photo of Flinders University (left), photo of Adelaide city (right)

The main campus is set in natural surroundings overlooking the Gulf of St Vincent and offers modern facilities, high-tech laboratories and collaborative spaces. Flinders University is known for its many international partnerships and internships, which enable students to gain valuable work experience and expand their horizons.



FLINDERS FACTS

- +26,000 students
- +2,300 staff
- +450 scholarships, worth $2.4m in total
- +500 undergraduate, postgraduate and research degrees
- 5 Rhodes Scholars
- 26 Fulbright Scholars
- One Fields Medallist
- One Australian of The Year

Flinders University

Figure n°3: Slide from Flinders University website with key numbers

Flinders University had >26,000 students registered in 2024. There were 83% of Australian students and 17% of international students on campus. The university also employs over 2,300 people. (1)(2)

Bibliography

1. Presentation of the Coorong and its Ecosystem

1) Geography

The Coorong is located where the waters of the Murray-Darling Basin (Fig. 3), meet the Southern Ocean. The Murray-Darling Basin is one of the largest freshwater basin in the world (include data on 1 061 469 km). It is almost twice larger than the size of France (**551 695 km²**) in surface area.

The Coorong is a coastal lagoon in South Australia about 150 km from Adelaide. It is separated from the ocean by a thin sand dune that extends for 140 km. Water that enters the Coorong is mainly supplied by the Murray river. It is an important place for the Ngarrindjeri aborigines who have lived there for thousands of years.

Figure n°4: Map of the Muray Bassin (left) and of the Coorong (from SARDI Aquatic Sciences)

2) Biome type

The Coorong is a coastal lagoon that shows large variations in salinity. Salt fluctuations in the Coorong are influenced by several factors including freshwater input from rivers, tides and evaporation. In the closest part of the Coorong, salinity is moderate because there is constant mixing between seawater and freshwater, whereas in more remote areas, especially during periods of drought, evaporation increases salt concentration so that some parts of the Coorong can become hypersaline, exceeding the salinity of the ocean. (3)

3) Weather and climate

The climate of the Coorong is of Mediterranean type with semi-arid influences. It is characterised by hot and dry summers, with temperatures often exceeding 30°C, and mild and humid winters with moderate rainfall concentrated between May and September; annual rainfall varies between 400 and 500 mm, limiting the supply of soft water in the lagoon. Evaporation is intense in summer, which accentuates the increase in salinity. Prevailing winds blow mainly from the south in summer and from the west in winter, influencing currents and sediment distribution; these climatic conditions play a key role in the ecological balance of the Coorong.

4) Biodiversity

The Coorong's biodiversity is rich and varied, thanks to its variety of aquatic and terrestrial habitats. *Ruppia megacarpa* and *Ruppia tuberosa* are important aquatic plants, providing food and shelter for many organisms; Over 240 bird species frequent the region, including Australian pelicans. The waters of the Coorong are home to euryhaline fish such as the big-headed mullet and various crustaceans that support the food chain. On land, there are grey kangaroos and reptiles adapted to sandy environments. The flora includes halophilous plants such as salicornia and herbaceous seepweed, and shrubs such as melaleuca. (4)(5)

2. Seagrass

1) Organism

Seagrasses are aquatic angiosperms and are descendants of terrestrial plants that colonised earth 100 millions years ago. Seagrass meadows are nowadays present in all continents except for Antarctica. Their phylogeny is diverse with about 70 species of basal monocots. They can live completely or partially submerged in water. However, the water depth to which seagrass can grow is limited by the amount of light penetrating the water column. (6)

2) Reproduction

The seagrass can reproduce both sexually and asexually like other terrestrial angiosperms. In the case of sexual reproduction, the male flower produces pollen which is distributed by the movement of water. The pollinated female flower develops into fruits and seeds. The seeds are then dispersed and carried away by the current until they find a place to germinate and create a new plant. The process of asexual reproduction of the plant involves elongation of rhizomes (or underground stems) which give new plants. These two ways of multiplying allow them to colonise space effectively and ensure genetic diversity. (7)

3) Blue carbon

Blue carbon is a term used to define the carbon acquired by oceans and coastal habitats. Seagrass plays a huge role in capturing and storing carbon: it’s a carbon sink. Carbon dioxide from the atmosphere, when dissolved in water, is used by seagrass for photosynthesis and can be converted into biomass. When the plant dies the leaves decompose slowly and the carbon is buried in the sediments. However, the destruction and disturbances of these ecosystems can re-suspend the sediments and release carbon dioxide. (8) (9)

4) Food source

Firstly, seagrass meadows are a pillar of the marine food chain. Indeed, seagrasses directly feed green turtles, manatees, dugongs or fish that graze the shoots. For example, manatees can consume up to 30-55 kg daily and dugongs between 28-40 kg. Other organisms may profit indirectly from seagrass meadows, such as small invertebrates that live in the leaves and eat small sediments or bacteria. Predators also benefit from it, such as water birds or sharks that can feed on preys living within the meadows. Furthermore, seagrass detritus and debris can be carried to the shore and be available for other organisms farther away. **(10)**

5) Other benefits

The seagrass meadows offer many more benefits to the submarine life. First, they are a nursery for several fish such as pollock, cod herring and whiting. Secondly, many pathogens can be eliminated or inhibited by the phytochemicals that seagrasses can produce. This can reduce bacterial loads and benefit other organisms. More importantly, they protect and stabilise shorelines by providing a barrier against harsh waves and also help to prevent the loss of the sediments.

6) Threats

Since 1879, seagrass has declined by 29% worldwide. (11) This decline can be explained by several factors. Firstly, the main cause is human activity: beach erosion and the deposition of fine sediments disturb some seagrass beds. Siltation can reduce the health of seagrasses by blocking sunlight due to the increase of particles in the water column. (12) Coastal development, damming and waste disposal also affect the health of seagrass beds. The reduction of meadows can have an impact on carbon dioxide emissions, as they are an important sink. But overall, the most destructive human activity is the eutrophication of coastal waters, which promotes the growth of algae that affect seagrass by blocking sunlight. (13)

Secondly, there are some natural threats, such as competition between seagrass and algae species for habitat and resources, and some diseases (14) or herbivores that overgraze the meadows. In addition, severe weather conditions such as hurricanes can thin seagrass beds and erode sediments. The impact on seagrasses also affects their microbial community and the overall health of seagrass meadows. (15).

Moreover, changes in weather can have destructive consequences. For example, during the Millennium drought (2001-2009) in South Australia (16), seagrasses such as *Ruppia* in the Coorong were particularly affected. Low freshwater availability and increasing salinity have created difficult conditions for these plants. *Ruppia*, which normally thrives in brackish environments, suffered from extreme salinity in some areas that became hypersaline due to the drought.

3. Holobiont

1) Definition

"A holobiont is an assemblage of a host and the many other species living in or around it, which together form a discrete ecological unit through symbiosis" (17)

It means all the interaction between microbial hosts with associated microorganisms and viruses functioning as a single biological unit. Some symbiotic microorganisms are essential for the health of the host by providing vitamins, energy, organic and inorganic nutrients. Several habitats in the same plants exist and harbor different communities including rhizoplane (surface of root tissue), rhizosphere (periphery of roots), the endosphere (inside plant tissue) and the phyllosphere (total above ground area). (18)

2) Symbiotic interactions

Microbiomes are involved in nitrogen/carbon fixation, on sulfur detoxification and organic matter decomposition. (19) Nitrogen fixing bacteria also called diazotrophic bacteria play a beneficial role for their host by providing up to 30-100% of the nitrogen requirements in the plant. Most of them are present in the rhizosphere and reside in anoxic microzones. (20)

Sulfate reduction produces sulfide that can act as a strong phytotoxin and can result in massive die-off events of seagrass meadows. (21) However, seagrass is able to detoxify small quantities of sulfide by oxygenating their roots and converting sulfide into thiols. Additionally, sulfur-oxidizing bacteria present in the rhizosphere can convert sulfide into non-toxic sulfate. Removal of phytotoxic compounds is an important function of the microbiome.

Similar mechanisms of bacterial detoxification are known for the ethanol produced by the plant itself when switching to fermentation. This ethanol can be used as an electron donor for sulfur reducing bacteria. (22)

Bacteria activity is also part of the carbon cycle. During photosynthesis the plant releases dissolved organic carbon that can be used in aerobic degradation to produce carbon dioxide. This carbon dioxide is used again by the plant to make photosynthesis. (23) Seagrasses microbiota are important for the fitness, growth, survival of plants and resistance to stressors [18-19], and sections of the seagrass plant have distinct bacterial communities commonly associated with them.

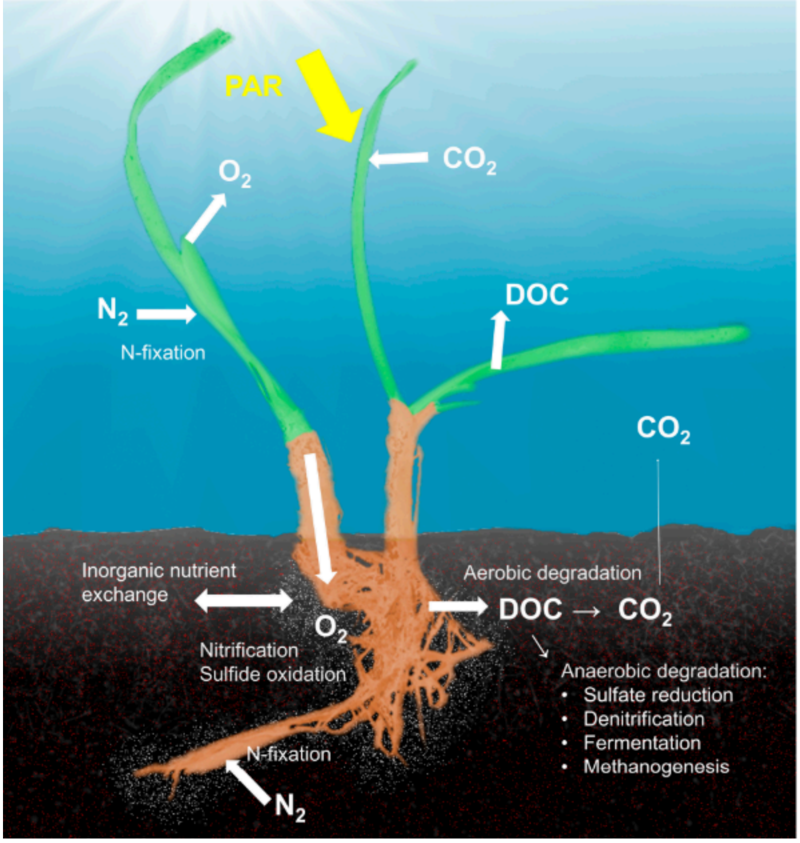


Figure n°5: Interconnected processes within the seagrass (from *Ugarelli, K., Chakrabarti, S., Laas, P., & Stingl, U. (2017). The Seagrass Holobiont and Its Microbiome.)*

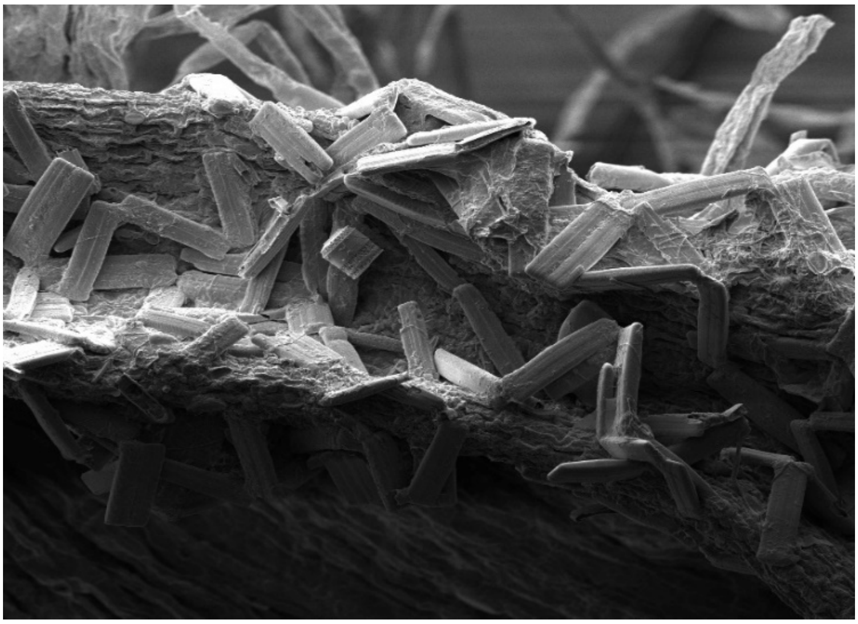


Figure n°6: Scanning Electron image of a leaf of *Ruppia Tuberosa* and of the diatoms attached to the leaf in a biofilm. (Photo by Sophie Leterme)

4. Ruppia tuberosa

1) Type of organism

Ruppia is a genus of monocotyledonous plants. It is the only genus in the family *Ruppiaceae*. It contains a few species of aquatic herbaceous plants that are usually perennial and grow submerged or floating in cold to tropical regions. *Ruppia tuberosa* is very resistant to high salinity and can colonise extreme environments. The morphology is thin and elongated leaves and discreet flowers under water.

Figure n°7: *Ruppia tuberosa* fruiting plant with coiled flower stalk (picture by Richard Schahinger)

2) Growth in the Coorong

Within the Coorong, *Ruppia tuberosa* is restricted to water no deeper than ~0.9m because the turbidity of the water limits light penetration to the bottom of the Coorong. Furthermore, although seeds and turions germinate in shallow water, those that grow in water less than ~0.3 m deep rarely survive to reproduce. This is due to Wind-induced changes in water levels from one day to the next can approach 0.3m in the southern Coorong, and when this happens, these plants are exposed to desiccation.

Annual water level changes in the southern Coorong are typically around 1m. During the dry season, when water levels are at their lowest, the plant remains on or in the mud surface as seeds and turions. When the water level rises again in late autumn and winter, most, if not all, of the turions that have survived the dry period germinate. The plants then grow over winter and, if water levels remain adequate, they reproduce sexually (producing seeds) and asexually (producing turions) in spring and early summer. (24)

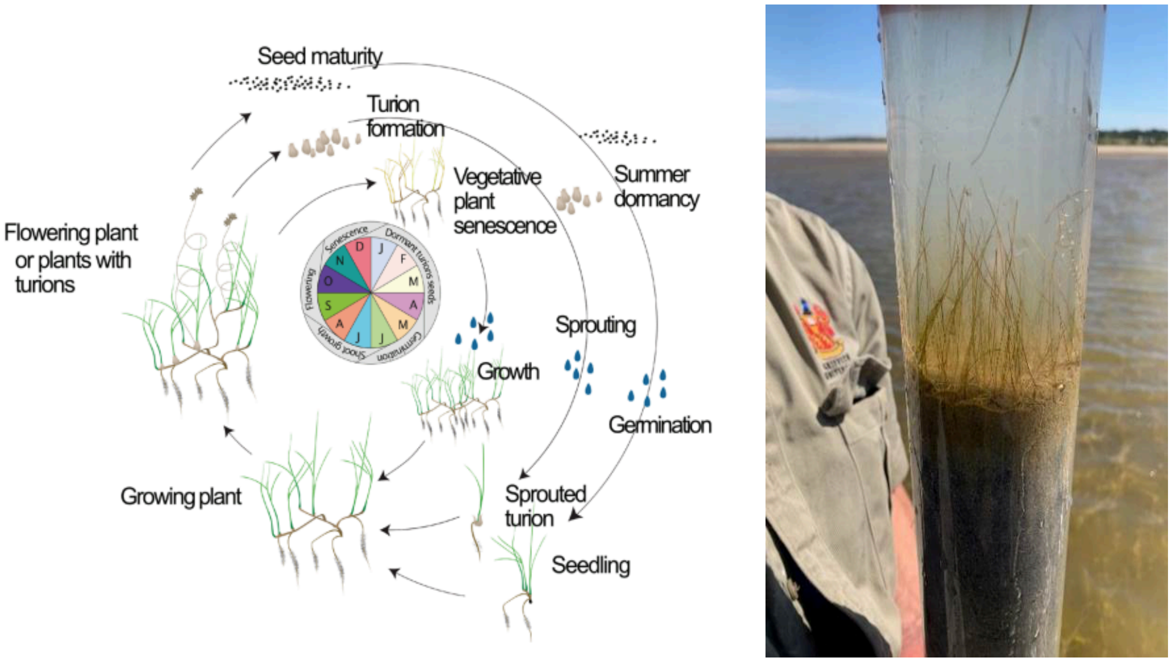


Figure n°8: Life cycle of *Ruppia Tuberosa* in the Coorong (left) and sediment core showing Ruppia when rooted into the sediment layer (right). (Waycott, et al. (2020) Goyder Institute for Water Research Technical Report Series No. 20/02.)

3) Role in the Coorong

As evocated earlier seagrasses have many benefits for the ecosystem they are part of and the same applies for *Ruppia tuberosa* in the Coorong. They constitute a habitat for small invertebrates and a nursery for many species. For migratory birds, including Black Swans (*Cygnus atratus*) and other species that pass through the Coorong, seeds and turions are an important food source. The invertebrates that live in the seagrass of Ruppia also provide food for fish and birds.(25)

Moreover, the presence of seagrasses can stabilize sediments and improve water quality by reducing turbidity as Ruppia meadows slow down strong currents. Oxygen and nutrient levels in the water are also influenced. The benefits of Ruppia tuberosa make its presence an indicator of water quality. Its sensitivity to changes in salinity or water level can lead to its decline, which can be a sign of ecological instability. (26)

5. Analysis

1) Definition of bioinformatics

Bioinformatics is an interdisciplinary field that uses computational and mathematical methods for the analysis, processing and interpretation of biological data. It has many applications. Firstly, it is used to store and manage biological data. For example, in the case of genomic sequences or amino acids, bioinformatics can better organise complex data and information.

This allows the identification of genes, proteins and other biological elements from genetic sequences. Bioinformatics makes biological modelling possible, but it also makes it easier to compare data and track developments.

2) Data analysis

The purpose of data analysis in an ecological study is to transform raw observations into meaningful information, allowing a better understanding of ecosystem functioning and the interactions between different components.

Ecological data can often be variable, complex, and multidimensional. Statistical analysis is used to identify significant relationships between variables, such as abiotic factors (temperature, salinity, depth) and species.

It can also test the validity of assumptions. Determine whether observations are due to real effects or simple random variation.

Finally it can reduce the complexity of the data while retaining most of the information needed to understand ecological dynamics.

Multivariate analyses are particularly suitable for ecological data because they allow simultaneous study of several factors, often interconnected.

Material and methods

Sampling was carried out by Flinders University researchers before the internship started. This is an extract from the report "Seasonal fluctuations of the seagrass holobiont in an environment with contrasting environmental conditions" (Tamar J., Mohsen C., Angélique G., Michelle W., Sophie C. L.; under review) (27). This extract describes the method of sample preparation.

1. Sampling Sites

Five sites were chosen along the Coorong, from Wild Dog Islands in the South Lagoon to Noonameena in the North Lagoon (*Figure n°2*), as the focus of this study. Sampling was conducted seasonally over a year: Spring (27 October 2020), Summer (16 December 2020), Autumn (9 March 2021), and Winter (15 June 2021).

Physico-chemical parameters including temperature, salinity, dissolved oxygen, turbidity, and dissolved nutrients (i.e Ammonium, NOx, phosphorus) were monitored at each site.

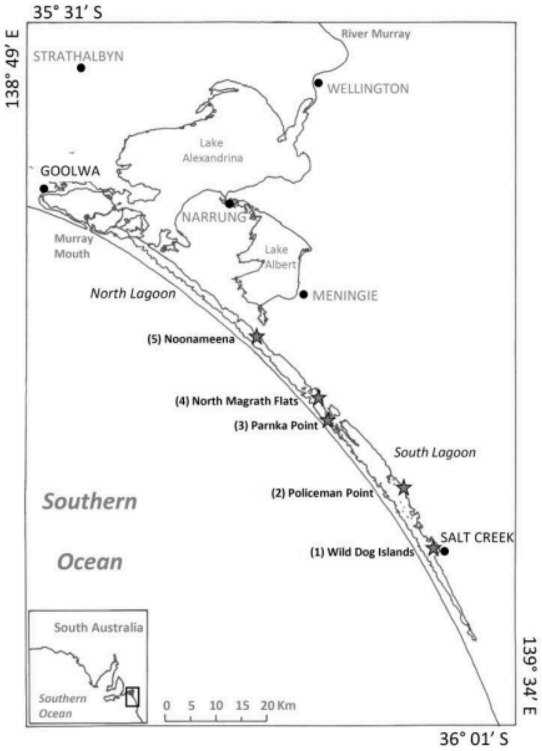


Figure n°9: Map of the Coorong South Lagoon and sampling sites : site 1, Wild Dog Islands; site 2, Policeman Point. Central Coorong: site 3, Parnka Point; site 4, North Magrath Flats. Coorong North Lagoon: site 5, Noonameena. From Microbial community composition of the southern Coorong including evaluating seasonal variation and sediment, water column, aquatic macrophytes and filamentous algae as substrates for microbial growth.

2. Water and Sediment microbiota

Triplicate 2 L water samples were collected for DNA extraction and passed through a 20 µm sieve before being filtered through 0.22 µm Millipore membrane filters (MF-Millipore™ Membrane Filters HAWP04700). Filters were placed in sterile and UV-treated petri dishes tightly closed using Parafilm and stored at -20°C until DNA extraction. Triplicate 2 mL surface sediment samples for DNA extraction were collected using a modified syringe along the waterline. The samples were stored at -20°C until DNA extraction.

3. The Ruppia Community

At each sampling site, three 45 mm diameter cores were taken randomly within a patch of Ruppia, to a depth of 10 cm. As plants of the Ruppia community were not present at all sampling sites, samples could not always be collected in triplicate. In the laboratory, the Ruppia community samples were dissected to separate the root systems from the leaves. When filamentous algae were present amongst the Ruppia Community, either by forming filamentous aggregates attached to the seagrass plants (PAA) or by forming floating mats in which seagrass plants were trapped, PAA and mats were also sampled.

All collected material was processed using the modified protocol of Ugarelli et al (19). The supernatant was sequentially filtered onto 0.22 µm Millipore membrane filters (MF-Millipore™ Membrane Filters HAWP04700). Filters were placed in petri dishes, enclosed using Parafilm and stored at -20°C until DNA extraction.

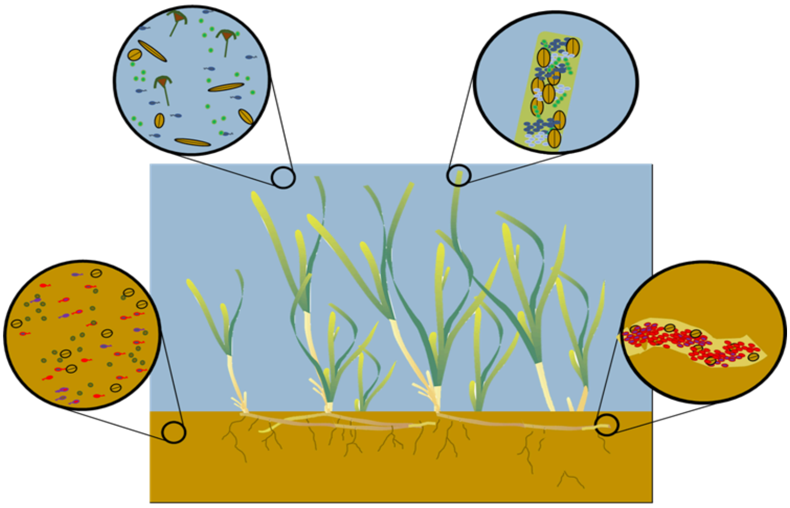


Figure n°10: Conceptual diagram of the project showing the different types of samples that were collected during the project ie. sediment, water, leaves and roots/rhizomes.

4. DNA extraction, amplicon formation, sequencing, and bioinformatics

DNA was extracted from the filters using the DNeasy PowerWater Kits (Qiagen, CA, USA). Whereas DNA from the sediment samples (0.25 g) was extracted using DNeasy PowerSoil Kit (Qiagen, CA,USA). The extracted DNA was used as a temple for formation of 16S rRNA gene and 18S rRNA gene amplicons. The v4 region of the 16S rRNA (515F/806R) and 18S rRNA (515FY/NSR951) was amplified by polymerase chain reactions using their respective primers.

The amplicons were sequenced using Illumina MiSeq™ System (2 x 250bp paired-end sequencing) at the Australian Genome Research Facility (AGRF, Melbourne, Australia). Sequencing analysis was inferred using the DADA2 package (version 1.26; Callahan et al. 2016) in R studio v.4.6.1 (R v.4.1.3) using amplicon sequence variants (ASVs) which created groups based on sequence similarities.
As this project focused on the Eukaryotes associated with Ruppia, the Taxonomy files obtained for the 16S rRNA and 18S rRNA gene sequences were curated and combined to obtain a single taxonomy file with the organisms of interest. In brief, raw sequences were demultiplexed with barcodes and adaptors removed by Australian Genome Research Facility (AGRF). The cutadapt program (Martin, 2011) was used to remove the primers from the amplicon reads. Sequences were filtered and trimmed following standard filter parameters, with the quality score of the sequence of less than or equal to 2 and with the maximum expected errors for forward reads equalling 2, and 5 for reverse reads. After filtering and trimming, the paired-end sequences were merged followed by denovo chimera removal. Taxonomy was inferred (98% similarity) for the 16S rRNA gene sequences using the SILVA database release 138.1 . Singletons and taxon such as Eukaryota, Mitochondria and Chloroplast were removed from the 16S rRNA dataset. Whereas, in the 18S rRNA datatset singletons and taxon such as Bacteria, Plantae and Mammalia (for example the genus *Capra)* were removed from the 18S rRNA dataset. This file was prepared with the help of Tamar Jamieson.

5. Statistical analysis

Statistical analysis was undertaken using PRIMER v.7 software +PERMANOVA add on. A factor was created that separated the community of eukaryotes into two groups: One was called “environment” and grouped the environmental biome of Eukaryotes from the water and sediment samples, the other one was called “biofilm” and grouped the biofilm biome of eukaryotes from the leaves and roots/rhizomes of Ruppia.

To begin the analysis of our data, a global overview was obtained by ploting the environmental data of water quality and the community of eukaryotes using boxplots. Then, the normalised environmental data of water quality was analysed using a Principal Component Analysis (PCA) to identify similarities in environmental conditions. Then, an analysis of diversity was conducted on the community of eukaryotes, separating samples according to the two different biomes (ie. environment and biofilm). The diversity analysis consisted of Pielou's evenness, species richness, and total individuals were calculated. Pielou's evenness (J') is a measure of equitability, a measure of how evenly the individuals are distributed among the different species; Species Richness (S) is the number of species in each sample. i.e. species with non-zero counts; Total individuals (N) is the number of individuals in each sample, it accumulates species counts. Significant differences were tested using PERMutational ANalysis Of VAriance (PERMANOVA).

In order to make further analyses clearer to understand the community of eukaryotes were transformed using log(x+1). Bray-Curtis dissimilarity was then used to quantify dissimilarities in species abundance between samples, it allowed to assess how similar or different the communities were. Analysis was made at class level.

A SIMPER analysis was used to identify which species contributed the most to the dissimilarities observed between the two biomes (environment and biofilm). It helps to determine the key species driving differences based on abundance data. This provides insights into how community composition varies between the factors studied. PCoA (Principal Coordinates Analysis) is used to visualize the differences between samples based on the dissimilarity-based matrix (Bray-Curtis) on the communities of eukaryotes. Pie charts are created on every sample of the PCoA with the class/family that explained most of the variation from the SIMPER analysis. The size of the pie is proportional to the relative abundance of the class/family.

A distance-based ReDundancy Analysis (dbRDA) was used to understand how environmental or experimental factors influence differences in community composition. The dbRDA first converts a dissimilarity-based matrix (Bray-Curtis on the communities of eukaryotes) into principal coordinates. Then, it constrains the ordination by fitting it to the explanatory environmental variables, allowing us to directly assess which factors explain the variation among samples. Euclidean distances were calculated to quantify differences in environmental conditions between samples. It allowed to assess how similar or different the conditions at each site were. Significant differences in environmental conditions between the sites and seasons were tested using PERMutational ANalysis Of VAriance (PERMANOVA) and the relationship between environmental conditions and the communities of eukaryotes were tested with the BEST procedure. Here, BEST selected environmental variables "best explaining" the community pattern, by maximising a rank correlation between their respective resemblance matrices.

Results

1. Water Quality analysis

The PCA displays the distribution of sampling points from four dates across two principal components (PC1 and PC2), with PC1 explaining 33.4% of the variance and PC2 explaining 19.8%. Sampling dates are color-coded: October 2020 (pink), December 2020 (yellow), March 2021 (orange), and June 2021 (blue).

Each group of points is spatially distinct on the plot, with some overlap between December 2020 and June 2021 samples, while October 2020 and March 2021 samples are more dispersed. The PCA shows that samples from the same trip tend to cluster together.

Environmental variables are represented by blue arrows. The vectors vary in length and direction, with temperature pointing in the negative direction of PC1 and PC2, while ammonium, phosphate, and nitrite align positively along PC1.

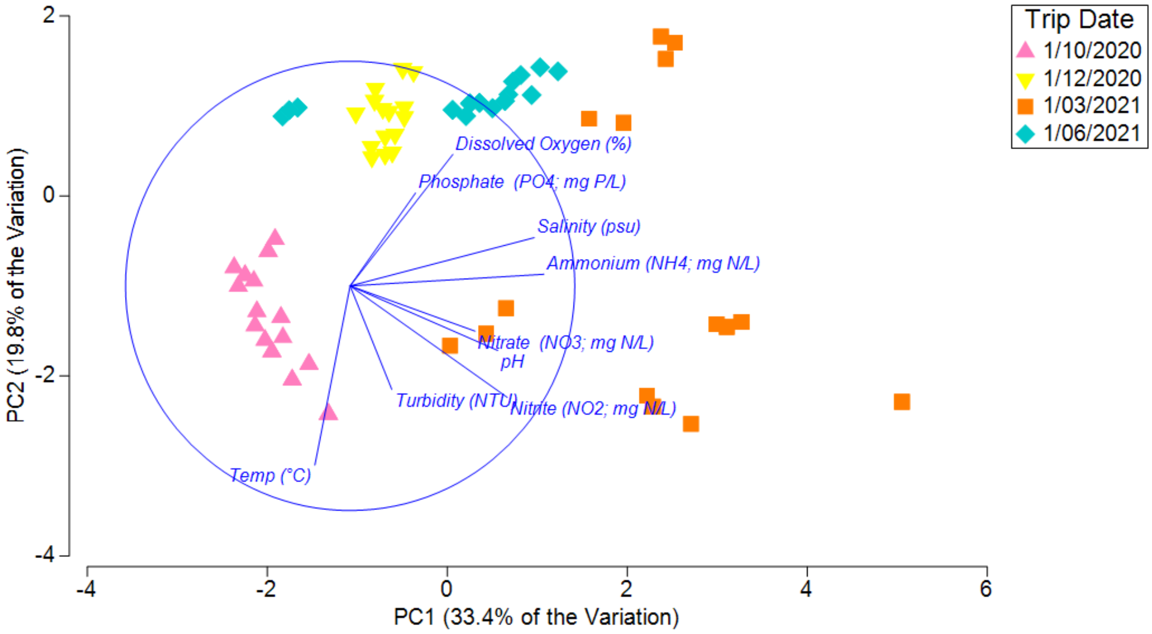


Figure n°11: Principal Component Analysis (PCA) plot based on the water quality parameters measured at each of the sampling sites in October 2020 (in pink), December 2020 (in yellow), March 2021 (in orange) and June 2021 (in blue). PC1 and PC2 explain 33.4% and 19.8% of the total variability, respectively. Pearson correlation vectors (blue vectors) represent the water quality parameters driving the differences between samples. The rayon of blue circle around the vectors represent a Pearson correlation of 1.

Table I : Pearson correlations calculated between the environmental variables and the Principal Component Analysis (PCA) axes

	pH	Temperature (°C)	Salinity (psu)	Turbidity (NTU)	Dissolved Oxygen (%)	Ammonium (NH4; mg N/L)	Phosphate (PO4; mg P/L)	Nitrite (NO2; mg N/L)	Nitrate (NO3; mg N/L)
PC1	0.657	-0.157	0.820	0.185	0.457	0.863	0.291	0.700	0.556
PC2	-0.288	-0.798	0.215	-0.461	0.585	0.052	0.414	-0.497	-0.202

According to the Pearson correlation table, PC1 is most strongly correlated with ammonium (0.863), phosphate (0.820), and nitrite (0.700). Temperature is negatively correlated with PC2 (−0.798), while dissolved oxygen shows a strong positive correlation with PC2 (0.585).

These results show clear temporal patterns in water composition, with samples clustering by date. PC1 mainly reflects nutrient levels (ammonium, phosphate, nitrite), while PC2 captures a temperature–oxygen gradient. Seasonal shifts are evident: colder periods show higher oxygen and lower nutrients, while warmer periods align with increased nutrient concentrations. This suggests that temperature and nutrient input are key factors driving temporal variability.

The boxplots in Figure 12 illustrate the variation of water quality parameters across the four sampling trips. These seasonal changes are consistent with the patterns previously observed in the PCA (Figure 11).

A PERMANOVA analysis conducted on the water quality data presented in Figure 14 showed a significant difference (p=0.001) between the sampling trips, highlighting the significant difference observed in water quality between seasons.

For example, **pH** remained relatively stable during most trips but showed a noticeable increase in March 2021. Temperature followed a clear seasonal trend, with the highest values recorded in March (late summer in the Southern Hemisphere) and the lowest in June 2021 (winter). This seasonal variation was also highlighted in the PCA, where **temperature** was one of the main drivers on the second principal component (PC2).

Salinity increased significantly in March 2021 and was lower during the other sampling periods. This observation aligns with the PCA, where salinity contributed strongly to the separation of the March samples. Dissolved oxygen showed the opposite pattern: very low in October 2020, then increasing over time. This trend was also visible in the multivariate analysis.

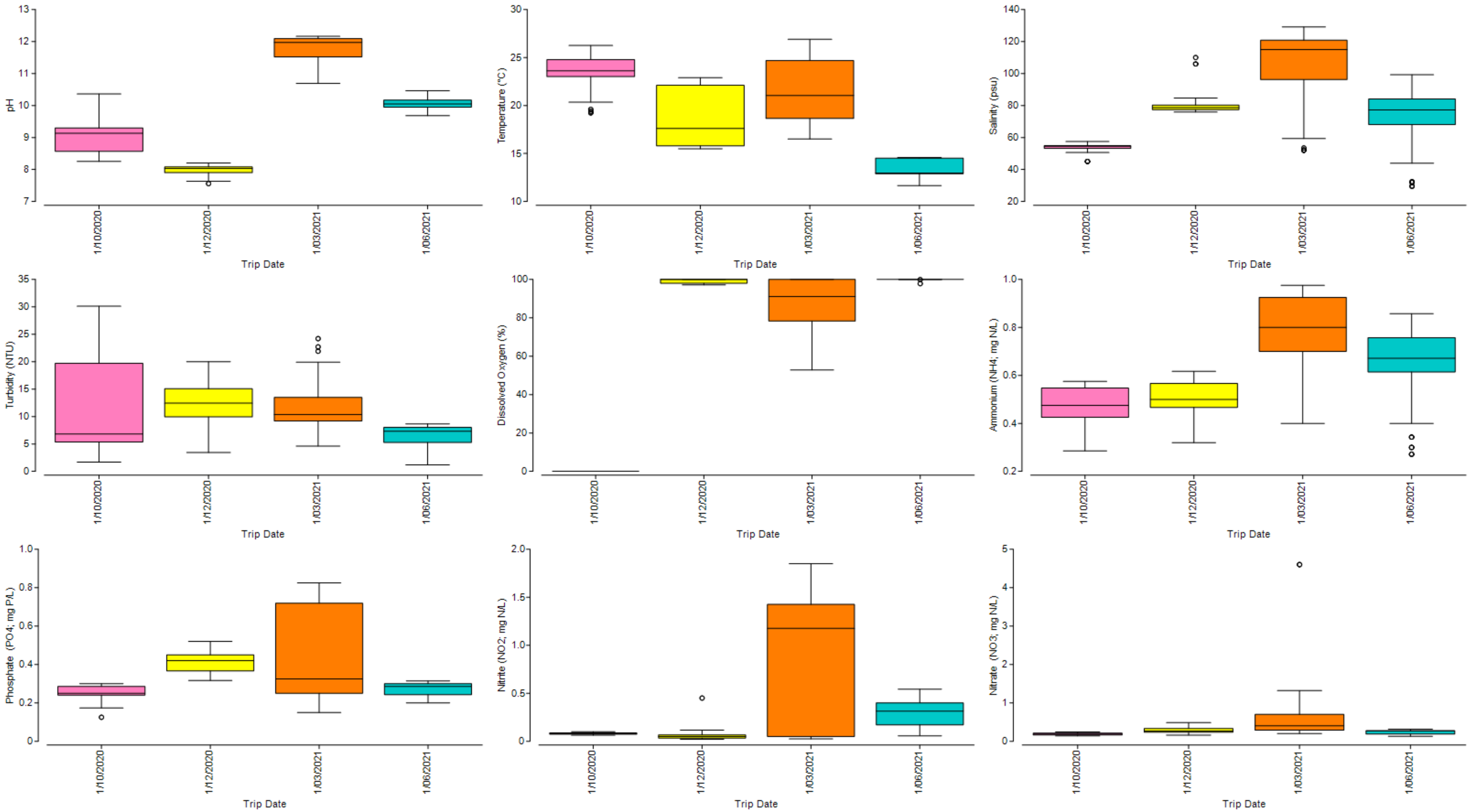


Figure n°12: Box plot based on water quality parameters measured at each of the five sampling sites in October 2020 (in pink), December 2020 (in yellow), March 2021 (in orange) and June 2021 (in blue). The box extends from the first to the third quartile, the line in the box represents the median. The whiskers are the minimum and maximum values. The outliers are represented by circles.

During the March 2021 sampling trip, **nutrient concentrations** showed a sharp increase, particularly for ammonium (NH_4), nitrite (NO_2), and nitrate (NO_3), as shown in Figure 12. This pattern suggests a significant eutrophication event. Such peaks in nutrient levels can be linked to reduced freshwater inflow into the Coorong system at that time, which limits water renewal and promotes nutrient accumulation (27).

This situation aligns with known eutrophication issues reported in the southern Coorong, where low flow conditions combined with high evaporation rates lead to hypersalinity and poor flushing (28). As a result, sediments release phosphorus and other nutrients back into the water, further fuelling algal growth (29).

The high ammonium and nitrate levels recorded in March, compared to other sampling periods, likely reflect this nutrient recycling and stagnation. These conditions are typical of eutrophic environments and pose risks to water quality and aquatic life. Overall, the data shown in this figure support the patterns observed in Figure 11 and reinforce the PERMANOVA results, which confirmed a statistically significant difference in water quality across the four sampling trips ($p = 0.001$). Together, these results highlight how environmental conditions and potential disturbances influenced the water quality over time.

2. Community analysis

The boxplots in Figure 13 show diversity metrics for the two biome types: the Environmental biome (E, in blue) and the Biofilm biome (B, in red). The top panel displays **Species Richness (S).** Values range from approximately 50 to over 600 for E samples, and from below 50 to around 300 for B samples. Median richness appears higher in the Environmental biome. The middle panel shows **Total Abundance (N)**. E samples range broadly, with values up to nearly 250,000 species, while B samples have generally lower values, with a maximum below 150,000. A few outliers are present in both biome types. The bottom panel represents **Pielou's Evenness (J')**. The range of J' is similar between biome types, but the distribution shows slightly different medians and spreads.

A PERMANOVA conducted on the Diversity data (S, N and J' together) presented in Figure 16 showed a significant difference in diversity ($p=0.001$) between the environmental biome and the biofilm biome.

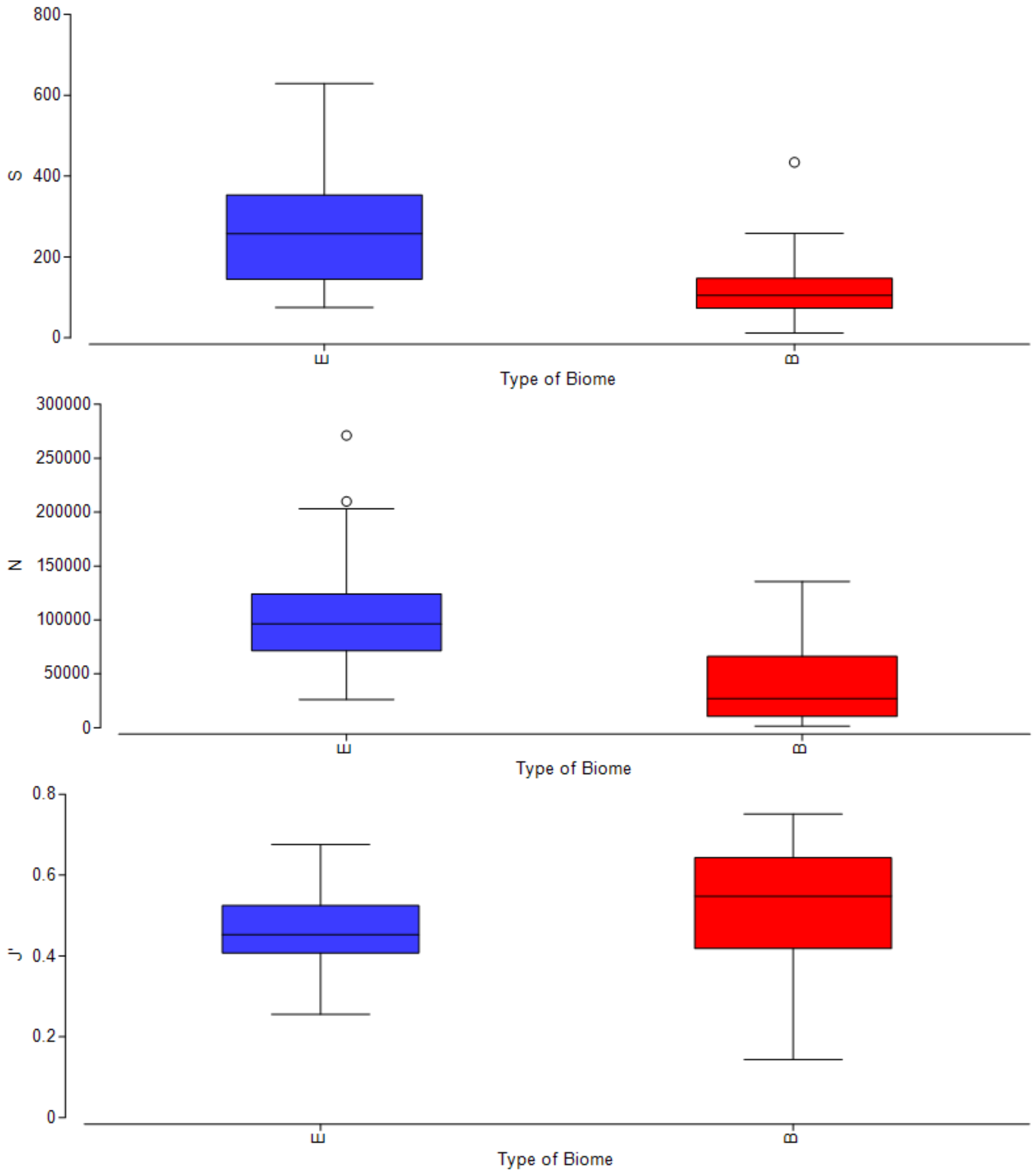


Figure n°13: Boxplots for the diversity analysis for (E) the Environmental biome (Eukaryote organisms in water and sediment samples) and (B) Biofilm biome (Eukaryote organisms in leaves and roots samples). Top panel: Species Richness (S); middle panel: Total Abundance (N); bottom panel: Pielou's Evenness (J'). The box extends from the first to the third quartile, the line in the box represents the median. The whiskers are the minimum and maximum values. The outliers are represented by circles.

These results highlight distinct ecological characteristics between the Environmental (E) and Biofilm (B) biomes. **Species richness (S)** and **total abundance (N)** are consistently higher in the Environmental biome, suggesting a more diverse and abundant microbial community in open water or sediment environments compared to the more specialized biofilm habitat. The lower richness and abundance in biofilms may reflect selective conditions favoring fewer, well-adapted species.

Pielou's evenness (J') shows similar ranges across both biomes, but the slight shift in medians suggests differences in how evenly species are distributed, possibly due to dominant taxa in biofilms. The significant PERMANOVA result (p = 0.001) confirms that the microbial communities in these two biomes are statistically distinct, supporting the idea that biome type strongly structures diversity patterns.

Analysis of Figures 14 to 17: Eukaryotic community structure across compartments
These four figures illustrate the relative abundance of eukaryotic taxa (>0.05% of sequences) at the class level across different compartments: water, sediment, and Ruppia leaves and roots, based on 18S rRNA sequencing data.
Figure 14 (water column):
The eukaryotic communities in the water appear relatively consistent across both sites and seasons, with dominant representation from phytoplanktonic groups such as Chlorodendrophyceae, Mamiellophyceae, Dictyochophyceae, and Chrysophyceae. The limited presence of benthic or plant-associated taxa confirms a planktonic profile typical of the water column.

Figure 15 (sediment):
Sediment communities are markedly more diverse and variable than those in the water, both spatially and temporally. Classes such as Nematoda, Annelida, Ciliophora, Discosea, and Bacillariophyceae are prominent, reflecting the complexity of the sediment habitat, which hosts a mix of metazoans, benthic protists, and microalgae. A high number of minor or less abundant taxa further highlights the richness of this compartment.

Figure 16 (Ruppia leaves):
Data is only available for October and June, as no samples could be collected in March and December due to the absence of Ruppia. Communities on the leaves were mainly composed of Nematoda and Annelida, suggesting close ecological associations with the plant surface, likely linked to organic matter processing or microbial grazing. Compared to sediment, the leaf biofilm appears less taxonomically diverse but more dominated by these metazoan groups.

Figure 17 (Ruppia roots):
Root-associated communities also exhibited a diverse range of taxa, including benthic metazoans (Annelida, Nematoda) and microbial eukaryotes (Ciliophora, Discosea). No data are available for December due to the absence of Ruppia at all sites. As with the leaves, the root microbiome differs from water and sediment, pointing toward a plant-specific assemblage likely shaped by belowground ecological interactions.

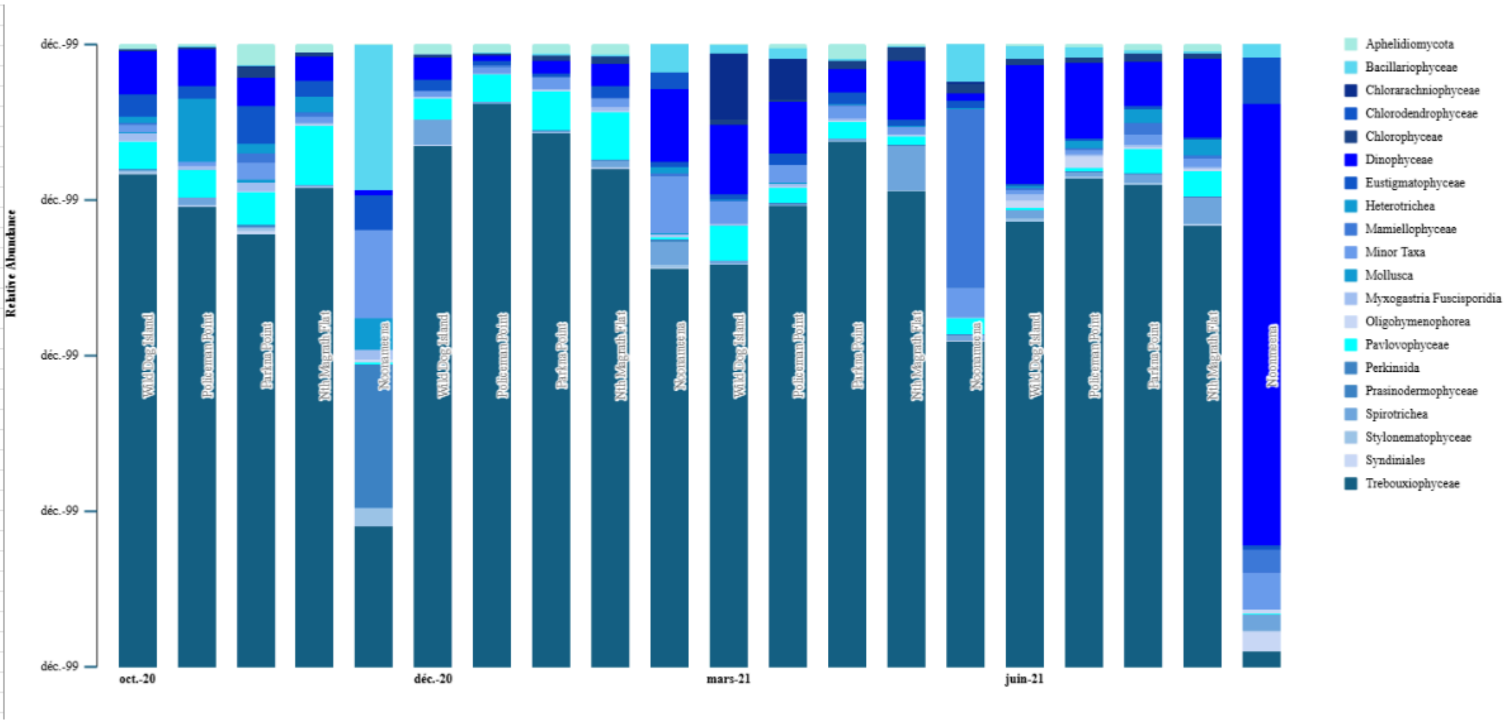


Figure 14 : Relative abundance of all the taxa that comprise more than 0.05% of the eukaryotic sequences in the water from 18S rRNA sequencing at the five sampling sites in October 2020, December 2020, March 2021, and June 2021. Sampling sites – Coorong South Lagoon: site 1, Wild Dog Islands; site 2, Policeman Point. Central Coorong: site 3, Parnka Point; site 4, North Magrath Flats. Coorong North Lagoon: site 5, Noonameena. The taxa are classified to the class level according to the PR2 database (version 5.0.0).

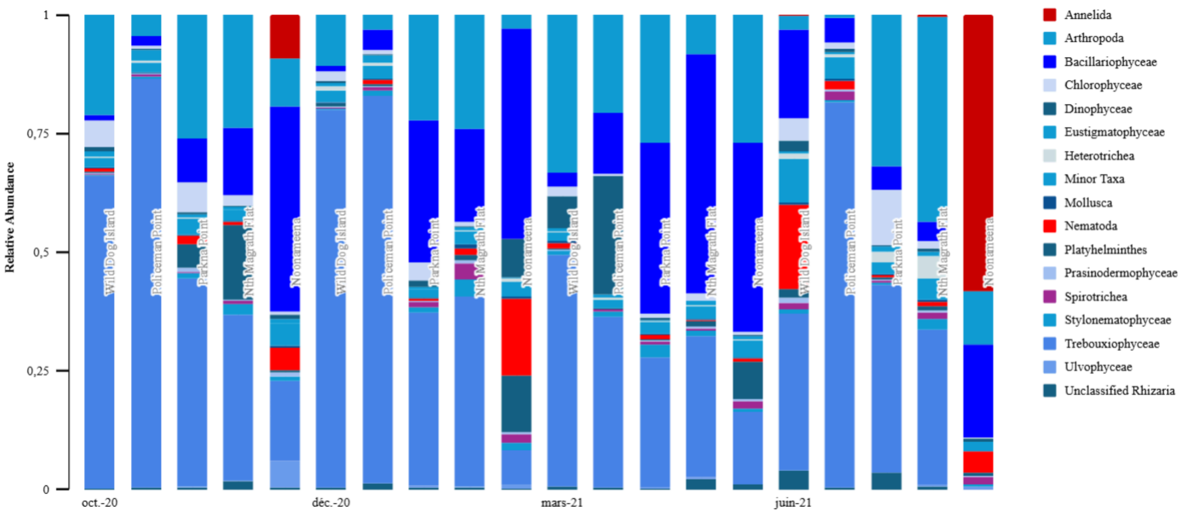


Figure 15: Relative abundance of all the taxa that comprise more than 0.05% of the eukaryotic sequences in the sediment from 18S rRNA sequencing at the five sampling sites in October 2020, December 2020, March 2021, and June 2021. Sampling sites – Coorong South Lagoon: site 1, Wild Dog Islands; site 2, Policeman Point. Central Coorong: site 3, Parnka Point; site 4, North Magrath Flats. Coorong North Lagoon: site 5, Noonameena. The taxa are classified to the class level according to the PR2 database (version 5.0.0).

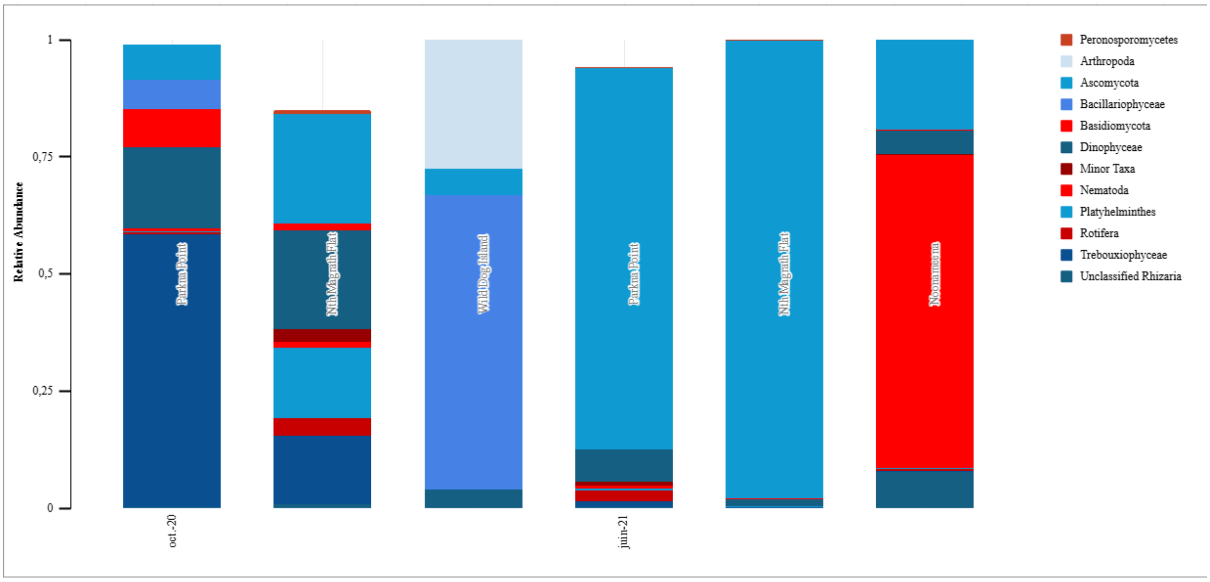


Figure 16 : Relative abundance of all the taxa that comprise more than 0.05% of the eukaryotic sequences on the Ruppia leaves from 18S rRNA sequencing. Sampling sites – Coorong South Lagoon: site 1, Wild Dog Islands; site 2, Policeman Point. Central Coorong: site 3, Parnka Point; site 4, North Magrath Flats. Coorong North Lagoon: site 5, Noonameena. The taxa are classified to the class level according to the PR2 database (version 5.0.0).

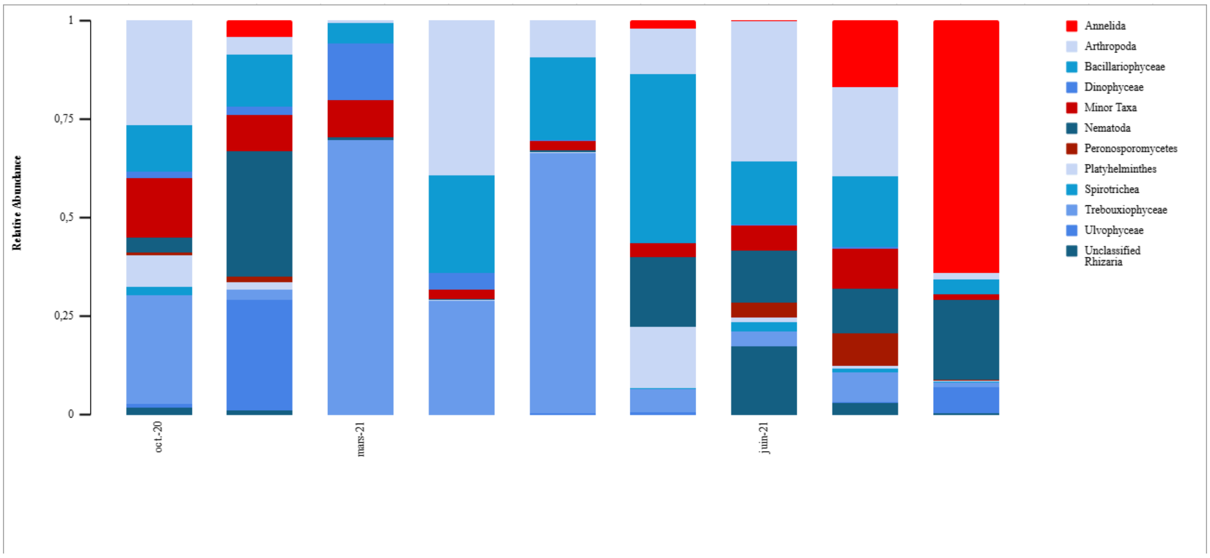


Figure 17: Relative abundance of all the taxa that comprise more than 0.05% of the eukaryotic sequences on the Ruppia roots from 18SrRNA sequencing. Sampling sites – Coorong South Lagoon: site 1, Wild Dog Islands; site 2, Policeman Point. Central Coorong: site 3, Parnka Point; site 4, North Magrath Flats. Coorong North Lagoon: site 5, Noonameena. The taxa are classified to the class level according to the PR2 database (version 5.0.0).

A PCoA (Principal Coordinates Analysis) was used to visualize the differences between samples based on the dissimilarity-based matrix (Bray-Curtis) of the communities of eukaryotes. The PCoA analysis (Figure 18) shows a clear separation between the environment (sediment and water) and biofilm (roots and leaves) communities, highlighting distinct eukaryotic assemblages (PCO1 = 27.8% variation; PCO2 = 17.2%). This distinction suggests a strong habitat-specific structuring of the communities. In particular, the biofilm biome was characterized by the dominance of taxa such as Nematoda and Annelida, which were absent from the water samples and less abundant in the surrounding sediment. These taxa are commonly associated with plant surfaces, where they can play roles in organic matter processing or microbial grazing. Their presence on *Ruppia* roots and leaves suggests a close ecological interaction.

Similar patterns were observed in other studies, although relatively few have focused directly on eukaryotic communities in seagrass habitats. For instance, Portas et al. (2023) (31) showed that marine biofouling systems, including biofilms, host diverse eukaryotes such as nematodes and annelids, which contribute significantly to the structure and function of these communities. Likewise, recent works on *Thalassia testudinum* (32) identified a consistent core microbiome across regions and tissue ages, but eukaryotic diversity remains underexplored compared to bacterial components. Interestingly, fungal endophytes like *Ascomycota* have been reported in *Thalassia* (33), supporting our detection of similar fungal groups in the *Ruppia* biofilm.

Moreover, studies on model organisms have emphasized the ecological relevance of these eukaryotes: nematodes have been shown to stimulate diatom biofilm growth (34), and annelid larvae settle preferentially on microalgal films (35), reinforcing the idea of biofilm-associated fauna playing active roles in shaping microbial landscapes. The presence of nematodes, annelids and fungi within the biofilm suggests that these communities could serve as potential bioindicators of *Ruppia* health. While a diverse and balanced assemblage may reflect a stable and functional environment, the dominance of stress-tolerant or opportunistic species could indicate early signs of ecosystem imbalance or plant decline. These patterns are consistent with previous findings suggesting that *Ruppia* beds can serve as hotspots for microbial diversity, especially in stressful and variable environments like the Coorong.

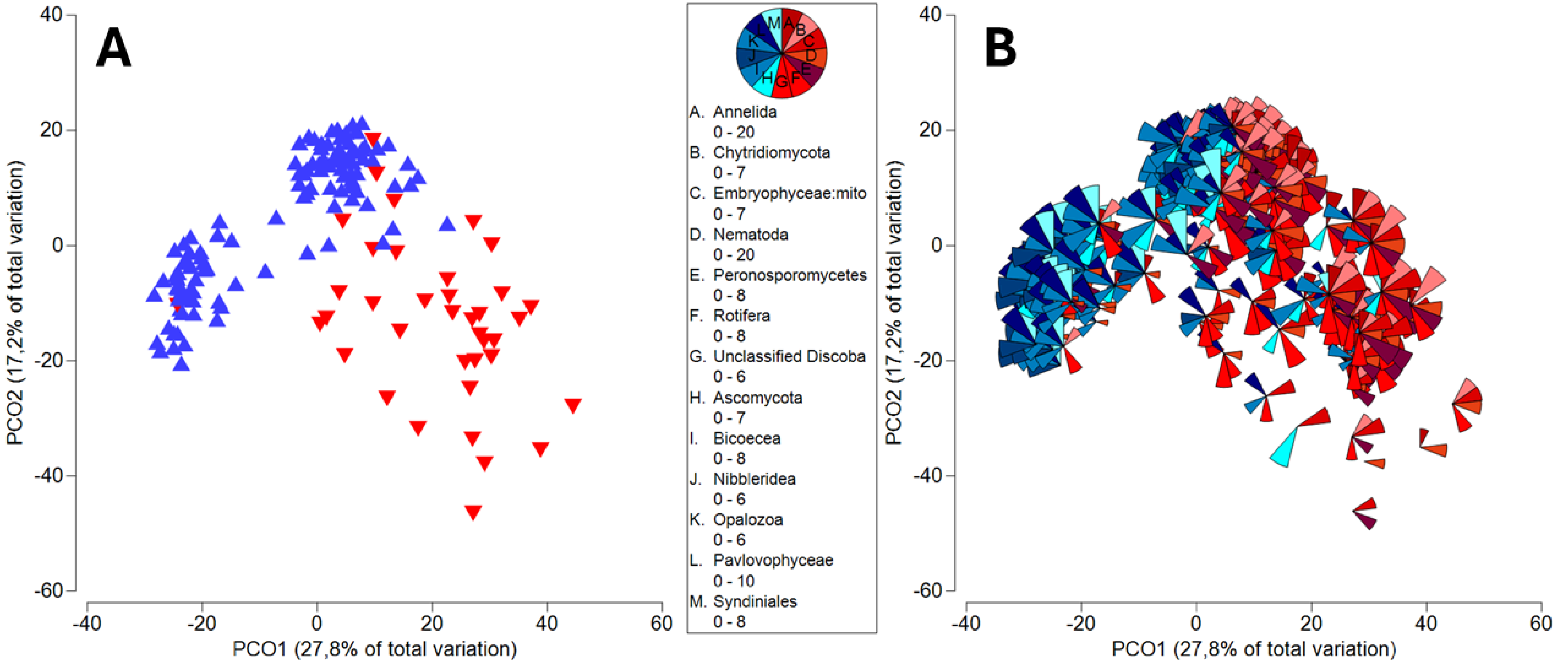


Figure n°18: (A) Principal Coordinates analysis (PCoA) of the communities of eukaryotes (class level) explaining most of the dissimilarities (SIMPER analysis) between the Environment biome (in blue: water and sediment samples) and the biofilm biome (in red: roots and leaves samples). (B) Principal Coordinates analysis (PCoA) of the communities of eukaryotes (class level) was overlaid with the classes of eukaryotes explaining most of the dissimilarities (SIMPER analysis) between the two biomes; In blue shades, organisms representing the environment biome, in red shades, organisms representing the biofilm biome

The comparison between the dbRDA of eukaryotic communities from the biofilm (leaves and roots, Figure 19) and from the sediment and water (Figure 20) shows some clear differences. The biofilm communities seem more influenced by environmental factors (explaining around 56% of the variation), while sediment and water communities are less explained by these variables (only around 36%). In the biofilm graph, samples are more separated, especially between seasons, which suggests that biofilm communities are more sensitive to changes like oxygen or salinity. In contrast, sediment and water samples are more grouped and show less seasonal variation. The main factors that affect the biofilm are dissolved oxygen and salinity, while temperature and turbidity have a bigger influence on sediment and water. This means biofilm communities, because they are closer to the plant, might be better indicators of changes in the plant's health, especially if oxygen levels drop. Sediment communities are more stable and may show broader environmental trends.

The relationship between environmental conditions and the communities of eukaryotes were tested with the BEST procedure; salinity and dissolved oxygen were found to be the combination of environmental variables (Spearman Rank correlation rho=0.425, $p<0.05$) best explaining the pattern observed in the eukaryotic communities of the biofilm biome, ie. associated with Ruppia.

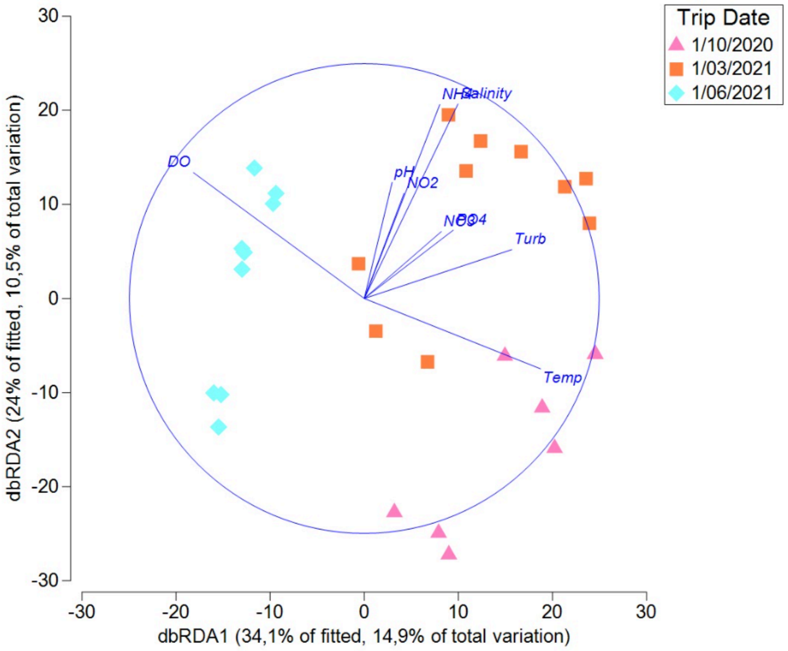


Figure n°19: Distance-based ReDundancy Analysis (dbRDA) of the communities of eukaryotes associated with the leaves and roots of Ruppia (B) in relation to water quality changes across the sampling sites over the duration of the study. Pearson correlations between the water quality parameters and the dbRDA axes are represented by the vectors in blue. The rayon of blue circle around the vectors represents a Pearson correlation of 1. The samples are represented by the date they were sampled: October 2020 (in pink), March 2021 (in orange) and June 2021 (in blue).

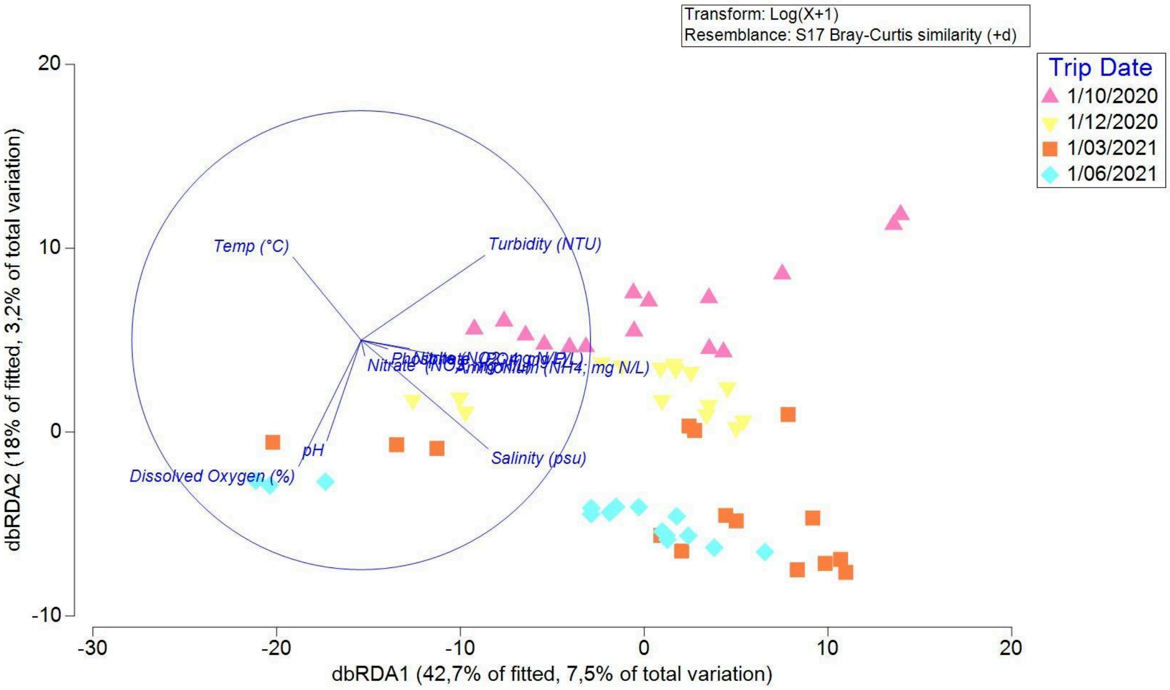


Figure n°20 : Distance-based ReDundancy Analysis (dbRDA) of the communities of eukaryotes associated with the sediment and water (E) in relation to water quality changes across the sampling sites over the duration of the study. Pearson correlations between the water quality parameters and the dbRDA axes are represented by the vectors in blue. The rayon of blue circle around the vectors represents a Pearson correlation of 1. The samples are represented by the date they were sampled: October 2020 (in pink), March 2021 (in orange) and June 2021 (in blue).

Conclusion

This study aimed to characterize the eukaryotic communities associated with *Ruppia tuberosa* in the Coorong lagoon (South Australia) and to understand how these communities are shaped by environmental conditions. Water quality analyses revealed strong spatial and temporal variability across the sampling period (October 2020, March 2021, and June 2021), especially in terms of salinity, temperature, dissolved oxygen and turbidity. The dbRDA results showed that these parameters had a clear influence on the structure of eukaryotic communities, particularly in water and sediment samples. Salinity and dissolved oxygen were identified as the main drivers of variation in the biofilm compartment, suggesting that these factors play a key role in shaping species composition in close association with *Ruppia*.

In parallel, the PCoA analysis highlighted a clear separation between environmental communities (water and sediment) and biofilm communities (roots and leaves), suggesting strong habitat filtering. The biofilms on *Ruppia* hosted distinct and diverse communities, notably dominated by nematodes, annelids and fungi. These organisms are known to interact with plant surfaces and microbial assemblages, potentially contributing to organic matter recycling, microbial regulation, or plant protection. Their presence suggests a complex web of interactions and supports the idea that biofilm communities may act as bioindicators of seagrass health.

While this study provides an important baseline, it also highlights the limited number of studies focusing on non-model eukaryotic organisms in seagrass systems. Yet, existing research in similar matrices has shown that nematodes, annelids and fungal taxa can play significant ecological roles. There is therefore a need for more targeted studies on these species and their interactions with seagrass hosts, particularly in variable and stressed environments like the Coorong.

Given these results, I plan to continue this project next year in Australia, focusing specifically on the species found on *Ruppia* and investigating their ecological roles. This future work would aim to better identify key eukaryotic taxa, explore how they interact with each other and with the host plant, and understand how they contribute to the stability and resilience of *Ruppia* ecosystems.

Internship experience

This third-year internship gave me valuable insight into the world of marine biology research. Although I was supposed to travel to Australia, visa issues prevented me from going, and I had to complete the internship remotely. This situation taught me how to work independently, manage my time effectively, and communicate regularly at a distance with my supervisor.

I developed strong skills in data analysis, bioinformatics, and ecological interpretation, especially regarding the eukaryotic communities associated with *Ruppia*. This experience confirmed my interest in marine science, and I've applied to master's programs in biology and marine biotechnology. I remain determined to continue this project next year in Australia, with a stronger focus on species interactions within *Ruppia* communities.

Finally, I would like to sincerely thank my supervisors, who were incredibly supportive throughout the internship. Their guidance and involvement helped me move forward despite the challenges, and I'll keep a positive memory of this experience.

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Resume

Understanding the ecological dynamics of *Ruppia*-associated communities is essential for assessing the health and resilience of estuarine ecosystems. In this study, we explored the eukaryotic diversity associated with *Ruppia tuberosa* in the Coorong lagoon system (South Australia), using 18S rRNA metabarcoding. Samples were collected from four compartments (water, sediment, *Ruppia* leaves, and roots) across five sites and multiple seasons. Environmental parameters, including salinity, dissolved oxygen, and nutrient concentrations, were also measured to contextualize community patterns.

Our results revealed strong compartmental differentiation, with water and sediment communities showing consistent contrasts in composition and diversity. Biofilm samples from roots and leaves exhibited distinct taxonomic profiles, characterized by high relative abundances of *Nematoda*, *Annelida*, and fungal classes such as *Ascomycota*, which were largely absent in surrounding matrices. These taxa may play key ecological roles and could serve as bioindicators of *Ruppia* health. PCoA and pie chart analyses confirmed the influence of habitat type over site location or temporal variation.

The ecological relevance of this work is further emphasized by the critical condition of the Coorong system, where reduced freshwater inflows have led to increased salinity and habitat degradation. As a result, several marine and estuarine species are under threat, highlighting the urgency to understand and preserve these ecosystems and their associated biodiversity.

Despite limited available data for some time points (no *Ruppia* in December and no leaves in March), the study highlights the complex interactions between *Ruppia* and its eukaryotic epibionts. These findings open perspectives for future research, particularly focusing on functional interactions between these organisms and their role in maintaining ecosystem stability. I intend to pursue this work during a future research stay in Australia, with a focus on identifying species-level interactions and their ecological significance.

Keywords

Ruppia tuberosa, Coorong, eukaryotic biofilms, 18S rRNA metabarcoding, benthic communities, salinity gradient, ecology, estuarine ecosystems, bioindicators, species interactions